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TESIS DOCTORAL

Repercusión del Tratamiento Ocular Crónico y la exposición a pantallas de ordenador sobre la Integridad de la Superficie Ocular. Diseño de Biomarcadores y Nuevas Terapias para Preservar la Transparencia Corneal y la Función Visual.

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CERTIFICAN:

Que la presente Tesis Doctoral titulada "Repercusión del tratamiento ocular crónico y la exposición a pantallas de ordenador sobre la integridad de la superficie ocular. Diseño de biomarcadores y nuevas terapias para preservar la transparencia corneal y la función visual" ha sido realizada por Dña. Carmen Galbis Estrada bajo nuestra dirección.

Hemos supervisado la parte experimental y revisado el formato y la redacción del trabajo, por lo que damos el consentimiento para que esta Tesis sea presentada y defendida ante tribunal para que pueda optar al grado Doctor.

Para que conste, en cumplimiento de la normativa vigente, firmamos el presente documento

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RESUMEN

Resumen

Nuestros ojos deben mantenerse humedos y lubrificados para permitir la transparencia corneal y la visión nítida. La lágrima es esencial para la estabilidad del epitelio corneal y conjuntival. Cuando es deficiente o se adhiere con dificultad a la superficie ocular, origina un amplio rango de signos y síntomas, cuyo máximo exponente es la xerosis, falta de transparencia corneal y pérdida de la visión. El tratamiento médico del paciente con ojo seco (OS) debe ser etiológico, sintomático, secretagogo y/o substitutivo. Sin embargo, la falta de respuesta de algunos sujetos y empeoramiento de otros, es incompatible con la vida habitual. El OS se produce por una serie de cambios en la composición lagrimal del paciente que alteran la superficie ocular, provocando signos y síntomas de mayor o menor envergadura, y cuyo máximo exponente es el dolor agudo o latente provocado por las ulceraciones corneales, acompañado por pérdida de visión como consecuencia de la pérdida de transparencia corneal. El OS se produce precisamente porque la película lagrimal no conserva su estabilidad. La incidencia es creciente en nuestra sociedad y de hecho, virtualmente, "todo el mundo tiene, ha tenido, o tendrá el ojo seco en algún momento de su vida". Las molestias que deriven de él dependen de la severidad del cuadro, y las molestias son causa de invalidez, pérdida de horas laborales, trastornos emocionales y pérdida de la calidad de vida.

La investigación en superficie ocular debe dirigirse hacia el conocimiento de las bases celulares y moleculares del OS. Por ello se diseñaron una serie de estudios con un objetivo común, utilizar las lágrimas como muestra biológica y analizar sus características en distintas situaciones causantes de OS.

- En el **primer estudio** se realizó en una muestra de pacientes procedentes de la consulta de Oftalmología de Hospital Universitario Dr. Peset de Valencia. Mediante un estudio prospectivo se investigó la relación entre los factores de riesgo, los resultados clínicos y los niveles de expresión de los mediadores de inflamación y respuesta inmune. Los participantes se distribuyeron en dos grupos mediante la realización del cuestionario Ocular Surface Disorder Index OSDI: pacientes diagnosticados con OS (GOS; n=30) y controles sanos (GC; n=36) que a su vez fueron asignados al azar a subgrupos homogéneos de acuerdo a la ingesta oral diaria (+ S) o no (NS) de antioxidantes (AOX) y ácidos grasos esenciales

poliinsaturados de cadena larga (AGPIs) durante 3 meses. Se recogieron las lágrimas reflejas de ambos ojos y se procesaron mediante un sistema multideterminación (Luminex ®). En la muestras del grupo OS se observó un aumento significativo de la expresión de las interleuquinas (IL) IL-1β, IL6 e IL10 y una disminución significativa del Factor de crecimiento endotelial vascular (VEGF) al compararlas con el GC. Los niveles de expresión de las IL-1β, IL6 e IL10 fueron significativamente menores en los pacientes con suplementación micronutricional.

- En el **segundo trabajo** se realizó un estudio multicéntrico transversal de casos, incluyendo pacientes con Glaucoma Primario de Angulo Abierto (GPAA) y controles sanos, que se llevó a cabo mediante selección de participantes de acuerdo a los criterios de inclusión y exclusión entre los sujetos que acudieron a las consultas de oftalmología de los centros del estudio en Andalucía y Comunidad Valenciana, donde se procedió a determinar los efectos de la suplementación oral con una formulación combinada de antioxidantes y ácidos grasos esenciales poliinsaturados en la expresión de citocinas y quimioquinas en las lágrimas de los sujetos de estudio (n = 97) que se distribuyeron en tres grupos: los individuos con OS no severo (GOS; n=30), (2) las personas con GPAA no avanzadas (GGL; n=31), y (3) los controles sanos (GC; n=36). Estos grupos fueron divididos aleatoriamente en dos subgrupos: uno de ellos recibió un antioxidante diario y suplemento de ácidos grasos poliinsaturados esenciales (dos pastillas) durante 3 meses (+ S), y el otro no (NS). Las concentraciones de citocinas y quimiocinas específicas en las lágrimas reflejo se determinaron por un sistema multideterminación (Luminex ®). Se hallaron diferencias significativas en la expresión de Factor de estimulación de colonias de macrófagos (GM-CSF) ($p = 0.008$), Factor de necrosis tumoral alfa (TNF- α) ($p= 0.005$), Factor de crecimiento endotelial vascular (VEGF) ($p= 0.038$), IL-4 ($p= 0.030$), IL-6 ($p= 0.044$) entre el GOS y el GGL. Los principales signos y síntomas del SOS mejoraron tras la suplementación con omega-3.
- En el **tercer artículo** de esta tesis se realizó un estudio prospectivo en trabajadores expuestos a pantallas de ordenador durante la jornada laboral, procedentes de la Tesorería General de la Seguridad Social de Valencia, cuyo ambiente oficinal está controlado, en cuanto a los parámetros medioambientales, en el cual se investigó la relación entre los factores de riesgo, los resultados clínicos, los niveles de

expresión de los mediadores de inflamación y respuesta inmune y el riesgo de aparición de síntomas relacionados con el OS. Los participantes se distribuyeron en dos grupos: sujetos expuestos a pantallas de ordenador durante la jornada laboral (GEP; n=88) dentro del cual se diagnosticó mediante cuestionario OSDI un 29,26% con OS leve y a un 70,73% con OS moderado; y sujetos no expuestos a pantallas de ordenador (n=36). Se realizó la extracción de la lágrima refleja por capilaridad y se determinó la concentración de citoquinas/quimioquinas mediante el sistema multideterminación (Luminex ®). Los sujetos del GEP presentaron niveles significativamente más elevados de las IL-1β, IL-6, IL-8 y VEGF. La expresión más significativa de los mediadores de inflamación y respuesta inmune del grupo de pacientes GEP clasificados como OS-leve correspondió a la IL-8 (p= 0,001) e IL-12 (p= 0,031), mientras que en el subgrupo clasificado como OS-moderado fue significativamente aumentada la expresión de la IL-6 (p= 0,032). Los factores ambientales estudiados en el puesto de trabajo (temperatura seca, humedad relativa, intensidad de luz, CO2) se hallaron dentro de los rangos normales

- En el **cuarto artículo** se utilizó la técnica de Resonancia Magnética Nuclear de protón, 1H RMN, para analizar el comportamiento metabólico de las lágrimas reflejas de los pacientes con trastornos de ojo seco. Se realizó un estudio prospectivo, clínico-experimental de casos-controles para analizar el comportamiento de variables clínico epidemiológicas y moleculares en 120 pacientes escogidos entre los asistentes a los centros del estudio, distribuidos conforme al cuestionario (OSDI) en: (1) pacientes con OS (GOS , n = 55) y (2) los sujetos sanos (GC , n = 35). El GOS se subdividió en 3 subgrupos según la gravedad del OS: leve, moderada y severa (n = 22, 18 y 15, respectivamente). Los principales metabolitos hallados en las muestras de lágrima fueron colesterol, N-acetilglucosamina, glutamato, creatina, amino-n-butirato, colina, acetilcolina, arginina, fosfoetanolamina, glucosa y fenilalanina. Las lágrimas de los dos grupos principales presentaron perfiles metabólicos diferentes, al igual que las lágrimas de los pacientes de los subgrupos: OS- leve ($p < 0,001$), OS- moderado ($p < 0,001$) y OS- severo ($p < 0,001$) .

En conclusión, el OS es una enfermedad muy prevalente, con factores de riesgo muy delimitados entre los que hay incluir la edad, el sexo femenino, la instilación crónica de colirios y los factores medioambientales (entre los que destaca la exposición laboral a las pantallas de visualización de datos).

Además, se demuestra la actividad inflamatoria en relación a un cambio metabólico en la superficie ocular que puede determinarse mediante análisis de las lágrimas e identificación de los biomarcadores correspondientes a la enfermedad de la superficie ocular de los pacientes afectos.

Entre las estrategias terapéuticas debería considerarse el tratamiento antiinflamatorio en adición a la instilación de sustitutivos de la lágrima y la suplementación oral con AOX y AGPIs omega-3 en los sujetos con mayor riesgo de padecer el OS, ya que esta suplementación micronutricional ha demostrado ejercer un efecto beneficioso sobre las manifestaciones del OS, disminuyendo la actividad inflamatoria sobre las estructuras de la superficie ocular, y mejorando los signos y síntomas del OS.

INTRODUCCIÓN

Introducción

La Superficie Ocular

Desde la antigüedad se hablaba de ojo seco cuando la superficie ocular era macroscópica y evidentemente seca, siendo objetivable el empeoramiento cuando causaba córneas opacas. En el mundo grecolatino, tanto Galeno como Pablo de Egida describieron la convergencia de xeroftalmía y reumatismo. Y entre las décadas de los años 40-70 del pasado siglo los oftalmólogos relacionaban la sequedad ocular con el síndrome de Sjögren. Debido a su elevada prevalencia, el estudio de la sequedad ocular ocupa una posición relevante dentro de la oftalmología [1, 2]. En este sentido, los esfuerzos se orientan esencialmente hacia dos grandes temas de interés:

1) Aspectos relacionados con la fisiopatología del Síndrome de Ojo Seco (SOS)

2) Desarrollo de nuevos y más eficaces tratamientos.

La superficie ocular consta de 5 estructuras:

- Córnea: Tejido translúcido con importante función refractiva, situada en el segmento anterior ocular. Es fundamental para la visión que la córnea siempre esté húmeda.

- Conjuntiva: Tejido membranoso semi-transparente con vasos sanguíneos que recubre la parte anterior del globo ocular (alrededor de la córnea) y los fondos de saco y cara interior de los párpados. Tiene que estar humedecida, pero no es tan sensible a la sequedad como la córnea.

- Película lagrimal: Red virtual de agua, sales, principios inmediatos, moléculas que se sitúa en la superficie ocular para nutrir, defender y proteger el segmento anterior ocular y que está producida de forma continua por las glándulas, atraviesa toda la superficie ocular, y se va eliminando a través de los puntos lagrimales y vías de drenaje.

- Párpados: Estructuras dérmicas móviles que recubren los ojos. Los párpados superiores e inferiores están compuestos por la conjuntiva *palpebral* (en contacto con el globo ocular), el tarso (una capa de tejido propio), el músculo orbicular (que le da su movilidad) y la piel exterior. En el borde libre se encuentran las pestañas. Su función es

la protección del ojo y la humectación del mismo mediante las secreciones lagrimales. Por otra parte, el reflejo del parpadeo protege al ojo de cuerpos extraños.

- Glándula lagrimal: tiene como función producir las lágrimas y se localiza en la fosa lagrimal, situada en la parte supero-externa de cada órbita. También existen varias glándulas accesorias situadas en el párpado, conocidas como glándulas de Meibomio, cuya secreción también forma parte de la película lagrimal. La glándula lagrimal se encuentra dividida por el tendón del músculo elevador del párpado superior en dos partes: una porción superior u orbitaria y una porción inferior o palpebral.

El Síndrome de Ojo Seco

El síndrome de ojo seco (SOS) corresponde al conjunto de manifestaciones subsecuentes a la alteración de la película lagrimal, que resultan de la disminución en la producción lagrimal, la evaporación precoz o anomalía de los componentes lipídico y/o mucinoso de la película lagrimal que protege la superficie ocular [1-7]. Esta alteración lagrimal se asocia a la persistencia de un proceso inflamatorio que empeora los síntomas y conduce al daño de la superficie ocular, provocando signos y síntomas de mayor o menor envergadura, y cuyo máximo exponente es el dolor agudo o latente provocado por las ulceraciones corneales, acompañado por pérdida de visión como consecuencia de la pérdida de transparencia corneal [4].

El SOS se produce precisamente porque la película lagrimal no conserva su estabilidad [6-13]. La incidencia es creciente en nuestra sociedad y de hecho, virtualmente, "todo el mundo tiene, ha tenido, o tendrá el ojo seco en algún momento de su vida". Las molestias que deriven de él dependen de la severidad del cuadro, y las molestias son causa de invalidez, pérdida de horas laborales, trastornos emocionales y pérdida de la calidad de vida.

Los datos epidemiológicos sobre la disfunción de la superficie ocular son limitados debido a que no existe uniformidad en los criterios diagnósticos de muchos estudios poblacionales. Además, no existe un patrón diagnóstico para esta patología. Se estima que existe una prevalencia cercana al 14 ó 17 % de la población, sólo en los países industrializados. Se ha descrito que el SOS es más frecuente en personas mayores

de 65 años y que predomina en mujeres [14-17]. Stern, afirma que aproximadamente el 35 % de los mayores de 60 años padecen ojo seco en España [18]. La Sociedad Española de Oftalmología afirma que aproximadamente el 35 % de los mayores de 60 años padecen ojo seco y similares resultados son expuestos en Estados Unidos, donde un trabajo afirma que la secreción lagrimal disminuye con la edad, siendo la incidencia de ojo seco en mayores de 65 años del 75%. El estudio Australiano Blue Mountains Eye Study, publicó que las mujeres afectas de ojo seco duplican a los hombres [19] Lo que es evidente es que el ojo debe mantener un nivel adecuado de humedad y lubrificación para desempeñar sus funciones, manteniendo la transparencia corneal y la visión nítida. Si está poco hidratado, deficientemente lubrificado, o seco las consecuencias incluyen un amplio rango de signos y síntomas, como ardor, picazón, sensación de cuerpo extraño, roces, dolor, visión borrosa, cuyo máximo exponente es la xerosis, pérdida de transparencia corneal y pérdida de visión [20-24]

Los ojos tienen que estar humectados y lubrificados para optimizar la transparencia corneal y la visión diáfana. Nuestras lágrimas son fundamentales para la integridad del epitelio corneal y conjuntival. Si son deficientes o se adhieren con dificultad, aparecen signos y síntomas, cuyo máximo exponente es la xerosis, pérdida de transparencia corneal y de la visión.

Las lágrimas humanas están formadas por tres capas: mucina (que tapiza la córnea); capa acuosa media (que proporciona hidratación y oxígeno, además de otros nutrientes importantes a la córnea); y la capa lipídica externa, que previene la evaporación de la lágrima.

Se han propuesto distintas clasificaciones para el ojo seco, aunque generalmente se utiliza: leve, moderado y grave [20]. El ojo seco grave se caracteriza por presentar una semiología marcada, que forma parte demasiado evidente de la vida diaria de estos enfermos, siendo muy molesta y acompañada de gran carga anímica de preocupación y sufrimiento, lo que logra alterar sus vidas y la de sus familiares, hasta tal punto que impide el normal desarrollo de sus actividades.

Los síntomas que ayudan a determinar un ojo seco son: picor, fatiga ocular, sensación de quemazón, ojo rojo, visión borrosa, que mejora con el parpadeo y lagrimeo

excesivo. El aumento de las molestias suele ser al final del día o cuando el paciente realiza actividades que acentuen el cansancio visual: lectura exhaustiva, televisión o trabajos con ordenador [20-22].

Las causas de ojo seco son variadas y las manifestaciones clínicas de la interacción entre estas y la superficie ocular dependen sobre todo de la capa de la película lagrimal que esté en déficit, así tenemos al ojo seco acuo-deficiente (las glándulas lagrimales principal y accesorias segregan deficitariamente), muco-deficiente (inicialmente la secreción mucosa es normal pero se altera secundariamente a otras modalidades de ojo seco), o lipo-deficiente (la capa lipídica es la afectada, y la causa más frecuente es la enfermedad de las glándulas lipídicas del márgen palpebral) [20-23].

El Taller Internacional sobre el Ojo Seco (DEWS, 2007)

La enfermedad del ojo seco es una afección clínica muy común, pero muchas veces no es reconocida, cuya etiologia y manejo representan un desafio para los médicos clínicos e investigadores. En los últimos 10 años, se han logrado avances en el entendimiento de esta enfermedad en las áreas de epidemiología, patogenia, manifestación clínica y posible terapia. El informe del Taller Internacional sobre el Ojo Seco (DEWS) representa el trabajo de muchos expertos durante un largo periodo de deliberación, a lo largo de un proceso repetitivo que incluyó la recolección de datos, la presentación de resúmenes en un formato de conferencia, así como la armonización de los informes [24]. Tiene como objetivo principal esclarecer la definición y las características de esta enfermedad, los factores de riesgo así como recomendar parámetros de confianza para realizar investigaciones clínicas y ensayos clínicos sobre el ojo seco. En cada capítulo se resumieron temas relevantes para entender el trabajo de los participantes sobre la enfermedad del ojo seco. La publicación combinada de todas las discusiones y conclusiones es un recurso valioso para los médicos clínicos, los epidemiólogos, los científicos básicos y clínicos, así como los miembros de la industria farmacéutica [24].

Factores de riesgo para el Síndrome de Ojo Seco

Como hemos indicado con anterioridad entre las causas de las alteraciones de la superficie ocular hay que considerar los factores externos; por su influencia en la conservación de las lágrimas y la exposición al exterior de los tejidos oculares. Entre ellos destacan los diversos cambios medio-ambientales, la exposición prolongada a pantallas, ordenadores, móviles, sistemas de juegos, la contaminación del aire, aires acondicionados, sequedad del ambiente, uso de lentillas o de colirios (sobre todo los de instilación crónica) y algunos medicamentos generales capaces de disminuir la producción de lágrima (antialérgicos, antiespasmódicos, antidepresivos, diuréticos, y otros) [3, 6, 11, 21]. También se debe tener en cuenta a los factores endógenos entre los que sobresalen los mecanismos ligados a la alteración del parpadeo (fijación extrema por lectura, ordenador, conducción), disfunción palpebral (ectropión, paresias, pacientes en coma, anestesias generales, dormir con los párpados entreabiertos), el envejecimiento, y también los factores hormonales (como en mujeres postmenopaúsicas). Además el SOS se asocia a otros factores como enfermedades sistémicas crónicas en especial colagenopatías y enfermedades autoinmunes [21,23]. El hábito tabáquico y ciertos trastornos de la conducta nutricional (como los consumidores de multivitaminas) también tienen que ser considerados en el contexto del paciente con SOS.

El diagnóstico de ojo seco se basa una secuencia de hechos que comienzan con obtención de datos a partir de las manifestaciones que el enfermo indica, por lo que es fundamental la entrevista personal apoyada en la evaluación de los cuestionarios validados para el SOS y las pruebas clínicas diagnósticas.

El tratamiento médico del paciente con ojo seco (OS) debe ser etiológico, sintomático y/o substitutivo. El tratamiento consiste básicamente en administrar sustitutivos de las lágrimas naturales en colirio, con o sin conservantes. Cuando esto no es suficiente existen otras opciones, como la oclusión de los puntos lagrimales de forma temporal o definitiva, para evitar que la lágrima que produce el paciente se drene hacia las fosas nasales y permanezca mas tiempo en contacto con la córnea; o bien, utilizar sustancias que incrementen la producción (secretagogos). No obstante, fisiológicamente

se favorece la integridad de la superficie ocular por diversos mecanismos**,** como el parpadeo, o la regulación de la producción de lágrimas (las necesidades de lágrimas cambian constantemente, y la producción tiene que adaptarse a esto). La conjuntiva y la córnea poseen terminaciones nerviosas sensoriales y motoras que actuan a este nivel detectando la sequedad y estimulando una mayor producción de lágrima.

