UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

Departamento de Bioquímica y Biología Molecular

TESIS DOCTORAL

Estrés oxidativo en el síndrome Down

MEMORIA PARA OPTAR AL GRADO DE DOCTOR
PRESENTADA POR

Carlos Campos Vaquero

DIRECTOR:

Ángela, dir Casado Moragon

Madrid, 2015

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

Departamento de Bioquímica y Biología Molecular I

ESTRÉS OXIDATIVO EN EL SÍNDROME DE DOWN

Memoria para optar al grado de Doctor
presentada por

CARLOS CAMPOS VAQUERO

Bajo la dirección de la Doctora

Ángela Casado Moragón

Centro de Investigaciones Biológicas, CSIC

Abril de 2011

Este trabajo ha sido realizado en el Departamento de Medicina Celular y Molecular del Centro de Investigaciones Biológicas (CIB, CSIC) y financiado por la Fundación Inocente, Inocente.

Centros colaboradores:

FUNDACIÓN SÍNDROME DE DOWN

Hospital Infantil Universitario Niño Jesús

SaludMadrid

Comunidad de Madrid

María Corredentora

Asociación de empleados de Iberia Padres de Minusválidos

C.P.E.E. Príncipe de Asturias de Aranjuez

C.P.E.E. Buenafuente de Madrid

C.P.E.E. Francisco del Pozo de Madrid

Fundación Nuestra Señora del Camino

« La investigación de las enfermedades ha avanzado tanto que cada vez es más difícil encontrar a alguien que esté completamente sano. »

Aldous Huxley

« Las proposiciones matemáticas, en cuanto tienen que ver con la realidad, no son ciertas; y en cuanto que son ciertas, no tienen que ver con la realidad. »

Albert Einstein

« Estuve tan ocupado escribiendo la crítica que nunca pude sentarme a leer el libro. »

Groucho Marx

AGRADECIMIENTOS

Siempre pensé que algún día escribiría estas líneas y sería uno de los momentos más felices de mi vida. Sin embargo, hecho la vista atrás y son tantos los buenos ratos y tantas las personas con los que los he compartido, que no es tanta la felicidad como la melancolía la que me invade. Después de 5 años de trabajo, en los que tanto he crecido, personal y profesionalmente, han sido muchas las personas que lo han hecho posible, y a las cuales tanto quiero y debo agradecer.

En primer lugar quisiera agradecer a mi directora de tesis, Ángela Casado, por darme la oportunidad de embarcarme en este mundo que llaman Ciencia. Por todo lo que me has enseñado, en lo científico y, más importante, en lo personal. Por tu gran paciencia, confianza y apoyo constantes. A ti te debo llegar al final de esta etapa con la "mochila llena".

A mi compañero de laboratorio y de viaje, Rodrigo, y amigo, Rorro. Hace ya más de 5 años de aquella llamada que ahora, por fin, te agradezco en el alma. Gracias también por todo lo demás, esto lo he disfrutado gracias a ti.

Y a Marien, por tu optimismo contagioso, y por toda tu ayuda; solo te faltó traerme unos percebes.

Al personal técnico de los servicios del CIB: química de proteínas, citometría de flujo, cromatografía de gases... por su ayuda y dedicación. En especial a Nieves Fonturbel, por los millones de artículos; ahora podré montar mi propia Biblioteca...

Por supuesto, a la Fundación Inocente, Inocente y a todos los voluntarios y centros colaboradores, en especial al C.P.E.E. Príncipe de Asturias, que por algo es de mi pueblo, y a Lorenzo y su familia. Nada de esto hubiera sido posible sin vuestra gran ayuda.

Un agradecimiento especial merecen todos mis compañeros y amigos del CIB & rurales & .... A Asier, Isa y Dina, con quien pase mis mejores momentos en el laboratorio. A Kike, Dey y Óscar, por tantas sepias y entrecots compartidos. Y por supuesto, a Eva y a Rubén, mis fieles y eficaces consejeros burocráticos estos últimos meses. No me olvido de Ana Kris, Lu, Mikel, Elisa, Afri y Blanco, muchos años de vecinos y ni una derrama. Los efedepés: Natalia, Patri V., Ana, Esther y

Alberto; Rubenes: Vir, Gonn, Blas, Javier, Ife, Iria, Juan y Andrés; y al resto de amigos rurales y demás chavalería: Cris, Ángela, Sergio, Raquel, Noemí, Carol, Marta, Edu, Rocío, Javi, Leo, Laura, Luque, Maripaz, Fer, Tere, Luis, Andrea, Benet, Fátima, Darío, Agustín, Inés, Mónica, Amaia y Karem. Y a los que ya no estáis, pero seguís ahí, aunque sea en Callejeros: Ernesto, Afri, Marian, Maricarmen, Fernando, Úrsula, Jimena, Esti, Gaizka, Carmen... Sois muy grandes. Gracias a todos y a todas, por vuestro apoyo y ayuda, pero sobretodo por vuestra amistad y vuestros disfraces. Gracias a vosotros esta etapa ha sido aún más especial.

A Víctor, Alberto, Julia y Sara, mis hermanos y hermanas de Rufus T. Firefly, mi válvula de escape, que no saben muy bien qué hago con mi vida, pero que, sin saberlo, tanto contribuyeron a que esto terminara y bien. Gracias por soportarme, sobretodo estos últimos meses. Y a Santi, filósofo universal, por sus consejos.

A mis compañeros de Casbega, que tanto me enseñasteis, de cocacolas y de la vida misma.

Y por último, a los más importantes. Los que lo vivieron más de cerca. A mis padres, que me dieron la moral de seguir adelante, por su cariño y deseo de felicidad. A mi hermana, que veo en ella todo lo que a mí me falta. Y a Noelia, que da sentido a mi vida, por su amor y paciencia infinitos. Os quiero.

"Soy consciente de la que decisión es irreversible.
Prefiero no tener que pensar
mientras cuento las mañanas sin final
y enferma mi curiosidad..."

Rufus T. Firefly

ÍNDICE DE CONTENIDOS

ÍNDICE DE FIGURAS

ABREVIATURAS

3-DN	3-deoxiglucosona
8-OHdG	8-hidroxi-2'-deoxiguanosina
ADA	adenosina deaminasa
ADN	ácido desoxirribonucleico
AGEs	productos finales de glicación avanzada (*advanced glycation end-products*)
AOPP	productos avanzados de oxidación proteica (*advanced oxidation protein products*)
AU	ácido úrico
BCS	sal disódica del ácido 2,9-dimetil-4,7-difenil-1,10-fenantrolindisulfónico (*bathocuproinedisulfonic acid disodium sal*)
CAT	catalasa
CEL	N-ε-(carboxietil)lisina
CML	N-ε-(carboximetil)lisina
COX	ciclooxigenasa
Cr	creatinina
CUPRAC	ensayo de reducción del cobre(II) o capacidad antioxidante de reducción del ión cúprico (*cupric ion reducing antioxidant capacity*)
CUPRAC-BCS	ensayo de reducción del cobre(II) utilizando sal disódica del ácido 2,9-dimetil-4,7-difenil-1,10-fenantrolindisulfónico (*copper(II) reduction assay using bathocuproinedisulfonic acid disodium salt*)
DiTyr	ditirosina
DPPH	ensayo 2,2-difenil-1-picrilhidrazil (*2,2-diphenyl-1-picrylhydrazyl assay*)
DOLD	dímero deoxiglucasona-lisina
ELISA	ensayo inmunoabsorbente ligado a enzimas (*enzyme-linked immunosorbent assay*)
FRAP	ensayo de capacidad del plasma de reducir el ión férrico (*ferric ion reducing ability of plasma*)
GOLD	dímero glioxal-lisina
GPx	glutation peroxidasa
GR	glutation reductasa
GSH	glutation reducido
GSSG	glutation oxidado
H_2O_2	peróxido de hidrógeno
$HO^{\bullet}$	radical hidroxilo
$HO_2^{\bullet}$	radical hidroperoxilo
HPLC	cromatografía líquida de alta resolución (*high-performance liquid cromatography*)

JaICA	*Japan Institute for the Control of Aging*
LOESS	*locally weighted scatter plot smooth*
LOOH	hidroperóxidos lipídicos
MOLD	dímero metilglioxal-lisina
MPO	mieloperoxidasa
NAD^+	nicotinamida adenina dinucleótido
NADH	nicotinamida adenina dinucleótido en forma reducida
$NADP^+$	nicotinamida adenina dinucleótido fosfato
NADPH	nicotinamida adenina dinucleótido fosfato en forma reducida
$NO^\bullet$	óxido nítrico
NOx	nitritos y nitratos
$O_2^{\bullet-}$	radical superóxido
OEPM	oficina española de patentes y marcas
$ONOO^-$	peroxinitrito
OxLDL	lipoproteínas de baja densidad oxidadas
PAO	poder antioxidante
$R^\bullet$	radical libre
ROOH	hidroperóxidos orgánicos
ROS	especies reactivas del oxígeno (*reactive oxygen species*)
RNS	especies reactivas del nitrógeno (*reactive nitrogen species*)
SD	síndrome de Down
SOD1	Cu/Zn superóxido dismutasa
SOD2	Mn superóxido dismutasa
TAC	capacidad antioxidante total (*total antioxidant capacity*)
TAC^{-AU}	capacidad antioxidante total sin la contribución relativa del ácido úrico
TBARS	sustancias reactivas del ácido tiobarbitúrico (*thiobarbituric acid-reacting substances*)
TEAC	ensayo de capacidad antioxidante equivalente de Trolox (*Trolox equivalent antioxidant capacity*)
UAF	unidades arbitrarias de fluorescencia
XDH	xantina deshidrogenasa
XO	xantina oxidasa
XOR	xantina oxidoreductasa

Resumen

El síndrome de Down (SD), causado principalmente por la presencia de una copia extra del cromosoma 21, es la anomalía cromosómica más frecuente observada en la especie humana. Los individuos con SD presentan una gran variedad de manifestaciones clínicas, tales como: defectos congénitos cardíacos, alteraciones del sistema inmune y endocrino, retraso mental, aparición temprana de la enfermedad de Alzheimer o un mayor riesgo de leucemia. Se ha observado, además, que los individuos con SD presentan un elevado estrés oxidativo y se ha sugerido que podría ser debido principalmente a un exceso de la actividad de la enzima Cu/Zn superóxido dismutasa (SOD1). La SOD1 está codificada por el gen *SOD1* situado en el cromosoma 21, si bien existe una larga lista de genes en éste cromosoma que también podrían estar implicados en el estatus oxidativo de los individuos con SD.

El estrés oxidativo en el SD se ha valorado en multitud de trabajos mediante la determinación de algunos biomarcadores. Sin embargo, muchos de estos biomarcadores no han sido analizados todavía en el SD, otros han sido poco estudiados y su determinación esta limitada a muestras de sangre, en grupos de edad de rango reducido y en muestras poblacionales pequeñas. Además, el set de biomarcadores determinados en cada estudio es, en general, muy limitado.

Por todo ello, en el presente trabajo de Tesis Doctoral, se han evaluado una serie de biomarcadores de estrés oxidativo en muestras de orina, por las ventajas que éstas presentan en estudios poblacionales. Los biomarcadores analizados han sido: 8-hidroxi-2'-deoxiguanosina (8-OHdG) (biomarcador de daño oxidativo al ADN), isoprostano 15-F_{2t}-IsoP y sustancias reactivas del ácido tiobarbitúrico (TBARS) (biomarcadores de peroxidación lipídica), productos finales de glicación avanzada (AGEs) (biomarcador de glicooxidación), ditirosina (biomarcador de daño oxidativo a proteínas y de estrés nitrosativo), peróxido de hidrógeno (H_2O_2), nitritos y nitratos (NOx), ácido úrico (AU), capacidad antioxidante total (TAC) y capacidad antioxidante sin la contribución del ácido úrico (TAC^{-UA}).

Los niveles de estos biomarcadores se analizaron en 104 individuos con SD, 31 de ellos estaban recibiendo tratamiento para el hipotiroidismo, y 84 controles. Se

establecieron los siguientes grupos de edad: 1) menores de 10 años, 2) entre 11 y 14 años, 3) entre 15 y 19 años, 4) entre 20 y 40 años, y 5) mayores de 40 años.

Entre los resultados más significativos: solo se obtuvieron diferencias significativas entre niños con SD y controles en los niveles de AU y TAC, siendo éstos mayores en SD. Por otra parte, AGEs, ditirosina, H_2O_2 y NOx podrían ser utilizados como biomarcadores de estrés oxidativo y/o nitrosativo en muestras de orina de adolescentes y adultos con SD, en contraposición a 8-OHdG, 15-F_{2t}-IsoP y TBARS. Se observó, además, un estrés oxidativo incrementado, con mayores niveles de AGES y ditirosina, en individuos con SD que recibían tratamiento para hipotiroidismo, si bien estos resultados podrían estar influidos por una posible disfunción renal en base a los menores niveles de creatinina obtenidos para estos individuos.

Para la determinación de TAC se evaluó una metodología, cuya realización solo podía realizarse mediante un *kit* comercial. Además, ya que se observó que los niveles de AU podrían influir en los resultados de TAC, se desarrollo un método para la determinación de TAC^{-UA}, que se presentó en la Oficina Española de Patentes y Marcas.

Por otra parte, en el presente trabajo también se realizó una revisión de la literatura relacionada con los estudios del estrés oxidativo en el SD, dado que éstos no han sido críticamente revisados y discutidos en profundidad.

Introducción

INTRODUCCIÓN

1. El síndrome de Down

El síndrome de Down (SD), o trisomía 21, es la alteración cromosómica más común observada en la especie humana, con una incidencia estimada a nivel mundial de 1 por cada 700-1000 nacimientos vivos (Hook, 1981; Mikkelsen, 1977; Sherman *et al.*, 2007). Fue clínicamente descrito por John Langdon Hayden Down en 1866 (Down, 1866), si bien no fue hasta casi un siglo después cuando, en 1959, Lejeune y colaboradores (1959) establecieron la asociación entre el SD y una copia extra del cromosoma 21 (Figura 1). En años sucesivos, se fueron encontrando otras formas cromosómicas de presentación del síndrome: trisomía por translocación del 21 a un cromosoma del grupo D (13-15), frecuentemente el 14, y trisomía por translocación del 21 a un cromosoma del grupo G (21-22). A estas formas se sumaron los casos de mosaicismo. Así, el 90-95% de los casos de SD es debido a trisomía regular (o completa) del cromosoma 21, el 2-6% a algún tipo de translocación y el 2-4% a mosaicismo (Hook, 1981; Mikkelsen, 1977; Sherman *et al.*, 2007).

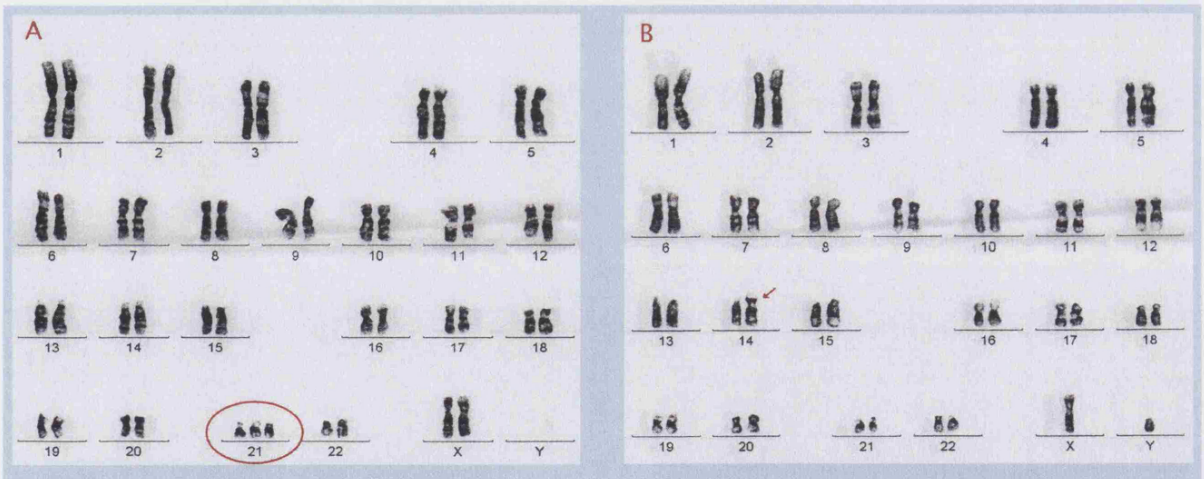


Figura 1. Cariotipos de individuos con síndrome de Down. A: mujer con trisomía regular del cromosoma 21 (♀, 47 XX,+21), y B: varón con translocación Robertsoniana entre los cromosomas 14 y 21,der (14;21), originada por fusión céntrica de dichos cromosomas (♂, 46,XY,der (14;21) (q10;q10)).

Los individuos con SD presentan unos rasgos fenotípicos muy característicos que aparecen en grado variable como, por ejemplo: corta estatura, pelvis y orejas displásicas, occipuccio plano, boca y nariz pequeñas, surco simiesco y epicanto.

Además de los rasgos físicos, el SD esta asociado con una gran variedad de características clínicas, tales como: defectos congénitos cardíacos, hipotonia, alteraciones del sistema inmune y endocrino, problemas digestivos o un mayor riesgo de leucemia, siendo una de las causas principales de retraso mental. Por otro lado, a pesar de que el SD es una de las condiciones genéticas más complejas compatible con una larga supervivencia, estos individuos manifiestan un envejecimiento prematuro y presentan con mayor frecuencia un gran número de patologías asociadas con la edad, como: aparición temprana de la enfermedad de Alzheimer, cataratas, osteoporosis, etc. (Hasle *et al.*, 2000; Pueschel, 1990). Sin embargo, el SD se puede considerar como un síndrome genético más que como una enfermedad, siendo la expresión fenotípica final muy variable de unas personas a otras. De hecho, ninguna de las características está presente en todos estos individuos, con excepción del retraso mental y la hipotonía. Se sabe también que ninguno de los rasgos observados en los individuos con SD es constante o patognomónico de este trastorno cromosómico. Es decir, no hay ninguna anomalía específica o única del SD (Shapiro, 1983).

Se ha propuesto que las alteraciones fenotípicas que caracterizan al SD podrían ser el resultado de un incremento de la expresión de genes específicos localizados en el cromosoma 21, así como de un desequilibrio entre estos genes y aquellos localizados en el resto de cromosomas. Teniendo en cuenta que numerosos genes localizados en el cromosoma 21 están implicados en el estado redox celular, un desequilibrio en su regulación debería generar estrés oxidativo. De hecho, se ha observado que los individuos con SD presentan un elevado estrés oxidativo (Jovanovic *et al.*, 1998; Pallardó *et al.*, 2006) que podría contribuir a la aparición de las numerosas patologías asociadas al síndrome así como también a la senescencia precoz o acelerada observada en estos individuos.

2. Estrés oxidativo y radicales libres

El término **estrés oxidativo** fue definido por Sies (1991a) como *"la perturbación en el balance entre agentes oxidantes y antioxidantes en la célula, en favor de los primeros"*. Posteriormente, se ha definido el estrés oxidativo como *"la alteración del control y la señalización redox"* (Jones, 2006) y al **daño oxidativo** como *"el daño biomolecular causado*

por el ataque de especies reactivas sobre los constituyentes de los organismos vivos" (Halliwell y Gutteridge, 2007; Halliwell y Whiteman, 2004). Los agentes oxidantes, principalmente especies reactivas del oxígeno (ROS) y del nitrógeno (RNS) (Tabla 1), se forman principalmente como resultado del metabolismo celular aeróbico. De esta forma, cuando la generación de ROS y/o de RNS excede la capacidad de los sistemas de defensa antioxidante para eliminarlos, se origina un desequilibrio que puede causar daño a constituyentes celulares (ADN, proteínas y lípidos) (Halliwell y Gutteridge, 2007; Sies, 1991b), siendo el grado de desequilibrio el que determinará el grado de estrés oxidativo y/o nitrosativo. El estrés oxidativo depende no solo de la agresividad química del propio oxidante, sino también de la cantidad de estos y del tiempo de exposición, así como del tipo de tejido que sufra el efecto y de la eficacia de las defensas antioxidantes disponibles (Finkel y Holbrook, 2000; Sies, 1991a).

Tabla 1. Algunas de las especies reactivas de mayor interés en el estudio del estrés oxidativo.

Radicales	**No radicales**
Especies reactivas del oxígeno (ROS)	
Superóxido, $O_2^{\bullet -}$	Peróxido de hidrógeno, H_2O_2
Hidroxilo, $HO^{\bullet}$	Peroxinitrito, $ONOO^-$
Hidroperoxilo, $HO_2^{\bullet}$	Ácido hipocloroso, HOCl
Peroxilo, $ROO^{\bullet}$	Ozono, O_3
Alcoxilo, $RO^{\bullet}$	Oxígeno singlete, $^1\Delta gO_2$
Carbonato, $CO_3^{\bullet -}$	Peróxidos orgánicos, ROOH
Dióxido de carbono, $CO_2^{\bullet -}$	
Especies reactivas del nitrógeno (RNS)	
Óxido nítrico, $NO^{\bullet}$	Peroxinitrito, $ONOO^-$
Dióxido de nitrógeno, $NO_2^{\bullet}$	Ácido nitroso, HNO_2
	Catión nitrosilo, NO^+
	Anión nitrosilo, NO^-
	Ácido peroxinitroso, ONOOH
	Peroxinitritos alkilo, ROONO
Especies reactivas del cloro (RCS)	
Cloro atómico, $Cl^{\bullet}$	Ácido hipocloroso, HOCl
	Cloraminas

Un radical libre es cualquier especie capaz de existencia independiente que contiene uno o más electrones desapareados (Halliwell y Gutteridge, 2007). Así, de

entre todos los agentes oxidantes, los radicales libres son las especies más reactivas y, por tanto, con un mayor potencial para causar daño oxidativo. Sin embargo, hay que tener en cuenta que las ROS, RNS y demás especies reactivas, son también importantes mediadores y reguladores de multitud de procesos fisiológicos, incluyendo: la señalización celular, la regulación redox de la transcripción de genes, la inmunidad celular y la apoptosis (Auten y Davis, 2009), siendo esenciales para la función fisiológica normal de las células. Por tanto, el equilibrio entre agentes oxidantes y antioxidantes es fundamental para el mantenimiento de la homeostasis.

Las ROS son generadas en las células por diversos procesos, principalmente por la mitocondria, como resultado de la reducción de O_2 en la cadena de transporte electrónico mitocondrial (Kowaltowski *et al.*, 2009) (Figura 2).

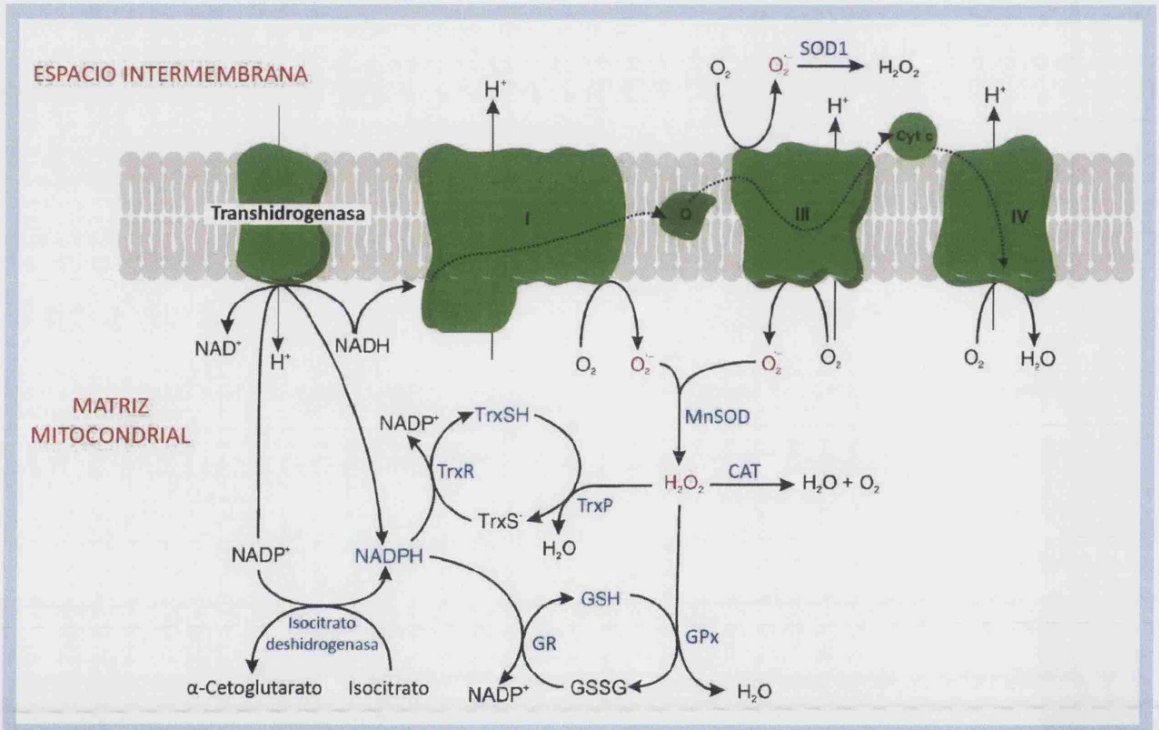


Figura 2. Metabolismo mitocondrial de las ROS. El radical superóxido ($O_2^{\bullet-}$) se forma por la reducción monoelectrónica del O_2, principalmente en los complejos I y III de la cadena respiratoria. $O_2^{\bullet-}$ es dismutado a peróxido de hidrógeno (H_2O_2) por la enzima Cu/Zn superóxido dismutasa (SOD1) en el espacio intermembrana mitocondrial y por la Mn superóxido dismutasa (MnSOD) en la matriz. H_2O_2 puede ser metabolizado por catalasa (CAT) o peroxidasas como la glutation peroxidasa (GPx) y la tiorredoxina peroxidasa (TrxP), utilizando como substratos glutation reducido (GSH) y tiorredoxina (TrxSH), respectivamente. Los productos oxidados del glutation (GSSG) y la tiorredoxina ($TrxS^-$) son reducidos por sus respectivas reductasas (glutation reductasa (GR) y tiorredoxina reductasa (TrxR), respectivamente), utilizando NADPH como fuente de electrones. $NADP^+$ se puede mantener en forma reducida por la actividad de la $NAD^+/NADP^+$ transhidrogenasa, con transporte de protones a la matriz, proporcionando una relación entre el potencial de membrana interno y la capacidad redox mitocondrial. Q = coenzima Q, Cyt c = citocromo c. [Adaptada de Kowaltowski y colaboradores (2009)].