Sin embargo, la falta de respuesta de algunos sujetos al tratamiento y empeoramiento de otros, es incompatible con la vida habitual. El número cada vez más elevado de pacientes con SOS, lo erige como uno de los más frecuentes motivos de consulta en la práctica oftalmológica diaria, y por ello la sequedad ocular es una de las líneas más actuales de investigación. Estudiar las características fisiopatológicas y clínicas, y nuevos aspectos del diagnóstico, pronóstico y tratamiento de este proceso adquieren una importancia indiscutible, puesto que el SOS provoca una alteración en la calidad de vida de los pacientes afectos.

- El Glaucoma y la Superficie Ocular

La neuropatia optica glaucomatosa es una enfermedad neurodegenerativa ocular que resulta del aumento de la presion intraocular y que en su curso espontaneo evoluciona crónica e indefectiblemente a ceguera por atrofia optica [27,28]. En este contexto, la instilación crónica de medicación hipotensora ocular, como en el caso de los pacientes con Glaucoma crónico o primario de angulo agudo abierto (GPAA), disminuye la presión pero es capaz de provocar cuanto menos el empeoramiento del SOS [11].

La primera línea de tratamiento del GPAA constituye un tratamiento médico tópico que, o bien disminuye la producción de humor acuoso y / o facilita su salida. Al igual que con otros muchos colirios, estos medicamentos generalmente contienen conservantes para garantizar su seguridad. La evidencia reciente ha demostrado que algunos de los ingredientes que se utilizan como conservantes en muchos medicamentos oftálmicos (incluso algunos para el tratamiento de SOS), son al menos parcialmente responsables del problema. En particular, los pacientes con glaucoma, que están expuestos a un uso crónico de conservantes en su tratamiento, representan una población muy susceptible.

El conservante más utilizado en preparaciones oftálmicas es cloruro de benzalconio (BAK). Es un agente que pertenece al grupo de amonio cuaternario (compuestos bipolares que son altamente hidrosolubles y tienen cualidades de agentes tensioactivos). Se comporta como un detergente, disolviendo las paredes de las células microbianas. El problema viene de este mismo efecto detergente que actúa sobre la superficie ocular debido a que existe un equilibrio delicado en la homeostasis de la película lagrimal y la función celular dentro de la conjuntiva y la córnea. Es bien sabido que la producción deficiente de lágrimas, la baja calidad de la película lagrimal y el aumento de la evaporación pueden conducir a SOS. Además, los efectos perjudiciales de los conservantes tales como BAK son responsables de gran parte de la inflamación que se produce en estos ojos [11].

La integridad de la superficie ocular de pacientes con glaucoma sujetos a medicación crónica con colirios se ve comprometida y expresa diversos marcadores de gran interés diagnóstico y terapéutico. Se ha descrito que expresan marcadores inflamatorios específicos para el reclutamiento de células T que podrían ser responsables de la generación de los primeros acontecimientos de la inflamación y la cicatrización de heridas. El perfil de proteínas inflamatorias en lágrimas presente en los pacientes con glaucoma tratados crónicamente parece ser diferente de la que se encuentra en el ojo seco primario [29, 30]. Por lo tanto, los pacientes que sufran estas exposiciones y además tengan que instilarse, de forma continua la medicación antiglaucomatosa, pueden agravar su situación inicial, empeorando los sígnos y síntomas que pueden conducir incluso a la pérdida de la visión por la lesion y descompensación de la superficie ocular [31-33].

- Las Pantallas de Visualización de Datos

En la actualidad, las pantallas de visualización de datos (PVD) constituyen una parte de nuestra cultura, tanto en el ámbito laboral como en el privado [34]. La participación en las redes sociales, el uso habitual de internet para realizar cualquier transacción o búsqueda relativa a productos, bienes y servicios hace que las PVD formen parte de lo cotidiano y su uso se ha generalizado de manera impensable hace 11 años, tal como demuestra la Encuesta de uso de las tecnologías de la información y

comunicación (TIC) y comercio electrónico de 2001 del Instituto Nacional de Estadística (INE) [35].

El uso generalizado que en nuestra vida extra laboral hacemos de sistemas de banca electrónica, adquisición de bienes y servicios por vía telemática hace que prácticamente un porcentaje muy elevado de la población seamos usuarios a todos los efectos de PVD. Por otra parte en España, la introducción de las nuevas tecnologías de la información, así como la de nuevos equipos y sistemas desde hace aproximadamente 20 años, hace que comencemos a ver los resultados de su exposición y manejo, es decir las consecuencias de la exposición laboral y no laboral en especial sus consecuencias para el globo ocular.

La Asociación Americana de Optometría, unió los diferentes trastornos del órgano visual bajo la denominación de "Computer Visión Sindrome", que en nuestro país conocemos como "Síndrome de la pantalla de visualización o Síndrome Visual del Ordenador (SPV) [36]. Este síndrome incluiría síntomas visuales y oculares. Entre las alteraciones de la función visual encontramos visón borrosa, visión doble o diplopía, hipersensibilidad a la luz, alteraciones en la percepción cromática, esfuerzo exagerado para visualizar objetos… todos ellos relacionados con la fatiga visual. Entre los signos y síntomas oculares destacan la sequedad de ojos, ojos irritados, sensación de quemazón y picazón, ojos rojos, sensación de arenilla, dificultad para el uso de lentes de contacto, pesadez, lagrimeo, ardor y malestar ocular.

Existe evidencia científica sobre las causas de la aparición de la sequedad ocular en los usuarios de PVD, aparte de los factores subyacentes en relación a la salud o la especial sensibilidad de cada individuo (37,38); y entre ellas destacan los siguientes hechos:

1- La mayor apertura de la hendidura palpebral: aumenta el área de exposición ocular. Cuando más hacia arriba se mira mayor apertura de hendidura palpebral y mayor evaporación.

2- La reducción de la frecuencia de parpadeo, frente a las PVD respecto a la visión lejana o la lectura de textos convencionales.

3- Los factores ambientales tales como la temperatura seca, la humedad relativa, la velocidad del aire, la calidad de renovación del mismo en base a la concentración de CO2 en los centros de trabajo.

4- Los factores ergonómicos:

a. Diseño del puesto de trabajo: distancias a PVD y planos de trabajo como ángulos de visión de los mismos.

b. Diseño y contenido de la tarea realizada Duránte la jornada laboral: complejidad, repetitividad, lectura, introducción de datos, concentración necesaria, posibilidad de realizar pausas periódicas.

Todos los aspectos anteriormente comentados, el largo tiempo de exposición, el uso generalizado en el mundo laboral, así como la imperiosidad legal y de sostenibilidad económica de nuestros sistemas de protección social con la prolongación de la vida laboral obliga a los servicios de prevención de riesgos laborales a empezar a considerar el ojo seco como un problema de salud en el mundo del trabajo importante.

Investigación en Superficie Ocular

- Estudios en animales de experimentación

Se han realizado muchos estudios en modelos animales para determinar la integridad de la superficie ocular expuesta a diferentes tóxicos o ambientes tales como el óxido nítrico en modelos animales [39], ozono en modelos de ratones donde se estudiaba la producción de citoquinas inflamatorias y los cambios en las células secretoras de mucina [41], los factores de regulación y diferenciación de las células caliciformes presentes en la cicatrización del epitelio conjuntival de la superficie ocular [42]. Los estudios principales realizados en modelos animales son los siguientes: 1) aquellos basados en la insuficiencia lagrimal los modelos de inflamación del Síndrome de Sjögren en ratones y modelos con autoanticuerpos en conejo y rata; el control mecánico de la secreción lagrimal en perros, gatos, conejos y ratones; el control endocrino de la secreción lagrimal en modelos de ratas; el control neuronal de la secreción lagrimal en conejo albino, 2) el ojo seco evaporativo en conejos, 3) la combinación de la insuficiencia lagrimal y el ojo seco

evaporativo en ratones y 4) la inducción de la keratitis seca (KCS) espontanea en perros [42].

- **Estudios epidemiológicos**

Los diferentes estudios epidemiológicos realizados a lo largo de los años con el fin de evaluar y determinar la prevalencia de los diferentes síntomas asociados al SOS y el estado de la película lagrimal mediante el análisis en pacientes con diferentes patologías como glaucoma [11,43]; el estudio del patrón proteico en lágrimas procedentes de pacientes con sintomatología subjetiva y con sintomatología clínica y el estudio de la relación existente entre el SOS y las enfermedades hormonales [44], son algunos de los ejemplos que se han llevado a cabo en este sentido. Destacan los Estudios multicentricos siguientes: Canadian Dry Eye Epidemiology Study, Salisbury Eye Evaluation Project, Melbourne Visual Impairment, Beaver Dam Eye Study y Women`s Health Study, todos ellos evaluados por Abelson et al [45].

- **Líneas de Investigación traslacionales. Los Biomarcadores.**

La necesidad de estrechar lazos entre la investigación básica y la clínica ha dado lugar a la investigación traslacional. La separación entre la investigación biomédica básica y la aplicación clínica ha crecido, y a pesar de una explosión de conocimiento sobre los mecanismos de los procesos biológicos, esto no se ha traducido en el incremento correspondiente de nuevos tratamientos. El conocimiento adquirido en la investigación básica ha tenido escaso impacto en la práctica clínica. Ni los estudios realizados en el laboratorio y en el quirófano experimental, ni los ensayos clínicos fase I reflejan el estado real de los pacientes para poder predecir la eficacia y seguridad de un nuevo tratamiento [46,50]. En este contexto surge el concepto de medicina traslacional con un objetivo tan fácil de definir como difícil de conseguir: facilitar la transición de la investigación básica en aplicaciones clínicas que redunden en beneficio de la salud [47].

Biomarcador o marcador biológico es aquella sustancia utilizada como indicador de concreto. Debe poder medirse objetivamente y ser evaluado como un indicador de un proceso biológico normal, estado patogénico o de respuesta a un tratamiento farmacológico. Los biomarcadores son medidos en los niveles molecular, bioquímico o celular, tanto en poblaciones naturales provenientes de hábitats contaminados, como en

organismos expuestos experimentalmente a contaminantes, y que indican que el organismo ha estado expuesto a sustancias tóxicas y la magnitud de la respuesta del organismo al contaminante. El uso de biomarcadores puede ser muy interesante en campos como el de la farmacogenómica, en concreto para poder alcanzar terapias personalizadas. El proceso por el que se encuentran biomarcadores es el siguiente: un fármaco puede ser aplicado a un grupo heterogéneo de pacientes, entre estos podremos distinguir a los que responden de manera adecuada a dicho tratamiento, y a los que no. De esta manera se obtiene un biomarcador que nos reportará qué individuos responderán bien a dicho tratamiento y podremos así aplicar una terapia personalizada [48]. Pero el mero hecho de descubrir el biomarcador no lo es todo, es decir, hay que validar dicho descubrimiento.

- **Identificación de moleculas implicadas en inflamación y respuesta inmune. Los sistemas multi-determinación . Luminex ®**

La inflamación se basa en una compleja serie de eventos celulares y bioquímicos que se producen Duránte una respuesta inmune a una lesión o un patógeno. Una reacción inflamatoria aguda implica aumento del flujo sanguíneo local y aumento de la permeabilidad vascular. Esto facilita la acumulación de leucocitos que son responsables de liberar al cuerpo del agente perjudicial. Esta respuesta inmune es crucial para la supervivencia. La inflamación esta, por lo general, bien regulado a través un balance de los procesos de pro-inflamatorias y anti -inflamatorias. Cuando no se controla, puede convertirse en crónica y puede producir daño en los tejidos. Inflamación sostenida ha sido vinculada a muchos trastornos, incluyendo la artritis, la diabetes, enfermedad cardíaca, y La enfermedad de Alzheimer. Las citoquinas son los reguladores primarios de ambas respuestas, pro-y respuestas anti - inflamatorias. Sus actividades pueden afectar directamente si la inflamación es controlada y beneficiosa o no regulada y patológica. En consecuencia, la investigación de la actividad de las citoquinas es esencial para la comprensión de los mecanismos que subyacen a una amplia gama de enfermedades humanas. El Fluorokine Multiplex kit puede ser usado simultáneamente para evaluar los niveles de doce biomarcadores de inflamación en una sola muestra.

Mediante el Sistema Multiplex (Luminex ® R- 200; Luminex Corporation, Austin, TX, EE.UU) es posible llevar a cabo el análisis de la expresión de un conjunto de

citoquinas/quimioquinas en las muestras de lágrima refleja obtenidas mediante capilaridad del menisco inferior [5,8,10,49]. Con 30-40 µl de cada muestra es posible determinar los siguientes marcadores de inflamación y respuesta inmune presentes en las lágrimas humanas: (IL) - 1β , IL-2 , IL-4 , IL-5 , IL-6 , IL7 , IL8 , IL10 , IL12, TNF – α, VEGF, GM-CSF e IF- γ. Para identificar y cuantificar cada reacción antígeno-anticuerpo particular se utiliza un sistema basado en el flujo de Bio- Plex ® array suspensión (Bio-Rad Laboratories , Hercules, CA , EE.UU.). La identificación de las moléculas ensayadas se basa en el color del grano y la fluorescencia, usando moléculas informadoras marcadas con fluorescencia asociados con cada proteína diana. Las concentraciones de citoquinas y quimioquinas desconocidas se calculan automáticamente por el software de Bio - Plex utilizando una curva estándar derivada de un estándar de citoquinas recombinantes. Una vez halladas las concentraciones de citocinas y de quimioquinas se corrigen para la concentración de proteína total inicial de cada muestra de lágrima humana Duránte el análisis [50].

- **Identificación de moléculas implicadas en el metabolismo celular. La metabolómica. La RMN.**

La metabolómica es la disciplina de investigación que engloba el estudio del metaboloma: conjunto de pequeñas moléculas o metabolitos que están presentes en las células, tejidos, órganos o fluidos orgánicos. Los metabolitos que analiza son pequeñas moléculas (productos intermedios del metabolismo), carbohidratos, péptidos y lípidos derivados de la dieta o de los procesos patológicos a través de los cuales es posible establecer una relación con las respuestas celulares [51]. La identificación, concentración y variación de estos compuestos es el resultado de la interacción compleja entre la expresión de los genes, las proteínas y el ambiente [52]. De la misma manera que la genómica estudia la expresión de los genes y la proteómica la expresión de las proteínas, la metabolómica estudia las consecuencias de la actividad de estos genes y estas proteínas con menor coste económico y en menos tiempo [53,54].

Una vez se ha recolectado la muestra, el estudio metabolómico comienza con la extracción de las pequeñas moléculas y su posterior análisis, mediante técnicas de espectroscopia avanzadas que separan y cuantifican las partículas de interés. Entre estas técnicas destacan la espectrometría de masa (EM), la RMN y la cromatografía de líquidos o gases.

La RMN permite el análisis de múltiples metabolitos rápida y simultáneamente, requiere muy poco tratamiento previo de la muestra (se añade solamente una solución estándar de referencia y óxido de deuterio) y no la altera, por lo que puede utilizarse para otros análisis [51,55]. La RMN ofrece un espectro que contiene señales de cientos de metabolitos que representan multitud de vías bioquímicas por lo que para analizar el espectro con mayor facilidad éste se suele segmentar en pequeñas regiones.

La gran cantidad de datos que se obtienen del estudio metabolómico limita mucho la utilidad de los métodos estadísticos tradicionales. El análisis estadístico de componentes principales (PCA), el análisis discriminativo lineal (LDA), el análisis discriminante por mínimos cuadrados parciales (PLS-DA) y el análisis por mínimos cuadrados parciales (PLS); que permiten analizar gran cantidad de información a partir de un número reducido de muestras (variables o rasgos) en un tiempo relativamente corto.

El estudio del metaboloma puede llegar a establecer un vínculo entre los componentes metabólicos y las correspondientes respuestas celulares. Duránte los últimos 30 años la metabolómica se ha utilizado en estudios clínicos y animales de varias enfermedades, incluyendo patologías oculares. Young and Wallace [51], revisaron las consecuencias metabólicas de las enfermedades oculares y explicaron que mediante el análisis multiplex inherente a la metabolómica es esperable obtener datos únicos aplicables al studio de la superficie ocular. Los avances biotecnológicos, como la aplicación de espectroscopía de RMN a muestras de tejidos y fluidos biológicos, han permitido la generación de datos sobre metabolitos de bajo peso molecular en los individuos de estudio [52-55]. Por ejemplo, Mildefart et al, [53] analizaron extractos de cornea de conejo y lentes por espectroscopia de RMN y observaron que la taurina y el glutatión se expresaban en niveles elevados, lo que sugiere la existencia de un entorno antioxidante potente en los tejidos oculares como sugieren otros autores [56]. Greiner et a., analizaron el humor acuoso y el vítreo de ojos de cerdo a través de 31P RMN [55]. Estos estudios y otros trabajos similares han demostrado la utilidad de la metabolómica para el seguimiento de enfermedades oculares como el SOS.

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ARTÍCULOS

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ORIGINAL RESEARCH

Effects of a nutraceutical formulation based on the combination of antioxidants and ω-3 essential fatty acids in the expression of inflammation and immune response mediators in tears from patients with dry eye disorders

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Background: Women, and those older than 65 years of age, are particularly susceptible to dry eye disorders (DEDs). Inflammation is clearly involved in the pathogenesis of DEDs, and there is mounting evidence on the antioxidant and antiinflammatory properties of essential polyunsaturated fatty acids (EPUFAs).

Objective: To analyze whether a combined formulation of antioxidants and long-chain EPUFAs may improve the evolution of DEDs.

Methods: We used a prospective study to address the relationship between risk factors, clinical outcomes, and expression levels of inflammation and immune response (IIR) mediators in human reflex tear samples. Participants included: (1) patients diagnosed with nonsevere DEDs (DED group [DEDG]); and (2) healthy controls (control group [CG]). Participants were randomly assigned to homogeneous subgroups according to daily oral intake (+S) or not (–NS) of antioxidants and long-chain EPUFAs for 3 months. After an interview and a systematized ophthalmic examination, reflex tears were collected simultaneously from both eyes; samples were later subjected to a multiplexed particle-based flow cytometry assay. A specific set of IIR mediators was analyzed. All data were statistically processed through the SPSS 15.0 software program.

Results: Significantly higher expressions of interleukin (IL)-1β, IL6, and IL10 and significantly lower vascular endothelial growth factor expressions were found in the DEDG as compared to the CG. In the DEDG, significant negative correlations were detected between the Schirmer test and IL-1β, IL6, IL8, and vascular endothelial growth factor levels, and between the fluorescein breakup time with IL6 and IL8 levels. However, levels of IL-1β, IL6, and IL10 in tears were significantly lower in the DEDG+S versus the DEDG–NS and in the CG+S versus the CG–NS. Subjective symptoms of dry eye significantly improved in the DEDG+S versus the DEDG–NS.

Conclusion: IIR mediators showed different expression patterns in DED patients, and these patterns changed in response to a combined formulation of antioxidant and EPUFAs supplementation. Our findings may be considered for future protocols integrating clinical/biochemical data to help manage DED patients.

Keywords: tears, cytokines, essential polyunsaturated fatty acids, nutraceutics, aging, women

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Introduction

Dry eye is a complex condition involving the lacrimal glands, eyelids, and tear film, as well as a variety of ocular surface tissues including epithelial, inflammatory, immune,

and goblet cells.[1] From a pathogenic viewpoint, there are two major types of dry eye: the aqueous-deficient clinical form that is due to lacrimal gland dysfunction, and the evaporative dry eye that is mainly due to meibomian gland disorder.[2,3] However, it is common to find dry eye patients (independently of the etiology) displaying a mixture of both aqueous-deficient and evaporative clinical manifestations. Dry eyes usually affect people aged ≥ 65 years;[3] moreover, dry eyes affect women selectively, as emphasized by the Women's Eye Health Organization and other large studies,[4–8] with an estimation of about 3.23 million American women suffering from dry eyes. The term "dry eye disorder" (DED) has recently been introduced to better define the ocular surface dysfunction that leads to tear film impairment and dry eye.[9]

Objective tests for the clinical diagnosis of DEDs include the following: (1) the Schirmer test, with or without anesthesia, which determines tear production; (2) tear breakup time, with or without fluorescein, which reflects tear film stability; and (3) dye staining tests for evaluating the ocular surface tissue integrity, by using conjunctival lissamine green or corneal fluorescein staining.[1–3,9] Reflex tears are the functional response to an agent irritating to the eyes, including bright light, foreign particles, and irritant substances, as well as vomiting, coughing, and yawning maneuvres.[2,3,9] Reflex tears can be collected either by capillary tubes or by Schirmer strips without using local anesthetic. Basal tears can be collected by Schirmer strips with the use of local anesthetic. Two sampling methods have been used to assess biochemical traits of tear fluid composition in healthy and pathologic conditions: yawn collection and eye-flush collection.[10,11] The glass capillary micropipettes extracting method has been proposed as the best means for obtaining reflex tear samples for laboratory assays.[11]

Recent advances in biotechnology strengthen our understanding of DED pathophysiology. Among the newest diagnostic techniques are the Optical Quality Analysis System (Visiometrics®, Terrassa, Barcelona, Spain) with double-pass measurement of diffusion and light scattering, masks and goggles for managing meibomian gland dysfunctions, LipiView (TearScience®, Morrisville, NC, USA) for stimulating meibomian gland secretion, and the TearLab Osmolarity Test (Tear Lab Corporation, San Diego, CA, USA) for measuring tear osmolarity. In parallel to these advances, much laboratory research has been done to better manage DED patients.[12–15] However, additional prospective studies are needed to elucidate other risk factors and molecular and cellular pathogenic mechanisms, discover new pharmacologic agents, improve eye health, and preserve better vision and quality of life.

Reactive oxygen species are chemically reactive molecules containing oxygen.[16] When reactive oxygen species formation becomes uncontrolled or the body's antioxidant defense barrier fails, reactive oxygen species accumulate and trigger lipids, proteins, and nucleic acid damage, subsequently leading to cell disease and death. From a biochemical viewpoint, oxidative stress (OS) is the result of an imbalance between prooxidants and antioxidants. Strong evidence indicates that OS plays a significant role in a variety of ocular conditions. For example, a significant decrease in antioxidant defenses and a significant increase in prooxidants in aqueous humor, vitreous body, ocular tissues, and plasma have been reported in relation to primary open-angle glaucoma, cataracts, diabetic retinopathy, age-related macular degeneration, and DEDs.[17–21] The initial steps of OS and whether this condition contributes to DED development and evolution remain unclear.

The eyes are exposed to external and internal damaging agents. Inflammation and immune response (IIR) mechanisms attempt to rescue the body from cell, tissue, and organ injuries and their downstream effects. The IIR mediators involve leukocytes and other innate immunity cells, as well as T, B, and natural killer lymphocytes (as adaptive immunity), which interact via cytokines, chemokines, and other molecules.[22] Tissue damage results from uncontrolled acute and chronic inflammation. Alteration of a wide spectrum of IIR mediators measured in blood, aqueous humor, vitreous body, or eye tissues supports an abnormal activity of the immune system in both anterior and posterior eye segment disorders.[21,23–27] New biotechnology strategies, such as multiplexed flow cytometry assays, metabolomics, and immunoproteomic profiling analyses, may improve our understanding of the pathogenesis and progression dynamics of eye diseases.[28–31]

The essential polyunsaturated fatty acids (EPUFAs) omega-3 (ω-3) and omega-6 (ω-6) play pivotal functions and display a wide spectrum of positive effects in the body, such as: helping to lower cholesterol and triglyceride levels; giving energy to the body; helping to reduce acute and chronic inflammation; reducing respiratory and asthma-like symptoms; enhancing appropriate pre- and postnatal development mainly of the central and peripheral nervous systems; helping in the regulation of blood pressure; reducing the odds of developing cancer, heart disease, and stroke; managing emotional distress and depression; and benefiting patients with neurodegenerative disorders, among others.[32–38] The n-3 derived eicosanoids exert antiinflammatory actions, while the n-6 derived eicosanoids are proinflammatories.[32,33] Recent studies have shed some light on the regulation of

biochemical mechanisms of acute inflammation, which are performed in part by endogenous polyunsaturated fatty acid-derived autacoids, such as the series of specialized proresolving mediators, the lipoxins, resolvins, protectins, and maresins.[34] Based on these findings, EPUFAs are attractive molecules in eye and vision research,[35–38] including DED treatment.[39]

In the present study, we evaluated the relationship between oral supplementation of a combined formulation of antioxidants and EPUFAs on the improvement of the signs and symptoms of patients diagnosed with nonsevere DEDs, as compared to DED patients not taking these supplements and healthy controls.

Materials and methods

This prospective, open-label, randomized study was performed under the approval of the Institutional Review Boards of the University Hospital Dr. Peset (Valencia, Spain), as a nonsignificant risk investigational device study (Re: CEIC 59/10), and all tenets of the Declaration of Helsinki for the protection of human subjects in medical research were strictly observed.

Study design

A total of 66 subjects of both sexes, aged 23–80 years, were enrolled during ophthalmologic appointments at the study centers in Jerez de la Frontera, Cádiz, Spain and Valencia, Spain between March 2011 and June 2011 according to the main inclusion/exclusion criteria listed in Table 1.

Prior to the baseline visit, subjects were required to discontinue use of nutritional supplements, systemic antihistamines, and dry eye (or meibomian gland disorder) related treatments such as antibiotics, nonsteroidal and antiinflammatory drugs, corticosteroids, as well as tears with vitamins, for at least 15 days, and participants were asked to strictly follow the recommendations of the ophthalmologists throughout the duration of the study. Ocular lubricants without nutritional agents were not restricted. Patients with obvious infection or significant eyelid inflammation were excluded from the present study. Patients diagnosed with mild-to-moderate DEDs (DED group [DEDG]) were enrolled. To classify our study participants, a systematized ophthalmologic examination and a questionnaire, with scores including objective/subjective criteria, were performed. Other signs and symptoms of DEDs were evaluated based on the personal viewpoint of the patients, as well as their comments and suggestions. According to this, and depending on their clinical form, patients were considered to be either aqueous deficient, evaporative (lipid layer insufficiency), or a combined form of both aqueous deficient/evaporative types. However, all of these clinical forms were enclosed in the DEDG, as a whole, and were compared to the group of healthy subjects. Further evaluation of the structure and function of the upper lid meibomian glands, as recently reported by Lane et al,[40] was not addressed in this study, but may be the subject of further research regarding the role of essential fatty acids in DED patients.

All participants (132 eyes) were randomized to the following groups: (1) patients diagnosed with DEDs (DEDG; n = 30); and (2) healthy individuals that constituted the control group ([CG]; n = 36). Two homogeneous subgroups were selected according to the oral intake of a supplement prescribed as two capsules a day (+S) or not receiving the oral supplement (–NS). The supplement formulation as reflected in Table 2 (Brudysec 1.5 g; Brudy Laboratories, Barcelona,

Table 1 Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Aged 23–80 years	Aged < 23 years or >80 years
Diagnosed with mild-to-moderate DED (DEDG)	Athopy, allergic disorders
Healthy subjects (CG)	Wearing contact lenses
Able to participate in the study	History of refractive surgery
Informed consent	Ophthalmic laser treatment (less than 3 months)
	Systemic diseases and general treatments
	Eyelid anomalies, severe blepharitis or meibomitis, punctal occlusion
	Ocular disorders and eye drops other than artificial tears
	Not able to participate in the study

Abbreviations: DED, dry eye disorder; DEDG, dry eye disorder group; CG, control group.

Table 2 Composition of Brudysec 1.5® (Brudy Laboratories, Barcelona, Spain) formula per capsule

Nutrient	Amount
DHA	350 mg
EPA	42.5 mg
Vitamin A	133.3 μg
Vitamin C	26.7 mg
Vitamin E	4 mg
Tyrosine	10.8 mg
Cysteine	5.83 mg
Glutathione	2 mg
Zinc	1.6 mg
Copper	0.16 mg
Manganese	0.33 mg
Selenium	9.17 μg
DPA	30 mg

Abbreviations: DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid.

Spain) was based on ω-3 EPUFA, vitamins, glutathione, amino acids, and oligoelements, in a combined nutraceutical formulation with (per capsule): docosahexaenoic acid (350 mg), eicosapentaenoic acid (42.5 mg), docosapentaenoic acid (30 mg), vitamin A (133.3 μg), vitamin C (26.7 mg), vitamin E (4 mg), tyrosine (10.8 mg), cysteine (5.83 mg), glutathione (2 mg), zinc (1.6 mg), copper (0.16 mg), manganese (0.33 mg), and selenium (9.17 μg).

Given that compliance with long-term self-administered medication therapy is approximately 50% for those who remain in care,[41] ophthalmologists were instructed to pay special attention to measure levels of compliance for participants in the personal interview, mainly in this case of prescribing self-administered doses of antioxidants and EPUFAs, in order to help patients to improve their compliance and increase the benefit they may receive from these supplements, as well as to ensure the validity of the final data of the study. Therefore, subjects were followed each month after the initial visit during the 3 months of follow-up.

Patient management

A personal interview was performed with all participants regarding their personal and familial backgrounds and characteristics of their disease, mainly symptoms of dry eyes and subjective sensations. The Ocular Surface Disease Index (OSDI) questionnaire assessed the subject's frequency of dry eye symptoms and problems with their ocular surface.[42] The OSDI questionnaire sections are divided into the following questions:

1. Have you experienced photophobia, a gritty feeling, soreness, or blurred vision during the last week?
2. Have your eye problems limited you in reading, driving at night, computer work, or watching TV during the last week?
3. Have your eyes felt uncomfortable in windy or dry situations or because of air conditioning during the last week?[42]

The questionnaire paid special attention to the comments of the interviewed participants regarding lifestyle and eye conditions.

A systematized ophthalmologic examination was carried out on all participants as follows: best corrected visual acuity (BCVA) in each eye, value of the eyelid Schirmer test, slit lamp examination for the eye adnexa and anterior eye segment, tear breakup time with fluorescein, and corneal surface details with fluorescein. All ophthalmologists completed a full sheath to enclose all data, and were advised to strictly follow the study protocol. The primary outcomes of the ophthalmologic measures for effectiveness of the oral nutraceutical formulation were the Schirmer test and fluorescein tear breakup time; the secondary measure was dry eye symptoms.[43]

Tear sampling procedures

The expression of a set of cytokines/chemokines in reflex tear samples obtained by the gentle rubbing method was assayed by the Multiplex System (Luminex® R-200; Luminex Corporation, Austin, TX, USA). Polystyrene beads coupled covalently to specifically directed antibodies (human cytokine/chemokine panel) were allowed to react with 30–40 μL of each tear sample containing an unknown amount of cytokine, or with a standard solution containing a known amount of cytokine, at room temperature for 1 hour, following the manufacturer's instructions. The cytokines/chemokines that were analyzed using this detection method were the interleukins (IL)-1β, IL2, IL4, IL5, IL6, IL7, IL8, IL10, and IL12; tumor necrosis factor-alpha (TNF-α); vascular endothelial growth factor (VEGF); granulocyte-macrophage-colony stimulating factor (GM-CSF); and interferon-gamma (IF-γ).

Briefly, a series of washes were carried out to remove unbound proteins. Then, a biotinylated detection antibody specific for a different epitope on the cytokine was added to the beads and incubated at room temperature for 30 minutes. Streptavidin-phycoerythrin (which binds to the biotinylated detection antibodies), was used to detect the reaction mixture. The flow-based Bio-Plex® (Bio-Rad Laboratories, Hercules, CA, USA) suspension array system was used to identify and quantify each antigen–antibody reaction. Identification of the assayed molecules was based on bead color and fluorescence, using fluorescently labeled reporter molecules associated with each target protein. Unknown cytokine/chemokine concentrations were calculated automatically by the Bio-Plex® Manager software (Bio-Rad Laboratories) using a standard curve derived from a recombinant cytokine standard. Cytokine/chemokine levels were corrected for the initial total protein concentration of each human tear sample during analysis.

Data are presented as the mean ± standard deviation for two or three determinations and expressed in picograms per milliliter per milligram.

Statistical analysis

Demographic, clinical, and biochemical data were recorded into a previously designed Excel spreadsheet (Microsoft Corporation, Redmond, WA, USA). A nonparametric Mann–Whitney *U*-test was selected for comparing two independent

sample groups by means of Statistical Package for the Social Sciences (SPSS) software (v15.0; IBM Corporation, Armonk, NY, USA). A value of $P < 0.05$ was considered to indicate a statistically significant difference between groups.

Results

The median age of the participants was 52 ± 15 years (DEDG) versus 50 ± 12 years (CG); 59% of the DEDG and 58% of the CG were older than 45 years. Regarding gender, men and women accounted for 28% and 72% of the DEDG versus 32% and 68% of the CG, respectively.

Our sample of mild-to-moderate DED patients had a history of dry eyes for more than 1 year. All of them reported one or more of the following symptoms: ocular irritation, soreness, burning, foreign body sensation, dryness, photophobia, and/or blurred vision. The DED patients had known dry eyes before starting our study, and most of them (89%) used eye drops regularly for treating their tear film alterations. None of the subjects of the DEDG suffered severe dryness or Sjögren's syndrome. By using the OSDI for measuring dry eye symptoms and severity in our study participants, and by taking into consideration all comments and suggestions from all of them, we found that scores significantly worsened when comparing healthy controls with dry eye patients ($P = 0.0015$).

All participants were examined under a slit lamp in relation to their anterior eye segment and media, and the DEDG displayed ocular surface disorder morphological alterations such as marginal blepharitis and stinging of the cornea. The interviews with the healthy subjects, the OSDI data, and the biomicroscopy examination of the anterior eye segment did not reveal any DED signs or symptoms in the CG.

The Schirmer test scores (wetting of the paper after 5 minutes) were significantly lower in the DEDG (4.26 ± 0.59 mm) than in the CG (13.25 ± 2.46 mm; $P = 0.0002$), reflecting the altered tear secretion in this mild-to-moderate group of DED patients. Regarding the age of the DEDG of our study participants, those younger than 45 years normally moistened 8–11 mm of each paper strip, while those older than 45 years usually wet about 5–8 mm in 5 minutes.

The fluorescein tear film breakup time was much shorter in the DEDG patients (4.35 ± 1.23 seconds) than that in the healthy group (14.24 ± 3.22 seconds), which reflects the statistically significant altered tear film stability in mild-to-moderate DED individuals as compared to the controls ($P = 0.0001$).

An overall amelioration in the signs and symptoms was observed in the DEDG+S as compared to the DEDG–NS groups at 3 months. Surprisingly, the CG+S group also reported an improvement of 50% or greater in ocular signs such as visual fatigue, heaviness, and improved eyelashes, nails, hair, and skin.

Neither the DEDG nor the CG showed significant differences in BCVA and intraocular pressure from baseline to 3 months, and no changes in these parameters were noted in the oral supplementation subgroups from pre- to postsupplementation. The mean changes in BCVA and intraocular pressure were less than 0.1 and 1 mmHg, respectively.

The gentle collection method of reflex human tears by means of the capillary tube was a noninvasive, useful, and a relatively easy procedure to achieve the main objectives of the present study. The responses were calculated by subtracting background cytokine concentrations from the cytokine concentrations in the tears. The standard curves for both the kit assay and the extraction buffers were similar for the 12 analyzed molecules. The amount of tear samples obtained from the participants in the study permitted detection in up to 90% of the sampling procedures. The set of assayed molecules showed a wide variety of expression levels in tear samples, with acceptable precision, as measured by the Luminex multianalyte profiling bioassay system (Luminex Corporation).

When comparing the main groups, significantly higher expressions of IL-1β ($P = 0.015$), IL6 ($P = 0.0001$), and IL10 ($P = 0.050$), and significantly lower VEGF expression ($P < 0.050$) were found in the DEDG as compared to the CG. In the DEDG, significant negative correlations were detected between the Schirmer test score and IL-1β ($P < 0.01$), IL6 ($P < 0.001$), and IL8 ($P < 0.05$) levels, and between the fluorescein breakup time and IL6 ($P < 0.001$) and IL8 ($P < 0.5$) levels. However, tear levels of IL-1β, IL6, and IL10 were significantly lower in DEDG+S versus DEDG–NS ($P = 0.05$), and in CG+S and CG–NS ($P = 0.001$; $P = 0.01$, respectively).

Subjective symptoms of DED significantly improved in the DEDG+S versus DEDG-NS group, as reflected in Figure 1. A significantly positive correlation was detected between cytokine/chemokine tear expression levels and aging when the participants were subclassified by age. In particular, the expression levels of IL4, IL10, IL6, IL12, IFγ, and GM-CSF were correlated with age in the DEDG patients 45 years of age or older (Figure 2). In this context, the inflammation/immune response mediators involved in tears from DED patients in relation to aging is shown in Table 3.

We found no significant correlation between cytokine/chemokine levels and gender in our study participants.

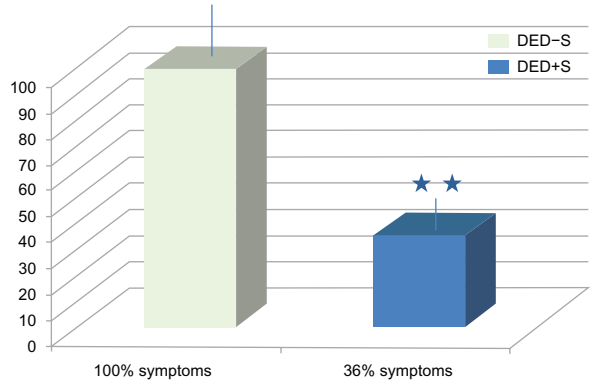


Figure 1 Remaining subjective symptoms of DEDs in DED–S or DED+S patients.
Note: $^{**}P < 0.001$.
Abbreviations: DED, dry eye disorder; DED–S, dry eye disorder without antioxidants/omega-3 fatty acid oral supplements; DED+S, dry eye disorder with antioxidants/omega-3 fatty acid oral supplements.

Discussion

DEDs are among the most common conditions observed in ophthalmic appointments, and are the ocular status with the highest prevalence rates worldwide. Among the hallmarks of DEDs are ocular discomfort and loss of vision, and there are derived complications for reading, cooking, working with computers, driving, and using makeup and eye cosmetics among the affected individuals.[1–3] The main goal of this work was to analyze possible changes in the expression of IIR mediators in tears of nonsevere DED patients (those pertaining to the three clinical types: the aqueous deficient, evaporative [lipid layer insufficiency], or combined form of both aqueous deficient/evaporative [lipid layer anomalies]) as compared to healthy controls, by using a new tool that is able to simultaneously measure various molecules in a single microplate well (the Luminex multianalyte profiling assay system).