Además, otras fuentes endógenas, como: la activación de los leucocitos, reacciones enzimáticas (como la catalizada por la xantina oxidorreductasa (XOR)) y el metabolismo de xenobióticos, así como diversas fuentes exógenas, como: la dieta, el ejercicio físico, iones metálicos, contaminantes, radiaciones o el consumo de tabaco, alcohol y determinados medicamentos, pueden dar lugar a la formación de ROS.

3. Biomarcadores de estrés oxidativo

Dado que la vida media de las ROS es extremadamente corta y los métodos disponibles para su detección requieren de equipamiento muy costoso y difícil de manejar, la valoración del daño oxidativo se realiza principalmente mediante la determinación de biomarcadores de estrés oxidativo. Los biomarcadores son síntomas físicos o medidas de laboratorio que se dan en asociación con una enfermedad específica y tienen un uso diagnóstico y pronóstico (Lesko y Atkinson, 2001). Idealmente, los atributos de un biomarcador deberían incluir los siguientes: 1) relevancia clínica, 2) especificidad y sensibilidad a los efectos de tratamientos, 3) fiabilidad, 4) practicabilidad y 5) simplicidad (Lesko y Atkinson, 2001). Algunos de ellos son indicadores directos de daño oxidativo, como los isoprostanos o la 8-hidroxi-2'-deoxiguanosina (8-OHdG), mientras que otros miden las defensas antioxidantes disponibles frente a la oxidación o los productos de oxidación de diversos antioxidantes no enzimáticos (Dalle-Donne *et al.*, 2006; Niki, 2010) (Figura 3).

En cualquier caso, el estado redox a nivel subcelular es una realidad compleja que no se puede medir ni definir con un solo parámetro aislado. De hecho, ninguno de los biomarcadores de estrés oxidativo disponibles consigue de forma aislada una valoración precisa y definitiva del estrés oxidativo que pueda ser directamente aplicado a la clínica humana (Pryor y Godber, 1991). Por ello, no es de extrañar que se hayan utilizado una gran diversidad de métodos en la aproximación a esta realidad del estado redox en el organismo humano.

Una de las principales dificultades en la evaluación del papel de los oxidantes en situaciones patológicas es la carencia de medidas precisas del estrés oxidativo *in vivo* (Gutteridge y Halliwell, 1990), siendo muchos de los métodos empleados poco específicos. Por otra parte, la determinación de biomarcadores empleando técnicas

sensibles y de elevada especificidad, como la cromatografía de gases o de masas, ofrecen una mayor garantía que otras como los ensayos inmunoabsorbentes ligados a enzimas o *enzyme-linked immunosorbent assay* (ELISA).

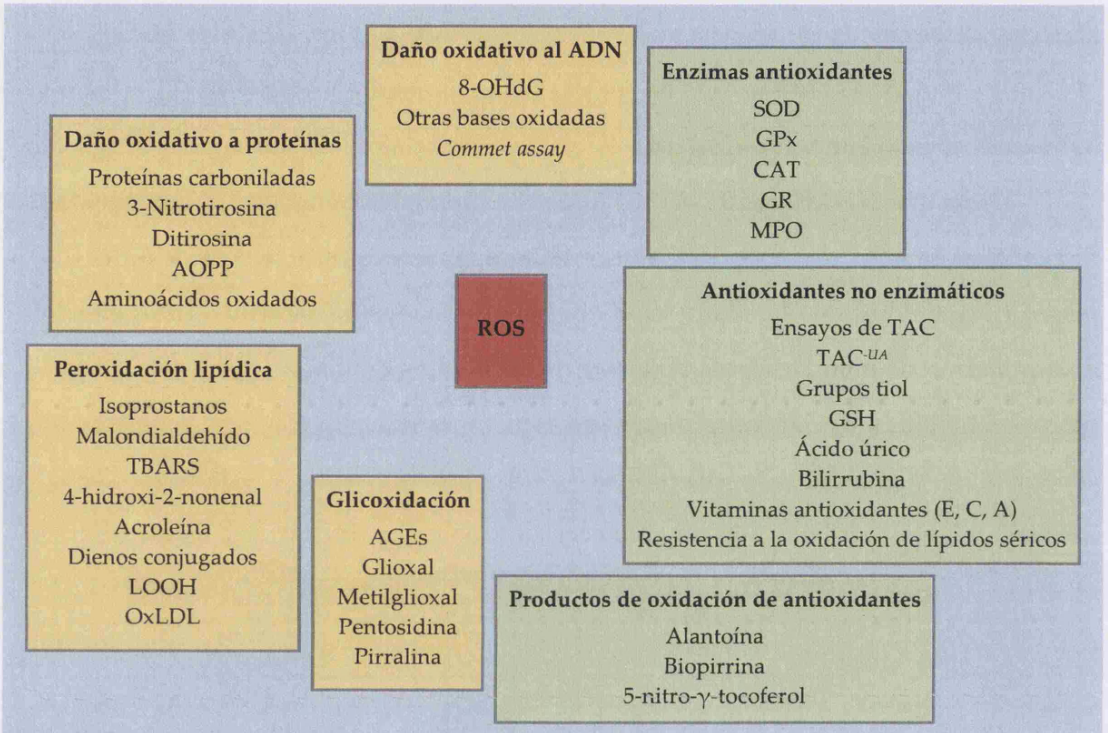


Figura 3. Ejemplos de biomarcadores de estrés oxidativo. 8-OHdG, 8-hidroxi-2'-deoxiguanosina; AGEs, productos finales de glicación avanzada; AOPP, productos avanzados de oxidación proteica; CAT, catalasa; GPx, glutation peroxidasa; GR, glutation reductasa; GSH, glutation reducido; LOOH, hidroperóxidos lipídicos; OxLDL, lipoproteínas de baja densidad oxidadas; MPO, mieloperoxidasa; ROS, especies reactivas del oxígeno; SOD, superóxido dismutasa; TAC, capacidad antioxidante total; TAC^{-AU}, capacidad antioxidante total sin la contribución relativa del ácido úrico; TBARS, sustancias reactivas del ácido tiobarbitúrico.

3.1. Biomarcadores de daño oxidativo al ADN

Hay una amplia evidencia sobre el hecho de que el ADN, aún siendo una molécula bien protegida, es atacada permanentemente por las ROS en las células vivas (Cadet *et al.*, 2010) (Figura 4). El radical hidroxilo ($HO^{\bullet}$) reacciona con todos los componentes de la molécula de ADN: el esqueleto de la desoxiribosa y las bases nitrogenadas. El ataque de $HO^{\bullet}$ al ADN da como resultado una gran variedad de productos de oxidación, siendo la interacción de $HO^{\bullet}$ con guanina la que genera 8-

OHdG (Valko *et al.*, 2004), aunque el ataque de otros radicales como el peroxinitrito ($ONOO^-$) puede también dar lugar a la formación de 8-OHdG (Kasai, 1997).

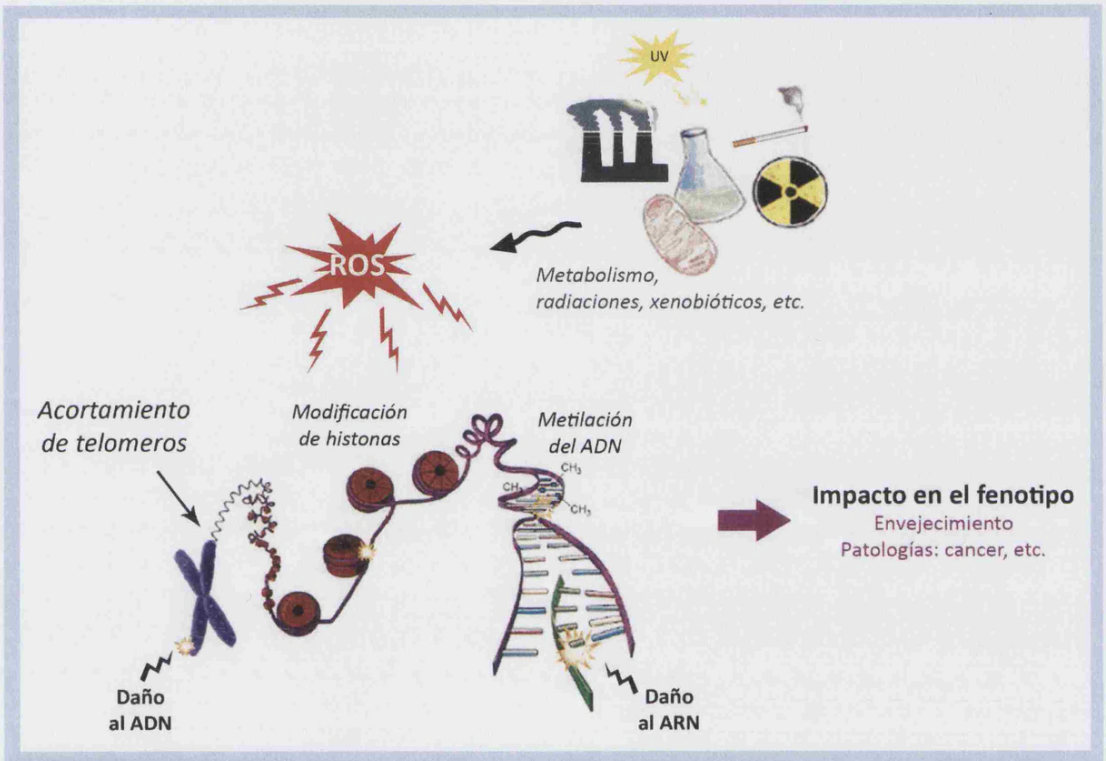


Figura 4. Vías de daño oxidativo a los ácidos nucleicos. Las especies reactivas del oxígeno (ROS), generadas endógenamente (metabolismo aeróbico, citocromo P450, etc.) o exógenamente (radiaciones, exposición a contaminantes, etc.), pueden dar lugar a daño oxidativo tanto al ADN como al ARN. En los cromosomas, las ROS pueden contribuir al acortamiento de los telómeros, los cuales parecen ser muy susceptibles al ataque de las ROS por su alto contenido en guaninas. Así mismo, son capaces de producir cambios en las histonas e inducir la metilación del ADN. Todo ello puede dar lugar, en último término, a envejecimiento o carcinogénesis.

De entre todos los productos de oxidación del ADN, la 8-OHdG (Figura 5) es el más estudiado, principalmente debido a que es el más abundante y a que es un mutágeno (Kasai, 1997). También porque se excreta en la orina, tras la reparación *in vivo* por exonucleasas del ADN dañado (Loft *et al.*, 1992), obviando la limitación que entraña la obtención de tejidos de los que poder obtener ADN. Así, la 8-OHdG ha sido utilizada para valorar el daño oxidativo al ADN "corporal" (Dalle-Donne *et al.*, 2006).

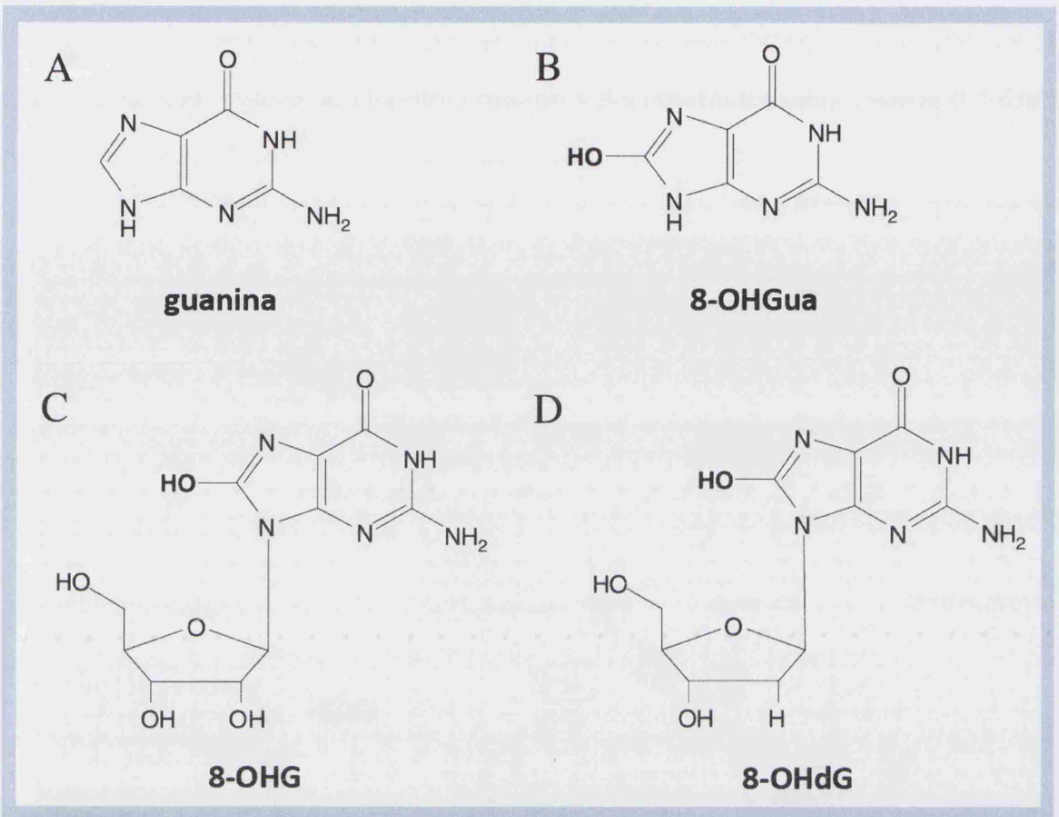


Figura 5. Estructura química de la base nitrogenada guanina y sus derivados de oxidación principales. A, Guanina; B, 8-hidroxiguanina (8-OHGua); C, 8-hidroxiguanosina (8-OHG); D, 8-hidroxi-2'-deoxiguanosina (8-OHdG).

La determinación de 8-OHdG puede ser realizada por técnicas cromatográficas, espectrometría de masas o por ELISA, aunque todas ellas presentan diversos problemas, bien de especificidad o bien problemas con diversos contaminantes (Evans *et al.*, 2010).

Otra metodología a destacar para la valoración del daño oxidativo al ADN es el ensayo del cometa. Es menos empleada que la valoración de 8-OHdG por ser una técnica más compleja y la metodología a seguir mucho más larga, si bien el ensayo del cometa tiene la ventaja de que detecta el daño al ADN en células individuales, normalmente linfocitos (Tice *et al.*, 2000). Además, se puede emplear para detectar una gran variedad de tipos de daño al ADN, tales como roturas de hebra sencilla o algunos tipos específicos de daño oxidativo mediante la utilización de enzimas de reparación de lesiones específicas.

3.2. Biomarcadores de peroxidación lipídica

Todo proceso de peroxidación de macromoléculas ocurre en tres etapas bien definidas: iniciación, propagación y terminación (Tabla 2). Es un proceso autopropagante que finaliza por la reacción de radicales contiguos, mediante la fragmentación del ácido graso o por la acción de los antioxidantes.

Tabla 2. Etapas del proceso peroxidativo *in vivo*.

Iniciación:	$R^{\bullet} + LH \rightarrow RH + L^{\bullet}$
Propagación:	$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$
	$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$
Terminación:	$L^{\bullet}\ AH \rightarrow LH + A^{\bullet}$
	$A^{\bullet} + LOO^{\bullet} \rightarrow LOO\text{-}A$

$R^{\bullet}$, radical libre; L, lípido; A, antioxidante.

Una de las moléculas más sensibles al ataque de los radicales libres son los ácidos grasos poliinsaturados cuya lesión oxidativa se denomina peroxidación lipídica, o lipoperoxidación, y resulta especialmente relevante cuando se afectan los lípidos constitutivos de las membranas biológicas, pues da lugar a alteraciones en las propiedades biofísicas de la membrana (permeabilidad, fluidez, integridad, etc.) pudiendo inactivar enzimas o receptores de membrana. Además, la generación de productos aldehído secundarios altamente reactivos (como el 4-hidroxi-2-nonenal) puede amplificar el daño celular debido a su capacidad para modificar covalentemente biomoléculas críticas. Por tanto, la medida de productos de peroxidación lipídica es muy utilizada para valorar el estrés oxidativo (Montuschi *et al.*, 2004).

Entre los biomarcadores más ampliamente utilizados se encuentran las sustancias reactivas del ácido tiobarbitúrico (TBARS), el malondialdehído (MDA), el 4-hidroxi-2-nonenal y los isoprostanos, entre otros. La valoración del MDA se lleva usando más de 4 décadas como biomarcador de peroxidacíon lipídica, siendo el TBARS el método analítico más empleado para su determinación. Sin embargo, su uso esta limitado debido a que presenta problemas de especificidad, entre otros.

De todos los biomarcadores de lipoperoxidación, los isoprostanos son, actualmente y desde hace ya varios años, los que más auge están teniendo. Existen multitud de isoprostanos que se han dividido en varias clases. Así, los F_2-isoprostanos, y en particular el 15-F_{2t}-Isop (también conocido como 8-*epi*-$PGF_{2\alpha}$, 8-*iso*-$PGF_{2\alpha}$ o $iPF_{2\alpha}$-

III), son los más conocidos y estudiados. El 15-F_{2t}-Isop es uno de los isoprostanos más abundantes, generado en tejidos y en fosfolípidos de las membranas celulares por el ataque de las ROS al ácido araquidónico (Figura 6), si bien ha sido descrito que este isoprostano también podría ser formado como un producto minoritario de las ciclooxigenasas (COX) en plaquetas y monocitos (Basu, 2008; Pratico *et al.*, 1994). Por otro lado, además de considerarse un buen biomarcador de estrés oxidativo, 15-F_{2t}-Isop está implicado en diversas funciones biológicas.

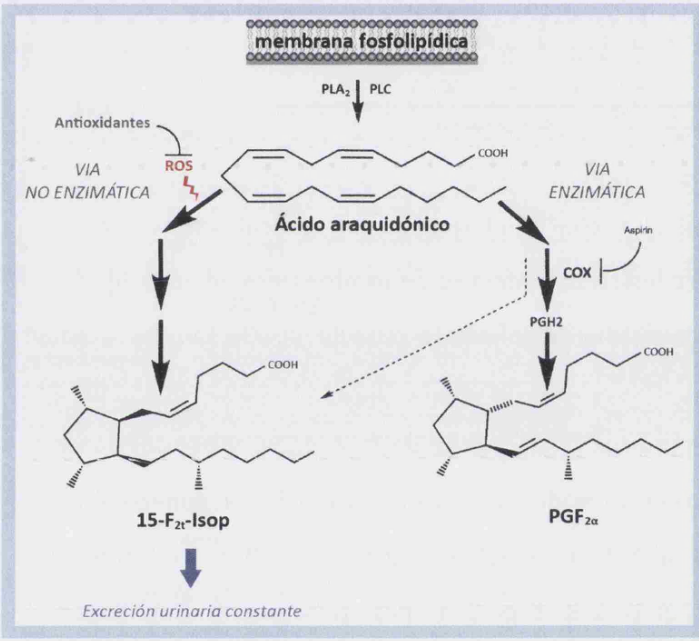


Figura 6. Vías metabólicas del ácido araquidónico y formación del isoprostano 15-F_{2t}-Isop. El ácido araquidónico, una vez liberado de la membrana fosfolipídica por la fosfolipasa A_2 (PLA2) o C (PLC), puede ser degradado vía enzimática mediante ciclooxigenasas (COX), que generarían prostaglandinas y tromboxanos, o no enzimática mediante el ataque de especies reactivas del oxígeno (ROS). El isoprostano 15-F_{2t}-Isop se formaría por la vía no enzimática o, de forma minoritaria, como producto de las COX. [Adaptada de Meydani (1997)].

3.3. Biomarcadores de daño oxidativo a proteínas

La modificación oxidativa de proteínas se inicia principalmente por el ataque de $HO^{\bullet}$; sin embargo, el proceso de oxidación esta determinado por la disponibilidad de O_2 y radical superóxido ($O_2^{\bullet -}$) o de su forma protonada, el radical hidroperoxilo ($HO_2^{\bullet}$) (Berlett y Stadtman, 1997). Todas las cadenas laterales de los aminoácidos que forman parte de las proteínas son susceptibles de ser atacadas por ROS, pudiendo conducir a modificaciones en la estructura terciaria y, por tanto, a la pérdida de la función biológica. Así, el daño a proteínas puede ocurrir por el ataque directo de ROS, pero también por un "daño secundario" que implica el ataque por productos de peroxidación lipídica, como el 4-hidroxi-2-nonenal. Además, el daño a proteínas puede

dar lugar, a su vez, a "daño secundario" a otras biomoléculas, por ejemplo aumentando los niveles de Ca^{2+} y activando nucleasas.

Como biomarcador de oxidación proteica más utilizado esta la determinación de grupos carbonilo. Los grupos carbonilo (aldehídos y cetonas), producidos en las cadenas laterales de las proteínas (principalmente de los aminoácidos prolina, arginina, lisina y treonina) cuando son oxidadas, son químicamente estables, permitiendo su uso como biomarcadores. Sin embargo, en los últimos años, el interés por la valoración del daño oxidativo a proteínas mediante la determinación de otros biomarcadores, como la 3-nitrotirosina o la ditirosina (diTyr), esta aumentando debido a los buenos resultados que se están obteniendo en multitud de estudios. Además, tanto la 3-nitrotirosina como la diTyr, pueden ser considerados no solo como biomarcadores de estrés oxidativo sino también de estrés nitrosativo. Los metabolitos activos del óxido nítrico ($NO^{\bullet}$) pueden reaccionar con $O_2^{\bullet -}$ dando lugar a peroxinitrito ($ONOO^-$), un potente agente oxidante y nitrante, el cual puede afectar tanto a la dimerización como la nitración de tirosina, dando lugar a la acumulación tanto de 3-nitrotirosina como de diTyr (Hensley *et al.*, 1998) (Figura 7).

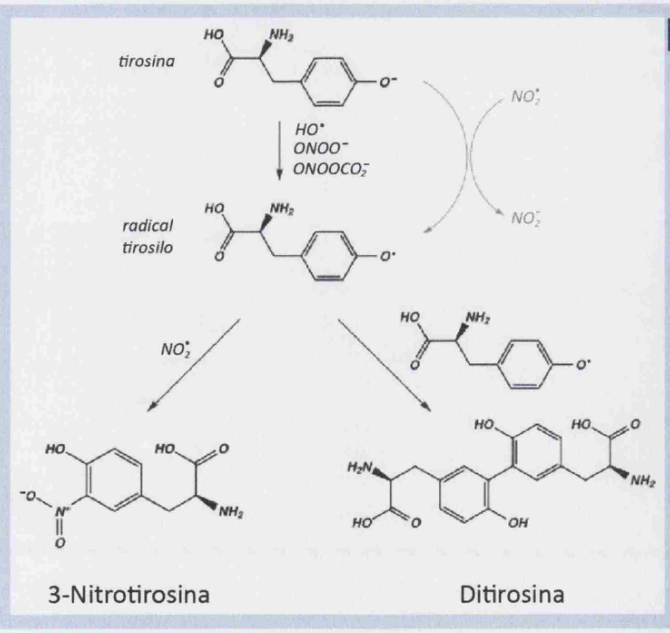


Figura 7. Mecanismos principales de formación de 3-nitrotirosina y ditirosina. La ditirosina es atacada por especies reactivas (hidroxilo ($HO^{\bullet}$), peroxinitrito ($ONOO^-$), nitrosoperoxicarbonato ($ONOOCO_2^-$) o dióxido de nitrógeno ($NO_2^{\bullet}$)) dando lugar al radical tiroxilo. Una vez generadas, tanto la 3-nitrotirosina como la ditirosina se excretan a través de la orina. Sin embargo, la nitrotirosina, a diferencia de la ditirosina, puede ser incorporada a la síntesis *de novo* de proteínas. [Adaptada de DiMarco y Giulivi (2007)].