A wide variety of cytokines and chemokines are present in human tears for maintaining the morphology and function of the ocular surface.[44–46] In the present study, tear samples from the DEDG (constituted by the aqueous deficient clinical form), evaporative (lipid layer insufficiency), or a combined type of both aqueous deficient/abnormal lipid layer clinical DED forms displayed significantly increased levels of IL-1β, IL6, and IL10 and decreased VEGF with respect to the CG. When comparing our results on the cytokine/chemokine expression in tears with other studies that consider different subtypes of dry eye, such as the one by Boehm et al,[47] several concordances exist, such as the significantly higher expression of IL-1β in the DEDG as compared to the CG. Brignole-Baudouin et al[48] reported that the destructive cell-signaling chemicals IL6, TNF-α, and VEGF were higher in DED patients. The proinflammatory cytokines, IL6 and TNF-α, have also been detected in tears and in the conjunctival epithelium of individuals with DEDs.[43–48] Enríquez-de-Salamanca et al[45] reported that IL6 was detected in only 65% of the tear samples from patients with mild-to-moderate DEDs, as collected by Schirmer strips, and that those concentrations did not differ from controls. In the

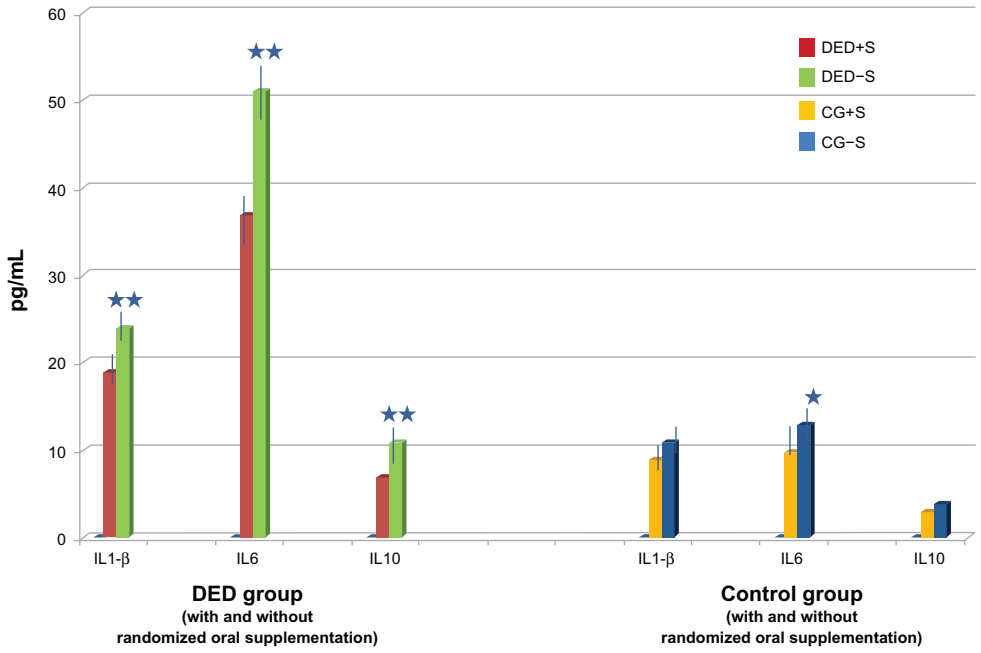
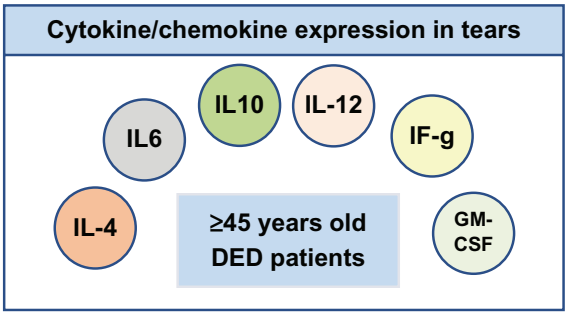


Figure 2 Subjective DED symptoms after supplementation with a combined formulation mainly containing antioxidants and docosahexaenoic acid, and its effects on human tear IL expression levels.
Note: $^{*}P < 0.01$; $^{**}P < 0.001$.
Abbreviations: DED, dry eye disorder(s); IL, interleukin.

Table 3 Cytokine/chemokine expression in tears



Abbreviations: IL, interleukin; DED, dry eye disorder; IF-γ, interferon-gamma; GM-CSF, granulocyte macrophage-colony stimulating factor.

same study, the authors emphasized that IL-1β and TNF-α were detected in 30% and 2% of their study patients, respectively.[45] With the gentle rubbing collection method used here, we detected IL-1β in 81.89% of the DEDG, IL6 in 73.77%, IL10 in 59.01%, and VEGF in 93.44%.

In our study sample, patients with mild-to-moderate DEDs showed positive results in the clinical tests and symptoms. It is possible that an underlying cytokine/chemokine-mediated inflammatory disorder is common to all ocular surface alterations, although the etiology of any DED in our patients is highly variable. The most dangerous proinflammatory cytokines and chemokines are TNF-α, IL-1β, IL6, and IL8, and among these IL-1β and IL6 levels were increased in the DEDG of our study participants, reflecting an inflammatory profile in the ocular surface of these patients.

Interesting data observed included the significantly lower VEGF expression ($P < 0.050$) found in our DEDG as compared to the CG. The expression of VEGF is tightly regulated. In fact, a wide range of signals also stimulate VEGF transcription, such as (inflammatory) cytokines and oncoproteins, including IL-1β, IL6, insulin-like growth factor (ILGF)-1, TGF-β1, c-Src, v-Raf, and Ras, among others. VEGF is the archetypical example of the cross-talk between nerves and vessels. Its role in conjunctival biopsies and tears in ocular chronic inflammatory diseases, such as vernal keratoconjunctivitis or atopic keratoconjunctivitis, has been recently revisited.[49] Moreover, it has been suggested that, in addition to its angiogenic actions, VEGF can also act as a direct proinflammatory mediator during the pathogenesis of rheumatoid arthritis.[50] Enríquez-de-Salamanca et al[45] stated that VEGF levels were increased in tear samples from mild-to-moderate DED patients. VEGF is the prototypic example of the crosstalk between nerves and vessels. The observation that reduced levels of VEGF are causally involved in neurodegeneration might have implications in anterior and posterior eye segment disorders.[51] Eye pain and soreness is a common symptom in dry eyes. It is likely that our patients suffered from a mild neurotrophic epitheliopathy that caused a reduced expression of the VEGF, as reflected in the tear analyses. This finding requires further research to elucidate the role of VEGF in dry eye syndrome.

The EPUFAs (ω-3 and ω-6) have to be consumed daily in order to cover all of their functions in an organism. Recent evidence has demonstrated that the intake of ω-3 fatty acids and the ratio of consumption with ω-6 fatty acids influences the expression of global inflammatory markers in humans.[52] These findings suggest a presumptive protective function of ω-3 supplementation in managing DED patients.[53–55] Therefore, we analyzed whether a combined formulation of antioxidants and long-chain EPUFAs may influence the evolution of DEDs. We found that oral supplementation with a combined formulation of antioxidants and EPUFAs significantly changed the expression pattern of cytokines/chemokines in tears from DED patients when compared with DED patients not taking the supplements. These results are in agreement with those of Roncone et al[53] and Brignole-Baudouin et al.[48] Furthermore, Cortina and Bazan[54] proposed that the eicosapentaenoic acid and docosahexaenoic acid derivatives, particularly resolvin E1 (RvE1) and neuroprotectin D1, appear to be responsible for the antiinflammatory effects of both EPUFAs. This was supported by a study from the same research group,[55] in which topical RvE1 resulted in decreased inflammation in a mouse dry eye model; RvE1 promoted tear production, corneal epithelial integrity, and decreased inflammatory inducible COX-2, suggesting that RvE1 and similar resolvin analogs have therapeutic potential in the treatment of DEDs.

Currently, the diagnosis of DEDs is still a challenging task due to the difficulties of identifying risk factors that may contribute to obscuring the diagnosis; in fact, this point requires further research. Moreover, the few available standardized clinical protocols for DED diagnosis, such as the Schirmer test, the fluorescein tear film breakup time, and the ocular surface staining probes, together with insufficient knowledge about the pathologic mechanisms of DEDs and the coexistence of other local and systemic conditions, contribute to the present situation and the inability to cure and eliminate the disease.

Differential expression of IIR mediators has also been detected in human tears in relation to aging. In this study, we confirmed a positive correlation of cytokine/chemokine tear expression with the aging process. Particularly, the expression levels of IL4, IL6, IL10, IL12, IFγ, and GM-CSF were correlated with age in members of the DEDG who were 45 years or older, as shown in Table 3. This relationship

should be considered when managing patients in this age range.[56] In spite of the fact that the main purpose of the present study was to compare the expression patterns of specific immune mediator molecules in the tears of DED patients, we detected significant changes in our older DED patients in respect to the healthy controls based on age, thus confirming previous findings from our group and others that age is the most common risk factor for dry eyes.[57–59] Taking this into consideration, dry eye has to be considered a normal part of aging. When specifically focusing on the topic of sex disparities in DEDs, we found no significant correlation between cytokine/chemokine levels and gender among our study participants.

It is interesting to consider that cytokines such as IL-1β and IL6, which act by inducing an inflammatory response (while others act on specific cell types in the immune response), were significantly augmented in tears from our sample of DED patients. IL6 has been described as one of the key molecules in DEDs.[44,60] This molecule was identified in the tears and conjunctival epithelium of DED patients, and its presence was correlated with some clinical parameters in patients with severe DED forms. Furthermore, the clinical and biochemical parameters related to DEDs, as well as the symptoms and subjective sensations of the patients were significantly reduced in members of the DEDG+S subgroup. Thus, our findings established that lower IIR mediators in tear levels were correlated with increased quality of life, this being an interesting point to appraise. Together, our findings suggest that oral supplementation with a combination of antioxidants and EPUFAs fully benefit DED patients.

Our findings also suggest that IL-1β and IL6 expression in tears can be used as presumptive biomarkers of DEDs and dry eye related-quality of life. Topical IL-1β and IL6 modulators may be a new therapeutic approach to DEDs. This topic offers ophthalmologists and researchers a great opportunity for future investigations. Larger trials with a greater number of participants may help to implement specific guidelines for the use of EPUFAs in DED patients.

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Disclosure

The authors report no conflicts of interest in this work.

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Clinical Interventions in Aging

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ORIGINAL RESEARCH

Patients undergoing long-term treatment with antihypertensive eye drops responded positively with respect to their ocular surface disorder to oral supplementation with antioxidants and essential fatty acids

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Background: Glaucoma and dry eye disorders (DEDs) are frequent comorbidities. The antioxidant and anti-inflammatory properties of essential polyunsaturated fatty acids have been extensively studied in relation to eye diseases.

Objective: Our objective was to determine the effects of oral supplementation with a combined formulation of antioxidants and essential polyunsaturated fatty acids on expression of cytokines and chemokines in tears from patients with DEDs or primary open-angle glaucoma (POAG).

Methods: Participants (n = 97) were distributed into three groups: (1) individuals with nonsevere DEDs (DEDG), (2) individuals with nonadvanced POAG (POAGG), and (3) healthy controls. These groups were randomized into two subgroups: one received a daily antioxidant and essential polyunsaturated fatty acid supplement (two pills) for 3 months (+S), and the other did not (–NS). Participants were interviewed and ophthalmologically examined. Concentrations of specific cytokines and chemokines in reflex tears were determined by multiplexed particle-based flow cytometry. The data were analyzed statistically (SPSS version 15.0).

Results: Comparison of the results from the DEDG and POAGG patients showed significant differences in tear expression of granulocyte-macrophage colony-stimulating factor ($P = 0.008$), tumor necrosis factor α ($P = 0.005$), vascular endothelial growth factor ($P = 0.038$), interleukin-4 ($P = 0.030$), and interleukin-6 ($P = 0.044$). The main signs and symptoms of dry eyes such as dryness, burning, photophobia, eye heaviness, and blurred vision, as well as positive changes in eyelashes, hair, nails and skin, were significantly improved in DEDG +S and POAGG +S patients relative to unsupplemented patients.

Conclusion: Inflammation biomarkers were differentially expressed in glaucomatous tears, but the differences changed upon antioxidant/essential polyunsaturated fatty acid supplementation. Chronic instillation of antihypertensive eye drops must be considered for integrating protocols to glaucoma standards of care.

Keywords: glaucoma, dry eye disorders, tears, cytokines, antioxidants, essential fatty acids

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Introduction

Owing to advances in disease prevention, detection, and treatment, life expectancy[1] has increased in most countries. From 2005 to 2010, the worldwide average life expectancy at birth was 67.88 years (70.14 years for females and 65.71 years for males).[2] As a result, biomedical research on the diseases of aging is especially relevant. Age is an important risk factor for ocular diseases such as glaucoma, dry eye disorders (DEDs), cataracts,

and age-related macular degeneration, and in elderly people, such diseases can occur together, significantly impairing visual acuity and thus reducing quality of life.

DEDs are complex pathological conditions involving the eyelids, lacrimal glands, tear film, and ocular surface tissues.[3] There are two major forms of DEDs – the deficient aqueous tear production form (due to lacrimal gland dysfunction) and the increased evaporative loss form (due to meibomian gland disorder) – but combinations of the two forms are usually seen in clinical practice.[4,5] DEDs are also classified according to severity, ranging from normal to mild-to-moderate to severe. Dry eyes usually affect people aged above 60 years old. Significant reduction in body water associated with aging may play a role in the onset or progression of DEDs. Meibomian gland dropout, reduced goblet cells, and laxity of the eyelids may also be contributing factors. In addition, diverse pathologic manifestations on the ocular surface may be triggered by external or internal injury, as well as by menopause, topical or systemic medications, light, computer use, environmental pollutants, and air conditioning.[3–5]

Glaucoma is a group of diseases characterized by increased intraocular pressure as the main risk factor, leading to progressive visual field defects and visual impairment secondary to loss of retinal ganglion cells and optic nerve fibers.[6] People with a glaucomatous family history, diabetes, hypertension, or myopia; African-Americans, Hispanics, and Asians; and people over forty years are at increased risk of glaucoma. Among the various types of glaucoma, the most prevalent is primary open-angle glaucoma (POAG), which accounts for almost 80% of all glaucomas.[7,8] The prevalence of POAG has been estimated as 1.1%–3.0% of Western populations aged 40 years or more. Glaucoma remains the second leading cause of blindness worldwide.[8]

Therefore, both glaucoma and DEDs are highly correlated with age. It is not atypical to see patients chronically treated with antihypertensive eye drops who also exhibit the clinical DED manifestations, from a lesser to a greater degree of severity categories. Recent studies have indicated that 66% of patients with severe DEDs also have glaucoma,[9] and approximately 60% of glaucomatous individuals undergoing topical antiglaucoma therapy report DED symptoms.[10] However, most cases of glaucoma and DED comorbidity remain undiagnosed, or misclassified as chronic eye irritation, and this is the most important point to be addressed in the present work.

Oxidative stress (OS) plays a significant role in a wide spectrum of ocular conditions.[11] OS, an imbalance between pro-oxidant and antioxidant species, can result from the accumulation or uncontrolled generation of reactive oxygen species, partially reduced byproducts of molecular oxygen,[11] which may trigger damage to lipids, proteins, and nucleic acids, resulting in cell lesions and death. A significant increase in oxidative activity and a decrease in antioxidant defenses in ocular fluids and tissues as well as in plasma samples have been associated with DEDs,[5] POAG,[12,13] cataracts,[14] diabetic retinopathy,[15,16] and age-related macular degeneration.[17] However, the pathogenesis of OS and the contribution of this condition to the initial stages or progression of DEDs in patients with POAG remain unclear.

The eyes are continuously exposed to environmental irritants, which stimulate inflammation and immune response (IIR) processes intended to prevent and heal external and internal injuries. The main mediators of IIR processes include leukocytes and other cells involved in the innate immunity system, as well as T, B, and natural killer (NK) lymphocytes (the major components of the adaptive immune system); and the interactions between these cells are modulated by cytokines, chemokines and other molecules.[18] Uncontrolled acute or chronic inflammation can lead to tissue damage. For example, altered levels of a wide spectrum of IIR mediators in blood, the aqueous humor, the vitreous body, and eye tissues support the idea that abnormal activity of the immune system is involved in both anterior and posterior ocular segment disorders.[19–22] Moreover, altered immune response regulation may shift the physiological equilibrium over a chronic-cumulative period, leading to a low-grade inflammatory degenerative process, known as para-inflammation, with the oxidative stress acting as a local trigger for retinal para-inflammatory responses.[23]

Advances in biotechnology, such as multiplexed flow cytometry assays, may improve our understanding of the pathogenesis and progression of eye diseases.[24,25]

Antioxidants (AOXs) and anti-inflammatory compounds, such as essential polyunsaturated fatty acids (EPUFAs), may have potential for the treatment of eye diseases. EPUFAs, such as omega-3 (ω-3) and omega-6 (ω-6) fatty acids have important effects in the body. Omega fatty acids provide energy and perform important functions in the body; enhance appropriate prenatal and postnatal development (mainly of the central and peripheral nervous systems), lower cholesterol and triglyceride levels, reduce acute and chronic inflammation, help in the management of emotional distress and depression, benefit patients with neurodegenerative disorders, reduce respiratory and asthma-like symptoms, regulate the blood pressure, and reduce the odds of developing cancer, heart disease, and stroke.[26–32] The ω-3-derived eicosanoids exert anti-inflammatory effects, whereas the ω-6-derived

eicosanoids are pro-inflammatory.[28,30] The regulation of the biochemical mechanisms of acute inflammation, which is at least in part performed by endogenous EPUFA-derived autacoids (including pro-resolving mediators such as lipoxins, resolvins, protectins, and maresins), has recently been the subject of diverse studies.[31–38] EPUFAs are expected to be useful molecules for studies of ocular health and disease, including DEDs and POAG.

In this study, our main goals were the following: (1) to assess the expression of IIR molecules in tears of patients diagnosed with DEDs or POAG and (2) to evaluate the effect of oral supplementation with a combined formulation of AOXs and EPUFAs on the signs and symptoms of dry eyes in patients diagnosed with nonsevere DEDs or POAG, by comparing the supplemented DED or POAG groups with the corresponding unsupplemented groups, as well as with healthy controls.

Materials and methods

This prospective, open-label, randomized study was approved by the Institutional Review Board of the University Hospital (Valencia, Spain), as a nonsignificant risk investigational device study (Ethics Committee approved reference 59/10), and all tenets of the Declaration of Helsinki for the protection of human subjects in medical research were strictly observed.

Study design

Using the main inclusion and exclusion criteria (Table 1), we enrolled a total of 97 participants of both sexes, aged 25–80 years, during ophthalmologic appointments at the study centers University and Polytechnic Hospital La Fe (Valencia, Spain), Ophthalmic Research Unit Santiago Grisolia (Valencia, Spain), and Hospital of Jerez (Jerez de la Frontera, Cadiz, Spain), between March 2012 and November 2012.

Table 1 Main inclusion and exclusion criteria among the study participants

Inclusion criteria	Exclusion criteria
Aged 25–80 years	Aged <20 years, or >80 years
Diagnosed of mild-to-moderate DED (DEDG)	Athopy, allergic disorders
Diagnosed of nonsevere POAG (POAGG)	Wearing contact lenses
Healthy subjects (CG)	History of refractive surgery
To be able to participate in the study	Ophthalmic laser treatment (less than 3 months)
Informed consent	Systemic diseases and General Treatments
	Eyelid anomalies, severe blepharitis or meibomitis, punctal occlusion
	Ocular disorders and eyedrops other than artificial tears
	Not able to participate in the study

Abbreviations: DED, dry eye disorder; POAG, primary open-angle glaucoma; CG, control group; G, group.

Prior to the baseline visit, participants were required to discontinue, for at least 1 month, use of nutritional supplements, systemic antihistamines, and treatments related to dry eyes (or meibomian gland disorder) such as antibiotics, nonsteroidal and anti-inflammatory drugs, and corticosteroids, as well as artificial tears containing vitamins. In addition, participants were asked to strictly follow the recommendations of the ophthalmologists throughout the duration of study. Ocular lubricants without nutraceuticals were permitted, and their use or nonuse was recorded. Antihypertensive eye drops were also allowed and their use or nonuse was also recorded. Patients with obvious infection or significant eyelid inflammation were excluded from the study, as were patients with advanced glaucoma and patients with a previous diagnosis of coexisting POAG and DEDs. We wanted to analyze the ocular surface characteristics and the expression of IIR molecules in tears for two specific groups of patients: those with a diagnosis of a nonsevere DED and those with a diagnosis of nonadvanced POAG.

Suitable participants (97 participants, 194 eyes) were assigned to one of the following groups: (1) patients diagnosed with nonsevere DEDs (DEDG; n = 30), (2) patients diagnosed of nonadvanced POAG (POAGG; n = 31), and (3) a control group of healthy participants (CG; n = 36). Two homogeneous subgroups were selected: one group was prescribed an oral supplement containing AOXs and EPUFAs (AOX/EPUFA, two capsules per day, +S) and the other received no supplement (–NS). The oral supplement was Brudysec 1.5® (Brudy Laboratories, Barcelona, Spain), each capsule of which contains the following components in a combined nutraceutical formulation: docosahexaenoic acid (350 mg), eicosapentaenoic acid (42.5 mg), docosapentaenoic acid (30 mg), vitamin A (133 μg), vitamin C (26.7 mg), vitamin E (4 mg), tyrosine (10.8 mg), cysteine (5.83 mg), glutathione (2 mg), zinc (1.6 mg), copper (0.16 mg), manganese (0.33 mg), selenium (9.17 μg).

Ophthalmologists were instructed to emphasize, during an initial personal interview with each participant, compliance with the oral supplementation regimen, to maximize the effectiveness of the supplements and, in turn, the reliability of the study data. After the initial visit, all participants were followed every 4 weeks for a total follow-up period of 3 months.

Participant management

At the beginning of the study, all participants underwent an interview during which information about their personal and familial background and the characteristics of their eye disease (DED or POAG) was collected; emphasis was placed on the signs and symptoms of dry eyes and the participants' subjective sensations. For each group of participants, the effectiveness of AOX/EPUFA supplementation was evaluated in terms of clinical and molecular changes from baseline to 3 months.

A systematized ophthalmologic examination was conducted on all participants: the examination included measurement of the best corrected visual acuity in each eye, the Schirmer's test, slit lamp examination of the eye adnexa and anterior eye segment, measurement of tear breakup time (BUT) with fluorescein, and examination of corneal surface details with fluorescein. All of the ophthalmologists were instructed to fill a specifically designed a fullsheath to enclose all data and were advised to strictly follow the study protocol. The primary outcome ophthalmologic measures of the effectiveness of the oral nutraceutical formulation were the Schirmer's test and fluorescein tear BUT, and the secondary outcome measures were dry eye symptoms and subjective sensations.

Tear sampling

We used the gentle rubbing method to obtain reflex tears from all participants, and we assayed the expression of a specific set of cytokines and chemokines in the tear samples by means of the Luminex® R-200 multiplex system (Luminex, Austin, TX, USA). Polystyrene beads coupled covalently to specifically directed antibodies (human cytokine/chemokine panel) were allowed to react with 30–40 μL of each tear sample containing unknown amounts of cytokines and chemokines, or with a standard solution containing known amounts of cytokines and chemokines, at room temperature for 1 hour, as previously described.[39] The specific set of IIR molecules that were determined by means of this assay, as standardized by the manufacturers, were interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, and IL-12; tumor necrosis factor alpha (TNF-α); vascular endothelial growth factor (VEGF); granulocyte-macrophage colony-stimulating factor (GM-CSF); and interferon gamma. Briefly, a series of washes were carried out to remove unbound protein. Then a biotinylated detection antibody specific for a different epitope on the cytokine was added to the beads and incubated at room temperature for 30 minutes. Streptavidin-phycoerythrin, which binds to the biotinylated detection antibodies, was used to detect the reaction mixture. We used a flow-based Bio-Plex® suspension array system (Bio-Rad Laboratories, Hercules, CA, USA) to identify and quantify each particular antigen–antibody reaction. Identification of the assayed molecules was based on bead color and fluorescence, using fluorescently labeled reporter molecules associated with each target protein. Unknown cytokine and chemokine concentrations were calculated automatically by the Bio-Plex Manager software using a standard curve derived from a recombinant cytokine standard. Cytokine and chemokine concentrations were corrected for the initial total protein concentration of each human tear sample during analysis.

All the results are shown as means ± standard deviations for two or three determinations and are expressed in picograms per milliliters per milligram.

Statistical procedures

Demographic, clinical, and biochemical data were recorded in a previously designed Excel sheet (Microsoft Corporation, Redmond, WA, USA). A nonparametric Mann–Whitney *U*-test was selected for comparing two independent sample groups by means of the SPSS software (version 15.0, SPSS Inc, Chicago, IL, USA). All results were statistically analyzed to detect differences between groups. For our purposes, $P < 0.05$ was established as statistically significant.

Results

The median ages of the participant groups were 52 ± 15 years (DEDG), 64 ± 17 years (POAGG), and 50 ± 12 years (CG). When the participants were aged over 40 years (pivotal risk factor for both the DEDs and POAG) the percentages attending each group were 64% (DEDG), 67% (POAGG), and 60% (CG). Regarding sex, the percentages of men and women in all groups were 28% and 72% (DEDG), 29% and 71% (POAGG), and 32% and 68% (CG) respectively. Patient characteristics and risk factors for DEDs are reflected in the Table 2.

All the DEDG patients reported a history of dry eyes before the start of the study; they suffered from at least one of the following signs and symptoms: scratchy sensation, soreness, itchiness, grittiness, foreign body sensation, dryness, burning, photophobia, eye fatigue and/or blurred vision, and redness. Most of the DEDG patients (89%) used eye drops regularly for treating their ocular surface alteration. None of the DEDG patients suffered from severe dryness or Sjögren's syndrome.

None of the POAGG patients had a previous diagnosis of a DED or used tear substitutes. Of the POAGG patients who

Table 2 Patient characteristics

	Duration years	MENOP %	DM %	HBP %	CVD %	SEDENT %	SMKING %	DRNKING %	AIR C %	POLLT %	PCU %
POAGG	3 ± 3	40	28	33	14	40	9	19	29	29	28
DEDG	3 + 1	56	19	26	11	33	15	11	89	85	86
CG	–	43	–	3	3	24	15	11	85	80	23

Abbreviations: AIR C, air conditioning exposure; CG, control group; CVD, cardiovascular disorders; DEDG, dry eye disorder group; DM, Diabetes Mellitus; DRNKING, drinking habits; HBP, high blood pressure; MENOP, menopause; PCU, personal computer use; POAGG, primary open-angle glaucoma group; POLLT, pollutants exposure; SEDENT, sedentary lifestyle; SMKING, smoking habits.

were under treatment with antihypertensive drops treatment, 52% reported at least one of the dry eye signs and symptoms listed above for the DEDG patients. The POAGG patients mainly presented with a burning, dryness, photophobia, and/or conjunctival hyperemia, usually in both eyes.

The three groups of participants were examined under a slit lamp in relation to their anterior eye segment, and almost every DEDG patient displayed ocular surface disorder morphological alterations such as marginal blepharitis and stinging of the cornea. Most of the POAGG patients showed DED-related morphological changes (38%). However, neither the interviews nor the ocular surface examination revealed significant DED-related manifestations in the healthy participants.

The Schirmer's test scores were significantly lower in the DEDG (4.26 ± 0.59 mm) than in the POAGG (7.82 ± 1.92 mm) or the CG (13.25 ± 2.46 mm; $P = 0.0002$), reflecting the altered tear secretion in both the DEDG and POAGG patients. DEDG and POAGG patients who were younger than 40 normally moistened 7–12 mm of each paper strip with 5 minutes of contacting the paper strip, whereas patients older than 40 usually moistened about 4–9 mm of each strip.

The fluorescein tear BUT was much shorter in the DEDG patients (4.35 ± 1.23 seconds) than in the POAGG and CG patients (6.14 ± 2.32 seconds and 14.24 ± 3.22 seconds, respectively), which strongly reflects the altered tear film stability in the DEDG and the POAGG patients as compared to that in the CG patients ($P = 0.0001$).

None of the three groups showed significant differences in best corrected visual acuity from baseline to 3 months, and no changes in this parameter was also noticed in the oral supplementation subgroups from pre- to postsupplementation.

Results from the tear-sampling procedure were calculated by subtracting background cytokine and chemokine concentrations from cytokine concentrations in the tear samples. The standard curves for both the kit assay and extraction buffers were similar for the 12 analyzed molecules. The set of assayed molecules showed a wide variety of expression levels in the tear samples, and the precision of the values as measured by the Luminex multianalyte profiling bioassay system was acceptable. The amounts of tear samples obtained from the study participants permitted detection in up to 92% of the samples.

Noticeable increases in the collected amount of tears (30% or more) were observed in the DEDG and POAGG subgroups taking the AOX/EPUFA supplement with respect to the amounts in the same groups not taking the supplement.

Comparison of the results from the DEDG and POAGG patients showed significant differences in tear levels of GM-CSF ($P = 0.008$), TNF-α ($P = 0.005$), VEGF ($P = 0.038$), IL-4 ($P = 0.030$), and IL-6 ($P = 0.044$). These results are shown in Figure 1.

Comparison of the data for the POAGG and CG patients demonstrated significant differences in the tear levels of IL-6 ($P = 0.014$) (Figure 2).

The POAGG +S patients showed a significant reduction in the tear concentrations of IL-6 ($P = 0.44$) and TNF-α ($P = 0.00001$) compared to the POAGG –NS patients (Figure 3).

The main signs and symptoms of dry eyes were significantly improved in the DEDG +S and POAGG +S patients compared to the corresponding patients who did not receive the supplement. At 3 months, an overall improvement in both the objective and the subjective manifestations was observed in the DEDG +S patients as compared to the DEDG –NS patients, and in the POAGG +S patients as compared to the POAGG –NS patients. Patients reported amelioration of at least 68% of ocular signs such as dryness, burning, photophobia, eye heaviness, and blurred vision, as well as noticeable improvements in eyelashes, hair, nails and skin.

Discussion

Dry eyes are usually due to environmental irritants and topical or systemic medications.[3–5,39,40] Because most patients with glaucoma are not aware of suffering any type of ocular surface disorder, special attention has to be paid to patients with chronic glaucoma who may develop DED signs and

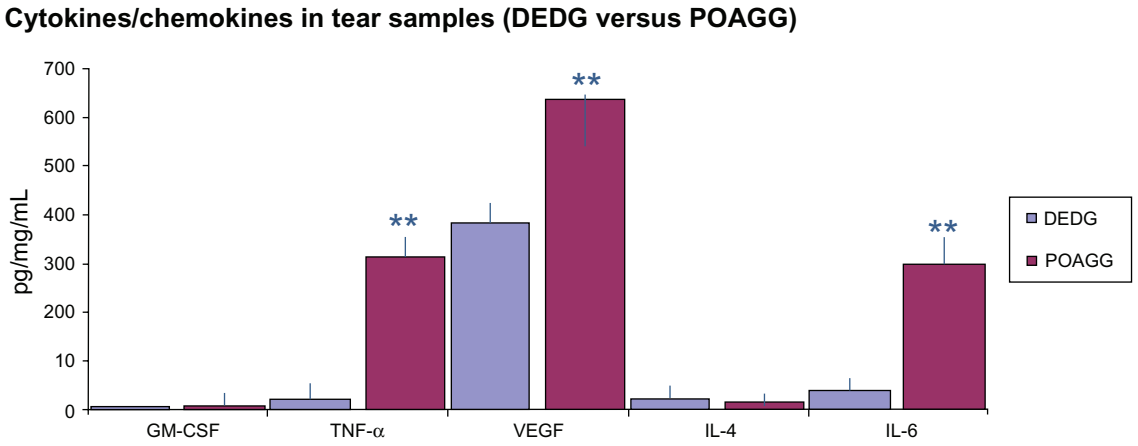

Figure 1 Comparison of the expression of cytokines/chemokines in tears from the DEDG and POAGG patients.
Notes: Data are mean ± standard deviation for three assays. **Indicates a *P* value < 0.001.
Abbreviations: DEDG, dry eye disorder group; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-4, interleukin-4; IL-6, interleukin-6; POAGG, primary open-angle glaucoma group; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.

symptoms during the course of their treatment. The main goal of this study was to determine whether there were any differences in tear expression of IIR mediators in patients with nonsevere DEDs and patients with POAG as compared to expression in healthy controls. We used the Luminex multianalyte profiling assay system, a new technique that allows simultaneous measurement of various molecules in a single microplate well.[41] We also evaluated the effect on patients with POAG or DED of oral supplementation with AOX/EPUFA over the course of 3 months for each group of participants.

Human tears contain a wide spectrum of cytokines and chemokines, the main function of which is to maintain the morphologic and physiologic properties of the ocular surface.[41–46] We found that tears collected from patients with DEDG and POAG showed differential expression of the assayed cytokines and chemokines compared to the tears of healthy controls. Specifically, the concentrations of IL-6 and TNF-α were significantly higher in the DEDG and POAGG than in the CG. Other investigators have also detected higher IL-6 and TNF-α levels in the conjunctival epithelium and in tear samples of patients with DEDs.[43,47,48] However, it has also been reported that the concentration of IL-6 is not higher in tear samples from patients with moderately dry eyes.[49] Because inflammatory processes mediated by cytokines and chemokines are commonly associated with ocular surface disorders, independently of their etiology, our data strongly suggest that the augmented expression of IL-6 and TNF-α in tears is worth noting, because these proinflammatory cytokines are most probably the underlying cause of most clinical manifestations of the ocular surface alterations in both the DEDG and POAGG patients.

Aging is a relevant factor in both DEDs and POAG. When all the participants were assembled as being aged

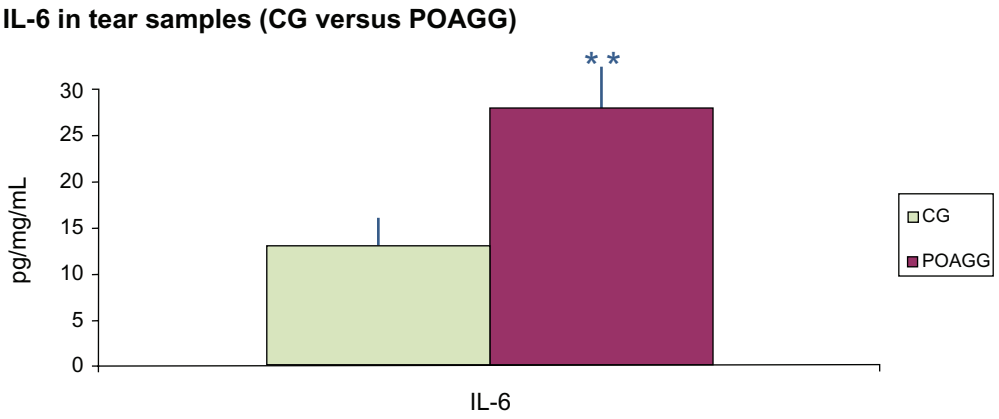

Figure 2 Comparison of the expression of IL-6 in tears from the CG and POAGG patients.
Notes: Data are mean ± standard deviation for three assays. **Indicates a *P* value < 0.001.
Abbreviations: CG, control group; IL-6, interleukin-6; POAGG, primary open-angle glaucoma group.

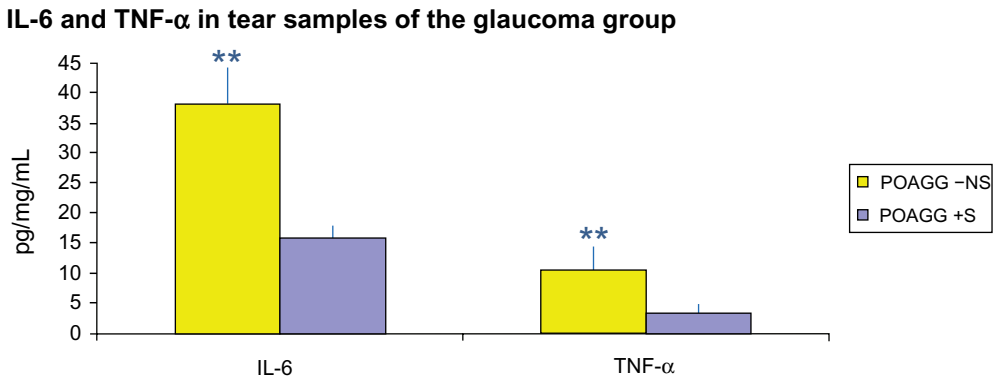

Figure 3 Comparison of the expression of IL-6 and TNF-α in tears from the POAGG +S compared to the POAGG –NS patients.
Notes: Data are mean ± standard deviation for three assays. **Indicates a *P* value < 0.001.
Abbreviations: IL-6, interleukin-6; POAGG –NS, primary open-angle glaucoma group without oral supplements; POAGG +S, primary open-angle glaucoma group with oral supplements; TNF-α, tumor necrosis factor alpha.

over 40 years, each group (DEDG, POAGG and CG) and subgroup (patients taking or not taking oral supplements) was homogeneous in their percentages.

Percentages of men and women in all groups were 28% and 72% (DEDG), 29% and 71% (POAGG), and 32% and 68% (CG) respectively, reflecting the differences in gender of both diseases in the general population.

Daily dietary intake of EPUFAs (ω-3 and ω-6) is necessary so that they can exert their effects in the body (eg, on the immune system) at both the cellular and the humoral levels.[30,31] Furthermore, the ω-3 EPUFA intake and the ω-3 EPUFA/ω-6 EPUFA intake ratio affect the expression of inflammatory biomarkers.[50] Therefore, it has been shown that ω-3 EPUFA supplementation can protect the ocular surface in patients at risk of DEDs.[33,51–53] In the present work, we determined whether the oral supplementation of a combined formulation of AOXs and EPUFAs (Brudysec 1.5®) influenced the progression of DED, and our data suggest that supplement intake significantly changed the expression patterns of various cytokines and chemokines in tears collected from patients with DEDs or POAG compared to the patterns in the corresponding unsupplemented patients. Other investigators previously reported similar results for AOX and EPUFA supplementation in patients with dry eyes.[51–53]

Patients with POAG are generally not aware of having ocular surface dysfunction, probably because they do not recognize some of the ocular signs and symptoms. As reported by Gilbard[54] other possible causes (and their symptoms) have to be considered for a patient's chronic eye irritation, as follows: iatrogenic, non-specific ocular irritation, tarsal foreign body, anterior blepharitis, obstruction of the lacrimal drainage system, meibomian gland dysfunction, allergic conjunctivitis, nocturnal lagophthalmos, superior limbic keratoconjunctivitis, superficial punctate Thygenson's keratitis, dry eyelid skin, normal eyes responding to abnormal environment, and blepharospasm. In these cases, careful questioning and specific clinical examination may reveal that patients are experiencing DED signs and symptoms rather than eye irritation.

All of the DED patients in our study reported a history of dry eyes before starting the study, whereas only half of the patients with POAG suffered symptoms related to dry eyes, mainly dryness, photophobia, burning, or conjunctival hyperemia. Furthermore, examination of the POAG patients revealed significant reductions in Schirmer's test scores and fluorescein tear BUT compared to those of the controls. In agreement with our data, other authors have also reported that around 60% of patients with POAG have dry eye signs and symptoms.[9,10] We speculate that POAG patients needing long-term treatment with antihypertensive eye drops are at risk of developing a DED (or exacerbating a latent DED). The significant role of aging in both DEDs and POAG may increase the probability of POAG and DED comorbidity.[55–57] Therefore, we strongly recommend that patients with POAG be carefully evaluated for dry eye symptoms prior to starting any topical antiglaucomatous therapy. Our results suggest that in addition to the use of eye drops to protect the ocular surface, oral supplementation with a combination of AOXs and EPUFAs, such as in the present study (Brudysec 1.5®), should be considered for elderly glaucomatous patients at risk for DEDs.

Disclosure

The authors report no conflicts of interest in this work. Part of this work was presented to the Meeting of the Society of Research in Retina and Visual Sciences (SIRCOVA) in Valencia, Spain (2013).

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Cytokine/Chemokine Expression in Reflex Tears from Employers Exposed to Computer Screens in a Healthy Office Environment

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Abstract

Objective: To inform employees exposed to computer screens (CSs) about the risk of dry eye disorders (DEDs) and how to prevent them.

Methods: From a total of 800 public-sector employees, eighty-eight CSs users were randomly selected to participate in an interview and an ophthalmic examination, and compared to thirty-six healthy volunteers no CSs users including family members, nurses and assistants. Environmental conditions in the workplace were documented. Reflex tear samples were collected simultaneously from both eyes and were later subjected to a multiplexed particle-based flow cytometry assay. A specific set of immune response biomarkers was analyzed.

Results: The mean age was 52.17 (5.17) years; 27% were men and 73% women. DEDs were newly diagnosed in 86% of the participants. Mean CS exposure was 4.8 (1.27) hours. Environmental workplace conditions complied with general standards. Schirmer test results and blinking frequency were pathologic in up to 2/3 of the employers exposed to CSs. Immune response biomarkers were detected in 90% of tear samples. Compared with records of healthy, non-exposed control subjects in a pre-existing database, tear samples of the participants exposed to CSs had significantly higher levels of interleukins (IL) (IL1B, IL2, IL6, IL8), GM-CSF, IFG, and VEGF.

Conclusion: Employee exposure to CSs was a major risk factor for DED, being inflammation a main contributor to ocular surface pathogenesis.

Keywords: Dry eyes; Computer screen; Employee; Immune response; Tears

Introduction

Temperature, humidity, wind, fumes, pollution, as well as air speed, CO_2 concentration, and light intensity play pivotal roles in vision outcomes [1]. Office employees spend many hours in front of a computer screen (CS). Adverse effects of such exposure have been referred to as computer-vision syndrome (CVS) [2]. In this context, computer users often present ocular signs and symptoms such as itchiness, soreness, foreign body sensation, irritation, photophobia, redness, eye strain, tired eyes, blurred vision blurred, and double vision [3-5]. A search of the scientific literature led us to several studies performed with subjects exposed to CSs in office environments [6-8], but there have been no reports of integrated data about working conditions, CSs exposure, external and internal risk factors, and inflammatory molecules related to ocular surface dysfunction.