3.4. Biomarcadores de glicoxidación

Los azúcares reductores, como la glucosa, reaccionan con los grupos amino de las proteínas, lípidos y ácidos nucleicos dando lugar a la formación de bases de Shiff y a los productos Amadori y, en último término, a compuestos covalentes conocidos como AGEs (productos finales de glicación avanzada) (Figura 8). La formación de AGEs comprende reacciones de deshidratación, condensación cíclica, entrecruzamientos intermoleculares y oxidación por ROS. Así, la glicoxidación es la combinación de las reacciones de glicación y oxidación, la cual genera productos que pueden ser especialmente aterogénicos (Baynes y Thorpe, 2000). Este proceso, conocido como reacción de Maillard, fue descrito a principios del siglo pasado cuando se observó que los aminoácidos calentados en presencia de azúcares reductores desarrollaban un color marrón-amarillo característico.

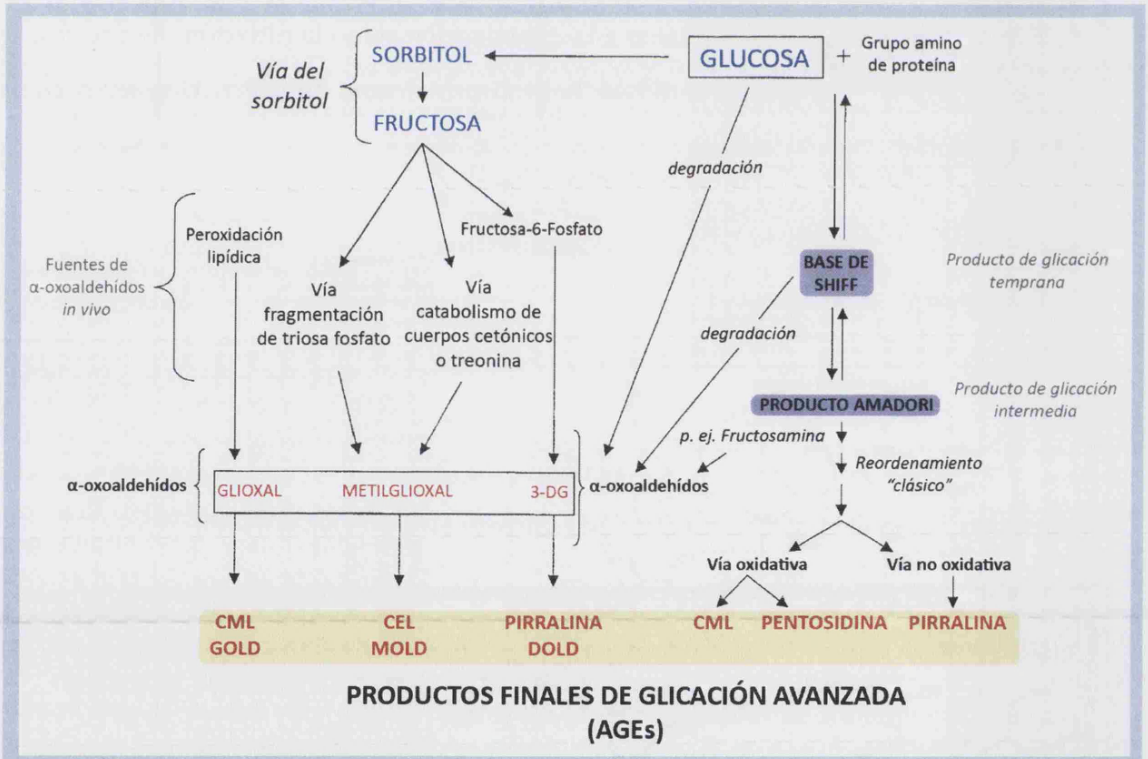


Figura 8. Formación de productos de glicación avanzada (AGEs). Vías de formación de AGEs. 3-DN, 3-deoxiglucosona; CEL, N-ε-(carboxietil)lisina; CML, N-ε-(carboximetil)lisina; DOLD, dímero deoxiglucasona-lisina; GOLD, dímero glioxal-lisina; MOLD, dímero metilglioxal-lisina. [Adaptada de Singh y colaboradores (2001)].

Los AGEs producen un gran número de efectos sobre células y tejidos, estando implicados en complicaciones asociadas a la diabetes. Así, causan estrés oxidativo disminuyendo la función de una de las principales enzimas antioxidantes del organismo, la Cu/Zn superóxido dismutasa (SOD1), y aumentando la producción de ROS, aumentan la vasoconstricción reduciendo el $NO^{\bullet}$ y aumentando la endotelina-1, producen cambios estructurales en el cristalino pudiendo dar lugar a la aparición de cataratas, etc. (Singh *et al.*, 2001). Además, el estrés oxidativo esta implicado en la formación acelerada de AGEs, por lo que estos compuestos son considerados biomarcadores de estrés oxidativo.

3.5. Antioxidantes

Los organismos aerobios han desarrollado sistemas eficientes de defensa antioxidante frente al estrés oxidativo a lo largo de la evolución. Los antioxidantes pueden ser definidos como todas aquellas sustancias que, presentes a bajas concentraciones en comparación con el sustrato susceptible de ser oxidado, retrasan o inhiben significativamente la oxidación de ese sustrato (Halliwell, 1997).

3.5.1. Tipos de defensa antioxidante

Los sistemas de defensa antioxidante pueden ser enzimáticos o no enzimáticos. Los antioxidantes enzimáticos constituyen la primera línea de defensa antioxidante, previniendo el daño interaccionando directamente con las ROS. La segunda línea de defensa antioxidante estaría representada por los antioxidantes no enzimáticos, los cuales pueden prevenir el daño oxidativo interaccionando tanto directa como indirectamente con las ROS. En una tercera línea de defensa antioxidante se encuentran varias enzimas, cuya función sería la de reparar y reconstituir la pérdida de función ocasionada por el daño oxidativo. Y, por último, una cuarta línea de defensa la formarían todos aquellos mecanismos de adaptación frente al estrés oxidativo (Niki, 2010). Además, hay un interés creciente en varios micronutrientes como el selenio y el zinc, ya que son importantes como constituyentes de determinadas enzimas antioxidantes, en aminoácidos específicos como la selenocisteina o la selenometionina,

o en componentes estructurales como los dedos de zinc (motivos estructurales de proteínas) o la zinc-metalotioneina.

Entre los antioxidantes enzimáticos se pueden destacar la SOD1, la glutation peroxidasa (GPx), la catalasa (CAT) o la glutation reductasa (GR):

- **SOD1** (EC 1.15.1.1): es una metaloenzima clave en el metabolismo de las ROS pues cataliza la conversión del radical superóxido ($O_2^{\bullet -}$) en peróxido de hidrógeno (H_2O_2). El gen *SOD1* se expresa constitutivamente en todos los tejidos y se localiza en el cromosoma 21. Así, los individuos con SD tienen sobreexpresado este gen y, por ello, una elevada actividad de la enzima SOD1.

- **GPx** (EC 1.11.1.9): es una enzima que cataliza la reducción de hidroperóxidos (H_2O_2 y ROOH) empleando glutation reducido (GSH) como donador de electrones. Presenta una forma enzimática dependiente de selenio, (elemento que es esencial para su actividad y que controla la síntesis de la proteína), y elimina hidroperóxidos tanto orgánicos (ROOH) como inorgánicos (H_2O_2). La otra forma no contiene selenio y sólo es capaz de eliminar ROOH. Ambas formas enzimáticas están presentes tanto en el citosol como en las mitocondrias.

- **CAT** (EC 1.11.1.6): es una metaloenzima que se encuentra ampliamente distribuida en el organismo humano, principalmente en el hígado, aunque en el tejido nervioso su actividad es prácticamente nula. A nivel celular se localiza en las mitocondrias y los peroxisomas, mientras que en los eritrocitos se encuentra en el citosol. Cataliza la descomposición del H_2O_2 en O_2 y H_2O, teniendo una alta capacidad de reacción pero relativamente poca afinidad por el sustrato.

- **GR** (1.6.4.2): reduce una molécula de glutation oxidado (GSSG) a dos de GSH, a expensas de la oxidación de NADPH. Esta enzima es de vital importancia para el funcionamiento de la eliminación de H_2O_2 por la vía de la GPx, así como para el mantenimiento del nivel de GSH intracelular.

Estas enzimas son las más empleadas en la valoración del estrés oxidativo. Así, una disminución en la actividad de alguna de ellas causaría un posible desequilibrio entre oxidantes y antioxidantes, es decir, un aumento del estrés oxidativo. Más correctamente, sería el ratio entre la actividad de SOD1 (generadora de H_2O_2) y la suma de las actividades de GPx y CAT la medida que mejor podría utilizarse como biomarcador de estrés oxidativo. Así, un ratio elevado daría lugar a la acumulación de H_2O_2 indicando un aumento del estrés oxidativo. Otras enzimas antioxidantes, que también se encargan de eliminar el H_2O_2, serían la mieloperoxidasa (MPO), las peroxirreductasas y la Mn superóxido dismutasa (SOD2).

Por otra parte, de entre la gran variedad de antioxidantes no enzimáticos, los más importantes por su actividad antioxidante y/o por las funciones biológicas que desempeñan, y que además se emplean como biomarcadores de estrés oxidativo, serían:

- **Glutation (GSH)**: es el antioxidante celular más abundante. Es un tripéptido cuyo enlace peptídico le confiere resistencia al ataque por peptidasas, y su grupo –SH reactivo, la capacidad de actuar como antioxidante. Actúa como cofactor de la GPx en la detoxificación de H_2O_2. Además, es capaz de interaccionar directamente con los radicales superóxido ($O_2^{\bullet -}$), hidroxilo ($HO^{\bullet}$), alcoxilo ($RO^{\bullet}$) y peroxilo ($ROO^{\bullet}$), así como con el ácido hipocloroso (HOCl). De hecho, su determinación es habitual en estudios de valoración del estrés oxidativo, para lo cual también se suele realizar el ratio entre los niveles de GSSG y GSH.

- **Ácido úrico (AU)**: el AU es uno de los antioxidantes de bajo peso molecular más importantes y abundantes en los fluidos biológicos, siendo capaz de eliminar $HO^{\bullet}$, $ROO^{\bullet}$ y oxígeno singlete ($^1\Delta gO_2$), e inhibir radicales generados por la descomposición de peroxinitrito ($ONOO^-$) (Glantzounis *et al.*, 2005). Existen dos fuentes de AU, una endógena por destrucción de tejidos del propio organismo y otra exógena proveniente de la alimentación. Se encuentra en todos los fluidos biológicos así como en el interior de las células. Ayuda a

proteger las membranas celulares, los eritrocitos, el ácido hialurónico y el ADN de la oxidación por ROS. Otra propiedad antioxidante importante del AU es la capacidad para formar complejos estables con iones de hierro, impidiendo reacciones tipo Fenton. Este proceso inhibe Fe^{3+}, que cataliza la oxidación del ácido ascórbico y la peroxidación lipídica, por lo que se ha sugerido que el AU es un protector del ácido ascórbico (Wayner *et al.*, 1987). Sin embargo, su uso como biomarcador de estrés oxidativo esta limitado, debido a que es generado en condiciones de isquemia-reperfusión (por ejemplo, tras realizar ejercicio físico intenso), por la enzima XOR. Esta enzima existe en dos formas, la xantina deshidrogenasa (XDH) y la xantina oxidasa (XO). En condiciones fisiológicas, la XOR se encuentra en la forma XDH (con mayor afinidad por NAD^+ que por el oxígeno), pero en condiciones de isquemia se produce un aumento en la conversión de XDH a XO (que reduce oxígeno y no NAD^+), la cual da lugar a la formación de $O_2^{\bullet -}$ (Figura 9), si bien se ha visto que la XDH también puede producir este radical libre (Glantzounis *et al.*, 2005; Sanders *et al.*, 1997). En este proceso, el AU generado podría servir como antioxidante local.

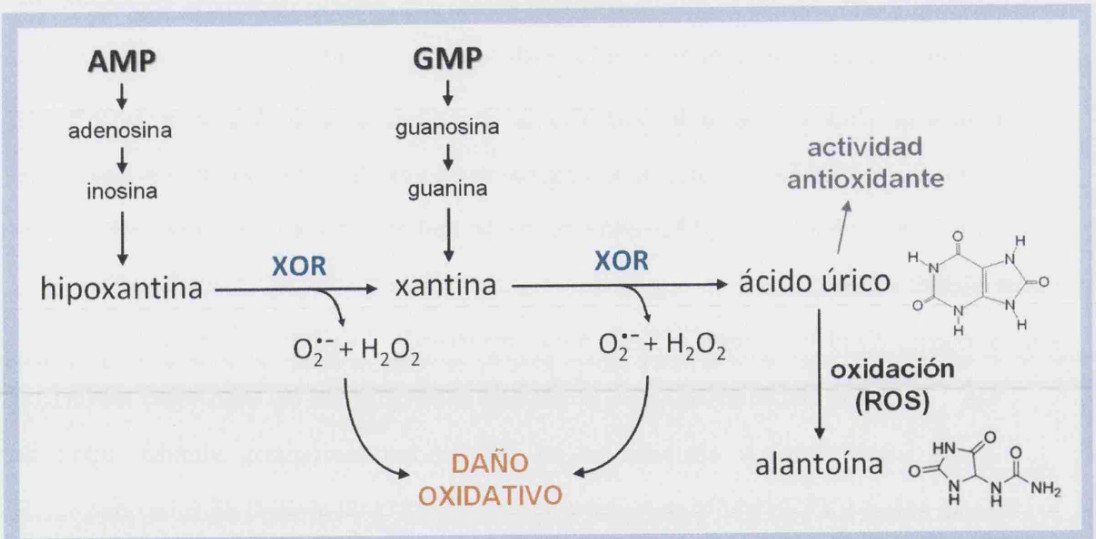


Figura 9. Esquema de la ruta metabólica de formación y degradación del ácido úrico en la especie humana. La xantina oxidoreductasa (XOR) es la única enzima en el organismo capaz de sintetizar ácido úrico. XOR reduce oxígeno o NAD^+ generando radical superóxido ($O_2^{\bullet -}$) y peróxido de hidrógeno (H_2O_2). La especie humana carece de la enzima urato oxidasa, por lo que el ácido úrico es su producto final en el metabolismo de las purinas (adenina y guanina). Su oxidación a alantoína solo tiene lugar por el ataque de especies reactivas del oxigeno (ROS).

En la especie humana, el AU es el producto final del metabolismo de las purinas, debido a que carecemos de la enzima urato oxidasa (EC 1.7.3.3) (Wu *et al.*, 1992), por lo que se ha sugerido que el producto de oxidación del AU, la alantoína, podría ser utilizado como biomarcador de estrés oxidativo (Zitnanová *et al.*, 2004).

- **α-tocoferol (vitamina E)**: es una molécula liposoluble que es transportada por las lipoproteínas plasmáticas. Ejerce su principal efecto antioxidante interrumpiendo la reacción en cadena de la peroxidación lipídica inducida por ROS, siendo el principal antioxidante en las membranas biológicas. La vitamina E incluye dos grupos de compuestos, los tocoferoles y los tocotrienoles, cada uno con los cuatro α-, β-, δ- y γ- análogos (Ricciarelli *et al.*, 2001). De todos ellos, el α-tocoferol es el más activo y ampliamente distribuido, siendo prácticamente la única forma activa en humanos.

- **Ácido ascórbico (vitamina C)**: es un antioxidante hidrosoluble que debe ser aportado exógenamente. Es capaz de reducir el radical semiquinona del α-tocoferol, los peróxidos y $O_2^{\bullet -}$, entre otras ROS.

3.5.2. Interacción entre antioxidantes

Algunos antioxidantes pueden evitar la oxidación de moléculas biológicas interaccionando directamente con otros antioxidantes, lo que se conoce como efecto sinérgico de los antioxidantes. Esto es, los antioxidantes reaccionan con ROS convirtiéndose a su vez en radicales menos reactivos, de forma que existen diversos sistemas antioxidantes que actúan de forma sinérgica reduciéndose los unos a los otros disminuyendo la reactividad del radical antioxidante formado, recuperando así el antioxidante reducido (Vertuani *et al.*, 2004). La interacción conocida más eficiente es la sinergia entre α-tocoferol y ácido ascórbico (Figura 10).

Por otra parte, se ha visto que el AU no reduce el radical semiquinona del α-tocoferol para regenerar α-tocoferol ni inhibe la acción prooxidante del α-tocoferol

(Niki, 2010), pero sí parece actuar como protector del ácido ascórbico (Wayner *et al.*, 1987).

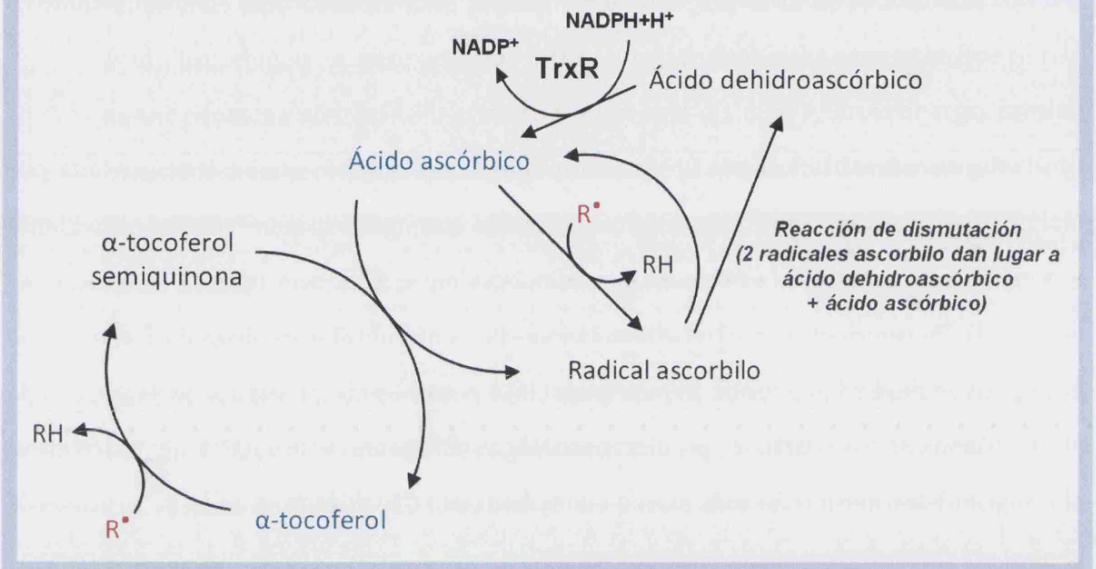


Figura 10. Interacción entre ácido ascórbico y α-tocoferol. El α-tocoferol puede reaccionar con radicales libres ($R^{\bullet}$), o especies reactivas del oxígeno, y transformarse en el correspondiente radical semiquinona, que puede atacar lípidos poliinsaturados e inducir otra cadena de oxidación. El ácido ascórbico puede reducir el radical semiquinona del α-tocoferol para regenerar α-tocoferol antes de que pueda producirse el ataque a los lípidos. Así, el ácido ascórbico se convierte a su vez en radical ascorbilo. A partir de dos de estos radicales, mediante dismutación espontánea, se puede formar una molécula de ácido dehidroascórbico y otra de ácido ascórbico, mientras que la tiorredoxina reductasa (TrxR) se encarga de reducir el ácido dehidroascórbico regenerando ácido ascórbico. [Adaptada de Nordberg y Arnér (2001)].

3.5.3. Capacidad antioxidante total

Debido a la dificultad de medir todos los antioxidantes de una muestra por separado, se han desarrollado multitud de metodologías para la determinación de lo que se ha llamado capacidad antioxidante total (TAC), tales como: el FRAP (capacidad del plasma de reducir el ión férrico), el TEAC (capacidad antioxidante equivalente de Trolox), el ensayo de quimioluminiscencia o el CUPRAC (ensayo de reducción del cobre(II) o capacidad antioxidante de reducción del ión cúprico) entre otros (Karadag *et al.*, 2009). Estas metodologías son muy sencillas, rápidas y económicas, pero, además, tienen la capacidad de considerar las posibles interacciones entre los diferentes antioxidantes, lo cual podría hacer que la determinación de antioxidantes de forma

individual sea menos representativa del estatus antioxidante general del individuo. Por ello, la TAC, que se define como la medida de la cantidad (en moles) de una ROS eliminada por los antioxidantes no enzimáticos presentes en una muestra (MacDonald-Wicks *et al.*, 2006), debe proporcionar una información más relevante para el estudio del estrés oxidativo que la obtenida de determinar cada antioxidante separadamente.

Entre las metodologías disponibles para la determinación de TAC, todas presentan alguna limitación. Así, las hay que requieren de reactivos poco estables, en otras la reacción no transcurre a pH fisiológico, algunas presentan problemas de interferencias, etc. De hecho, no existe ninguna metodología ampliamente aceptada para su aplicación a una razonable variedad de matrices.

3.6. Biomarcadores de estrés oxidativo en el síndrome de Down

El estrés oxidativo en el SD se ha valorado mediante la determinación de algunos biomarcadores de estrés oxidativo en multitud de trabajos (Carratelli *et al.*, 2001; Casado *et al.*, 2007; Jovanovic *et al.*, 1998; Muchová *et al.*, 2001; Pallardó *et al.*, 2006; Praticò *et al.*, 2000; Zitnanová *et al.*, 2004). Sin embargo, muchos biomarcadores no han sido analizados todavía en el SD, otros han sido poco estudiados y su determinación esta limitada a muestras de sangre y en grupos reducidos tanto en edad como en número de individuos. Además, el set de biomarcadores determinados en cada estudio es, en general, muy reducido. Por otro lado, a pesar de que los resultados obtenidos para cualquier biomarcador deben de ser interpretados de forma distinta según la edad de la población estudiada, se pueden encontrar multitud de trabajos en los que analizan conjuntamente individuos en edad infantil con adultos. De hecho, con frecuencia se encuentran discrepancias en la literatura, de forma que muchas cuestiones sobre la relación existente entre el estrés oxidativo y el SD permanecen todavía sin aclarar. Por tanto, es evidente la necesidad de realizar más investigaciones respecto al estrés oxidativo en este síndrome, que, por otra parte, bien podría servir de modelo para el estudio de multitud de patologías.

4. Causas del estrés oxidativo en el síndrome de Down

Se ha sugerido que el estrés oxidativo incrementado en el SD es debido principalmente a un exceso de la actividad de la enzima SOD1, que está codificada por el gen *SOD1* situado en el cromosoma 21 (región 21q22.1). Así, en el SD, la expresión de este gen y la actividad de SOD1 están incrementadas aproximadamente un 50% (Anneren y Edman., 1993; De La Torre *et al.*, 1996; Sinet, 1982). La SOD1 es una enzima antioxidante y un exceso de ella debería ser beneficioso para el organismo. Sin embargo, se ha visto que ratones transgénicos que sobreexpresan *SOD1* muestran un elevado estrés oxidativo, que es atribuido a la generación de $HO^{\bullet}$ en cerebro y músculos (Peled-Kamar *et al.*, 1997). La SOD1, localizada en el citosol celular y en el espacio intermembrana mitocondrial (Okado-Matsumoto y Fridovich, 2001), cataliza la dismutación de $O_2^{\bullet -}$ (Halliwell y Gutteridge, 2007), según la siguiente ecuación:

$$2\,O_2^{\bullet -} + 2\,H^+ \xrightarrow{\text{SOD1}} O_2 + H_2O_2$$

El H_2O_2 generado, que es citotóxico, es descompuesto por las enzimas GPx y CAT. En el SD, debido al exceso de SOD1, el ratio SOD1/(GPx+CAT) estaría aumentado dando lugar a una acumulación de H_2O_2 no metabolizado. El H_2O_2 no es un radical libre, pero tiene capacidad para inducir estrés oxidativo (Coyle *et al.*, 2006). Gran parte de los efectos citotóxicos del H_2O_2 son debidos a su conversión en $HO^{\bullet}$, que posee elevada reactividad para reaccionar con macromoléculas y producir daño celular (de Haan *et al.*, 1997; Halliwell y Gutteridge, 2007), vía reacción de Fenton o de Haber-Weiss:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + OH^- \text{ (reacción de Fenton)}$$
$$O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + HO^{\bullet} + OH^- \text{ (reacción de Haber-Weiss)}$$

En definitiva, un aumento en la actividad de SOD1 puede perturbar el equilibrio de las ROS en las células causando daño oxidativo en moléculas biológicas tales como ADN, proteínas y lípidos, aunque en el SD existen otras vías que se encuentran alteradas y también podrían dar lugar a una mayor producción de ROS (Figura 11).

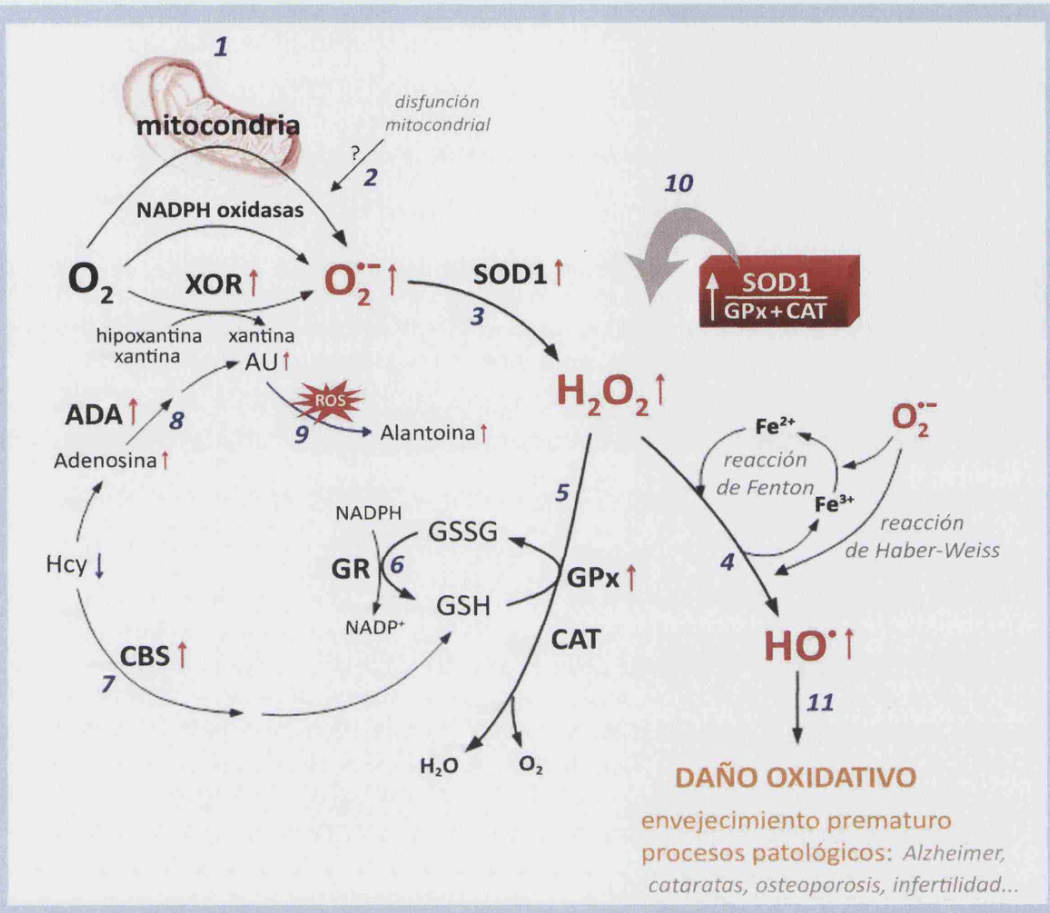


Figura 11. Vías principales de formación de especies reactivas del oxígeno (ROS) en el síndrome de Down (SD). Las flechas rojas y azules indican niveles elevados o reducidos encontrados en individuos con SD, si bien en algunos casos los estudios son insuficientes y en otros hay discrepancias. En aquellos casos en los que estas discrepancias son muy notables o en los que no se han encontrado diferencias entre SD y controles, el metabolito o enzima aparece sin flecha.

1: El radical superoxido ($O_2^{\bullet-}$) es formado por el proceso de reducción del oxígeno molecular mediado por NADPH oxidasas y xantina oxidoreductasa (XOR) o de forma no enzimática en la cadena de transporte de electrones mitocondrial. **2**: En el SD se ha visto una disfunción mitocondrial que, en principio, origina una mayor producción de $O_2^{\bullet-}$. Sin embargo, esto podría dar lugar a una respuesta adaptativa (conocida como hormesis o mitohormesis) por la que aumentarían las defensas del organismo frente a las ROS. **3**: La Cu/Zn superóxido dismutasa (SOD1) cataliza la dismutación de $O_2^{\bullet-}$ a peróxido de hidrógeno (H_2O_2). **4**: H_2O_2 puede dar lugar al radical hidroxilo ($HO^{\bullet}$) vía reacción de Fenton o de Haber-Weiss. **5**: H_2O_2 es descompuesto por las enzimas glutation peroxidasa (GPx) y catalasa (CAT). **6**: El glutation oxidado (GSSG) es reducido (GSH) por la enzima glutation reductasa (GR), que utiliza NADPH como donador de electrones. **7**: A través de la vía de la transulfuración, la enzima cistationina-β-sintetasa (CBS) sintetiza cistationina a partir de homocisteína (Hcy). La cistationina liasa metaboliza la cistationina a cisteína, la cual es precursora de GSH. **8**: La enzima adenosina deaminasa (ADA) cataliza la desaminación de adenosina a inosina en el metabolismo de las purinas. La adenosina puede también proceder de la hidrólisis de S-adenosilhomocisteína (a adenosina y Hcy) en la vía de la transulfuración. **9**: La especie humana, entre otras, carece de la enzima urato oxidasa. Por ello, la oxidación de ácido úrico se debe únicamente al ataque de las ROS. Así, su producto de oxidación, la alantoina, es considerado un biomarcador de estrés oxidativo. **10**: Es un elevado ratio SOD1/(GPx+CAT), y no solo una mayor actividad de SOD1, el que debe dar lugar a un aumento en los niveles de H_2O_2. Este ratio se ha visto que esta aumentado en SD en varios estudios, aunque otros no encontraron diferencias significativas entre SD y controles. **11**: La mayor producción de ROS ($O_2^{\bullet-}$, H_2O_2 y $HO^{\bullet}$) debe dar lugar a estrés oxidativo (oxidantes > antioxidantes) y, consecuentemente, a daño oxidativo, siendo $HO^{\bullet}$ la especie potencialmente más dañina por su elevada reactividad.

Se sabe que el estrés oxidativo está implicado en los mecanismos patofisiológicos de multitud de enfermedades (Dalle-Donne *et al.*, 2006) y se piensa que debe jugar un papel muy importante en la patogénesis del SD. Sin embargo, todavía no esta clara la medida en la que el aumento en el ratio SOD1/(GPx+CAT) influye sobre el estrés oxidativo en el SD. Así, quizá otros factores puedan estar influyendo, en mayor o menor grado, a elevar el estrés oxidativo en estos individuos. De hecho, se han descrito posibles alteraciones de la función mitocondrial en el SD (Arbuzova *et al.*, 2002). Además, en el presente trabajo de Tesis Doctoral, se han identificado cerca de 20 genes localizados en el cromosoma 21 que han sido relacionados con el estrés oxidativo y deben estar también implicados en el estrés oxidativo en el SD.

Justificación y objetivos

JUSTIFICACIÓN Y OBJETIVOS

El síndrome de Down (SD) es la anomalía genética más frecuente y la causa más común de discapacidad mental. Además, las personas con SD tienen una probabilidad algo superior a la de la población general de padecer algunas patologías, especialmente de corazón, sistema digestivo y sistema endocrino, y presentan también una senilidad prematura.

Hay evidencias sustanciales sobre la presencia de un estrés oxidativo anormalmente elevado en individuos con SD, que podría ser, al menos en parte, la causa de la patogénesis que presentan. El estudio del estatus oxidativo se realiza mediante la determinación de biomarcadores de estrés oxidativo, existiendo un gran número de investigaciones y trabajos publicados al respecto. Sin embargo, las causas de este estrés oxidativo inusual en el SD están todavía por dilucidar, en parte debido al gran número de resultados controvertidos.

Normalmente, el estudio de biomarcadores de estrés oxidativo en el SD se ha realizado en muestras de sangre (plasma o suero), siendo los estudios en muestras de orina muy poco frecuentes. Sin embargo, este tipo de muestras presenta un gran número de ventajas frente a las de sangre. Así, las muestras de orina no requieren de técnicas invasivas, son más adecuadas para la determinación de ciertos biomarcadores como los isoprostanos o la ditirosina, etc.

De entre los biomarcadores de estrés oxidativo, la capacidad antioxidante total es uno de los que más interés esta suscitando en los últimos años. Sin embargo, el ácido úrico presenta una contribución relativa muy elevada en la capacidad antioxidante total, y además es generado en condiciones de isquemia-reperfusión (por ejemplo, tras realizar ejercicio físico intenso). Por ello, la determinación de la capacidad antioxidante total sin la contribución relativa del ácido úrico podría ser una determinación mucho más relevante para el estudio del estatus antioxidante y el estrés oxidativo en estudios experimentales, epidemiológicos o clínicos.

Por otro lado, se pueden encontrar en la literatura científica numerosas revisiones sobre la relación entre el estrés oxidativo y enfermedad para la mayoría de las patologías conocidas, pero no existen para el SD.

En base a lo expuesto, los objetivos generales de la presente Tesis Doctoral fueron:

1. Revisión y actualización de la bibliografía existente en relación al estrés oxidativo en el SD.
2. Evaluación de biomarcadores de estrés oxidativo y nitrosativo en muestras de orina de individuos con SD.

y los objetivos particulares:

1. Evaluación y optimización del método CUPRAC para la determinación de la capacidad antioxidante total en muestras de orina y de plasma.
2. Desarrollo de una metodología para la determinación de la capacidad antioxidante total sin la contribución relativa del ácido úrico en muestras biológicas.

Resultados, materiales y métodos

RESULTADOS, MATERIALES Y MÉTODOS

Los resultados, materiales y métodos de este trabajo de Tesis Doctoral se encuentran descritos en los apartados correspondientes de los siguientes artículos:

1. **Campos, C.** y Casado, A. Oxidative stress in Down syndrome: A review. (Enviado a *"Free Radical Biology & Medicine"*).

2. **Campos, C.**, Guzmán, R., López-Fernández, E. y Casado, Á. (2009). Evaluation of the copper(II) reduction assay using bathocuproinedisulfonic acid disodium salt for the total antioxidant capacity assessment: The CUPRAC–BCS assay. *Anal Biochem* **392**:37-44.

3. **Campos, C.**, Guzmán, R., López-Fernández, E. y Casado, Á. (2010). Urinary uric acid and antioxidant capacity in children and adults with Down syndrome. *Clin Biochem* **43**:228-233.

4. **Campos, C.**, Guzmán, R., López-Fernández, E. y Casado Á. Evaluation of urinary biomarkers of oxidative/nitrosative stress in children with Down syndrome. (Enviado a *"Life Sciences"*, en evaluación).

5. **Campos, C.**, Guzmán, R., López-Fernández, E. y Casado, Á. Evaluation of urinary biomarkers of oxidative/nitrosative stress in adolescents and adults with Down syndrome. *BBA-Mol Basis Dis.* (In Press). DOI: 10.1016/j.bbadis.2011.03.013.

6. **Campos, C.**, Guzmán, R., López-Fernández, E. y Casado, Á. Método para valorar la concentración y la capacidad antioxidante del ácido úrico. Solicitud de patente en la Oficina Española de Patentes y Marcas (OEPM) nº P201030674.*

7. Resultados adicionales.

* Los materiales y métodos del método para valorar la concentración y la capacidad antioxidante del ácido úrico no se muestran en la presente Tesis Doctoral, debido a que aún no se ha resuelto su solicitud de patente.

C. Campos y Á. Casado

Cada vez son más numerosas las evidencias que indican que los individuos con síndrome de Down (SD) se encuentran bajo un estrés oxidativo anormalmente elevado, el cual debe estar implicado en la mayor prevalencia y severidad de un gran número de patologías asociadas con el síndrome, así como en el envejecimiento acelerado observado en estos individuos. Numerosas enfermedades parecen relacionadas con el daño oxidativo en su patogénesis y progresión. De hecho, existen en la literatura biomédica revisiones que tratan sobre la relación que existe entre el estrés oxidativo y enfermedad para casi todas las patologías conocidas. Sin embargo, los trabajos acerca del estrés oxidativo en el SD no han sido críticamente revisados y discutidos.

En este trabajo, se realiza una revisión crítica de la literatura científica sobre la relación entre estrés oxidativo y SD. Se analizan los resultados de los trabajos realizados con biomarcadores de estrés oxidativo y aquellos que estudian el efecto del ejercicio y terapias de prevención frente al daño oxidativo en individuos con SD. Se muestra una visión global de la relación entre el estrés oxidativo y diversas patologías que aparecen con mayor frecuencia en estos individuos. Además, se discuten las causas del fenotipo SD y se identifican cerca de 20 genes localizados en el cromosoma 21 e implicados en el estrés oxidativo.

Entre las evidencias que muestran un estrés oxidativo incrementado en el SD se pueden destacar: la sobreexpresión de un gran número de genes implicados en el estrés oxidativo, multitud de biomarcadores de estrés oxidativo que se han encontrado incrementados en individuos con SD y en modelos de ratón, o niveles intracelulares de radicales libres elevados y aumento de la apoptosis de neuronas en fetos con SD.

A pesar del elevado número de estudios valorando el estrés oxidativo en el SD, existen todavía multitud de cuestiones e hipótesis por determinar antes de poder comprender el papel que desempeña el estrés oxidativo en el fenotipo SD.

Review Article

Oxidative stress in Down syndrome: A review

Carlos Campos*, Ángela Casado

Department of Cellular and Molecular Medicine, Centre for Biological Research - Spanish National Research Council (CIB-CSIC). Madrid, Spain.

ABSTRACT

Increasing evidence has shown that Down syndrome (DS) individuals are under unusual increased oxidative stress, which may be involved in the higher prevalence and severity of a number of pathologies associated with the syndrome, as well as in the accelerate aging observed in these individuals.

In this work, we critically review the scientific literature concerning the link between oxidative stress and DS. We analyze the findings of studies concerning oxidative stress biomarkers, exercise-induced oxidative stress and oxidative damage prevention therapies in DS individuals. Moreover, we give an overview of the link between several pathologies and oxidative stress in DS. In addition, the causes of DS phenotype are discussed and the environment within an epigenetic context as another possible cause for DS phenotype is introduced. We also identified almost 20 genes located on chromosome 21 which are involved in oxidative stress.

Evidences for increased oxidative stress in DS include: gene dose overexpression of a number of genes involved in oxidative stress, increased biomarkers of oxidative stress in individuals with DS and in DS mice models, or increased intracellular free radicals and enhanced apoptosis in neurons of DS fetuses.

In spite of the large number of studies addressing oxidative stress in DS, many aspects remain to be elucidated and further research is still required to understand the role of oxidative stress in the DS phenotype.

Keywords: Down syndrome, oxidative stress, oxidative damage, Cu/Zn superoxide dismutase, biomarker, aging.

Contents

Abbreviations: 8-OHdG, 8-hydroxy-2'-deoxyguanosine; 8-OHG, 8-hydroxyguanosine; Aβ, β-amyloid; ADNF-9, peptide SALLRSIPA, derived from the activity-dependent neurotrophic factor ADNF; AGEs, advanced glycation end-products; APP, amyloid precursor protein; CAT, catalase; Cr, creatinine; CUPRAC, cupric ion reducing antioxidant capacity; DiTyr, dityrosine; DS, Down syndrome; ELISA, enzyme-linked immunosorbent assay; FRAP, ferric reducing ability of plasma; G6PDH, glucose-6-phosphate-dehydrogenase; GC-MS, gas chromatography-mass spectroscopy; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; HPLC, high-performance liquid chromatography; HSA21, human chromosome 21; JaICA, Japan Institute for the Control of Aging; LDL, low density lipoprotein; MDA, malondialdehyde; MPO, myeloperoxidase; NAP, peptide NAPVSIPQ, derived from the activity-dependent neuroprotective protein ADNP; NOx, total nitrite and nitrate; NS, not significant; PMNs, polymorphonuclear neutrophils; RNS, reactive nitrogen species; RONS, reactive oxygen and nitrogen species; ROS, reactive oxygen species; SGS-111, N-phenyl-acetyl-l-prolyl-glycine ethyl ester; SOD1, Cu/Zn superoxide dismutase; TAC, total antioxidant capacity; TAC^{-UA}, total antioxidant capacity without relative contribution of uric acid; TBARS, thiobarbituric acid-reacting substances; TEAC, Trolox equivalent antioxidant capacity; XO, xanthine oxidase.

* Corresponding author: Phone: +34 91 837 31 12. Fax: +34 91 536 04 32.
E-mail address: ccampos@cib.csic.es (C. Campos)

1. Introduction

Down syndrome (DS), often referred to as trisomy 21 due to the fact that it is caused mainly by the triplication of the whole or part of human chromosome 21 (HSA21), is found to be one of the more common and complex genetic abnormalities which is also compatible with a large survival. This condition was first described by John Langdon Hayden Down in 1866 [1], but the cause remained unknown for nearly one century, when Lejeune *et al.* [2] reported the association of DS with one extra copy of HSA21 in 1959. However, the earliest evidence that we had of this syndrome seems to be observed much earlier (Figure 1). Trisomy 21 is now accepted to be the major cause of DS, accounting for about 90-95% of cases. The other 5-10% are caused by other genetic abnormalities including chromosomal translocations (2% to 6%) and mosaicism (2% to 4%) [3, 4]. Thus, scientists are interested in assessing whether there are critical regions of HSA21 specifically responsible for the symptoms of DS.

DS is one of the most common birth defects affecting about one in every 700-1000 live births [3-6], depending mainly on the country or region. Despite the medical and research advances, the preventive measures and the availability of advanced prenatal screening tests, the overall life birth prevalence of DS seems to have increased worldwide in recent years [6]. Several reasons have been proposed to explain this trend. It has been found that the increase in average maternal age at childbirth is the major reason for the increased number of pregnancies affected by DS [5, 6]. On the other hand, the life birth prevalence of DS also depends on sociocultural variables, such as the legality or illegality of abortion and the attitude towards the selection of life in cultures. Thus, the influence of maternal age on prevalence must exceed that of DS pregnancy terminations. However, due to the introduction of prenatal screening programs, the life birth prevalence of DS is decreasing in several developed countries, such as the United States [7], France [8], Taiwan [9], Singapore [10] or Australia [11].

The clinical features of DS include a variety, chronicity and severity of abnormalities that include intellectual disability, dysmorphic features and systemic anomalies, such as: cardiovascular, immunological, haematological, musculoskeletal, nervous and endocrinal defects. Besides, there is evidence of accelerated aging in individuals with DS, being this disorder considered as a progeroid syndrome, and it has been postulated that it may be the result of an increased oxidative stress. Thus, the trisomy is associated with an early onset and an increased risk of age-related manifestations such as: Alzheimer disease, leukemia, menopause, osteoporosis, osteoarthritis, hypogonadism, visual impairment, senile cataracts, and skin changes, hearing loss, premature graying and alopecia [12-14].

The biomedical literature contains a multitude of claims that oxidative stress is involved in DS. Thus, it has been proposed that the increased oxidative stress observed in these subjects is mainly caused to an excessive activity of Cu/Zn superoxide dismutase (SOD1), an enzyme coded on HSA21 (21q22.1). Besides, several abnormalities in mitochondrial function have been found in DS and also in mouse models of this pathology. Indeed, in addition to SOD1, there are several genes or predicted genes on HSA21 with a role in mitochondrial energy generation and reactive oxygen species (ROS) metabolism [15]. However, it is frequently to find discrepancies in literature in this field and even the finding of "false positives" and "false negatives" may be very common, mainly due to the fact that oxidative stress occurs naturally in all cells. In fact, the role of oxidative stress in DS remains uncertain.

In Scopus database (http://www.scopus.com) only 248 papers contain the terms "Down syndrome" and "oxidative stress" in their title, abstract or keywords, which accounts for 0.97% of the 26,019 citations that can be found on the topic "Down syndrome" (updated on Apr 08th, 2011). This ratio is much lower than for other pathologies such as Alzheimer disease (5.16%), Parkinson disease (5.08%), atherosclerosis (5.09%), diabetes mellitus (2.22%) or even progeria (3.30%), although it seems to be increasing up to 2.77% last year (2010). A number of outstanding reviews discuss about oxidative stress and the link between this topic and disease has been assessed for almost all known pathologies, however the studies concerning oxidative stress in DS have not been critically overviewed and discussed.

The aim of this article is to critically review the scientific literature concerning the link between oxidative stress and DS. We review the extent of information available in scientific literature regarding oxidative stress in DS, providing evidence against and in favour of increased oxidative stress in this condition. We present data from 82 original investigations about oxidative stress biomarkers in a detailed table and discuss the information available about oxidative stress in exercise and thyroid disorders. To identify relevant articles, a comprehensive search, conducted between September 2010 and March 2011, was performed using the following databases: PubMed, Scopus, Google Scholar and SciFinder. Although we believe we have identified the bulk of original investigations within this area, some investigations may have escaped our search. We apologize to the authors whose work is not described or referenced here.

Figure 1. Major items and trends in the research of Down syndrome (DS) throughout the history with especial attention in oxidative stress findings.

Neolithic figurine dated ~5000 BCE

~5000 BCE – Neolithic figurine from Greece resembling an individual with DS [16].

~1500 to 400 BCE – Carvings representing "were-jaguars" or jaguar gods from the Mexican Olmec civilization are thought to be DS individuals representations [17].

Jaguar gods from the Mexican Olmec civilization.

~500 BCE – Ceramic with suggestive characteristics of DS in Tumaco-La-Tolita culture (Colombia-Ecuador) [18].

Ceramic from Tumaco-La-Tolita ~500 BCE

~900 to 1200 CE – Terra-cotta sculpture of a DS infant from the Tolteca culture of Mexico [19].

15th and 16th centuries – Paintings depicting individuals with DS features [20].

1838 – First phenotypic description of a child who presumably had DS by Étienne Esquirol [21].

1843 – Another description is make by Édouard Séguin [22].

1866 – Duncan describes a girl with DS features [23].

1866 – John L. Down describes DS clinically [1].

1894 – The use of H_2O_2 and Fe^{2+} mixtures as an oxidant is described by Fenton [24].

John L. Down (1828-1896) British physician

Sculpture from Tolteca culture ~900-1200 CE

1932 – The generation of $HO^{\bullet}$ from H_2O_2 by Fe^{2+} is described by Haber and Weis [25].

1958 – First assessment of antioxidants, vitamin A and uric acid, in DS [26].

1959 – The association of DS with one extra copy of HSA21 is reported by Jérôme Lejeune [2].

1969 – McCord and Fridovich discover the enzyme SOD [27].

1973 – The fact that the clinical picture of trisomy 21 was essentially due to the trisomy for the single distal segment of the chromosome long arm is suggested [28].

Prof. Jérôme Lejeune (1926-1994) French geneticist

1960s
Nutritional therapies in DS

1970s
DS mouse models become available

1974 – First report on SOD1 activity in DS individuals [29].

1976 – Sinet et al. [30] put forward the hypothesis that the chromosome segment containing the *SOD1* gene is also responsible for the clinical features of DS. The locus for SOD1 appears to be located in band 21q22.

1984 – First report on lipid peroxidation assessment in vitro in DS [31].

1985 – The term "oxidative stress" is defined by Helmut Sies [32].

1986 – First report on lipid peroxidation assessment in vivo in DS [33].

1986 – Trisomy 16 mice, a model for DS, are used to assess SOD1 and GPx activities [34].

Museum of Fine Arts in Boston. Andrea Mantegna (1430-1506) used a DS infant as the model for his Christ child.

Metropolitan Museum of Art, New York. Painting entitled *The Adoration of the Christ Child*. The paint, dated ~1515 and attributed to an unknown follower of Jan Joest of Kalkar, shows several individuals with DS features.

1996 – Erythrocyte SOD1 activity is increased by 42% in complete trisomy 21 [35].

1996 – Protein damage is firstly measured in DS [36].

1997 – DNA damage is firstly measured in vitro in DS [37].

1997 – First study assessing exercise-induced oxidative stress in DS [38].

1998 – The ratio SOD1/(GPx+CAT) is assessed in DS [39].

1999 – Vitamin E could reduces chromosomal damage of lymphocytes of DS [40].

2000 – Sequencing of the long arm of chromosome 21 by Hattori et al. [41].

2000 – First study suggesting a possible relation between oxidative stress and thyroid hypofunction in DS [42].

2001 – Total antioxidant capacity is firstly measured in DS [43].

2003 – A list of genes on chromosome 21 with a role in energy and reactive oxygen species metabolism is given [15].

2008 – Randomized controlled trial with DS children assessing the effect of antioxidants (selenium, zinc and vitamins A, E and C) and folinic acid supplementation on SOD1 and GPx activities, onSOD1 to GPx ratio and on urinary isoprostane concentrations [44].

2011 – Firsts studies assessing oxidative stress biomarkers in DS subjects treated for hypothyroidism [45, 46].

2. Oxidative stress and Down syndrome: the link

A free radical is any species capable of independent existence, containing one or more unpaired electrons [47], being the most important ones those derived from either oxygen and/or nitrogen. Both the radicals and the non-radical species generated via interaction with free radicals, are referred to as reactive oxygen/nitrogen species (RONS) [48]. RONS are highly reactive and extremely short-lived agents generated mainly as by-products of aerobic metabolism, playing a dual role as both deleterious and beneficial species.

When the generation of RONS exceeds the ability of antioxidant defence systems to remove them, such imbalance can cause oxidative/nitrosative damage to cellular constituents (DNA, proteins, lipids and sugars), which is defined as oxidative/nitrosative stress [47, 49]. Thus, the degree of balance between ROS or reactive nitrogen species (RNS) production and antioxidant defences determines the degree of oxidative or nitrosative stress, respectively. When the system becomes unbalanced (free radicals > antioxidant defences) that can potentially result in a change in the intracellular redox balance towards a more oxidizing environment, which may result in direct DNA damage, lipid peroxidation and protein damage with the possibility to cause ill-health and disease. However, despite their excess leads to oxidative/nitrosative stress, RONS are involved in several important biological processes, including cell signaling, redox regulation of gene transcription, cellular immunity and apoptosis, being essential for normal physiological function [48].

Oxidative stress is a process induced by endogenous as well as exogenous factors. Endogenous factors include normal physiological processes, such as oxidative phosphorylation or cytochrome P450 metabolism. Several environmental factors, including smoking, diet or exposure to ambient air pollution, represent exogenous sources of RONS.