Ocular surface dysfunction refers to complex conditions involving the eyelids, cornea, conjunctiva, lacrimal glands, and tear film [1-5]. However, the term *dry eye disorder/s* (DED/s) has been more recently introduced to better distinguish the ocular surface dysfunction that induces tear film impairment and dry eye [3].

We attempt to shed light on ocular surface dysfunction and working conditions to improve awareness of workers and help them prevent chronic DEDs and visual impairment. For reaching this objective in a better way we have determined: 1) the main risk factors for DEDs; 2) the prevalence and severity of DEDs, and the relationship between CS exposure duration and DED severity; and 3) the expression of biomarkers of immune response in tear samples and its relationship to DED severity.

Methods

Men and women were randomly selected from among the employees of the General Treasury of Social Security in Valencia, Spain. Inclusion and exclusion criteria are reflected in Table 1. Eighty-eight CSs users and thirty-six CSs non users were enrolled when signing the informed consent. The Ethics Committee of the Ophthalmic Research Unit "Santiago Grisolia" Center approved the present study (2012) that was conducted according to Declaration of Helsinki principles.

We conducted personal interviews and assigned scores for

inclusion criteria	Exclusion criteria
Unawareness of ocular surface disorders	Previous diagnosis of ocular surface disorders
Ability to participate	Sjögren syndrome
	Use of contact lenses
	Other ocular disorders
	Use of eye drops other than artificial tears
	Recent history of ocular laser treatment/ophthalmic SURGERY
	Systemic diseases and general treatments
	Atopy or allergic disorders
	No ability to participate

Table 1: Inclusion and exclusion criteria for the study participants.

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objective and subjective criteria (ocular surface disease index [OSDI] questionnaire). The questionnaire sections are divided into three main questions:

1. Have you experienced photophobia, a gritty feeling, soreness, or blurred vision during the last week?
2. Have your eye problems limited you in reading, driving at night, computer work, or watching TV during the last week?
3. Have your eyes felt uncomfortable in windy or dry situations or because of air conditioning during the last week?

Interviewers paid special attention to the comments of the participants regarding lifestyle and eye conditions.

In addition, a systematized ophthalmic examination was performed on all participants as follows: value of the eyelid Schirmer test, slit lamp examination for the eye adnexa and anterior eye segment and corneal surface details with fluorescein. Examiners completed a full sheath to enclose all data, and were advised to strictly follow the study protocol. Primary outcomes of the ophthalmic examination measures for determining the ocular surface status were the Schirmer test and fluorescein ocular surface details, as well as assessment of blinking frequency (near and far); the secondary measure was dry eye symptoms. Other signs and symptoms of DEDs were evaluated based on self-reports. Depending on clinical manifestations, patients were considered to be free of DEDs or to have mild, moderate, or severe DEDs.

Reflex tear samples were obtained with a Pasteur micropipette from the inferior lid cul-de-sac of both eyes by gentle rubbing, as shown in the Figure 1. The expression of a set of immune mediators was assayed with the Multiplex System (Luminex® R-200, Luminex Corporation; Austin, TX, USA). Polystyrene beads coupled covalently to specifically directed antibodies (human cytokine/chemokine panel) were allowed to react with 20–30 μL of each tear sample containing an unknown amount of cytokines, or with a standard solution containing a known amount of cytokines, at room temperature for 1 hour, according to the manufacturer's instructions. The following cytokines/chemokines were analyzed: interleukin (IL)-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, and IL-12; tumor necrosis factor alpha; vascular endothelial growth factor (VEGF); granulocyte-macrophage colony-stimulating factor; and interferon gamma, as previously described [9]. Briefly, a series of washes was carried out to remove unbound proteins. Then, a biotinylated detection antibody specific for a different epitope on the cytokine was added to the beads and incubated at room temperature for 30 minutes. Streptavidin-phycoerythrin (which binds to the biotinylated detection antibodies), was used to detect the reaction mixture. The flow-based Bio-Plex® (Bio-Rad Laboratories; Hercules, CA, USA) suspension array system was used to identify and quantify each antigen–antibody reaction. Identification of the assayed molecules was based on bead color and fluorescence, using fluorescently labeled reporter molecules associated with each target protein. Unknown cytokine/chemokine concentrations were calculated automatically by the Bio-Plex® Manager software (Bio-Rad Laboratories) using a standard curve derived from a recombinant cytokine standard. Levels of these molecules were corrected for the initial total protein concentration of each tear sample during analysis. We previously created a database with values for the above set of immune response molecules [9,10]. Values for expression of molecules in samples obtained from non-exposed, healthy controls were used for comparison with data from employees participating in the current study. Data are reported as means (standard deviations) of two or three values and expressed in picograms per milliliter per milligram.

Special attention was paid to the workplace conditions in the office that were evaluated by the following homologized systems: heat stress monitor, indoor air quality (IAQ), Microtherm IAQPROBE DAE 504002, luxometer GOSSEN MAVOLUX 5032 C/B nº serie 0C60759, and CO_2 concentration analyzer Ex 2000 Oldham/CO_2.

Demographic, lifestyle, working conditions, environmental, clinical, and molecular data were independently gathered. We used nonparametric Mann–Whitney U test to compare two independent sample groups with Statistical Package for the Social Sciences (SPSS) software (v15.0; SPSS Inc; Chicago, IL, USA). A value of $p \leq 0.05$ was considered an indication of a statistically significant difference between groups.

Results

The participants' mean age was 52.17 (5.17) years in the CSs exposed individuals and 50.10 (12.24) years in the non-exposed controls; 26.82% were men and 73.17% women in the CSs exposed group vs. 32% and 68% of the controls. Regarding ethnics, all participants were Caucasian.

Of the CCs exposed group 96% wear glasses all the time vs. 62% of the controls.

DEDs were newly diagnosed (based on the OSDI questionnaire and ophthalmic examination) in 86% of the CSs exposed subjects. Among these, 29.26% had mild DED and 70.73% had moderate DED. No severe DED were detected within this group of participants. Their main complaints were itchiness, dryness, tired eyes, red eyes, and blurred vision. Mean exposure time to computer screens was 4.8 (1.27) hours (8 hours maximum). However, no significant CSs exposure was recorded within the healthy volunteers.

The most significant molecules in tear samples from the mild DED group were IL-8 (P=0.001) and IL-12 (p=0.031). The outstanding immune mediator in samples from the moderate DED group was IL-6 (p=0.032).

Environmental conditions detected in the workplace (dry temperature, relative humidity, light intensity, CO_2 parts per million) were within normal ranges at all times (Table 2).

Background cytokine concentrations were subtracted from the cytokine concentrations detected in the tears. The standard curves for both the kit assay and the extraction buffers were similar for the 12 analyzed molecules. In up to 90% of the sampling procedures, the volume of tears obtained was sufficient.

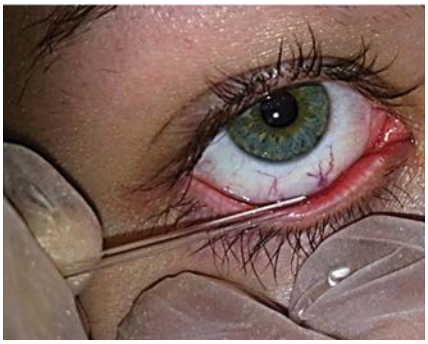

Figure 1: Illustrating the tear reflex sample collecting method.

The tear samples showed a wide variety of expression levels of the set of assayed molecules. Precision, as measured by the Luminex multianalyte profiling bioassay system (Luminex Corporation), was acceptable. In fact, CS-exposed study participants had significantly higher levels of cytokines/chemokines than non-exposed controls (Table 3).

Discussion

During interviews, the main complaints were itchiness, dryness, tired eyes, red eyes, and blurred vision, similar to results of other studies [11-13]. Considering that the mean CS exposure time was 4 hours, these signs and symptoms were similar to those in several reports of video-terminal users [11-16]. Interestingly, 86% of our participants had mild or mild-to-moderate dry eyes not previously diagnosed.

Cytokines are extracellular signaling proteins [17]. The expression of cytokines/chemokines such as regulated on activation, normal T-cell expressed and secreted, may underlie the clinical manifestations observed in some DEDs patients. In fact, tear samples from the DEDs affected subjects displayed significantly higher expression levels of immune response molecules. Moreover, the cytokine profile seen in chronic mild DED patients was different from that observed in chronic moderate DEDs. Concentrations of IL-8 and IL-12 released from macrophages in the mild DED group were significantly higher than those from healthy controls and from those in patients with moderate DEDs. Those molecules play an important role in the inflammation processes underlying airway dysfunction [18-20], as well as in response to smoking habits [21]. Nevertheless, the role of these cytokines needs to be defined and there is a potential for anticytokine therapy in chronic ocular surface disease.

In addition, in the moderate DED patients there were an upregulation of IL-6. It has been suggested that IL-6, a major pro-inflammatory cytokine, is, by itself or in combination with other similar molecules, a potential biomarker of DED progression [9,10,22-27].

Under the current study's environmental conditions, CSs exposure induced a significant increase in the immune mediator tear levels. In a similar manner it has also been described the effects of oxidant air pollutants on the respiratory system [18]. Although the importance of immune mediators in ocular surface anomalies is still growing, this study provides enough evidence that the tear expression profiles of inflammatory mediators in the mild and moderate phases of the DEDs can be useful for further understanding of the complex DEDs pathology.

Data strongly indicate that screening employees exposed to CSs, with or without eye signs or symptoms, may be useful. Answers to the three sets of questions in Table 4 can shed light on the status of the ocular surface, regardless of the subject's knowledge of eye disorders.

DEDs and their downstream effects contribute negatively to quality of life. This confers special relevance to the care process, as previously recognized [20-26]. The following measures are recommended for employees exposed to CSs for ≥ 4 hours: 1) Use ample, uncluttered screens with flexible contrast and brightness and establish a distance of 50–80 cm from the CS, with the CS slightly below the plane of the eyes. 2) To prevent reflections and glare, avoid positioning the CS in front of or behind a light source or window. 3) Adjust environmental light to 200–500 lux, depending on the type of work. 4) Close eyes for a few seconds after ≤ 30 minutes of CS exposure. 5) Squint hard 5 times after ≤ 1 hour of continuous CS exposure, and increase blinking frequency. 6) Look away from the computer and toward the infinite after ≤ 2 hours of CS exposure. 7) Without moving the head, direct eyes up, down, right, then left 5-10 times (midway through work period and again at the end of work period). 8) Whenever possible use glasses and avoid contact lenses. 9) Use artificial tears or hydrating eye drops whether suffering dryness or not. 10) Take supplements with antioxidants and omega-3 fatty acids, which help in maintaining the oxidative and antioxidant balance for the ocular surface and promote healthy eyes and vision.

Environmental Parameters	Measurements
Air Speed m/s	0.11 ± 0.031
Relative Humidity %	32.673 ± 5.13
Dry Temperature ºC	24.56 ± 0.60
Light Intensity Lux	500
CO2 parts per million	2370.71 ± 646.89

Environmental parameters in the work place (measured by homologized systems)

Table 2: Environmental parameters in the workplace.

Set of immune mediator molecules assayed in tear samples	GECS (Group exposed to computer screens)	GC (Group of non exposed to computer screens)	p valor*
IL1β	36,40 ± 35,30	10,98 ± 15,42	0.00001
IL-2	0,67 ± 0,36	3,91 ± 1,21	0.034
IL-6	28,10 ± 71,7	13,16 ± 8,71	0.05
IL-8	1138, 47 ± 915,45	398,75 ± 270,43	0.00001
IL-10	0,59 ± 0,69	4,20 ± 1,51	0.00001
GM-CSF	2,96 ± 2,58	7,60 ± 3,24	0.00001
IFγ	0,26 ± 0,43	2,077 ± 0,67	0.00001
VEGF	1367,13 ± 591,51	542,29 ± 276,73	0.00001

Table 3: Expression levels of immune mediators in tear samples from employees exposed to computer screens. Data are means (standard deviations) of two values and are expressed in picograms per milliliter per milligram. Tear expression levels of immune mediators in employers exposed to CS. Data are mean ± standard deviation for two/three determinations, expressed in picograms per milliliter per milligram.

Usually I become aware of my eyes when exposed to:	In general, I resent of my eyes with the following symptoms:	I try to meliorate my eye disturbing sensations by:
Sunlight	Dryness	Coliriums
Brightness	Soreness	Home remedies
Wind	Itchiness	Tear substitutive eye drops
Heaters/Ventilators	Tired eyes	Saline solutions
Air Conditioning	Foreign body sensation	Vitamin eye drops
Fumes/Pollution	Photofobia	Antioxidant oral supplementation
Smoke	Burning	Essential fatty acid supplementation
Tv/Computer Screens	Redness	Clossing eyes
Reading	Blurred vision	Restricting activities
Driving at night	Visual loss	Nothing
Total Score 1st Column	*Total Score 2nd Column*	*Total Score 3rd Column*

Table 4: Awareness of the integrity of the ocular surface among employees exposed to computer screens: 30-item self-scored questionnaire for adults. *Convert each response to numerical data: mark each affirmative answer 1 and each negative answer 0. Visit an eye doctor if you have a sum of 4 or more in each of the three columns (or a global score of 12 or more)*. Being aware of the integrity of the ocular surface in people exposed to computer screens during the working time. 30-item self-scoring questionnaire for adults. Converting each response to numerical data as follows: each affirmative answer has to be marked with 1 while the negative ones as 0. Ranking 4 or more in each of the three columns (or obtaining a global score of 12 or more) it can be convenient to visit an eye doctor.

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Differential Effects of Dry Eye Disorders on Metabolomic Profile by 1H Nuclear Magnetic Resonance Spectroscopy.

Differential Metabolomics in dry eyes

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Abstract

In this study, we used ^{1}H NMR spectroscopy to analyze the metabolomic profile of reflex tears from patients with dry eye disorders (DEDs). 90 subjects were divided into 2 groups: (1) patients with DEDs (DEDG; *n* = 55) and (2) healthy subjects (CG; *n* = 35). Additionally, the DEDG was subdivided into 2 subgroups based on DED severity: mild-to-moderate, and moderate (*n* = 22 and n=33 respectively). Personal interviews and systematized ophthalmologic examinations were carried out. Reflex tears (20–30 µl) were collected by gently rubbing in the inferior meniscus of both eyelids with a microglass pipette and stored at -80°C until analysis. NMR spectra were acquired using a standard one-dimensional pulse sequence with water suppression. Data were processed and transferred to MATLAB for further chemometric analysis. Main differences in tear composition between DEDG and CG were found in cholesterol, *N*-acetylglucosamine, glutamate, creatine, amino-*n*-butyrate, choline, acetylcholine, arginine, phosphoethanolamine, glucose, and phenylalanine levels. This metabolic fingerprint helped also to discriminate between the three additional subgroups of DEDG. Our results suggest that tear metabolic

differences between DEDG and CG identified by NMR could be useful in understanding ocular surface pathogenesis and improving biotherapy.

Keywords: Dry eyes, Metabolomics, Ocular surface, Tears, Nuclear Magnetic Resonance Spectroscopy

Introduction

The ocular surface (OS) is a functional unit consisting of the eyelids, lacrimal glands, cornea, conjunctiva, and tear film. Main roles of the OS are to preserve the anatomical and physiological properties of its components, to maintain the optic conditions of the eye surface dioptric, and to protect the eyes against injury by exogenous and endogenous agents [1–3]. A healthy tear film is fundamental for supporting OS integrity and refractive functions [1].

Dry eye disorders (DEDs) are complex pathological conditions involving the OS [2, 3]. Approximately 20–50% of the patients who visit an ophthalmology clinic complain of dryness, making DEDs one of the most frequent ocular morbidities. In fact, DEDs are a growing public health problem worldwide [3]. DEDs usually affect the elderly and postmenopausal women [4, 5]. However, the prevalence of DEDs may be higher than suspected, because some patients may not report their eye problems to ophthalmologists and thus remain undiagnosed. The main symptoms of DEDs include dryness, itchiness, burning, stinging, grittiness, foreign body sensation, tearing, tired eyes, redness, and blurred vision [2–4]. Among the recognized risk factors for DEDs are aging,

being female, smoking, the use of topical or systemic medications, contact lenses wearing, laser excimer refractive surgery, weather conditions, environmental pollutants, air conditioning, hormone disorders, immune system diseases, and the use of video display terminals [6]. There are two major clinical forms of DEDs—the deficient aqueous tear production type (due to lacrimal gland dysfunction) and the increased evaporative loss type (due to meibomian gland disorder)—but combinations of the two forms are usually seen in clinical practice [2, 3, 7, 8]. DEDs are also classified according to severity, ranging from mild to moderate to severe forms. Significant reduction in body water content associated with aging may play a pivotal role in DEDs. Meibomian gland dropout, a reduction in the number of goblet cells, and laxity of the eyelids may also be contributing factors [7, 8].

According to a report from the 2007 International Dry Eye Workshop [2], the main pathogenic mechanisms of DEDs are tear film instability and hyperosmolarity. Oxidative stress, inflammation and apoptosis must also be considered in this process. All conditions lead to a chain of events that induce a series of OS clinical manifestations that taken together are referred to as DEDs.

Recent reports have proposed certain molecules and genes, as well as various clinical parameters, as presumptive DED biomarkers [7, 8], including oxidant and antioxidant activities, apoptotic mediators, antibodies, cytokines and chemokines, and hormone tear levels. However, lacks of specificity of these biomarkers justify the need of a further research on DED pathogenesis.

The relevance of the metabolome—the complex array of small-molecule metabolites and metabolic by-products, including carbohydrates, peptides, and lipids, present in cells, tissues, organs, and body fluids as a result of the expression and activity of genes and proteins [9–14] to both disease and health has long been recognized. The study of the metabolome, metabolomics, may help to establish a link between the components of the metabolome and corresponding cellular responses [15, 16]. During the past 30 years, metabolomics has been used in clinical and animal studies of several diseases, including ocular pathologies. Young and Wallace reviewed the metabolic consequences of ocular diseases and explained why the multiplexed analysis inherent to metabolomics can be expected to provide data that are uniquely useful for the assessment of ocular diseases [17].

Metabolomics is defined as “the quantitative measurement of the metabolic response of living systems to pathophysiological stimuli or genetic modification” [18,19] and is based on analytical platforms such as proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) and mass spectrometry (MS), particularly, gas chromatography (GC) and liquid chromatography (LC)-MS [18,19]. The metabolites and their concentrations provided a dataset on which multivariate statistical analysis was performed.

The NMR technique is based on the measurements of the magnetic properties of certain atomic nuclei, e.g. ^{1}H, ^{31}P, ^{13}C, in the metabolites. Each chemical group (CH, CH_2, CH_3, etc.) of each metabolite will have a unique chemical shift (in ppm). The metabolite peak will have an integral value that is

directly proportional to the metabolite concentration in the sample. In MS experiments compounds are ionized to form positively or negatively charged molecules which are separated and detected according to their mass-to-charge ratio.

^{1}H NMR spectroscopy analysis of biofluids provides information on both the structure and the composition of low-molecular-mass metabolites in biological fluids, and is a rapid and low-cost technique for exploring pathological metabolic processes. The major advantages of NMR spectroscopy include its unbiased metabolite detection, quantitative nature and highly reproducibility [18]. Unfortunately, this technique is associated with a low sensitivity compared to the other analytical methods such as GC-MS or LC-MS. The MS analysis requires more labor-intense (and destructive) tissue preparation. Detectable compounds are limited to those that can be derivatized, which can be time consuming, costly, and carries a risk of metabolite loss. NMR-based metabolomics could be appropriate as a cost effective solution for high-throughput analysis [20].