Increasing evidence suggests that oxidative stress is linked to either the primary or secondary pathophysiologic mechanisms of multiple human diseases, including DS [50]. Beginning in the 1970s, theories that ROS might be implicated as a cause for central nervous system injury in DS began to emerge in the context of similar beliefs about the cause of a number of degenerative diseases [51]. Since then, the number of investigations in this area is remarkable; being now the fact that DS individuals are under unusual increased oxidative stress unquestionable.

An excess of the enzyme SOD1 activity has been considered as the main responsible for the increased oxidative stress found in this condition. The gen encoding SOD1 is located on HSA21, so DS individuals are trisomic for SOD1. Indeed, SOD1 is overexpressed ~50% in these individuals [52]. This enzyme plays a key role in the metabolism of ROS, being part of the first line of antioxidant defense by catalysis the dismutation of superoxide radical ($O_2^{\bullet -}$), mainly generated by oxidative metabolism, into oxygen plus hydrogen peroxide (H_2O_2) [53] (equation 1). Indeed, SOD1 is the major cytoplasmic superoxide scavenger, being also located in the intermembrane space of the mitochondria [54].

$$2\,O_2^{\bullet -} + 2\,H^+ \xrightarrow{\textbf{SOD1}} O_2 + H_2O_2 \quad \text{(Eq. 1)}$$

In a second step, H_2O_2 is metabolized to water via glutathione peroxidase (GPx) or catalase (CAT) [47]. However, it has been reported that, in DS individuals, SOD1 is increased in a variety of cell types and organs [29, 31, 34, 35, 55-57]. This increase in SOD1 activity leads to an increase in the SOD:GPx ratio and this imbalance may lead to the accumulation of unmetabolized H_2O_2 which is not itself considered an oxygen free radical, but has the ability to induce oxidative stress [58]. Much of the cytotoxic effects of H_2O_2 are due to its ultimate conversion into the hydroxyl radical ($HO^\bullet$), the most reactive oxygen-containing species known, via Fenton and Haber-Weiss reactions as follows:

Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^\bullet + OH^- \quad \text{(Eq. 2)}$$

Haber-Weiss reaction:

$$O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + HO^\bullet + OH^- \quad \text{(Eq. 3)}$$

The highly reactive hydroxyl radical readily interacts with macromolecules such as DNA, proteins and lipids, which result in damage of cellular structures, membranes and organelles.

However, many questions remain about the link between oxidative stress and DS. Indeed, conflicting results are found in literature. Thus, no significant differences in SOD1 activity between DS and controls in samples of cerebral cortex has been reported [36]. Moreover, it has been reported that SOD1, although overexpressed in DS, is a compensated gene without significant differences between controls and DS [59]. Nonetheless, in addition to SOD1, other genes on HSA21 have a role in mitochondrial energy generation and ROS metabolism (see next section above), suggesting a genetic based association between oxidative stress and DS.

It can be mentioned and discussed several pathologies and features associated with DS in which a convincing link between the occurrence of increased oxidative stress and the development and/or progression of disease has been demonstrated.

2.1. Down syndrome and aging

Aging, the physiological process intimately linked with decline and characterised by the accumulation of damage to molecules, cells and tissues over a lifetime, is the biggest risk factor for many diseases.

Many theories have been proposed to explain the cause of aging [60-62]. From them, the "free radical theory of aging" is currently the most studied and popular one. The "free radical theory of aging", proposed by Denham Harman in the mid-1950s [63], postulates that endogenous ROS play a role in the aging process because of an increase in oxidative damage to macromolecules. Consistent with this theory, there are numerous age-related diseases having an important free radical component. The theory has since been modified to the "oxidative stress theory of aging" because ROS such as peroxides and aldehydes, which are not free radicals, play also a role in oxidative damage to cells.

DS is characterized by multiple signs of early senescence, including many age-related conditions such as: Alzheimer disease, cataracts, osteoporosis, premature graying and alopecia. In fact, DS is considered a progeroid syndrome, defined as that genetic disorder in which multiple major aspects of the senescent phenotype appear [64]. Besides, since DS has been associated to an increased oxidative stress, in addition to studies with animal models and those using oxidative stress biomarkers which support the "oxidative stress theory of aging", DS has been used as a model for the study of physiological and pathological senescence.

The investigation of the physiological role of SOD1 in the phenomenon of premature aging comes from research on the normal aging process. Continuing to today, numerous studies in humans provided evidence to support a role for SOD1, alterations in the SOD1:GPx ratio and oxidative stress in the aging process (reviewed in [65]). Thus, for example, a concomitant increase in GPx:CAT activity with an increase in SOD1 activity in most organs tested as a function of age has been found [66, 67]. In addition, it has been reported that various organs (brain, lung, heart and thymus) have an altered SOD1:GPx ratio in DS, which may contribute to the premature aging observed in these individuals [68].

In contrast, the overall studies of the effect of genetic manipulation of antioxidant defenses on age-related disease in challenged animal models suggest that oxidative stress may play a role in health span. However, if oxidative stress plays a role in aging, it is much more limited than previously thought [69]. Nonetheless, the effect of SOD1 overexpression on lifespan may considerably vary with different genetic backgrounds.

It has been proposed that the core statement of the free radical (or oxidative stress) theory of aging, i.e. the production of mitochondrial ROS is the cause of aging, is wrong [70]. It should be understood that aging is a complex process, which is probably determined by multiple genetic and environmental factors. Thus, multicausal or unifying theories of aging often proposed that oxidative stress might result from the failure of one particular maintenance system of the organism and thus participate in causing aging, but no more [70]. For example, it has been suggested that age-related accumulation of somatic DNA mutations in DS contributes to oxidative stress that exacerbates the imbalance in gene expression [71].

On the other hand, the 'telomere hypothesis of cellular aging', which proposes that loss of telomeric DNA may ultimately cause cell-cycle exit during replicative senescence, has also been used to explain the accelerate aging observed in DS individuals. Telomeres are chromosome ends consisting of highly conserved TTAGGG repeats found in most vertebrates, whose predominant function is the protection of chromosome extremities from fusion and degradation [72]. Individuals with DS exhibit a higher rate of telomere loss compared with age-matched controls [73]. However, in addition to physiological, replication-related reactions, telomeres may shorten due to DNA damage, mainly caused by oxidative stress [74]. Indeed, Jenkins *et al.* [75, 76] found shorter telomeres in T lymphocytes of individuals with DS and dementia or mild cognitive impairment, which are related to oxidative stress, compared to that in individuals with DS but without dementia or mild cognitive impairment. Therefore, the accelerated loss of telomeres in DS individuals could also be the result of an increased oxidative stress.

In addition, dysregulation of "aging genes" could also have an influence on lifespan in DS individuals. For example, it has been recently reported that *ADARB1* gene (also *ADAR2*) is associated with extreme old age [77]. This gene, which encodes a dsRNA-specific adenosine deaminase, is located on HSA21 and, therefore, could be involved in the accelerate aging in DS.

Life expectancy of individuals with DS has significantly improved during the last years. Hence, keeping those elderly individuals healthy must be a major goal for medical research. However, our understanding of the molecular basis of aging remains largely unknown and further research is still required. For that, further research advances in DS could unlock the mysteries of the aging process or even of several age-related pathologies such as the Alzheimer disease.

2.2. Oxidative stress, Alzheimer disease and Down syndrome

DS phenotype is characterised by abnormalities in mental development. Learning and memory problems that begin to emerge in late infancy become

considerably more noticeable in adolescence and lead to mild-to profound impairment in intellectual functioning [78]. Besides, DS is the most common genetic cause of mental retardation. The intelligence quotient decreases in the first decade of life and cognitive function reaches a plateau in the adolescent years that continues into adulthood [79]. In addition, these individuals are much more likely to have cognitive deterioration and develop dementia at an earlier age than individuals without DS. Thus, adults with DS over 40 years have a higher incidence of early-onset Alzheimer disease compared to the euploid population and clinical signs and symptoms of this type of dementia are noted in 75% of such individuals over 60 years of age [80], being its age-specific prevalence rates similar but 30-40 years earlier in life in DS than in the general elderly population [81]. In addition, neurodegenerative phenotypes have also been observed in DS mouse models [82].

The pathology of Alzheimer disease is characterized by brain atrophy, extracellular β-amyloid (Aβ) deposits, the accumulation of neurofibrillary tangles and synapse loss, and oxidative stress may play a major role in its pathophysiology. A link between oxidative stress and Alzheimer disease in DS can be established. The amyloid precursor protein (APP), from which Aβ is produced, is encoded on HSA21. Aβ is able to generate ROS *per se*, increasing cellular oxidative stress [83]. It leads to deleterious oxidative effects such as the modification and inhibition of several neuronal and glial transmembrane transport systems [84]. Thus, the overproduction of Aβ by genetic or other mechanisms leads to Aβ-associated free-radical oxidative stress which seems to be responsible, at least in part, for the premature neuronal dysfunction in DS. Indeed, the triplication of APP is proposed to be the underlying mechanism through which DS individuals show an increased frequency of dementia of the Alzheimer type which also appear early in life [85, 86].

Experimental evidence supports a prominent role for oxidative stress in neuropathogenesis of DS. Sinet *et al.* [87] found a highly significant positive correlation between GPx activity and intelligence quotient in DS individuals and concluded that GPx may play an important role in preserving the cerebral status of these individuals. Brooksbank and Balazs [31] reported an increase in SOD1 activity as well as an increased lipid peroxidation in the cerebral cortex of fetuses with DS. In fact, it has been reported that fetal DS neurons generate increased levels of ROS and exhibit increased lipid peroxidation leading to apoptosis [88]. Besides, increased brain glycoxidative stress occurs very early in the life of DS individuals [89]. Advanced glycation end-products (AGEs) also tend to accumulate in the brains of those who develop Alzheimer disease [90, 91]. Therefore, the development of this cognitive disability in DS may also be due to early accumulation of these products, which may play an adverse role in prenatal and postnatal brain development.

The imbalance of ROS homeostasis resulting from *SOD1* and *APP* genes overexpression and its involvement in the neuropathogenesis of DS has been demonstrated through a number of investigations. Nonetheless, a long list of other HSA21 genes may also contribute to neurodegenerative mechanisms, including: *DSCR1* [92], *ETS2* [93], *DYRK1A* [94], *S100β* [95], *PCP4*, *DSCAM* and *GRIK1* [15], as well as a short length of telomeres [75, 76]. Moreover, it has been suggested that shorter telomeres may indicate dementia status in older individuals with DS [75, 76], denoting the highly complexity of the neuropathogenesis for which DS could be a model of study in cognitive disorders.

2.3. Oxidative stress, cataracts and Down syndrome

Cataracts, the opacification of the eye lens, are the leading cause of blindness worldwide. Substantial evidence has been gathered to support the conclusion that oxidative stress contributes to the development of cataracts [96, 97]. In fact, it has been suggested that opacity of the lens is a direct result of oxidative stress [98].

Early-onset cataracts occur with high frequency in DS individuals [99]. A possible link between oxidative stress and cataractogenesis in DS was first suggested by Brás *et al.* [100]. Later, it has been reported that APP is involved in fiber cell formation and may contribute to early-onset cataracts in DS [101]. In lens organ cultures oxidative stress stimulates APP expression and increases Aβ peptides [102] which can, in turn, contribute to additional oxidative stress to form a "vicious cycle" [103], suggesting an oxidative stress-related cataract formation in DS. Moreover, although it has been reported that overexpression of SOD1 may prevent H_2O_2-induced oxidative damage to the lens [104], high levels of SOD1 have been found to be associated with increased risk of cataract in non-Down individuals [105, 106]. In fact, it has been found that DS individuals with cataracts have higher SOD1 and GPx activities than controls [107].

However, given the limited information available in this field, further evidence should be provided in future works, even more when other genes such as *CRYAA* and *CRYZL1* encoding crystallins which may be involved in cataractogenesis are also located on HSA21 [108].

2.4. Oxidative stress, thyroid dysfunction and Down syndrome

Thyroid hormones are associated with oxidative stress and antioxidant status. They regulate proteins, vitamins and antioxidant enzymes synthesis and

degradation as well as oxygen consumption and mitochondria energy metabolism, playing an important role in free radical production [109]. In fact, it has been suggested that variations of thyroid hormones levels can be one of the main physiological modulators of in vivo cellular oxidative stress [110].

Thyroid dysfunction is the most frequent endocrine abnormality in subjects with DS, with a prevalence varying between 0% and 66%, depending on variations in population size, age, laboratory assays or definitions of thyroid dysfunction used, being the more common rates >20% [111]. Hypothyroidism is the most frequent thyroid abnormality in DS [112, 113]. It can be either congenital, with an incidence in babies with DS of 1:141 live births [112] compared with an incidence ranging between 1:2,500 and <1:5,000 among newborn babies without DS [114], or acquired at any age after birth.

The available data concerning oxidative stress in both hypothyroidism and hyperthyroidism are scarce and controversial. In hypothyroidism, a low free radical generation is expected because of the metabolic suppression brought about by the decrease in thyroid hormone levels [115]. However, there are some studies reporting an increased oxidative stress in patients with hyperthyroidism as well as with hypothyroidism [109, 115-119], even in subclinical hypothyroid states. In addition, thyroid hormone (T_3) has been shown to down regulate the expression of SOD1 and, conversely, progressive hypothyroidism leads to an increase of SOD1 activity in the brain of rats [120].

Only a handful of investigations have been conducted addressing the link between thyroid dysfunction and oxidative stress in DS, all of them in hypothyroid subjects. Kanavin *et al.* [42] were the first studying the link between oxidative stress and thyroid dysfunction in DS, suggesting that hypothyroidism is linked to decreased levels of selenium in DS subjects. Recently, oxidative and nitrosative stress have been assessed in hypothyroid DS subjects receiving levothyroxine for treatment of hypothyroidism by measuring a set of urinary biomarkers: 8-hydroxy-2'-deoxyguanosine (8-OHdG), isoprostane 15-F2t-IsoP, thiobarbituric acid-reacting substances (TBARS), AGEs, dityrosine (diTyr), H_2O_2 and nitrites and nitrates (NOx), in children [45] and in adolescents and adults [46]. In these works, significantly higher levels of diTyr in children with DS receiving levothyroxine for hypothyroidism has been found compared to their non-Down healthy siblings. Besides, subjects with DS receiving levothyroxine showed increased levels of diTyr in the early adulthood (from 15 to 19 years) and increased levels of diTyr, AGEs and TBARS in the adulthood (from 20 to 40 years) than those without hypothyroidism diagnosed. Both hypothyroid and hyperthyroid patients are characterized by higher levels of low density lipoprotein (LDL) oxidation when compared to healthy normolipidemic control subjects [117], which could explain the increased levels of urinary TBARS. However, whereas in hyperthyroid patients increased lipid peroxidation was strictly related to free thyroxine levels, in hypothyroidism it was strongly influenced by serum lipids [117]. Therefore, lipid composition must be studied in hypothyroid DS subjects before any conclusion can be reached.

Nevertheless, urinary levels of creatinine (Cr) decreased in DS children receiving levothyroxine compared to their non-Down healthy siblings [45]. Besides, lower levels of urinary Cr has been found in the early adulthood (from 15 to 19 years) of DS subjects receiving levothyroxine than DS without hypothyroidism diagnosed [46]. Hence, renal impairment due to hypothyroidism may bias the results in these patients as it has been suggested by the authors in these previous works. It is well known that levels of Cr are influenced by thyroid hormones. Hypothyroidism enhances serum Cr levels because it reduces glomerular filtration rate and increases production of Cr [121]. In fact, impaired renal function has been reported in subjects with hypothyroidism [122]. Moreover, it has been recently reported that non-Down children with congenital hypothyroidism have an increased prevalence of congenital renal and urologic anomalies [123], and renal impairment has been also described in DS based on decreased Cr clearance [124, 125].

Previous works reported reduced Cr clearance in non-Down patients with hypothyroidism, but normal values were obtained when they were treated with thyroid hormones [122]. However, the same has not been found in DS [45, 46], suggesting that factors contributing to the etiology of hypothyroidism may be different in DS than in non-Down individuals.

Some abnormalities reported in DS may influence the thyroid function: *1*) decreased levels of selenium [126], which is required for thyroid hormone synthesis and metabolism, acts as an antioxidant protecting the thyrocyte against peroxides and is part of selenium-dependent antioxidant enzymes (e.g., GPx and thioredoxin reductase), *2*) an impairment in the activity of phenylalanine hydroxylase [127], which converts the phenylalanine in tyrosine, and *3*) overexpression of DYRK1A kinase [128], which could reduce availability of tyrosine. These factors may lead to several anomalies related to thyroid disorders, even in DS subjects with "normal" thyroid hormones levels. However, further investigation is required to ascertain the mechanisms underlying these findings.

On the other hand, signs and symptoms of hypothyroidism can be difficult to discriminate from those found in the natural course of DS itself. They are overlapped to some extent in both DS and

hypothyroidism (e.g. hypotonia, lethargy, mental retardation, growth failure, prolonged neonatal jaundice, delayed closure fontanellae, macroglossia, obesity, etc) [129]. Moreover, although it has been reported that mild plasma TSH elevation is extremely prevalent in DS: 80-90% in early infancy and 30-50% thereafter [130], untreated subclinical hypothyroidism is present in DS at birth and persists throughout life [131]. In summary, more studies linking thyroid disorders and oxidative stress in DS are clearly needed.

2.5. Oxidative stress, immune dysfunction and Down syndrome

DS is associated with various immunologic impairments [132-134]. Thus, individuals with DS have a 10 to 20-fold increased overall risk of developing leukaemia [135]. Moreover, increased susceptibility to viral and bacterial infections, particularly respiratory infections, and high rates of malignancies and autoimmune phenomena occur in DS. The reasons for these facts are multifactorial, but it has been hypothesised that abnormal metabolism of ROS may contribute to the defective immunity and increased susceptibility to infections of these individuals. Besides, the possibility that the overexpression of SOD1 is the cause of decreased immune function in this condition has been speculated.

It has been reported that overproduction of SOD1 in intraperitoneal macrophages of mice results in an inhibition of extracellular release of superoxide radicals, an increased intracellular production of H_2O_2 and in the significant reduction of their microbicidal activity [136]. Moreover, superoxide levels were found lower in polymorphonuclear neutrophils (PMNs) of DS individuals [137] and whole blood leukocytes of DS subjects were found to produce less superoxide than those of healthy controls [138]. It may in turn contribute to the susceptibility to infections in DS, particularly those caused by pathogens that require superoxide to be killed efficiently. Indeed, DS patients are not particularly prone to infections with catalase-negative bacteria which are not dependent on superoxide for efficient killing [139].

In summary, it can be hypothesised that an increase in SOD1 activity, which may lead to a damage of immune cells due to an excess of H_2O_2, and impair normal signal transduction processes involved in phagocyte activation can reduce immunity in DS.

2.6. Oxidative stress, osteoporosis and Down syndrome

Osteoporosis is an age-related disease characterised by low bone mass and deterioration of bone tissue, leading to enhanced bone fragility and an increase in fracture risk. People with intellectual disability are at higher risk of developing osteoporosis, being the presence of DS an independent risk factor for low bone mineral density [140, 141].

A link between increased oxidative stress and reduced bone density has been established [142, 143], being found that mice deficient in SOD1 ($SOD1^{-/-}$ mice) have elevated oxidative stress and decreased muscle mass and strength compared to wild-type mice.

Although a number of conditions commonly presented in DS may contribute to the development of osteoporosis in this population (e.g. thyroid dysfunction, abnormalities of sexual development, anticonvulsant medication, insufficient daily dairy intake, generalized hypotonia, delay of development of gross motor skills, sedentary lifestyle and low muscle strength) [140], the role of oxidative stress in osteoporosis development in DS has not yet been studied.

3. Molecular genetics of Down syndrome and oxidative stress

HSA21, the smallest human autosome, represents only around 1.0-1.5% of the human genome and the complete genomic sequence of HSA21q was published in 2000 by Hattori *et al.* [41]. Nonetheless, the sequence of the short (p) arm is not yet known and annotation for gene discovery continues. Hattori *et al.* predicted that HSA21 contained just 225 genes, whereas a recent review identified more than 500 genes [144] which are accessible through the "human chromosome 21 gene function and pathway database" (from the University of Colorado Denver) at the website http://chr21.egr.vcu.edu/.

It has been proposed that the phenotypic alterations that characterize DS are the result either of the increased dosage and hence expression of specific genes on HSA21 (the "gene dosage effect" hypothesis) or of the phenotypic instability due to the extra genetic information (the "developmental instability" hypothesis) [145]. Therefore, three copies of any functional element in the genome might be responsible for specific trisomy-related phenotypes [146]. However, gene-environment interactions and epigenetic influence on DS phenotype must be also taken into account.

Epigenetics can be defined as the heritable changes in gene expression that are not coded in the DNA sequence itself but by post-translational modifications in DNA and histone proteins [147]. That is, genetic information is encoded not only by the linear sequence of DNA, but also by epigenetic modifications of chromatin structure [148]. Epigenetic systems have a major role in the pathogenesis of a number of human diseases [149] and can be modified by environmental factors [150] leading to a genetic instability. Indeed, each cell type has its own epigenetic signature which

reflects genotype and environmental influence, and is ultimately reflected in the phenotype of the organism [151]. Thus, most genetic findings must be considered within in an epigenetic and environmental context. Gene-environment interactions are thought to be mediated by epigenetic modifications of the genome, and epigenetic changes of the genome often arise in response to environmental changes [152]. Therefore, DS phenotype may be the result not only of the increased dosage of HSA21 genes and the dosage imbalance between HSA21 and non-HSA21 genes (DS genotype), but also of the interaction between this genotype and the environment in an epigenetic context (Figure 2).

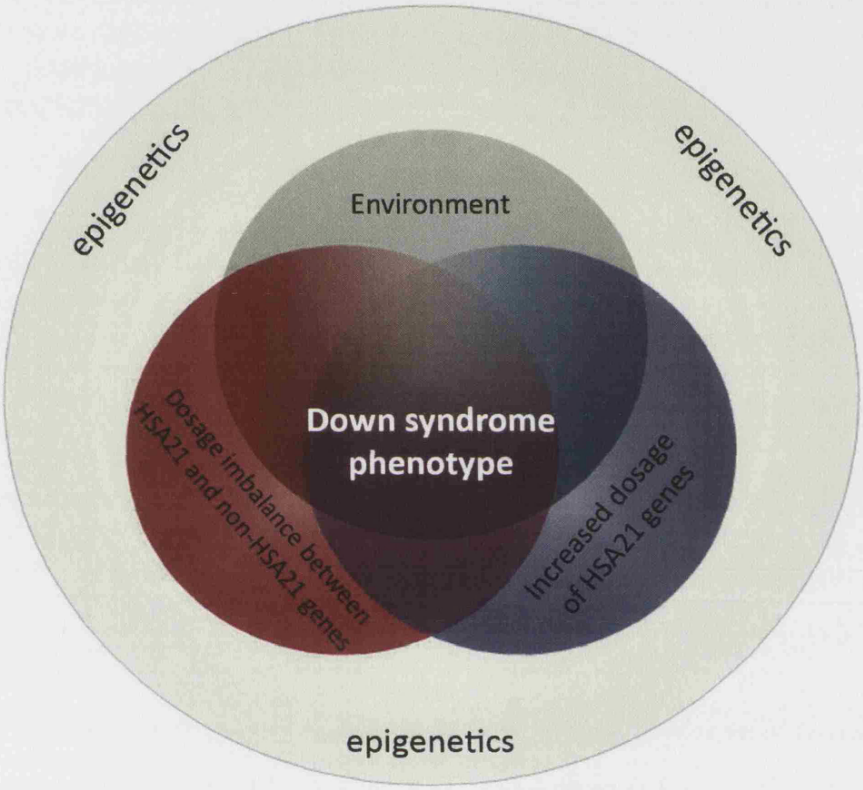


Figure 2. The causes of Down syndrome phenotype.

Transcriptional regulation, which is potentially perturbed by post-translational modification that is directly or indirectly affected by HSA21 proteins, seems to be dysregulated in DS [153]. Thus, in DS, oxidative stress-related genes and genes involved in growth factor signaling and transcriptional regulation are often already dysregulated in fetal tissue but grow more abnormal in adult brains [145]. On the other hand, in a classification of HSA21 genes according to their level of DS lymphoblastoid cell lines expression, among the expressed transcripts: 29% are sensitive to the gene-dosage effect or are amplified, 56% are compensated (including SOD1) and 15% are highly variable among individuals [154]. Thus, most of the expressed transcripts are compensated for the gene-dosage effect, so they are not involved in the DS phenotype, although it is only like that for lymphoblastoid cell lines.

Consistent with the evidences obtained from oxidative stress and mitochondrial dysfunction studies, multiple genes on HSA21q have a direct and indirect role in oxidative stress, such as *SOD1*, *DSCR1*, *S100B*, *APP* or *ETS2* (Table 1). Thus, deregulation of these genes may generate oxidative stress.

In addition, a number of genes on HSA21 are involved in energy/mitochondria (ATP5J, ATP5O, C21orf33, HLCS, MRPL39, MRPS6, C21orf2, RPL23AP4, RPL23P2, RPL31P1, RPL3P1, RPS5L or RIMKLP). Their deregulation may lead to mitochondrial dysfunction and, therefore, to increased oxidative stress.

x

Table 1. Genes on HSA21 involved in oxidative stress pathways.

STCH	Stress 70 protein chaperone, microsome-associated, 60kDa	q11.2	May be involved in the protection of cells against OS. Elevated levels could lead to perturbations in the intracellular redox state and in the apoptotic process.	F	[155]
GABPA	GA binding protein transcription factor, alpha subunit 60kDa	q21.3	Also named NRF2. Induces transcriptional activation of several antioxidant genes. Regulates the synthesis of glutathione. Induced by cellular stressors including endogenous ROS or exogenous electrophiles.	T	[156, 157]
APP	Amyloid beta (A4) precursor protein (protease nexin-II, Alzheimer disease)	q21.3	Amyloid precursor protein, source of neurotoxic Aβ, which is able to generate ROS *per se*. Regulates ROS homeostasis. Involved in the regulation of intracellular Ca^{2+}.	S	[144]
BACH1	BTB and CNC homology 1, basic leucine zipper transcription factor 1	q21.3	OS-regulated transcription factor. Inhibits the expression of oxidative response genes. Increases ROS.	T	[144, 158]
SOD1	Superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult))	q22.11	Catalyzes the dismutation of superoxide anions to H_2O_2.	E	[144, 155]
GART	Phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	q22.11	Involved in *de novo* purine biosynthesis. Its overexpression may cause accumulation of the antioxidant uric acid.	E	[159]
CRYZL1	Crystallin, zeta (quinone reductase)-like 1	q22.11	Involved in the synthesis of crystallin, a major component of the eye lens. Associated with increased susceptibility to oxidative damage.	E	[160]
DSCR1	Down syndrome critical region gene 1	q22.11	Regulator of calcineurin. DSCR1 activity is modulated by OS. Can stimulate production of SOD1 and mitochondrial ROS. Its overexpression is protective to cells, but chronic upregulation is associated with cellular damage.	F	[92, 155]
CBR1	Carbonyl reductase 1	q22.12	Protects cells against lipid peroxidation.	E	[161, 162]
CBR3	Carbonyl reductase 3	q22.12	Potential role in the detoxification of ROS.	E	[161, 163]
ETS2	V-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	q22.2	Upregulated by OS. Its overexpression may contribute to the increased rate of apoptosis of neurons in DS.	T	[93, 164]
SH3BGR	SH3 domain binding glutamic acid-rich protein	q22.2	Could be involved in the control of redox dependent processes as factor of oxidative protection.	F	[165]
ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	q22.3	Protects against OS-induced macrophage apoptosis during the phagocytosis of apoptotic cells.	F	[166]
NDUFV3	NADH dehydrogenase (ubiquinone) flavoprotein 3, 10kDa	q22.3	Involved in mitochondrial function. Its dysfunction may lead increased basal generation of superoxide.	E	[167]
CBS	Cystathionine-beta-synthase	q22.3	Catalyzes the condensation of Hcy with serine to form cystathionine. Elevated Hcy induces OS, so its deficiency promotes OS. Its overexpression may cause hyperuricemia.	E	[168]
CRYAA	Crystallin, alpha A	q22.3	Protects the lens against stress, particularly against OS, acting as a small heat-shock protein.	S	[169]
TRPM2	Transient receptor potential cation channel, subfamily M, member 2	q22.3	Multifunctional Ca^{2+}-permeable non-selective cation channel activated by ROS, including H_2O_2. Involved in OS-induced cell death.	F	[170, 171]

Symbol	Name	Cytogenetic location	Function related to oxidative stress	Cat*	Refs.
SUMO3	SMT3 suppressor of mif two 3 homolog 3 (yeast)	q22.3	Its expression can be down by OS. Implicated in cellular OS response.	T	[172, 173]
S100B	S100 calcium binding protein, beta (neural)	q22.3	Ca^{2+}-binding protein whose function is regulated by oxidative state, promoting neuronal survival in the oxidized state and apoptosis and neurotoxicity in the reduced state. Increases NO release by stimulating ROS production and activating the stress-activated kinases, p38 and JNK. Suppresses OS induced by cupper.	F	[144, 174]

Genes are listed in order from the centromere to the telomere on HSA21. Since annotation for gene discovery continues and the function of most of them are not well known, other genes on HSA21 might also contribute to perturbations in the ROS metabolism and/or have their expression affected by the ROS balance in DS. Genes set in black type are those which have been extensively studied.

* Category of genes is as follows: E for an enzyme, S for a structural protein, F for a functional protein, T for a gene related to transcription, translation, replication or gene regulation and P for a pseudogene.

Aβ, amyloid β peptide; DS, Down syndrome; H_2O_2, hydrogen peroxide; Hcy, homocysteine; NO, nitric oxide; JNK, c-Jun NH_2 terminal protein kinase; OS, oxidative stress; ROS, reactive oxygen species; SOD1, Cu/Zn superoxide dismutase.

3.1. Oxidative stress in Down syndrome mouse models

The use of DS mouse models to identify which genes contribute to DS phenotypes is increasing in an intend to develop pharmacological or genetic therapeutic interventions. Several DS mouse models have been generated, such as Ts16, Ts65Dn or Ts1Cje mice, based on the fact that regions on HSA21q are syntenically conserved with three regions located on mouse chromosomes 10 (MMU10), 16 (MMU16) and 17 (MMU17). DS mice are limited in their applicability, although these models are continually evolving. Indeed, a novel mouse model has been recently generated which carries duplications spanning the entire HSA21 syntenic regions on all three mouse chromosomes (i.e. Dp(10)1Yey/+;Dp(16)1Yey/+;Dp(17)1Yey/+ mice) [175]. Although studies using mouse models can not replace human studies, the pace of research advances helping to understand the etiology of DS phenotype is increasing.

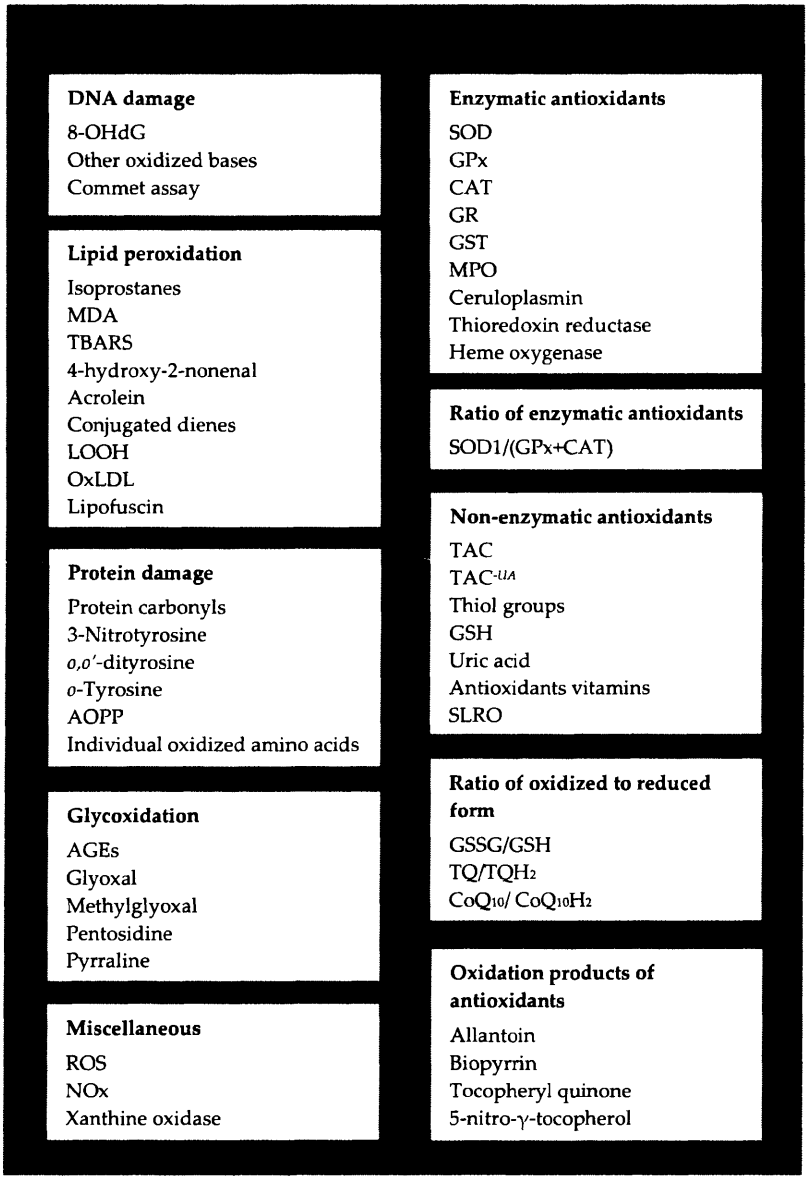
Studies addressing oxidative stress in DS mouse models have revealed increased oxidative damage in a number of tissues and fluids. Thus, for example, it has been recently found an increased lipid peroxidation in the brains of two DS mouse models, Ts1Cje and Ts2Cje [176], as well as increased levels of 4-hydroxy-2-nonenal and 3-nitrotyrosine in the soleus muscle of Ts65Dn mice [177]. In addition, it has been reported an increased basal generation of superoxide in Ts16 neurons, probably caused by a dysfunction of complex I of the mitochondrial electron transport chain [167], and an abnormal elevation in superoxide anion produced by immune-activated microglia from the cerebral cortex of Ts16 mice [178]. Ts16 embryos also show abnormal neuronal migration and evidence of oxidative stress [179]. Indeed, mice that over-express SOD1 develop several features of DS phenotype. Long-term overexpression of SOD1 in the transgenic SOD1 mice (Tg-CuZnSOD) causes oxidative stress, showing enhanced formation of hydroxyl radical in brain and muscle extracts, which leads to muscle function impairment [180].

4. Biomarkers of oxidative and nitrosative stress in Down syndrome

The main difficulty in measuring oxidative and/or nitrosative stress *in vivo* is in capturing the extremely short-lived ROS and/or RNS. Alternatively, several biomarkers are available, in addition to RONS themselves, for the assessment of oxidative/nitrosative damage in biological samples. Biomarkers are physical signs or laboratory measurements that occur in association with a specific disease and have diagnostic or prognostic use [181]. Ideally, the attributes of a biomarker should include the following: *1*) clinical relevance, *2*) sensitivity and specificity to treatment effects, 3) reliability, 4) practicality and *5*) simplicity [181]. However, available biomarkers of oxidative/nitrosative stress are not equivalent indicators at all. Thus, biomarkers of oxidative and/or nitrosative damage to DNA, proteins, lipids or carbohydrates can be found, i.e. the major targets of RONS in the cellular components. Under conditions in which RONS production is increased, the capacity of

the antioxidant defense system may be overwhelmed by the oxidant attack. Therefore, oxidative stress can also be assessed by measuring the antioxidant status. For that, the activity of certain antioxidant enzymes or the levels of non-enzymatic antioxidants can be measured as an index of oxidative stress. In addition, a large number of total antioxidant capacity (TAC) assays are also available, such as: the cupric ion reducing antioxidant capacity (CUPRAC), the Trolox equivalent antioxidant capacity (TEAC) and the ferric reducing ability of plasma (FRAP) [182]. Direct assessment of free radical production is also possible via electron spin resonance spectroscopy or by chemiluminescence [50]. However, these techniques are cumbersome and highly sophisticated, rendering them very difficult to apply clinically.

Suitable oxidative stress biomarkers include 8-OHdG, isoprostanes, 3-nitrotyrosine, AGEs or TAC (Table 2). Many of them have been assessed in a number of *in vivo* studies such as those performed in Alzheimer disease [183, 184], Parkinson disease [185], and smokers [186] or athletes [187].



8-OHdG, 8-hydroxy-2'-deoxyguanosine; AGEs, advanced glycation end-products; AOPP, advanced oxidation protein products; CAT, catalase; CoQ_{10} , ubiquinone-10; $CoQ_{10}H_2$, ubiquinol-10; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; LOOH, lipid hydroperoxides; MDA, malondialdehyde; MPO, myeloperoxidase; NOx, total nitrite and nitrate; OxLDL, oxidized low density lipoproteins; ROS, reactive oxygen species; SOD, superoxide dismutase; SLRO, serum lipid resistance to oxidation; SOD1, Cu/Zn superoxide dismutase; TAC, total antioxidant capacity; TAC^{-UA}, total antioxidant capacity without relative contribution of uric acid; TBARS, thiobarbituric acid-reacting substances; TQ, tocopheryl quinone; TQH_2, tocopheryl hydroquinone.

In DS, a large number of biomarkers have been assessed, as it is shown in Table 3. However, controversial results have been obtained for most of them.

For example, levels of 8-OHdG, the most commonly used marker of oxidative DNA damage, have been found increased in urine samples of children with DS [188], although no significant differences between DS and non-Down individuals were found by our research group in recently published studies performed in children [45] and in adolescents and adults [46], which is in accordance with the results of Lloret *et al.* [189]. Besides, controversial results has been found in lipid peroxidation studies when levels of malondialdehyde (MDA) or TBARS are assessed in plasma, serum or urine samples, with several works reporting increased levels, others reporting no significant differences and others reporting decreased levels of this biomarker in DS compared to controls. In fact, Campos *et al.* [46] also reported a negative correlation between lipid peroxidation and age in DS, and suggested that it could be due to the altered levels of lipids and lipoproteins or to the increased levels of the enzyme carbonyl reductase 1 observed in these individuals.

Although some works reported the opposite, results obtained for antioxidant enzymes activity, MDA/TBARS levels in erythrocytes or plasmatic uric acid seem to be more similar among studies. Thus, in most works, SOD1 activity has been found significantly increased in DS compared to controls. In addition, cell cultures from DS individuals also showed increased levels of SOD1 including fibroblast [34], neurons and astrocytes [57]. More important, the ratio of enzymatic antioxidants involving SOD1 (i.e. SOD1/GPx, SOD1/(GPx+CAT) or SOD1/(GPx+CAT+MPO)) also seems to be increased in DS individuals [190-192]. That is, increased activity of SOD1 in DS leads to an imbalance in the enzymatic antioxidants, which could increase ROS production in this condition. Hence, an excess of H_2O_2 is expected in DS individuals. Indeed, levels of H_2O_2, which was postulated to be a biomarker of oxidative stress [193], have been found increased in adolescents and adults with DS [46].

In addition, uric acid has been extensively assessed in DS in a wide number of matrices, being reported that DS individuals usually have hyperuricemia. Indeed, two genes (*GART* and *CBS*) involved in uric acid accumulation are located on HSA21 (Table 1). It is known that free radicals are generated in uric acid production by xanthine oxidase (XO), whose activity has been found increased in DS individuals [194]. Therefore, being a powerful antioxidant, the deleterious effects of free radicals generated during its production could also contribute to increase oxidative stress in DS. Besides, since uric acid represents a high relative contribution of TAC in biological samples [195], its involvement in TAC measurements may be remarkably important. For this reason, total antioxidant capacity without relative contribution of uric acid (TAC^{-UA}) has been assessed in DS [195, 196] and may give more reliable results than TAC.

On the other hand, for the collective results of the relative studies assessing oxidative/nitrosative stress biomarkers in DS individuals, it should be pointed out that: *1*) most biomarkers are related not only to trisomy 21 but also to age, diseases and nutritional status; *2*) since oxidative conditions have been suggested to be of importance in developmental and maturational processes, biomarkers of oxidative stress may need to be differently interpreted in childhood and adulthood, nonetheless we can found several works in which age groups are formed by both children and adolescents or even children and adults; *3*) methodological shortcomings are frequently found, although they are not usually taken into account in the approach to discussion; *4*) it has been suggested that DS individuals could suffer from renal impairment, therefore oxidative stress biomarkers must be determined not only in plasma or serum samples but also in urine samples of DS individuals; *5*) most of biomarkers have been assessed in a reduced number of matrices and/or in a limited age range; *6*) a large number of oxidative stress biomarkers have not been analysed in DS yet, such as 3-nitrotyrosine or biopyrrins; *7*) consensus and establishment of reference intervals and values are requirements for suitable biomarkers. Therefore, further studies assessing oxidative/nitrosative stress biomarkers in DS may help to fully elucidate the underlying etiology and pathophysiology as well as the development of effective therapies.

Table 3. Results of the studies that measured biomarkers of oxidative stress in DS individuals.

DNA	8-OHdG	Urine	↑	62 DS vs. 62 CS (n.a.; children)	Jovanovic *et al.* (1998) [188]
			NS	6 DS vs. 47 C (n.a.)	Lloret *et al.* (2008) [189]
			NS	26 DS vs. 19 CS (3-14 years)	Campos *et al.* (2011) [45]
			NS	77 DS vs. 45 C (15-59 years)	Campos *et al.* (2011) [46]
			NS	29 DS vs. 10 C (15-19 years)	
			NS	33 DS vs. 23 C (20-40 years)	
			NS	15 DS vs. 12 C (41-59 years)	
		Leukocytes	↑	28 DS vs. 63 C (1-57 years)	Pallardó *et al.* (2006) [194]
			↑	11 DS vs. 4 C (1-11 years)	
			↑	4 DS vs. 14 C (11-20 years)	
			NS	6 DS vs. 11 C (21-30 years)	
			NS	7 DS vs. 24 C (31-57 years)	
			↑	n.a. (1-10 years)	Lloret *et al.* (2008) [189]
			↑	n.a. (11-20 years)	
			NS	n.a. (21-30 years)	
			NS	n.a. (>30 years)	
		Cerebral cortex	NS	10 DS vs. 10 C (44-69 years)	Seidl *et al.* (1997) [37]
	8-OHG	Cerebral cortex	↑	22 DS vs. 10 C (0.3-65 years)	Nunomura *et al.* (1999) [197]
			↑	10 DS vs. 10 C (0.8-6.2 years)	Kantar *et al.* (1999) [198]
	Comet assay	Lymphocytes	↑	40 DS vs. n.a. C (4-13 years)	Tiano *et al.* (2005) [199]
			NS	25 DS vs. 25 C (3-58 years)	Zana *et al.* (2006) [200]
			NS	7 DS vs. 7 C (3-15 years)	
			NS	18 DS vs. 18 C (18-58 years)	
			↑	40 DS vs. 17 C (<5 years)	Jayaprakash *et al.* (2010) [201]
Lipid	MDA/TBARS	Urine	↑	68 DS vs. 73 CS (n.a.; children)	Jovanovic *et al.* (1998) [188]
			NS	26 DS vs. 19 CS (3-14 years)	Campos *et al.* (2011) [45]
			NS	78 DS vs. 65 C (15-59 years)	Campos *et al.* (2011) [46]
			NS	29 DS vs. 15 C (15-19 years)	
			NS	33 DS vs. 31 C (20-40 years)	
			↓	16 DS vs. 19 C (41-59 years)	
		Plasma	↓	6 DS vs. 68 C (6-16 years)	Gromadzinska *et al.* (1988) [202]
			↓	8DS vs. 9 C (17-30 years)	
			NS	10 DS vs. 6 C (0.75-22 years)	Bras *et al.* (1989) [100]
			NS	12 DS vs. 12 C (22-62 years)	Shah & Johnson (1989) [203]
			NS	46 DS vs. 43 C (1-8 years)	Capone *et al.* (2002) [204]
		Serum	↑	31 DS vs. 30 CS (1.7-46.8 years)	Muchová *et al.* (2001) [190]
			NS	n.a. (1-5 years)	
			NS	n.a. (6-12 years)	
			NS	n.a. (13-20 years)	
			NS	n.a. (20-46.8 years)	
			↑	50 DS vs. 50 C (3-24 years)	Garcez *et al.* (2005) [205]
			NS	42 DS vs. 48 C (n.a.; children)	Meguiz *et al.* (2010) [206]
		Erythrocytes	↑	9 DS vs. 9 C (0.75-22 years)	Bras *et al.* (1989) [100]
			↑	12 DS vs. 12 C (22-62 years)	Shah & Johnson (1989) [203]
			NS	37 DS vs. 33 CS (1.7-46.8 years)	Muchová *et al.* (2001) [190]
			NS	n.a. (1-5 years)	
			NS	n.a. (6-12 years)	
			NS	n.a. (13-20 years)	
			NS	n.a. (20-46.8 years)	
			↑	42 DS vs. 24 C (n.a.)	Garaiová *et al.* (2004) [191]
			↑	16 DS vs. 20 C (Newborn-1 year)	Casado *et al.* (2007) [207]
			↑	17 DS vs. 20 C (2-4 years)	
			↑	18 DS vs. 18 C (5-9 years)	
			↑	14 DS vs. 15 C (10-14 years)	
			↑	14 DS vs. 15 C (15-19 years)	
			↑	11 DS vs. 12 C (20-29 years)	
		Cerebral cortex	↑	5 DS vs. 12 C (fetuses)	Brooksbank *et al.* (1984) [31]

Lipid	MDA/TBARS	Cerebral cortex	↑	4 DS vs. 4 C (n.a.)	Reynolds & Cutts (1993) [208]
			NS	9 DS vs. 14 C (n.a.)	Hayn *et al.* (1996) [36]
			↑	8 DS vs. 4 C (fetuses)	Odetti *et al.* (1998) [89]
	Isoprostanes 8,13-iso-iPF$_{2\alpha}$-VI	Urine	↑	33 DS vs. 33 C (1-15 years)	Praticò *et al.* (2000) [209]
	Isoprostanes 15-F$_{2t}$-IsoP	Urine	NS	26 DS vs. 19 CS (3-14 years)	Campos *et al.* (2011) [45]
			NS	78 DS vs. 44 C (15-59 years)	Campos *et al.* (2011) [46]
			NS	29 DS vs. 10 C (15-19 years)	
			NS	33 DS vs. 21 C (20-40 years)	
			↓	16 DS vs. 13 C (41-59 years)	
	4-hydroxy-2-nonenal	Plasma	NS	20 DS vs. 18 CS (n.a.; children)	Žitňanová *et al.* (2006) [210]
		Cerebral cortex	↑	8 DS vs. 4 C (fetuses)	Odetti *et al.* (1998) [89]
	Lipofuscin	Serum	NS	31 DS vs. 30 CS (1.7-46.8 years)	Muchová *et al.* (2001) [190]
			NS	n.a., (1-5 years)	
			NS	n.a., (6-12 years)	
			NS	n.a. (13-20 years)	
			NS	n.a. (20-46.8 years)	
			NS	34 DS vs. 18 C (n.a.)	Garaiová *et al.* (2004) [191]
	LOOH	Plasma	↑	14 DS vs. 81 C (n.a.)	Kedziora *et al.* (1986) [33]
			NS	46 DS vs. 43 C (1-8 years)	Capone *et al.* (2002) [204]
			↑	23 DS vs. 80 C (18-60 years)	Massaccesi *et al.* (2006) [211]
			↑	23 DS (2-14 y.) vs. 20 C (5-18 y.)	Licastro *et al.* (2007) [212]
			↑	14 DS (20-50 y.) vs. 20 C (5-18 y.)	
			NS	13 DS (>60 y.) vs. 20 C (5-18 y.)	
	OxLDL	Plasma	↑	23 DS (2-14 y.) vs. 20 C (5-18 y.)	Licastro *et al.* (2007) [212]
			↑	14 DS (20-50 y.) vs. 20 C (5-18 y.)	
			NS	13 DS (>60 y.) vs. 20 C (5-18 y.)	
Protein	Protein carbonyls	Plasma	↑	20 DS vs. 18 CS (n.a.; children)	Žitňanová *et al.* (2006) [210]
		Cerebral cortex	↑	8 DS vs. 4 C (fetuses)	Odetti *et al.* (1998) [89]
	Dityrosine	Urine	↑	26 DS vs. 19 CS (3-14 years)	Campos *et al.* (2011) [45]
			↑	75 DS vs. 61 C (15-59 years)	Campos *et al.* (2011) [46]
			↑	29 DS vs. 14 C (15-19 years)	
			↑	31 DS vs. 30 C (20-40 years)	
			↑	15 DS vs. 17 C (41-59 years)	
	o-Tyrosine	Cerebral cortex	NS	9 DS vs. 14 C (n.a.)	Hayn *et al.* (1996) [36]
Antioxidant enzymes	SOD1	Plasma	↑	10 DS vs. 25 C (n.a.; children)	Tanabe *et al.* (1994) [213]
			↓	20 DS vs. 15 C (3-16 years)	Teksen *et al.* (1998) [214]
		Serum	↑	50 DS vs. 50 C (3-24 years)	Garcez *et al.* (2005) [205]
		Blood	↑	16 DS vs. 20 C (Newborn-1 year)	Casado *et al.* (2005) [215]
			↑	17 DS vs. 20 C (2-4 years)	
			↑	18 DS vs. 18 C (5-9 years)	
			↑	14 DS vs. 15 C (10-14 years)	
			↑	14 DS vs. 15 C (15-19 years)	
			↑	11 DS vs. 12 C (20-29 years)	
		Erythrocytes	↑	10 DS vs. 10 C (>4 years)	Sinet *et al.* (1974) [29]
			↑	33 DS vs. 33 C (4-29 years)	Frants *et al.* (1975) [216]
			↑	8 DS vs. 9 C (n.a.; children)	Gilles *et al.* (1976) [217]
			↑	28 DS vs. 26 C (16-24 years)	Crosti *et al.* (1976) [218]
			↑	13 DS vs. 19 C (16-26 years)	Kedziora *et al.* (1977) [219]
			↑	70 DS vs. 67 C (n.a.)	Garber *et al.* (1979) [220]
			↑	24 DS vs. 24 C (n.a.)	Fischer *et al.* (1981) [221]
			↑	15 DS vs. 36 C (n.a.)	Jeziorowska *et al.* (1982) [222]
			↑	29 DS vs. 32 C (5-35 years)	Nève *et al.* (1983) [126]
			↑	14 DS vs. 81 C (n.a.)	Kedziora *et al.* (1986) [33]
			↑	59 DS vs. 50 C (n.a.; children)	Jeziorowska *et al.* (1988) [223]