Biotechnological advances, such as the application of NMR spectroscopy to tissue and biofluid samples, have permitted the generation of data on low-molecular-weight metabolites in samples from individuals [9–11]. For example, Greiner et al. analyzed the aqueous humor and vitreous body of pig eyes by means of ^{31}P NMR [15]. Midelfart et al. analyzed extracts of rabbit corneas and lenses by NMR spectroscopy and observed that some metabolites suggested the existence of a powerful antioxidant environment in the ocular tissues [21–

23]. Our group reported previous experience on the metabolomics study by NMR of the aqueous humor in a rat model of hyaluronic acid–induced ocular hypertension [24]. Compounds identified in these glaucomatous samples correlated well with data obtained in similar glaucoma models by means of conventional techniques. Moreover, Chen et al. published a detailed work on the characterization of human tear metabolome using LC-MS/MS [25]. Although NMR study of tear composition remains incomplete, some works using this biofluid [23-24,27-29] have demonstrated that metabolomics can be useful for monitoring eye diseases such as DEDs.

Proteomic assays have recently been reported in tear samples of dry eye patients by 2D electrophoresis (2DE) and Differential Gel Electrophoresis (DIGE). Presumptive biomarkers of DEDs included proline rich 4 protein (LPRR4) which appeared downregulated in all types of DEDs [30]. Other authors have suggested the higher expression of apoptotic and inflammation proteins in tears from diabetic dry eye patients as performed by two-dimensional nano-liquid chromatography coupled with tandem mass spectrometry (MS)-based proteomics [31].

In the present study, our goal was to improve knowledge on human tear composition by using ^{1}H NMR spectroscopy–based metabolite profiling, followed by multivariate statistical analysis, in order to explore metabolite imbalances between DEDG. In addition, we investigated the possibility to build-up a metabolic discriminating model that help us to identify the three

established severity ranges subgroups of DED, with the ultimate goal of improving the management of these patients.

Material and Methods

All participants firmed the informed consent and all procedures of this prospective study were subjected to the Declaration of Helsinki for the protection of human subjects in medical research. The study was approved by the Institutional Review Board of the University and Polytechnic Hospital La Fe (Valencia, Spain), (Ref:2013/0417).

Patients and Groups

A total of 90 subjects of both sexes, aged 25–80 years, were enrolled during ophthalmologic appointments at the study center (University and Polytechnic Hospital La Fe, Valencia, Spain) between February 2013 and September 2013 according to the main inclusion and exclusion criteria listed in Table 1.

Inclusion criteria	Exclusion criteria
Aged 23-80 years	Aged ≤ 23 years or ≥ 80 years
Diagnosed with DEDs (DEDG)	Athopy, allergic disorders
Healthy subjects (CG)	Wearing contact lenses
Able to participate in the study	Not able to participate in the study
Informed consent obtained	Sistemic diseases and general treatments
	Ocular disorders and use of eye drops other than artificial tears
	History of refractive surgery, Ophthalmic laser treatment (≤3 months)

Table 1. Inclusion and exclusion criteria.

Suitable subjects were assigned to one of the following groups: (1) patients diagnosed with DEDs (DEDG; *n* = 55) and (2) healthy subjects as a control group (CG; *n* = 35). From the DEDG, three subgroups were formed on the basis of clinical OS data and OSDI questionnaire: patients diagnosed with mild-to moderate (*n* = 22) and moderate (*n* = 33) DEDs. Participants pertaining to the severe forms were excluded of the present study.

Clinical assessment and tear sampling

Each subject was interviewed about their personal and familial background, their personal characteristics and lifestyle, as well as disease data (symptoms of dry eyes and subjective sensations, duration, and treatments). We used the Ocular Surface Disease Index (OSDI) questionnaire as part of the interview. This clinically validated questionnaire was designed to evaluate DEDs in terms of ocular symptoms, visual function, and environmental factors. Each of the 12

questions has to be responded to with a rating from 0 to 4), and a final score (0–100) is then calculated, with higher scores indicating more-severe DEDs [24].

Each subject was given a systematized ophthalmologic examination consisting of the following tests: measurement of best corrected visual acuity in each eye, Schirmer test, slit-lamp examination of the eye adnexa and anterior segment, and measurement of tear break-up time (BUT) with fluorescein.

Reflex tear samples were obtained by gently rubbing the inferior meniscus of both eyelids with a micro Pasteur pipette, without damaging the OS tissues, as previously described [25]. Tears from both eyes (total volume 20–30 µl) were deposited in one labeled cryotube per subject and stored at –80°C until metabolomics analysis. Patients for whom the volume of the collected tear sample was less than [10 µl] were rejected, because of this, it was difficult to get a significant number of severe DEDs participants.

^{1}H NMR spectroscopy

For NMR analysis, 20 µl of tear were mixed with 2.5 µl of 0.05 mM sodium-3-trimethylsilylpropionate-2,2,3,3-d4 (TSP,) in deuterium oxide (D_2O). A total of 20 µl of the mixture from each subject was then transferred to a 1mm high-quality NMR tube. All 1H NMR spectra were acquired using a standard one-dimensional pulse sequence with water suppression on a Bruker Avance 600 spectrometer operating at 600.13 MHz with a 1-mm 1H/13C/15N TXI probe. A total of 256 free induction decays were collected into 64k data points with a

spectral width of 14 ppm and a recycle delay of 1 s. The water signal was saturated by weak irradiation during the recycle delay. Before Fourier transformation, the free induction decay was multiplied by a 0.3 Hz exponential line broadening. Spectral chemical shift referencing on the TSP signal at 0 ppm was performed for all spectra. Spectral regions between 0.5 and 4.4 ppm and between 5.5 and 9.5 ppm were binned in segments of 0.01 ppm width (6 Hz) for multivariate analysis. Binned data were normalized to total aliphatic spectral area. We used available spectral databases and two-dimensional NMR experiments to facilitate structural identification of relevant metabolites. Spectra were processed using MestRenova 6.2 software (Mestrelab Research S.L., Santiago de Compostela, Spain) and transferred to MATLAB 7.6 (The MathWorks Inc, Natick, MA, USA) for additional processing and further analysis. Signals belonging to selected metabolites were integrated and quantified using semi-automated in-house MATLAB peak-fitting routines based on Levenburg-Marquard optimization procedures. Resonances were assigned according to the previous literature, the Human Metabolome Database (http://www.hmdb.ca) and characteristic cross-peak from previously mentioned 2D spectra to unequivocal assignation of the resonances.

Statistical analyses

Chemometrics statistical analyses were performed using in-house MATLAB scripts and PLS Toolbox 6.7 (Eigenvector Research Inc., Wenatchee, WA, USA). Principal Component Analysis (PCA) and Partial Least Squares

Discriminant Analysis (PLS-DA) were applied to NMR spectra data matrix. PLS-DA is a classification technique that combines the properties of partial least-squares (PLS) regression with the discrimination power of discriminant analysis (DA) [20]. The main advantage of PLS-DA models is that the main sources of variability in the data are modeled by the so-called latent variables, and consequently, in their associated scores and loadings, allowing the visualization and understanding of different patterns and relations in the data. The PLS-DA model was tested using a leave-one-out cross-validation (CV) algorithm.

All data are expressed as mean ± standard deviation (SD). Finally, one-way analysis of variance was used for the determination of statistical significance between group means of the corresponding integrals. A difference was considered significant when $p < 0.05$.

Results

Median ages of the subjects were 52±18 years old (DEDG) and 36±11 years old (CG). The percentages of men and women in the two groups (DEDG and CG) were 75% and 58%, 25% and 42%, respectively.

On the basis of data collected from the personal interview and the OSDI questionnaire, the ophthalmologic examination, and the reported symptomatology and subjective sensations, patients were classified according to the degree of DED severity as follows: 42 % mild-to moderate DEDs, 58 % moderate DEDs.

The Schirmer test scores were lower in patients with mild-to moderate [9.80 ±1.71 mm], and moderate DEDs [6.13 ± 2.42 mm] than in subjects in the CG [18.88 ± 5.56 mm], reflecting the altered tear secretion in the DEDG patients. The BUT scores were lower in patients with mild-to-moderate [7.52 ± 0.87 s], and moderate [5.89 ± 0.65 s] than in subjects in the CG [7.98 ±0.06 s], which strongly suggests that the tear film stability was altered in DEDG patients.

Data from the ^{1}H NMR spectra of the tear samples from all the study subjects permitted a visual comparison between the average spectra of tears from CG subjects and the DEDG patients. Average spectra for the two groups are shown in Figure 1 with some of the most intense metabolite peaks labeled.

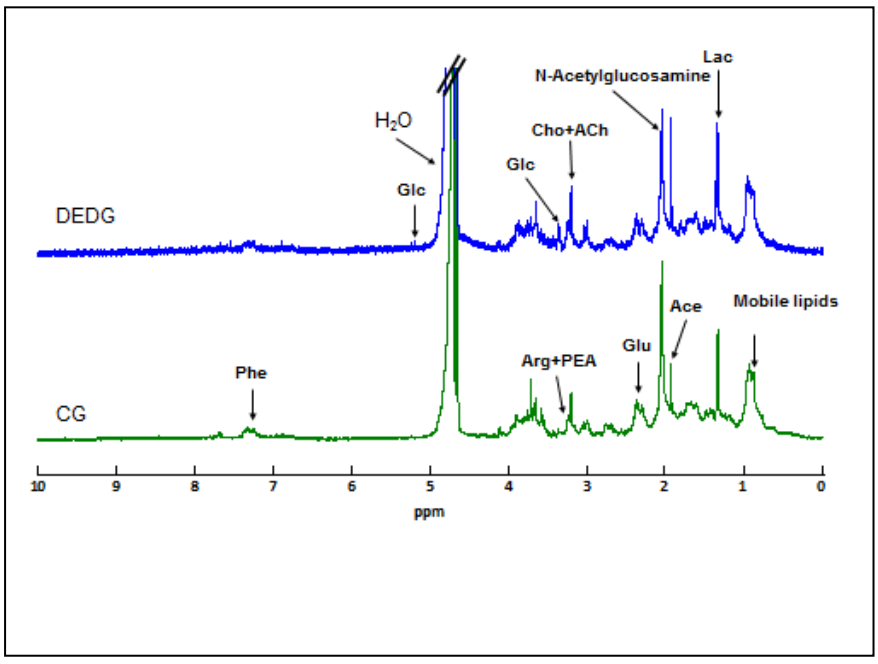

Figure 1: Representative spectra of tears from DEDG patients (upper spectrum) and CG subjects (lower spectrum).

The metabolic profiles of the groups were compared by means of principal component analysis, which is an unsupervised test for homogeneity of the set of samples (detecting the existence of possible outliers). As part of this process we determined the presence of 9 samples (3 from the CG and 6 from the DEDG) that were excluded from further analysis. Outliers are defined as those samples that are situated outside the 95% confidence interval of the Hotelling`s T-squared distribution in the score scatter plot. Because the samples did not cluster spontaneously, we performed a PLS-DA that maximized the separation between the groups (Fig. 2). The PLS-DA score plot clearly indicated an incipient separation with minimum overlap between DEDG and the CG samples, confirming the existence of significant differences in metabolic profile between the two groups.

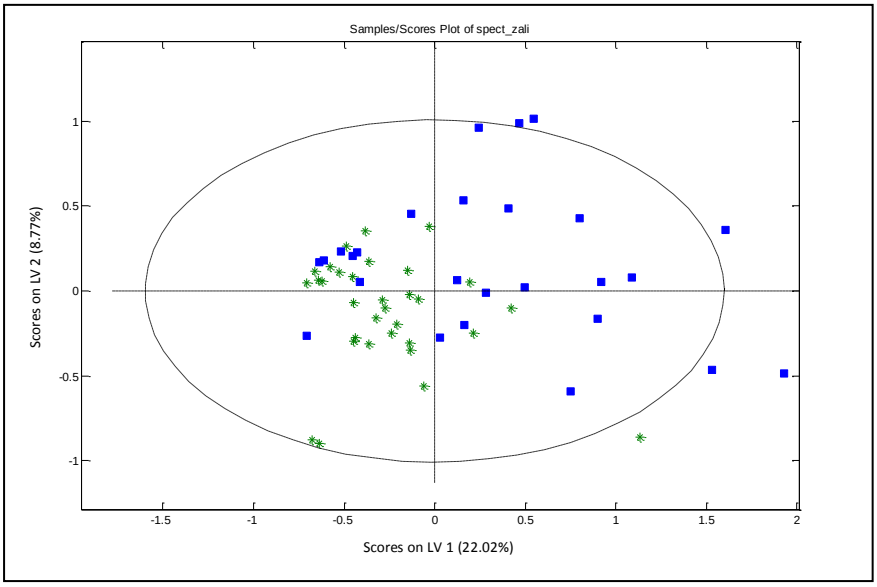

Figure 2: PLS-DA score plot of the DEDG (blue squares) and the CG (green asterisks).

Quantification of the most contributing regions in the PLS-DA model allowed us to determine the major metabolites differences in the tears of the two study groups (Table 2).

Metabolites	Region (ppm)	p-value	CG (au)	DEDG (au)
-CH_3 Lipids	*0.84-0.88*	3e-6	0.0264 ± 0.006	0.0202 ± 0.006
Cholesterol/Lipids	*0.90-0.93*	7e-7	0.0248 ± 0.005	0.0184 ± 0.006
N-acetylglucosamine	*2.0-2.08*	8e-9	0.079 ± 0.02	0.052 ± 0.02
Glutamate	*2.325-2.415*	0.001	0.0313 ± 0.01	0.0266 ± 0.004
Total Creatine	*3.02-3.05*	9e-9	0.0053 ± 9e-4	0.0067 ± 0.001
Amino-n-butyrate	*2.95-3.025*	0.0002	0.0135 ± 0.003	0.0157 ± 0.002
Choline/acetilcholine	*3.18-3.21*	0.01	0.0089 ± 0.002	0.010 ± 0.002
Arginine+Phosphoetanolamina	*3.21-3.28*	3e-6	0.0147 ± 0.003	0.0189 ± 0.004
Choline	*4.05-4.09*	1e-7	0.0036 ± 0.001	0.0057 ± 0.002
Glucose	*5.17-5.29*	8e-8	0.0064 ± 0.005	0.0177 ± 0.01
Phenylalanine	*7.20-7.40*	2e-7	0.0245 ± 0.005	0.0333 ± 0.008

Table 2: Mean spectral intensity on the metabolites included in the discriminating model between DEDG and CG subjects.

Additionally, based on previous metabolites differences found between DEDG and CG, we proceeded with a deeper analysis in order to build-up a model that help us to discriminate between the three DED subgroups. Firstly, we constructed a PLS-DA model using the two extreme groups (severe-DED and mild-DED). After that, the intermediate group (moderate DEDs) was projected in the same discriminating space. We obtained a multivariate space where the three subgroups presented a clear differential grouping. The mild-to-moderate and the moderate DED samples were distributed as shown in the figure 3.

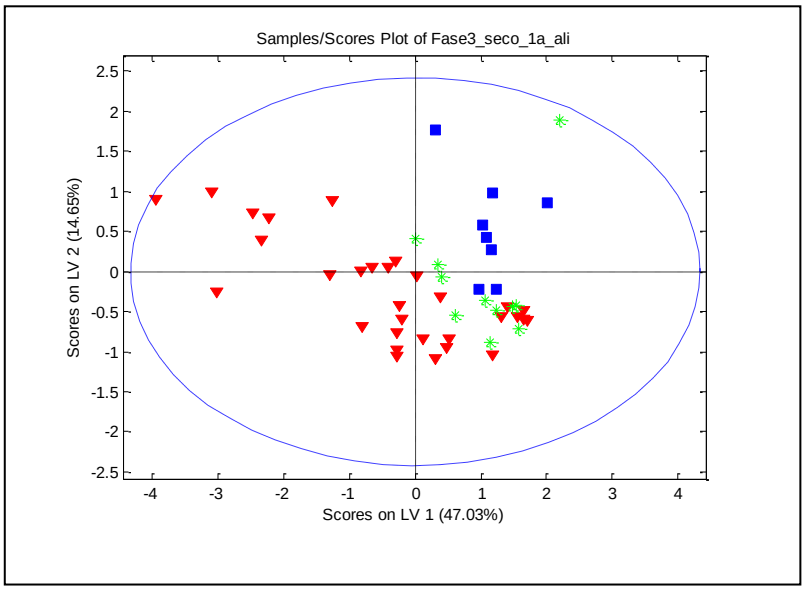

Figure 3: Score plot of the PLS-DA discriminatory model to differentiate between mild-to-moderate dry (red and blue) and moderate dry eyes (green).

In the Table 3 relative values of the most contributing regions in the PLS-DA discriminating model for each subgroups samples are reflected.

Metabolites	Region (ppm)	p-value	Mild-to-moderate DEDG (au)	Moderate DEDG (au)
-CH_3 Lípids	*0.84-0.88*	0.007	0.0263±0.005	0.0233±0.007
Cholesterol/Lipids	*0.90-0.93*	0.01	0.025±0.004	0.021±0.006
N-acetylglucosamine	*2.0-2.08*	0.004	0.077±0.01	0.059±0.02
Glutamate	*2.325-2.415*	0.02	0.031±0.002	0.027±0.004
Amino-n-butyrate	*2.95-3.025*	0.04	0.0141±0.001	0.0142±0.002
Choline	*4.05-4.09*	0.02	0.004±0.001	0.005±0.002
Glucose	*5.17-5.29*	0.007	0.0084±0.005	0.015±0.009
Phenylalanine	*7.20-7.40*	0.08	0.028±0.004	0.034±0.006
Formate	*8.45-8.475*	0.02	0.0018±0.0008	0.003±0.001

Table 3: Relative concentration of tear discriminating metabolites from mild-to-moderate DEDG, and moderate-DEDG patients. Values are mean ± SD. Only significant metabolites are shown ($p<0.05$, two-sample *t* test).

Discussion

The anatomy and function of the OS must be preserved by appropriate tear secretion and availability [1–4]. The tear film is formed by various layers that are secreted, in turns, by several ocular glands and tissues. DEDs are complex pathological conditions involving the OS and are among the most frequent ocular morbidities worldwide [1–8]. This research was conducted to shed light on the metabolite composition of the human tears in relation to DED manifestations and severity.

Mean age of the DED patients was 52 ±18 years, and women made up 75% of the DEDG, in agreement with previous reports [1–5], confirming that age and sex are relevant factors in DED initiation and progression, as previously reported [5, 6]. Statistically significant differences in the Schirmer and BUT scores were observed between the two main groups, as previously reported [1–8]. We subdivided the DEDG patients according to the severity of clinical DED manifestations (mild, moderate, or severe). The results of the Schirmer and BUT tests showed different degrees of alteration that were closely related to the intensity of clinical signs and symptoms.

^{1}H NMR spectroscopy allowed us to identify and quantify sets of metabolites in human tears with little sample preparation and quite small sample volumes. In addition, the metabolite profile could be acquired relatively rapidly (5 min with a short routine), with sensitivity sufficient to evaluate even subtle differences. By using the combination of NMR metabolomics and chemometric approaches, we were able to obtain and include in the analysis a large amount of data, which contribute to obtaining robust models that can be expected to provide useful information for further ophthalmic research [9–16].

The ^{1}H NMR metabolic profiles of the DEDG and the CG were clearly different from each other. The major differences in tear composition, between CG and the DEDG samples, were in *N*- acetylglucosamine, glutamate, creatine, amino-*n*-butyrate, choline, acetylcholine, arginine, phosphoethanolamine, cholesterol/lipids, glucose, and phenylalanine levels. Many of these metabolites have been cited in previous works which were carried out in human tears

samples, as substrate (or by-products) of the ocular surface metabolism. In the next table are summarized each metabolite and their bibliographical reference.

Metabolite	Ref.	Metabolite	Ref.
Cholesterol	*Butovich, 2008*	Creatine	*Lei Zhou et al., 2012*
Glutamate	*Nakatsukasa et al., 2011 Lei Zhou et al., 2012*	Acetilcholine	*Lei Zhou et al., 2012*
Arginine	*Nakatsukasa et al., 2011 Lei Zhou et al., 2012*	Phenilalanine	*Nakatsukasa et al., 2011; Lei Zhou et al., 2012*
Glucose	*Taormine et al., 2007*	Choline	*Lei Zhou et al., 2012*

Table 4: **Tear metabolites in the literature.**

The meibomian glands secretions comprise the lipid layer of the tear film which is essential for preventing rapid evaporation of the tears [31]. The results presented herein demonstrated lower lipid levels in tears from the DEDG as compared to the controls.