Antioxidant enzymes	SOD1	Erythrocytes	↑	20 DS and C (31-70 years)	Percy *et al.* (1990) [224]
			NS	4 DS vs. 5 C (0-3 years)	Ibarra *et al.* (1990) [225]
			↑	13 DS vs. 8 C (4-6 years)	
			↑	29 DS vs. 30 C (7-15.6 years)	
			↑	10 DS vs. 25 C (n.a.; children)	Tanabe *et al.* (1994) [213]
			↑	57 DS vs. 2139 C (2-45 years)	De La Torre *et al.* (1996) [35]
			↑	6 DS vs. 255 C (2-4 years)	
			↑	13 DS vs. 206 C (5-9 years)	
			↑	38 DS vs. 96 C (10-14 years)	
			↑	72 DS vs. 72 C (1-50 years)	Pastor *et al.* (1998) [39]
			↑	8 DS vs. 15 C (0.75-3 years)	Meguiz *et al.* (2001) [226]
			↑	29 DS vs. 25 CS (1.7-46.8 years)	Muchová *et al.* (2001) [190]
			↑	n.a., (1-5 years)	
			↑	n.a., (6-12 years)	
			NS	n.a. (13-20 years)	
			NS	n.a. (20-46.8 years)	
			↑	33 DS vs. 33 C (18-53 years)	Torsdottir *et al.* (2001) [227]
			↑	12 DS with cataracts vs. 12 C (n.a.)	Cengiz *et al.* (2002) [107]
			↑	46 DS vs. 64 C (n.a.; children)	Pastore *et al.* (2003) [192]
			↑	42 DS vs. 26 C (n.a.)	Garaiová *et al.* (2004) [191]
		PMNs	↑	6 DS vs. 7 C (n.a.)	Feaster *et al.* (1977) [56]
		Lymphocytes	↑	5 DS vs. 7 C (n.a.)	Feaster *et al.* (1977) [56]
		Neutrophils	↑	31 DS vs. 28 CS (1.7-46.8 years)	Muchová *et al.* (2001) [190]
			NS	n.a. (1-5 years)	
			NS	n.a. (6-12 years)	
			NS	n.a. (13-20 years)	
			NS	n.a. (20-46.8 years)	
		Platelets	↑	11 DS vs. 9 C (n.a.)	Sinet *et al.* (1975) [55]
		Cerebral cortex	NS	9 DS vs. 14 C (n.a.)	Hayn *et al.* (1996) [36]
			↑	5 DS vs. 12 C (fetuses)	Brooksbank *et al.* (1984) [31]
	GPx	Plasma	↓	6 DS vs. 78 C (6-16 years)	Gromadzinska *et al.* (1988) [202]
			↓	8DS vs. 32 C (17-30 years)	
			↓	20 DS vs. 15 C (3-16 years)	Teksen *et al.* (1998) [214]
		Blood	↑	8 DS vs. 15 C (0.75-3 years)	Meguiz *et al.* (2001) [226]
		Erythrocytes	↑	12 DS vs. 18 C (>3 years)	Sinet *et al.* (1975) [228]
			↑	47 DS vs. 46 C (n.a.)	Agar *et al.* (1980) [229]
			↑	24 DS vs. 24 C (n.a.)	Fischer *et al.* (1981) [221]
			↑	29 DS vs. 32 C (5-35 years)	Nève *et al.* (1983) [126]
			↑	14 DS vs. 81 C (n.a.)	Kedziora *et al.* (1986) [33]
			↑	6 DS vs. 78 C (6-16 years)	Gromadzinska *et al.* (1988) [202]
			NS	8DS vs. 32 C (17-30 years)	
			↑	20 DS and C (31-70 years)	Percy *et al.* (1990) [224]
			NS	10 DS vs. 25 C (n.a.; children)	Tanabe *et al.* (1994) [213]
			↑	72 DS vs. 72 C (1-50 years)	Pastor *et al.* (1998) [39]
			↑	37 DS vs. 30 CS (1.7-46.8 years)	Muchová *et al.* (2001) [190]
			NS	n.a., (1-5 years)	
			↑	n.a., (6-12 years)	
			NS	n.a. (13-20 years)	
			NS	n.a. (20-46.8 years)	
			↑	12 DS with cataracts vs. 12 C (n.a.)	Cengiz *et al.* (2002) [107]
			↓	46 DS vs. 64 C (n.a.; children)	Pastore *et al.* (2003) [192]
			↑	43 DS vs. 26 C (n.a.)	Garaiová *et al.* (2004) [191]

Antioxidant enzymes	GPx	Erythrocytes	↑	16 DS vs. 20 C (Newborn-1 year)	Casado *et al.* (2005) [215]
			↑	17 DS vs. 20 C (2-4 years)	
			↑	18 DS vs. 18 C (5-9 years)	
			↑	14 DS vs. 15 C (10-14 years)	
			↑	14 DS vs. 15 C (15-19 years)	
			↑	11 DS vs. 12 C (20-29 years)	
		Neutrophils	↑	32 DS vs. 27 CS (1.7-46.8 years)	Muchová *et al.* (2001) [190]
			NS	n.a. (1-5 years)	
			NS	n.a. (6-12 years)	
			NS	n.a. (13-20 years)	
			NS	n.a. (20-46.8 years)	
		Cerebral cortex	NS	7 DS vs. 9 C (fetuses)	Brooksbank *et al.* (1984) [31]
			NS	9 DS vs. 14 C (n.a.)	Hayn *et al.* (1996) [36]
	CAT	Serum	↑	50 DS vs. 50 C (3-24 years)	Garcez *et al.* (2005) [205]
			NS	14 DS vs. 81 C (n.a.)	Kedziora *et al.* (1986) [33]
			NS	20 DS and C (31-70 years)	Percy *et al.* (1990) [224]
			NS	10 DS vs. 25 C (n.a.; children)	Tanabe *et al.* (1994) [213]
			NS	72 DS vs. 72 C (1-50 years)	Pastor *et al.* (1998) [39]
		Erythrocytes	NS	32 DS vs. 29 CS (1.7-46.8 years)	Muchová *et al.* (2001) [190]
			NS	n.a. (1-5 years)	
			NS	n.a. (6-12 years)	
			NS	n.a. (13-20 years)	
			NS	n.a. (20-46.8 years)	
			NS	41 DS vs. 25 C (n.a.)	Garaiová *et al.* (2004) [191]
			NS	16 DS vs. 20 C (Newborn-1 year)	Casado *et al.* (2005) [215]
			NS	17 DS vs. 20 C (2-4 years)	
			NS	18 DS vs. 18 C (5-9 years)	
			NS	14 DS vs. 15 C (10-14 years)	
			NS	14 DS vs. 15 C (15-19 years)	
			NS	11 DS vs. 12 C (20-29 years)	
		Neutrophils	NS	29 DS vs. 29 CS (1.7-46.8 years)	Muchová *et al.* (2001) [190]
			NS	n.a. (1-5 years)	
			NS	n.a. (6-12 years)	
			NS	n.a. (13-20 years)	
			NS	n.a. (20-46.8 years)	
			NS	22 DS vs. 24 C (n.a.)	Fischer *et al.* (1981) [221]
	GR		↑	72 DS vs. 72 C (1-50 years)	Pastor *et al.* (1998) [39]
		Erythrocytes	NS	36 DS vs. 32 CS (1.7-46.8 years)	Muchová *et al.* (2001) [190]
			NS	n.a. (1-5 years)	
			NS	n.a. (6-12 years)	
			NS	n.a. (13-20 years)	
			NS	n.a. (20-46.8 years)	
			NS	46 DS vs. 64 C (n.a.; children)	Pastore *et al.* (2003) [192]
			NS	16 DS vs. 20 C (Newborn-1 year)	Casado *et al.* (2005) [215]
			NS	17 DS vs. 20 C (2-4 years)	
			NS	18 DS vs. 18 C (5-9 years)	
			NS	14 DS vs. 15 C (10-14 years)	
			NS	14 DS vs. 15 C (15-19 years)	
			NS	11 DS vs. 12 C (20-29 years)	
		Neutrophils	NS	29 DS vs. 30 CS (1.7-46.8 years)	Muchová *et al.* (2001) [190]
			NS	n.a., (1-5 years)	
			NS	n.a., (6-12 years)	
			NS	n.a. (13-20 years)	
			NS	n.a. (20-46.8 years)	
	MPO	Neutrophils	NS	23 DS vs. 30 CS (1.7-46.8 years)	Muchová *et al.* (2001) [190]
	GST	Erythrocytes	NS	12 DS with cataracts vs. 12 C (n.a.)	Cengiz *et al.* (2002) [107]
			↓	46 DS vs. 64 C (n.a.; children)	Pastore *et al.* (2003) [192]
	SOD1/GPx	Erythr./Blood	NS	8 DS vs. 15 C (0.75-3 years)	Meguiz *et al.* (2001) [226]
		Erythrocytes	↑	46 DS vs. 64 C (n.a.; children)	Pastore *et al.* (2003) [192]

Antioxidant enzymes	SOD1/(GPx+CAT)	Erythrocytes	NS	72 DS vs. 72 C (1-50 years)	Pastor *et al.* (1998) [39]
			↑	24 DS vs. 19 CS (1.7-46.8 years)	Muchová *et al.* (2001) [190]
			NS	n.a. (1-5 years)	
			NS	n.a. (6-12 years)	
			NS	n.a. (13-20 years)	
			NS	n.a. (20-46.8 years)	
			↑	38 DS vs. 24 C (n.a.)	Garaiová *et al.* (2004) [191]
	SOD1/(GPx+CAT+MPO)	Neutrophils	↑	16 DS vs. 19 CS (1.7-46.8 years)	Muchová *et al.* (2001) [190]
			NS	n.a. (1-5 years)	
			NS	n.a. (6-12 years)	
			NS	n.a. (13-20 years)	
			NS	n.a. (20-46.8 years)	
	Ceruloplasmin	Serum	NS	35 DS vs. 35 C (18-53 years)	Torsdottir *et al.* (2001) [227]
	Ceruloplasmin oxidative activity	Serum	NS	35 DS vs. 35 C (18-53 years)	Torsdottir *et al.* (2001) [227]
	Ceruloplasmin specific oxidative activity	Serum	NS	35 DS vs. 35 C (18-53 years)	Torsdottir *et al.* (2001) [227]
Antioxidants	GSH	Plasma	↓	42 DS vs. 36 CS (n.a.; children)	Pogribna *et al.* (2001) [230]
			NS	60 DS vs. 29 CS (0.5-17 years)	Pinto *et al.* (2002) [231]
		Serum	NS	12 DS with cataracts vs. 12 C (n.a.)	Cengiz *et al.* (2002) [107]
		Blood	NS	24 DS vs. 24 C (n.a.)	Fischer *et al.* (1981) [221]
			↓	46 DS vs. 64 C (n.a.; children)	Pastore *et al.* (2003) [192]
			NS	40 DS vs. 26 C (n.a.)	Garaiová *et al.* (2004) [191]
			NS	10 DS vs. 31 C (0.16-14 years)	Pallardó *et al.* (2006) [194]
			NS	7 DS vs. 29 C (15-57 years)	
	GSSG	Plasma	NS	60 DS vs. 29 CS (0.5-17 years)	Pinto *et al.* (2002) [231]
		Blood	↓	40 DS vs. 25 C (n.a.)	Garaiová *et al.* (2004) [191]
			↑	10 DS vs. 31 C (0.16-14 years)	Pallardó *et al.* (2006) [194]
			NS	7 DS vs. 29 C (15-57 years)	
	Total GSH	Plasma	NS	60 DS vs. 29 CS (0.5-17 years)	Pinto *et al.* (2002) [231]
		Blood	↓	46 DS vs. 64 C (n.a.; children)	Pastore *et al.* (2003) [192]
			NS	10 DS vs. 31 C (0.16-14 years)	Pallardó *et al.* (2006) [194]
			NS	7 DS vs. 29 C (15-57 years)	
	GSSH/GSH	Blood	↓	46 DS vs. 64 C (n.a.; children)	Pastore *et al.* (2003) [192]
			↑	10 DS vs. 31 C (0.16-14 years)	Pallardó *et al.* (2006) [194]
			↓	7 DS vs. 29 C (15-57 years)	
			↑	10 DS vs. 43 C (<16 years)	Lloret *et al.* (2008) [189]
			↓	7 DS vs. 29 C (>14 years)	
	GSH/GSSG	Blood	NS	40 DS vs. 25 C (n.a.)	Garaiová *et al.* (2004) [191]
	Uric acid	Urine	NS	35 DS vs. 35 C ♂ (n.a.)	Coburn *et al.* (1967) [124]
			NS	25 DS vs. 13 C ♂ (0-24 years)	Howell *et al.* (1973) [232]
			↑	18 DS vs. 17 C ♀ (0-24 years)	
			NS	20 DS vs. 25 C ♂ (25-39 years)	
			NS	17 DS vs. 14 C ♀ (25-39 years)	
			NS	22 DS vs. 24 C ♂ (>40 years)	
			NS	11 DS vs. 13 C ♀ (>40 years)	
			↑	19 DS vs. 14 C (1-13 years)	Campos *et al.* (2010) [195]
			NS	13 DS vs. 15 C (43-61 years)	
		Plasma	↑	35 DS vs. 35 C ♂ (n.a.)	Coburn *et al.* (1967) [124]
			↑	15 DS vs. 15 C (15-25 years)	Appleton *et al.* (1969) [233]
			NS	20 DS vs. 16 C (8-43 years)	Nishida *et al.* (1979) [125]
			↑	20 DS vs. 20 C (8-33 years)	Puuka *et al.* (1982) [234]
			NS	8 DS vs. 5 C (0.75-22 years)	Bras *et al.* (1989) [100]
			↑	16 DS vs. 16 C (n.a.; children)	Žitňanová *et al.* (2004) [235]
			↑	13 DS vs. 7 C (0.16-14 years)	Pallardó *et al.* (2006) [194]
			↑	5 DS vs. 9 C (15-57 years)	

Antioxidants	Uric acid	Plasma	NS	23 DS vs. 98 C (n.a.)	Lloret *et al.* (2008) [189]
			↑	30 DS vs. 30 C (4-12 years)	Tiano *et al.* (2008) [196]
		Serum	NS	15 SD vs. 18 C (5-14.5 years)	Sobel *et al.* (1958) [26]
			↑	10 DS vs. 10 C (n.a.)	Appleton *et al.* (1964) [236]
			↑	107 DS vs. 107 C (<20 years)	Kaufman & O'Brien (1967) [237]
			↑	280 DS vs. 298 C (3-66 years)	Pant *et al.* (1968) [238]
			↑	9 DS vs. 9 C ♂ (3-5 years)	
			NS	9 DS vs. 9 C ♀ (3-5 years)	
			↑	20 DS vs. 18 C ♂ (6-10 years)	
			↑	14 DS vs. 14 C ♀ (6-10 years)	
			↑	19 DS vs. 21 C ♂ (11-15 years)	
			↑	14 DS vs. 18 C ♀ (11-15 years)	
			↑	17 DS vs. 19 C ♂ (16-20 years)	
			↑	15 DS vs. 18 C ♀ (16-20 years)	
			↑	18 DS vs. 20 C ♂ (21-25 years)	
			↑	13 DS vs. 13 C ♀ (21-25 years)	
			↑	10 DS vs. 10 C ♂ (26-30 years)	
			↑	15 DS vs. 15 C ♀ (26-30 years)	
			↑	16 DS vs. 18 C ♂ (31-35 years)	
			↑	18 DS vs. 18 C ♀ (31-35 years)	
			↑	13 DS vs. 13 C ♂ (36-40 years)	
			↑	10 DS vs. 10 C ♀ (36-40 years)	
			↑	14 DS vs. 13 C ♂ (41-45 years)	
			↑	12 DS vs. 10 C ♀ (41-45 years)	
			↑	8 DS vs. 6 C ♂ (46-50 years)	
			NS	5 DS vs. 7 C ♀ (46-50 years)	
			↑	4 DS vs. 8 C ♂ (51-66 years)	
			NS	9 DS vs. 13 C ♀ (51-66 years)	
			↑	25 DS vs. 13 C ♂ (0-24 years)	Howell *et al.* (1973) [232]
			↑	18 DS vs. 17 C ♀ (0-24 years)	
			↑	20 DS vs. 25 C ♂ (25-39 years)	
			↑	17 DS vs. 14 C ♀ (25-39 years)	
			↑	22 DS vs. 24 C ♂ (>40 years)	
			↑	11 DS vs. 13 C ♀ (>40 years)	
			↑	29 DS vs. 29 C (21-72 years)	Hestnes *et al.* (1991) [239]
			↑	25 DS vs. 25 CS (n.a.; children and adolescents)	Nagyová *et al.* (2000) [240]
			↑	30 DS vs. 25 C (n.a.)	Garaiová *et al.* (2004) [191]
			↑	50 DS vs. 50 C (3-24 years)	Garcez *et al.* (2005) [205]
	Vitamin C (ascorbic acid)	Plasma	NS	5 DS vs. 6 C (0.75-22 years)	Bras *et al.* (1989) [100]
			↑	13 DS vs. 19 C (0.16-14 years)	Pallardó *et al.* (2006) [194]
			NS	5 DS vs. 9 C (15-57 years)	
			NS	n.a. (n.a.)	Lloret *et al.* (2008) [189]
		Serum	↓	42 DS vs. 48 C (n.a.; children)	Meguiz *et al.* (2010) [206]
	Vitamin E (α-tocopherol)	Plasma	NS	9 DS vs. 7 C (0.75-22 years)	Bras *et al.* (1989) [100]
			↓	12 DS vs. 12 C (22-62 years)	Shah & Johnson (1989) [203]
			NS	22 DS vs. 25 C (n.a.; children)	Tanabe *et al.* (1994) [213]
			NS	n.a. (n.a.)	Lloret *et al.* (2008) [189]
		Serum	NS	42 DS vs. 48 C (n.a.; children)	Meguiz *et al.* (2010) [206]
		Erythrocytes	↓	12 DS vs. 12 C (22-62 years)	Shah & Johnson (1989) [203]
			NS	22 DS vs. 25 C (n.a.; children)	Tanabe *et al.* (1994) [213]
			NS	72 DS vs. 72 C (1-50 years)	Pastor *et al.* (1998) [39]
		Platelets	NS	22 DS vs. 25 C (n.a.; children)	Tanabe *et al.* (1994) [213]
		MNs	NS	22 DS vs. 25 C (n.a.; children)	Tanabe *et al.* (1994) [213]
		PMNs	NS	22 DS vs. 25 C (n.a.; children)	Tanabe *et al.* (1994) [213]

Antioxidants	Vitamin E (α-tocopherol)	BMs	NS	22 DS vs. 25 C (n.a.; children)	Tanabe *et al.* (1994) [213]
		Cerebral cortex	NS	8 DS vs. 8 C (fetuses)	Metcalfe *et al.* (1989) [241]
	Vitamin A (β-carotene)	Plasma	↑	12 DS vs. 12 C (22-62 years)	Shah & Johnson (1989) [203]
			NS	22 DS vs. 25 C (n.a.; children)	Tanabe *et al.* (1994) [213]
		Serum	↓	10 DS vs. 10 C (n.a.)	Appleton *et al.* (1964) [236]
			NS	44 DS vs. 40 C (1-34 years)	Barden (1977) [242]
			NS	44 DS vs. 21 C (1-19 years)	Storm (1990) [243]
			NS	71 DS vs. 41 C (0.16-25 years)	Ercis *et al.* (1996) [244]
		Blood	NS	33 DS vs. 14 C (6-33 years)	Pueschel *et al.* (1990) [245]
	Thiol groups	Serum	↓	40 DS vs. 20 C (2-7 years)	Carratelli *et al.* (2001) [43]
	SLRO	Plasma	↑	23 DS vs. 80 C (18-60 years)	Massaccesi *et al.* (2006) [211]
			NS	30 DS vs. 30 C (4-12 years)	Tiano *et al.* (2008) [196]
		Serum	↑	25 DS vs. 25 CS (n.a.; children and adolescents)	Nagyová *et al.* (2000) [240]
	TAC	Urine	↑	19 DS vs. 14 C (1-13 years)	Campos *et al.* (2010) [195]
			NS	13 DS vs. 15 C (43-61 years)	
		Plasma	NS	20 DS vs. 18 CS (n.a.; children)	Žitňanová *et al.* (2006) [210]
			↑	30 DS vs. 30 C (4-12 years)	Tiano *et al.* (2008) [196]
		Serum	↓	40 DS vs. 20 C (2-7 years)	Carratelli *et al.* (2001) [43]
			↓	42 DS vs. 25 C (n.a.)	Garaiová *et al.* (2004) [191]
	TAC^{-UA}	Urine	NS	19 DS vs. 14 C (1-13 years)	Campos *et al.* (2010) [195]
			↓	13 DS vs. 15 C (43-61 years)	
		Plasma	NS	30 DS vs. 30 C (4-12 years)	Tiano *et al.* (2008) [196]
Miscellaneous	Allantoin	Plasma	↑	16 DS vs. 16 C (n.a.; children)	Žitňanová *et al.* (2004) [235]
			NS	30 DS vs. 30 C (4-12 years)	Tiano *et al.* (2008) [196]
	AGEs	Urine	NS	26 DS vs. 19 CS (3-14 years)	Campos *et al.* (2011) [45]
			NS	75 DS vs. 61 C (15-59 years)	Campos *et al.* (2011) [46]
			↑	29 DS vs. 14 C (15-19 years)	
			NS	31 DS vs. 30 C (20-40 years)	
			NS	15 DS vs. 17 C (41-59 years)	
	Glyoxal	Plasma	↑	9 DS vs. 13 C (0.16-14 years)	Pallardó *et al.* (2006) [194]
			NS	14 DS vs. 10 C (15-57 years)	
			NS	23 DS vs. 98 C (n.a.)	Lloret *et al.* (2008) [189]
	Methylglyoxal	Plasma	NS	9 DS vs. 13 C (0.16-14 years)	Pallardó *et al.* (2006) [194]
			↓	14 DS vs. 10 C (15-57 years)	
			NS	23 DS vs. 98 C (n.a.)	Lloret *et al.* (2008) [189]
	Pentosidine	Cerebral cortex	↑	8 DS vs. 4 C (fetuses)	Odetti *et al.* (1998) [89]
	Pyrraline	Cerebral cortex	↑	8 DS vs. 4 C (fetuses)	Odetti *et al.* (1998) [89]
	GS-Hb	Erythrocytes	↓	46 DS vs. 64 C (n.a.; children)	Pastore *et al.* (2003) [192]
	XO	Plasma	↑	7 DS vs. 19 C (n.a.; young)	Pallardó *et al.* (2006) [194]
	CoQ_{10}	Plasma	↑	14 DS vs. 12 C (n.a.; children)	Milles *et al.* (2007) [246]
			NS	30 DS vs. 30 C (4-12 years)	Tiano *et al.* (2008) [196]
		Platelets	↓	30 DS vs. 30 C (4-12 years)	Tiano *et al.* (2008) [196]
		Lymphocytes	↓	30 DS vs. 30 C (4-12 years)	Tiano *et al.* (2008) [196]
	Total CoQ_{10}	Plasma	NS	14 DS vs. 12 C (n.a.; children)	Milles *et al.* (2007) [246]
	$CoQ_{10}H_2$	Plasma	NS	14 DS vs. 12 C (n.a.; children)	Milles *et al.* (2007) [246]
	$CoQ_{10}H_2$/Total CoQ_{10}	Plasma	↓	14 DS vs. 12 C (n.a.; children)	Milles *et al.* (2007) [246]
	Hcy	Plasma	↓	8 DS vs. 8 C (6-10 years)	Chadefaux *et al.* (1988) [247]
			↓	42 DS vs. 36 CS (n.a.; children)	Pogribna *et al.* (2001) [230]