The increased levels of essential amino acids such as arginine and phenylalanine in the DED reflect the pro-inflammatory response to this type of ocular surface alteration [32]. However it is difficult to clearly discuss the presence and function of these amino acids, due to the variability of them found in individuals with or without alteration of the ocular surface. Furthermore, oxidative stress occurring in the cellular compartments of the ocular surface, as described in subjects with DEDs, could generate increased levels of glucose

and creatine in the tear film, in a similar manner as it has been described in our work [33].

There is a strong regulatory action of parasympathetic autonomous system stimulation on the secretion of lacrimal and salivary glands. Important changes in the stimulus/secretion process associated with parasympathetic stimulation have been previously described by Bacman et al., [34]. In fact, higher levels of neurotransmitters choline and acetylcholine in tears of our DEDs patients constituted an interesting finding but elucidation of its role in dry eyes is far from complete.

In an attempt to obtain additional data on OS alterations and tear composition in patients with DEDs, we discriminated the metabolome of DED tears in three subgroups of progression (mild, moderate, and severe). Our data showed that the metabolomic profiles of each one of these DEDs subgroups differed significantly. In this scenario we suggest for the first time that DED differences in severity have a significant effect in tear metabolic content, suggesting that the tear metabolomic profile may be utilized as biomarkers and surrogated endpoints of DEDs.

In summary, metabolomics is a feasible tool for measuring the metabolite profile of human tears. The results presented herein strongly suggest that further metabolomic analyses of OS pathologies should be conducted. The identification of specific metabolites associated with particular situations (oxidative stress, inflammation, apoptosis) will enhance the usefulness of this technique in a better understanding of the pathogenic mechanisms of eye

disorders and design of new therapeutic strategies [35, 36]. Finally, the combination of all this metabolomic information in a discriminating model gives us the opportunity of developing a fast, reliable and cheap methodology for severity classification of DED patients using a small sample of tears.

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RESULTADOS PRINCIPALES Y DISCUSIÓN

Resultados Principales y Discusión

Diseño del Estudio

Nos planteamos la utilización de las lágrimas humanas como muestras biológicas experimentales para analizar las características de las mismas en condiciones patológicas, tomando como referente una patología ocular muy prevalerte, como es el SOS, y compararlas con las lágrimas procedentes de sujetos controles sanos. Pretendemos investigar moléculas que se expresen en las lágrimas de pacientes con el SOS mediante técnicas de multideterminación por citometría de flujo y metabolómica por RMN. Además, tratando de profundizar en el conocimiento de los factores de riesgo medioambientales y su repercusión en la susceptibilidad individual a la presentación y progresión del SOS pretendemos evaluar pacientes expuestos a la utilización crónica de colirios para tratar su Glaucoma, e individuos que se ven obligados a exponerse a pantallas de visualización de datos durante la jornada laboral.

En relación a nuestros objetivos hemos realizado cuatro estudios que han sido publicados en revistas internacionales. A continuación exponemos de forma individual los resultados de estos trabajos y resumiremos los resultados de otros estudios publicados por diversos autores sobre el tema que nos ocupa.

1. **Effects of a nutraceutical formulation based on the combination of antioxidants and ω-3 essential fatty acids in the expression of inflammation and immune response mediators in tears from patients with dry eye disorders.**

En este estudio hemos analizado la expresión de moléculas implicadas en inflamación y respuesta inmune en lágrimas de pacientes con SOS (GOS; n=30) y controles sanos (GC; n=36) destaca el aumento significativo de la expresión de la IL-1beta (p= 0,015), IL-6 (0,0001) e IL-10 (0,050) y la disminución significativa de la expresión del VEGF (p= 0,05) en el GOS frente al GC. En las lágrimas humanas existe una amplia variedad de citoquinas/quimioquinas implicadas en el mantenimiento de la morfología y la función de la superficie ocular [1-3].

Al comparar nuestros resultados con otros estudios encontramos varias concordancias como el aumento de la expresión de IL-10 en pacientes con OS tal y como indica Boehm et al [4]. Por otra parte Brignole-Baudouin et al [5] descubren

mayores niveles de citoquinas de señalización celular, conocidas como moléculas destructivas, entre las que destacan la IL-6, TNF-alfa y VEGF en los pacientes con SOS. De hecho, las citoquinas/quimioquinas proinflamatorias más peligrosas son el TNF-alfa, IL-1beta, IL-6 y la IL-8. Los niveles de IL-1beta e IL-6 se incrementaron en nuestros participantes del GOS, como indican otros autores [5,6], reflejando un perfil inflamatorio en la superficie ocular de estos pacientes. Es interesante remarcar que las IL-1beta y la IL-6, que actúan induciendo respuesta inflamatoria (mientras que otras actúan sobre tipos específicos de células en la respuesta inmune) aumentaron significativamente en las muestras de nuestros pacientes. La IL-6 ha sido descrita como una de las moléculas clave en la patogenia del SOS [1, 15].

La disminución de los niveles de expresión del VEGF en nuestras muestras de lágrimas es contraria a los resultados obtenidos por Enríquez -de- Salamanca [2]. Sugerimos que podría estar implicado causalmente en los procesos neurodegenerativos y esto podría tener implicaciones en las alteraciones del segmento anterior (inervación) y posterior del ojo [7]. Un síntoma común en el SOS es el dolor y por ello es probable que estos pacientes presentaran una epitelopatía neurotrófica leve que pudo generar esta disminución del VEGF. Este hallazgo requiere de una mayor investigación para elucidar el papel del VEGF en el SOS.

En cuanto a la función de la suplementación micronutricional observamos que los niveles de expresión de la IL-1beta, IL-6 e IL-10 fueron significativamente menores en el GOS + S que en el NS ($p= 0,05$). Evidencias recientes apoyan el hecho de que la ingesta de ácidos grasos omega-3 influye en la expresión global de los marcadores inflamatorios en humanos [8]. Estos resultados sugieren una presunta función protectora del omega-3 frente al desarrollo del SOS [9-11]. La suplementación oral con una formulación combinada de AOX y AGPIs de cadena larga puede influir en la evolución del síndrome. En este sentido, nuestros resultados coinciden con los obtenidos por Brignole- Baudouin [5] y por Roncone et al [9] que también utilizaron suplementación oral en pacientes con SOS.

Y respecto a la prevalencia del SOS en la tercera edad, en este estudio confirmamos que existe una correlación positiva entre la expresión de mediadores de inflamación y respuesta inmune y la edad. En concreto los niveles de IL-4, IL-6, IL-10, IL-12, IFNgamma, GM-CSF aumentaban en los pacientes mayores de 45 años. Esto

confirma hallazgos previos de nuestro grupo y de otros en los que se proponía que la edad es el factor de riesgo más común para el desarrollo del SOS [12-14].

2. **Patients undergoing long-term treatment with antihypertensive eye drops responded positively with respect to their ocular surface disorder to oral supplementation with antioxidants and essential fatty acids.**

En este trabajo evaluamos la expresión de moléculas relacionadas con inflamación, y el hecho de instilarse diariamente colirios hipotensores para tratar el glaucoma y las comparaciones con pacientes diagnosticados de SOS. Los principales resultados obtenidos en este estudio fueron las diferencias significativas en la expresión de GM-CSF (p= 0,008), TNF-alfa (p= 0,005), VEGF (p= 0,038), IL-4 (p=0,030), IL-6 (p= 0,044) entre el GOS y el GGL. Las muestras de lágrima procedente de los pacientes de los grupos GOS y GGL mostraron una expresión diferencial en las citoquinas/quimioquinas en comparación con las muestras de lágrima de los sujetos sanos. En concreto, las concentraciones del TNF-alfa y la IL-6 fueron significativamente mayores en estos grupos que en el grupo control. Otros estudios han revelado niveles elevados de estas moléculas de inflamación y respuesta inmune en el epitelio conjuntivas y en lágrimas de pacientes con SOS [16,17], sin embargo también se han detectado niveles más bajos de expresión de IL-6 en muestras de lágrimas de pacientes con SOS moderado [18]. Dado que los procesos inflamatorios mediados por citoquinas/quimioquinas están asociados con las alteraciones de la superficie ocular, independientemente de su etiología, nuestros datos sugieren que el aumento de su expresión es debido a que estas moléculas son posiblemente la causa subyacente de la mayoría de las manifestaciones clínicas de las alteraciones en la superficie ocular, tanto en el SOS como en el GPAA.

La ingesta diaria de una formulación combinada de AOXs y AGPIs influyó en la progresión del SOS y cambió significativamente (disminución) los patrones de expresión del TNF-alfa (p= 0,00001) y la IL-6 (p= 0,044) en las muestras de lágrima de pacientes del GGL + S, como se ha descrito en otros estudios [19-21].

3. **Cytokine/Chemokine Expression in Reflex Tears from Employers Exposed to Computer Screens in a Healthy Office Environment**

El trabajo analiza la expresión de citoquinas y quimioquinas en lágrimas de empleados expuestos a pantallas de ordenador en un ambiente laboral controlado mediante las determinaciones constantes de los parámetros ambientales. Uno de los principales hallazgos de este estudio fue el diagnostico de OS en el 86% de los participantes expuestos a pantallas de ordenador, de entre los cuales un 29,26% presentaban OS-leve y un 70,73% OS-moderado. Todos ellos presentaban durante las entrevistas los siguientes síntomas relacionados con la patología: picazón, sequedad, ojos cansados, ojos rojos y visión borrosa, resultados similares a otros estudios [22-24]. La media de exposición a las pantallas de ordenador durante la jornada laboral fue de 4,8±1,27 horas.

Las muestras de lágrimas del grupo de sujetos expuestos a pantallas de ordenador (GEP) presento niveles de expresión significativamente más elevados de las IL-1β (P= 0,00001), IL-6 (P= 0,05), IL-8 (0,00001) y VEGF (0,00001), siendo la IL-1β, IL-8 y VEGF más significativa en el subgrupo de OS-leve y la IL-6 en el subgrupo de OS-moderado. La expresión de estas citoquinas/quimioquinas, que son proteínas de señalización extracelular, podría ser la base de las manifestaciones clínicas observadas en los sujetos con OS [25], y en concreto la IL-6 se ha sugerido que esta citoquina proinflamatoria es muy importante ya que por sí misma o en combinación con otras moléculas es un biomarcador de progresión del OS. [26-33].

Las condiciones ambientales detectadas en el puesto de trabajo (temperatura seca, humedad relativa, intensidad de la luz y CO2) se encontraban en los rangos normales.

En esta publicación recomendamos una serie de actuaciones para mejorar la superficie ocular en los empleados expuestos a las pantallas de ordenador. Además diseñamos una autoevaluación para que los propios individuos puedan conocer el estado de sus ojos respecto a la superficie ocular.

Nuestro trabajo refuerza el hecho de estar expuesto diariamente a las pantallas de ordenador como factor de riesgo como factor de riesgo para el desarrollo del SOS.

4. **Optimized metabolite extraction from human tears by 1H Nuclear Magnetic Resonance Spectroscopy.**

Esta publicación se realizó sobre los resultados obtenidos de las lágrimas de pacientes con SOS y controles. Mediante la 1H RMN pudimos identificar y cuantificar grupos de metabolitos en las lágrimas humanas, enfatizando que precisamos poca cantidad y relativamente poca preparación de la muestra. Mediante el uso de esta técnica hemos sido capaces de obtener e incluir en el análisis una gran cantidad de datos que contribuyen a la obtención de modelos que pueden proporcionar información útil para futuras investigaciones oftalmológicas [34-41]. Las principales diferencias en la composición de la lágrima entre los sujetos del GC y los pacientes del GOS fueron: colesterol, N-acetilglucosamina, glutamato, creatina, amino-n-butirato, colina, acetilcolina, arginina, fosfoetanolamina, –CH3 lípidos, glucosa y fenilalanina. Muchos de estos metabolitos han sido citados en trabajos anteriores que se llevaron a cabo en muestras de lágrimas humanas, como sustrato (o subproductos) del metabolismo de la superficie ocular. Los perfiles metabólicos de las muestras de los pacientes del GOS y del GC fueron claramente diferentes entre sí. Muchos de estos metabolitos han sido citados en trabajos anteriores que se llevaron a cabo en muestras de lágrimas humanas, como sustrato (o subproductos) del metabolismo de la superficie ocular. Las glándulas de Meibomio secreciones comprenden la capa lipídica de la película lagrimal, que es esencial para prevenir la evaporación rápida de las lágrimas [42]. Los resultados presentados en el presente documento demostraron los niveles de lípidos más bajos en las lágrimas de la DEDG en comparación con los controles. Los lípidos juegan un papel fundamental en la estabilidad de la película lagrimal y son los principales componentes de las secreciones de la glándula de Meibomio [42]. Una de las causas del Síndrome de ojo Seco es la disminución de lípidos procedentes de estas glándulas, como reflejan nuestros resultados en el caso del colesterol en las muestras de lágrimas procedentes de pacientes con Síndrome de Ojo seco. El aumento de los niveles de aminoácidos esenciales tales como arginina y fenilalanina en los DED reflejan la respuesta pro-inflamatoria ante este tipo de alteración de la superficie ocular [43]. Sin embargo es difícil discutir con claridad la presencia y función de estos aminoácidos, debido a la variabilidad presente en los individuos con o sin alteración de la superficie ocular.

El estrés oxidativo presente en las células de la superficie ocular en los sujetos con DED podría generar el aumento de los niveles de glucosa y creatina en la película lagrimal como hemos observado en nuestros resultados [44]. En relación a los niveles superiores de colina y acetilcolina en los DEDs reafirma el efecto antiinflamatorio que a nivel neural ejerce el sistema autónomo parasimpático a través de estos neurotransmisores [45].

Para obtener datos adicionales sobre la composición de la lágrima en relación al SOS, analizamos el metaboloma de las muestras de lágrimas de los tres subgrupos de OS (leve, moderado, severo) y hallamos que los perfiles metabólicos diferían significativamente. Estos resultados indicaron, por primera vez, que la gravedad del SOS juega un papel significativo en el contenido metabólico de la lágrima y sugiere que estos pueden ser utilizados como biomarcadores de la enfermedad y de la progresión de la misma.

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CONCLUSIONES

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1. La susceptibilidad personal a desarrollar el Síndrome de Ojo Seco puede agravarse por factores exógenos que modifiquen las condiciones naturales del privilegio inmune en la superficie ocular.
2. Las lágrimas presentan cambios estructurales y moleculares en relación directa a la alteración de la superficie ocular. Son pues una muestra biológica muy válida para abordar estudios sobre el Síndrome de Ojo Seco.
3. Las lágrimas de los pacientes con el ojo seco expresan moléculas relacionadas con inflamación que pueden ser utilizadas como biomarcadores de la enfermedad. La suplementación oral con antioxidantes y ácidos grasos omega-3 ejerce un efecto beneficioso sobre la sintomatología objetiva y subjetiva del Síndrome de Ojo Seco.
4. Los pacientes sometidos a tratamiento crónico con colirios antihipertensivos desarrollan una alteración de la superficie ocular, demostrada por la presencia de biomarcadores de inflamación en las lágrimas. La suplementación oral con antioxidantes y ácidos grasos omega-3 disminuye la actividad inflamatoria y favorece la estabilización del proceso en los pacientes glaucomatosos.
5. Los trabajadores expuestos a pantallas de visualización de datos durante su jornada laboral presentan un mayor riesgo de desarrollar el ojo seco, independientemente de otros factores de riesgo medioambientales.
6. La resonancia magnética nuclear de protón es una técnica útil para estudiar el perfil metabólico de las lágrimas humanas en condiciones normales y patológicas.
7. El Síndrome de Ojo Seco induce modificaciones en los metabolitos presentes en la superficie ocular en función del grado de progresión de la enfermedad.
8. Las moléculas relacionadas con inflamación y respuesta inmune, y los metabolitos identificados en las lágrimas de los pacientes con el síndrome de ojo seco, sugieren un nuevo abordaje terapéutico como alternativa a la actual terapia sustitutiva de las lágrimas.

ANEXO

CONCLUSIONES

Anexo I

Abreviaturas

- AGPIs: Ácidos grasos poliinsaturados
- BAK: Cloruro de Benzalcoino.
- DEWS: International Dry Eye WorkShop.
- EM: Espectometría de Masa.
- GC: Grupo Control.
- GEP: Grupo expuestos a pantallas.
- GM-CSF: Factor de Estimulación de Colonias de Macrófagos.
- GOS: Grupo Ojo Seco.
- GGLA: Grupo Glaucoma .
- GPAA: Glaucoma Primario de Angulo Abierto.
- H1 RMN: Resonancia Magnética Nuclear de Protón.
- P31 RMN: Resonancia Magnética Nuclear de Fósforo 31.
- IF: Interferón.
- IL: Interleukinas.
- INE: Instituto Nacional de Estadística.
- KCS: Keratitis seca
- LDA: Análisis Discriminante Lineal.
- NS: No Suplementación.
- OS: Ojo Seco.
- OSDI: Ocular Surface Disorder Index.
- PCA: Análisis de Componentes Principales.
- PLS-DA: Análisis Discriminante por mínimos cuadrados.
- PLS: Análisis por mínimos cuadrados Principales.
- PVD: Pantalla de Visualización de Datos.
- RMN: Resonancia Magnética Nuclear.
- +S: Suplementación.
- SOS: Síndrome de Ojo Seco.
- SPV: Síndrome Pantalla de Visualización.
- TNF: Factor de Necrosis Tumoral.
- TIC: Tecnología de la Información y Comunicación.
- UV: Ultravioleta.
- VEGF: Factor de Crecimiento Endotelial Vascular.

Anexo II

Cuestionario OSDI

Ocular Surface Disease Index© (OSDI©)[2]

Ask your patients the following 12 questions, and circle the number in the box that best represents each answer. Then, fill in boxes A, B, C, D, and E according to the instructions beside each.

Have you experienced any of the following *during the last week*?	All of the time	Most of the time	Half of the time	Some of the time	None of the time
1. Eyes that are sensitive to light?	4	3	2	1	0
2. Eyes that feel gritty?	4	3	2	1	0
3. Painful or sore eyes?	4	3	2	1	0
4. Blurred vision?	4	3	2	1	0
5. Poor vision?	4	3	2	1	0

Subtotal score for answers 1 to 5 (A)

Have problems with your eyes limited you in performing any of the following *during the last week*?	All of the time	Most of the time	Half of the time	Some of the time	None of the time	N/A
6. Reading?	4	3	2	1	0	N/A
7. Driving at night?	4	3	2	1	0	N/A
8. Working with a computer or bank machine (ATM)?	4	3	2	1	0	N/A
9. Watching TV?	4	3	2	1	0	N/A

Subtotal score for answers 6 to 9 (B)

Have your eyes felt uncomfortable in any of the following situations *during the last week*?	All of the time	Most of the time	Half of the time	Some of the time	None of the time	N/A
10. Windy conditions?	4	3	2	1	0	N/A
11. Places or areas with low humidity (very dry)?	4	3	2	1	0	N/A
12. Areas that are air conditioned?	4	3	2	1	0	N/A

Subtotal score for answers 10 to 12 (C)

Add subtotals A, B, and C to obtain D (D = sum of scores for all questions answered) (D)

Total number of questions answered (do not include questions answered N/A) (E)

Please turn over the questionnaire to calculate the patient's final OSDI© score.

Anexo III

Cuestionario de auto-evaluación para identificar el Síndrome de Ojo Seco.

Usually I become aware of my eyes when exposed to:	**In general, I resent of my eyes with the following symptoms:**	**I try to meliorate my eye disturbing sensations by:**
Sunlight	Dryness	Coliriums
Brightness	Soreness	Home remedies
Wind	Itchiness	Tear substitutive eye drops
Heaters/Ventilators	Tired eyes	Saline solutions
Air Conditioning	Foreign body sensation	Vitamin eye drops
Fumes/Pollution	Photofobia	Antioxidant oral supplementation
Smoke	Burning	Essential fatty acid supplementation
Tv/Computer Screens	Redness	Clossing eyes
Reading	Blurred vision	Restricting activities
Driving at night	Visual loss	Nothing
Total Score 1st Column	*Total Score 2nd Column*	*Total Score 3rd Column*

Being aware of the integrity of the ocular surface in people exposed to computer screens during the working time. 30-item self-scoring questionnaire for adults.

Converting each response to numerical data as follows: each affirmative answer has to be marked with 1 while the negative ones as 0. Ranking 4 or more in each of the three columns (or obtaining a global score of 12 or more) it can be convenient to visit an eye doctor.