Miscellaneous		Hcy	Plasma	NS	25 DS vs. 160 C (15-46 years)	Fillon-Emery *et al.* (2004) [248]
				NS	5 DS vs. 47 C (15-46 years)	
				NS	10 DS vs. 62 C (15-46 years)	
				NS	9 DS vs. 46 C (15-46 years)	
				NS	1 DS vs. 4 C (15-46 years)	
			Serum	↓	42 DS vs. 48 C (n.a.; children)	Meguiz *et al.* (2010) [206]
		Zinc	Plasma	NS	29 DS vs. 32 C (5-35 years)	Nève *et al.* (1983) [126]
				NS	16 DS vs. 16 C (<6 years)	Noble & Warren (1988) [249]
				NS	20 DS vs. 15 C (3-16 years)	Teksen *et al.* (1998) [214]
				NS	8 DS vs. 15 C (0.75-3 years)	Meguiz *et al.* (2001) [226]
			Serum	↓	16 DS vs. 16 C (4-23 years)	Kadrabova *et al.* (1996) [250]
				↓	42 DS vs. 48 C (n.a.; children)	Meguiz *et al.* (2010) [206]
			Erythrocytes	↑	29 DS vs. 32 C (5-35 years)	Nève *et al.* (1983) [126]
		Selenium	Plasma	↓	29 DS vs. 32 C (5-35 years)	Nève *et al.* (1983) [126]
				NS	65 DS vs. 90 C (0-56 years)	Annerén *et al.* (1985) [251]
				↓	14 DS vs. 81 C (n.a.)	Kedziora *et al.* (1986) [33]
				↓	6 DS vs. 77 C (6-16 years)	Gromadzinska *et al.* (1988) [202]
				↓	8DS vs. 32 C (17-30 years)	
				NS	20 DS vs. 15 C (3-16 years)	Teksen *et al.* (1998) [214]
			Serum	↓	16 DS vs. 16 C (4-23 years)	Kadrabova *et al.* (1996) [250]
			Blood	↓	6 DS vs. 77 C (6-16 years)	Gromadzinska *et al.* (1988) [202]
				↓	8DS vs. 32 C (17-30 years)	
				↓	8 DS vs. 15 C (0.75-3 years)	Meguiz *et al.* (2001) [226]
			Erythrocytes	NS	47 DS vs. 46 C (n.a.)	Agar *et al.* (1980) [229]
				NS	29 DS vs. 32 C (5-35 years)	Nève *et al.* (1983) [126]
				↑	65 DS vs. 90 C (0-56 years)	Annerén *et al.* (1985) [251]
				↓	6 DS vs. 74 C (6-16 years)	Gromadzinska *et al.* (1988) [202]
				↓	8DS vs. 32 C (17-30 years)	
		NOx	Urine	NS	26 DS vs. 19 CS (3-14 years)	Campos *et al.* (2011) [45]
				↑	74 DS vs. 64 C (15-59 years)	Campos *et al.* (2011) [46]
				↑	29 DS vs. 15 C (15-19 years)	
				↑	31 DS vs. 31 C (20-40 years)	
				NS	14 DS vs. 18 C (41-59 years)	
	ROS	d-ROMs test	Serum	↑	40 DS vs. 20 C (2-7 years)	Carratelli *et al.* (2001) [43]
		superoxide	Leukocytes	↑	46 DS vs. 43 C (1-8 years)	Capone *et al.* (2002) [204]
		H_2O_2	Urine	NS	26 DS vs. 19 CS (3-14 years)	Campos *et al.* (2011) [45]
				↑	72 DS vs. 58 C (15-59 years)	Campos *et al.* (2011) [46]
				↑	29 DS vs. 14 C (15-19 years)	
				↑	31 DS vs. 29 C (20-40 years)	
				NS	12 DS vs. 15 C (41-59 years)	

8-OHdG, 8-hydroxy-2'-deoxyguanosine; 8-OHG, 8-hydroxyguanosine; AGEs, advanced glycation end-products; BMs, buccal mucosal cells; C, controls; CAT, catalase; CoQ_{10}, coenzyme Q_{10} or ubiquinone-10; $CoQ_{10}H_2$, ubiquinol-10; CS, control siblings of Down syndrome subjects; DS, Down syndrome; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GS-Hb, glutathionyl-hemoglobin; GSSG, oxidized glutathione; GST, glutathione S-transferase; H_2O_2, hydrogen peroxide; Hcy, homocysteine; LOOH, lipid hydroperoxides; MNs, mononuclear cells; MDA, malondialdehyde; MPO, myeloperoxidase; n.a., not available in the cited reference; NOx, total nitrite and nitrate; NS, not significant; OxLDL, oxidized low density lipoproteins; PMNs, polymorphonuclear leukocytes; ROS, reactive oxygen species; SLRO, serum lipid resistance to oxidation, lag-time to inhibit peroxidation, plasma susceptibility to peroxidation; SOD1, Cu/Zn superoxide dismutase; TAC, total antioxidant capacity; TAC^{-UA}, total antioxidant capacity without relative contribution of uric acid; TBARS, thiobarbituric acid-reacting substances; XO, xanthine oxidase; ♂, males; ♀, females; ↑, significantly increased in DS group; ↓, significantly decreased in DS group.

5. Oxidative stress, exercise and Down syndrome

Since Dillard *et al.* [252] first reported in 1978 that exercise increases lipid peroxidation, the link between exercise and oxidative stress has received considerable attention and the studies about this topic are increasing markedly in number. Thus, it is now well established that exercise of enough intensity and duration increases the formation of RONS which could result in increased oxidative stress [253], as indicated by an increase in oxidized molecules in a variety of tissues and body fluids. However, whether or not an increase in RONS resulting from acute exercise causes oxidative damage remains still unclear.

The effect of exercise on the levels of oxidative stress biomarkers has been extensively studied for both healthy and diseased subjects [254]. These studies have reported inconsistent results concerning the effect of exercise on the levels of specific oxidative stress biomarkers, a fact that, at least partially, may be attributable to differences in the training status, dietary intake, age and gender of subjects, type of exercise test (mode aerobic or anaerobic, duration and intensity), sampling time points, tissue sampled and the assays employed among the studies.

The mitochondrial respiratory chain is believed to be the major cellular source of free radical generation during exercise [255, 256]. Mitochondrial anomalies and dysfunctions are present in DS, which are directly related to the presence of oxidative stress [257]. Moreover, in addition to generation through mitochondrial electron transport, RONS can be produced through a number of alternative pathways during exercise. This is the case of the enzyme XO, whose activity has been found increased in DS [194] and it has been suggested that this enzyme may be more important than mitochondria as a source of exercise-induced free radicals [258]. Thus, mitochondrial dysfunction and the excess in XO activity found in DS lead to think that exercise may exacerbate oxidative stress and tissue damage in these subjects compared to healthy controls.

However, results reported in literature seem to show a beneficial effect of exercise on DS subjects, although the effect of exercise on oxidative stress parameters has been poorly studied in this condition. Monteiro *et al.* [38] found significant increases in plasma TBARS as well as in plasma and erythrocyte GSH and GSSG in adults with DS after a 16-week training program. Besides, they also found that exercise did not significantly alter erythrocyte SOD1 activity, as it was also reported in a later study [259]. Other antioxidant enzymes have been evaluated in DS subjects after an exercise program. Thus, increased activity of erythrocyte glucose-6-phosphate-dehydrogenase (G6PDH) [260], GPx [261] and glutathione reductase (GR) [262] has been found in adolescents with DS, whereas not significant changes have been reported for erythrocyte CAT [263]. Lipid peroxidation was also evaluated by Ordoñez *et al.* [264] in adolescents with DS and, in contrast to Monteiro *et al.* [38], they found decreased levels of MDA after a very similar exercise program, so discrepancies in results could be due to methodological differences. Supporting the results of Ordoñez *et al.* [264], it has been reported that lipid hydroperoxides decreased in saliva samples of DS subjects after an exercise program of slight intensity [265]. On the other hand, it has been recently reported that exercise significantly reduced protein oxidation, measured as plasma protein carbonyl content, in adolescents with DS [266] and also plasma allantoin in the same population [267]. Since both plasma protein carbonyls and allantoin levels have been found increased in children with DS [210, 235], exercise may improve healthy of this subjects.

The effect of exercise on TAC has been also evaluated in DS and no significant effect was reported, in blood [268] or in saliva [265] samples. Moreover, levels of the ratio thiols/total proteins in plasma samples remained stable after exercise in DS, although these levels were lower over the entire physical activity test in DS compared with non-Down controls [268]. In contrast, blood TAC was higher in DS subjects than in non-Down controls after exercise [268]. Authors suggest that the greater TAC in DS participants during exercise might reflect a greater ability to mobilize blood-borne antioxidants due to chronic oxidative stress. In our opinion, increased levels of uric acid during exercise could be another possible explanation for these results, even more when XO activity is increased in DS. In fact, it has been reported that high uric acid concentration increases serum antioxidant capacity and reduces exercise-induced oxidative stress in healthy subjects [269]. However, Zambrano *et al.* [265] found that exercise had no effect on salivary uric acid of DS participants. Therefore, more studies are necessary to clarify these hypotheses.

Data pointed to a greater ability to mobilize blood-borne antioxidants and/or a significant increase of antioxidant enzymes activity (G6PDH, GPx and GR) by exercise in DS subjects as the possible causes of the decrease oxidative stress by exercise. Thus, exercise does not alter CAT and SOD1 activities, but induces increased GPx activity in erythrocytes of these subjects. Since erythrocyte SOD1 activity seems to be increased in this condition [29, 31, 34, 35, 55-57] and GPx is more efficient in scavenging H_2O_2 than CAT [270], exercise could compensate the imbalance of this enzymes protecting against oxidative stress in DS.

On the other hand, resistance exercise and endurance training cause adaptive responses of gene

expression in nuclear and mitochondrial genomes in the skeletal muscle [271]. However, changes of gene expression induced by physical activities and its relation with the gene-dosage imbalance in DS have not yet been studied. Therefore, more well-conducted studies which examine long-term physical outcomes, adverse effects and psychosocial outcomes are needed to reach firm conclusions.

6. Methodological aspects in the assessment of oxidative stress in Down syndrome

Levels of oxidative stress biomarkers are influenced by a number of factors related to the subject population as well as the analytical procedures used in testing the outcome measures. Thus, it should be understood that a large number of studies addressing oxidative stress in DS may be biased by methodological and/or statistical shortcomings and limitations. However, these aspects are not usually taken into account in the approach to discussion.

A wide number of analytical methodologies are available for the assessment of oxidative stress in several body fluids as well as tissues, ranging from simple spectrophotometric assays to more complex assays using gas chromatography-mass spectroscopy (GC-MS) and high-performance liquid chromatography (HPLC). However, many of them, although routinely employed, have not been extensively validated. As such, the measurement of a specific biomarker is usually performed in numerous variations, often resulting in a high degree of variance from one laboratory to another.

Chromatographic techniques are generally more accurate and sensitive than spectrophotometric ones, but suffer from being time-consuming and expensive in apparatus, reagents and man-power requirements, rendering them very difficult to apply clinically in population studies.

Assessment of oxidative stress biomarkers using reliable, sensitive, specific, practical and simple methodologies with clinical relevance seems to be a useful pursuit. Several commercial assay kits have been made available for the measurement of oxidative stress, with many new kits emerging each year. However, the lack of validation of these assays could be responsible for the differences in findings across studies. Thus, for example, it has been recently reported that urea and other substances contribute to the overestimation of urinary 8-OHdG due to cross-reaction with the commercial enzyme-linked immunosorbent assay (ELISA) of Japan Institute for the Control of Aging (JaICA) [272]. In a similar manner, commercially available ELISA assays against 15-F_{2t}-IsoP have not been tested for cross reactivity with most isoeicosanoids and most of their metabolites, that is why they are of uncertain quantitative reliability in biological fluids. Therefore, simple and validated assays must be developed for the accurate determination of oxidative stress biomarkers, even more when the establishment of reference intervals and values are required to define suitable biomarkers.

On the other hand, variations in population size, age or gender could also bias the results. Since oxidative conditions have been suggested to be of importance in developmental and maturational processes, biomarkers of oxidative stress may need to be differently interpreted in childhood and adulthood. Nonetheless, we can found several works studying them in DS where age groups formed by both children and adolescents or even children and adults are compared, so discrepancies in results are frequently found.

Besides, DS subjects suffer from a large number of oxidative stress-related diseases. For example, adults with DS over 40 years are at higher risk for dementia. However, the diagnosis of Alzheimer disease in people with DS is far from an easy task and oxidative damage may increase as a consequence of this disease process in addition to aging, therefore biasing the results.

In addition, DS subjects with trisomy 21 caused by translocation and mosaic trisomy 21 may have a different dosage of both HSA21 genes and imbalance between HSA21 and non-HSA21 genes than those with regular trisomy 21. Therefore, the amount of oxidative damage in these subjects may depend on their kariotype. Indeed, it has been reported that, in individuals with DS due to mosaic, the higher the percentage of diploid cells is, the lower are erythrocyte MDA levels are [207]. Nonetheless, further studies involving individuals with translocation and mosaic DS are needed.

Other potential biasing factors, such as dietary or exercise habits, antioxidant intake, exposure to pollutants, etc., should be considered in future works studying oxidative/nitrosative stress biomarkers in DS. Indeed, improvements in life patterns of DS individuals could contribute to decrease oxidative stress, biasing the results, as it has been recently suggested [46].

7. Therapies for oxidative damage prevention in Down syndrome

Many types of therapies have been used in DS, most of them to prevent and treat the cognitive problems associated with the syndrome, and several revisions can be found in literature [273-275]. In the 1960s, nutritional therapies were used to improve intelligence and appearance of children with DS but without satisfactory results [276]. Since then, advances in medical treatment and social inclusion have

significantly improved the quality of life and life expectancy of individuals with DS. Indeed, their average life expectancy that was barely 12 years in the 1940s is now around 60 years in developed countries [277, 278].

The number of studies conducted to examine interventions focused on decreasing oxidative damage is steadily increasing, and antioxidants have received much attention as potential, non-toxic, treatments for oxidative stress-related pathological conditions.

In a theoretical framework, antioxidants may reduce oxidative damage, improving clinical problems associated with DS and derived from the increased oxidative stress typical of this condition. In fact, studies using antioxidant supplementation often aimed to improve directly some of the oxidative stress-related diseases associated with DS. However, clinical trials with antioxidants have given disappointing results. Thus, Ellis *et al.* [44] reported no significant effect of antioxidant (selenium, zinc, vitamin A, vitamin E and vitamin C) or folinic acid (leucovorin) supplementation on SOD1 and GPx activities or on the SOD1 to GPx ratio or on urinary isoprostane concentrations or on developmental, motor and language outcomes in a randomized controlled trial with DS children. Besides, treatment with ubiquinone-10 (oxidized form of CoQ_{10}) was not able to revert cellular DNA damage in a double blind randomized controlled trial performed in DS children [279].

In contrast, uncontrolled trials support a beneficial effect of antioxidant supplements. Gualandri *et al.* [280] found that L-cysteine and α-lipoic acid supplementation increases levels of thiol groups and TAC of serum samples as well as decreases serum total and septic ROS concentrations in DS children. Besides, Pincheira *et al.* [40] reported an increase in chromosomal damage in lymphocytes of individuals with DS compared with non-Down individuals, which could be reduced by more than 50% by the addition of vitamin E to the cell culture. It has been also reported that the piracetam analog SGS-111 (GVS-111/DVD111/Noopet) (*N*-phenyl-acetyl-l-prolyl-glycine ethyl ester) prevents oxidative damage and apoptosis in both normal and DS human cortical neurons [281]. Moreover, the peptides ADNF-9 (the peptide SALLRSIPA, derived from the activity-dependent neurotrophic factor ADNF) and NAP (the peptide NAPVSIPQ, derived from the activity-dependent neuroprotective protein ADNP) possess potent neuroprotective properties against oxidative damage in DS cortical neurons [282]. Therefore, vitamin E, SGS-111, ADNF-9 and NAP could be useful to preserve neuronal function and prevent neuronal death associated with chronic neurodegenerative disorders in DS.

Trace elements supplementations with selenium and zinc have demonstrated beneficial effects for oxidative damage prevention in DS. Thus, the oral administration of these minerals, which act as cofactors for the action of antioxidants, is frequently included in nutritional supplements advocated for individuals with DS such as Nutravene-D.

Selenium is a component of GPx, which is part of the body's endogenous antioxidant system [283]. Levels of this mineral are decreased in DS individuals [42, 126, 250], suggesting a link with oxidative stress. Indeed, it has been reported a 25% increase in the erythocyte GPx activity and a 24% reduction in the SOD:GPx ratio in selenium supplemented DS individuals from 1 to 54 years old compared to those unsupplemented [284]. In contrast, Anneren *et al.* reported that adults with DS and controls did not differ in their mean plasma and erythrocyte selenium concentrations [251] as well as that selenium supplementation decreases erythrocyte GPx activity in children with DS [285]. However, these studies were carried out in different age groups of DS individuals.

Zinc is part of the SOD1 enzyme. Most studies showed significantly lower serum levels of this mineral in DS individuals compared to those found in controls [250, 274]. It has been recently reported that zinc concentrations are significantly lower in plasma and urine and higher in erythrocytes of DS children [286] and adolescents [287] as compared with those without DS, concluding that altered zinc nutritional status of individuals with DS contributes to clinical disturbances that usually appear with aging in these patients [286]. Moreover, the activities of serum acetyl- and butyrylcholinesterase, key enzymes taking place in pathogenesis of Alzheimer's disease, were found to be reduced in DS children compared to controls and significantly increased after six months of zinc supplementation in combination with antioxidant vitamins and minerals [288], which also improved the cognitive skills and behavioural patterns. Moreover, zinc supplementation has shown its capacity to reduce oxidative stress and incidence of infections [289] and it could enhance the anti-oxidative defences of peripheral blood lymphocytes [290] in elderly non-Down individuals.

On the other hand, it has been suggested that oxidative stress reduces available selenium levels and is thus a major contributor to hypothyroidism in DS [42]. Selenium acts as an antioxidant protecting the thyrocyte from peroxides [291] and is essential for the production of optimum thyroid hormone levels. Therefore, selenium supplementation may be helpful to improve thyroid impairment in DS individuals. In contrast, intervention with zinc showed to be effective in the stabilization of the concentrations of this mineral in plasma and erythrocytes, but seemed to have no influence on the metabolism of thyroid

hormones [292]. In addition, it has been hypothesised that an increase in SOD activity can reduce immunity in DS [274]. Although controversial, several studies have reported reduced infections in DS individuals when they received supplementation with zinc [293] and vitamin A [294]. Therefore, supplementation with selenium could improve thyroid function as well as zinc, selenium and vitamin A could improve immune system through reducing oxidative stress in DS individuals. However, more studies are clearly needed in this area until their supplementation can be recommended in DS individuals.

Novel antioxidant therapies appear each year as attractive candidates to be used as potential therapeutic interventions for oxidative damage prevention. For example, it has been recently reported that dietary astaxanthin decreased 8-OHdG as well as enhanced immune response and inflammation in young healthy females [295]. It has been reported that hydropinic therapy obtains major benefits from sulfurous water consumption in reducing biomolecule oxidation, possibly furnishing valid protection against oxidative damage commonly associated with aging and age-related degenerative diseases [296].

In summary, some studies assessing antioxidant supplements had shown beneficial effects over oxidative damage in DS. This is encouraging because it suggests that effective therapeutic interventions to improve clinical problems associated with DS are possible, improving the quality of life and life expectancy of these individuals, even when the current knowledge about the molecular pathogenesis of DS is remarkably limited. However, there are several issues that reduce that enthusiasm. Studies in this area are scarce, and most of them are uncontrolled trials and present major methodological shortcomings. Moreover, most studies reporting a beneficial effect of a supplement have their analogous which failed to provide the same evidence. Therefore, there is a need for well-conducted randomized controlled trials to evaluate possible therapies for oxidative damage prevention in DS, which is undoubtedly a truly remarkable challenge in this pathology. For that, it should be taken into account several concerns. First, there are few risks for most nutritional supplements because what is not needed is excreted or metabolized. However, a number of antioxidants carry significant risks of overdose such as prooxidant effects. Second, there is a lack of information about the interactions between supplements and other medications of about their long-term effects. Third, of particular importance in the development of potential therapeutic interventions is the fact that, although some aspects of DS are congenital, others appear in infancy and childhood and others still occur in adulthood or in the elderly [297].

8. Future directions

Despite the high number of studies appraising the role of oxidative stress in DS, conflicting results have been reported.

1) Other genes, not only SOD1, may be involved in the enhancement of oxidative stress in DS. In fact, more evidence has been provided by other genes than the results of studies concerning SOD1 in last years.
2) There are controversial data about oxidative stress in DS. A high variability occurs in DS subjects, which may be the reason for the inconsistency found in literature.
3) Individuals with DS could have renal impairment, leading to a diminished excretion of metabolites such as oxidative/nitrosative stress biomarkers. Therefore, a comparative study assessing a comprehensive set of these biomarkers in both urine and plasma or serum samples must be performed in DS to clarify this hypothesis.
4) Thyroid dysfunction may be considered in studies concerning oxidative stress biomarkers in DS due the fact that thyroid hormones are involved in oxidative stress and a possible renal dysfunction in DS subjects taking levothyroxine for hypothyroidism has been found.
5) Potential biasing factors, such as dietary or exercise habits, exposure to pollutants, etc., must be considered in future works studying oxidative/nitrosative stress biomarkers in DS. Improvements in life patterns of DS individuals could contribute to decrease oxidative stress, biasing the results.
6) Adults with DS over 40 years are at higher risk for dementia. However, the diagnosis of Alzheimer disease in people with DS is far from an easy task and oxidative damage may increase as a consequence of this disease process in addition to aging biasing the results. Improvements in this area are required before the causes of increased oxidative stress in senescent DS subjects can be well established.
7) Studies addressing biomarkers of oxidative stress in DS have indicated conflicting results. Methodological aspects are not usually taken into account in the approach to discussion. Moreover, since oxidative conditions have been suggested to be of importance in developmental and maturational processes, biomarkers of oxidative stress may need to be differently interpreted in childhood and adulthood. Nonetheless, we can found several works studying them in disease states, including DS, where they compare age groups that are formed by both children and

adolescents or even children and adults, so discrepancies in results are frequently found. Thus, despite the numerous investigations, it remains unclear what biochemical measurements of oxidative stress biomarkers truly represents.

Search strategy and selection criteria

A literature search was performed using the electronic databases PubMed, Scopus, Google Scholar and SciFinder (1866 onwards). We focused on recently publications, but did not exclude commonly referenced and highly regarded older publications. Further PubMed searching was performed by using the "see all related citations" function by manual scanning of the reference lists of several review articles, as well as original investigations. Articles from all languages were included. The following search terms were included in multiple combinations: "Down syndrome", "trisomy", "oxidative stress", "oxidative damage", "nitrosative stress", "lipid peroxidation", "antioxidant", "aging", "senescence", "lifespan", "progeria", "hydrogen peroxide", "ROS", "SOD1", "phenotype", "epigenetics", "environment", "mitochondria", "transgenic", "mouse model", "chromosome 21", "gene", "Alzheimer", "dementia", "APP", "thyroid", "hypothyroidism", "hyperthyroidism", "cataracts", "immune", "infections", "osteoporosis", "renal impairment", "prevalence", "excretion", "biomarker", "creatinine", "8-OHdG", "comet assay", "MDA", "TBARS", "isoprostane", "4-hydroxy-2-nonenal", "lipofuscin", "hydroperoxydes", "oxLDL", "carbonyls", dityrosine", "homocysteine", "glutathione peroxidase", "catalase", "glutathione reductase", "antioxidant enzyme", "glutathione", "uric acid", "vitamin", "thiol", "total antioxidant capacity", "allantoin", "AGEs", "xanthine oxidase", "xanthine oxidoreductase", "CoQ10", "3-nitrotyrosine", "biopyrrin", "zinc", "selenium", "nitric oxide", "NOx", "exercise", "physical activity" or "therapy". Besides, genes on chromosome 21 were identified by searches at the website http://chr21.egr.vcu.edu/ (from the University of Colorado Denver). The search was conducted between September 2010 and March 2011.

9. Concluding remarks

In summary, abundant data from oxidative stress biomarkers and mouse DS models have indicated a major role for oxidative stress as an important factor in DS pathogenesis, being an increased oxidative stress in DS individuals under undisputed observation. Increased oxidative stress in DS may be due not only to increased SOD1 activity but also to overexpression of other specific genes located on HSA21. In addition, epigenetics may contribute to DS phenotype. Although the genetic mysteries of DS are gradually being solved, a limited understanding of the biological basis of the etiology of DS phenotype still remains. However, many aspects that are crucial for the health and well-being of people with this condition remain to be elucidated and require further research.

Acknowledgements

The authors would like to thank Dra. Noelia Cañas for her valuable advices, María Burgos for English correction of the manuscript, and Nieves Fonturbel for her helpful with bibliographic compilation. We also thank Fondation Jérôme Lejeune, for permission to report photograph of the Professor Jérôme Lejeune, and to John Wiley & Sons publisher, for permission to use images of figure 1. This study was supported in part by Fundación Inocente, Inocente.

References

[1] Down, J. L. Observations on an ethnic classification of idiots. *London Hospital Clinical Lectures and Reports* **3**:259-262; 1866.

[2] Lejeune, J.; Gautier, M.; Turpin, R. Etude des chromosomes somatiques de 9 enfants mongoliens. *C.R. Acad Sci Paris* **248**:1721-1728; 1959.

[3] Mikkelsen, M. Down syndrome: cytogenetical epidemiology. *Hereditas* **86**:45-50; 1977.

[4] Hook, E. B. Down syndrome: Its frequency in human populations and some factors pertinent to variation in rates. In: de la Cruzz, F. F.; Gerald, P. S. (eds.). Trisomy 21 (Down syndrome): research perspectives. Baltimore, USA: University Park Press; p. 3-68; 1981.

[5] Sherman, S. L.; Allen, E. G.; Bean, L. H.; Freeman, S. B. Epidemiology of Down syndrome. *Ment Retard Dev Disabil Res Rev* **13**:221-227; 2007.

[6] Weijerman, M. E.; van Furth, A. M.; Vonk Noordegraaf, A.; van Wouwe, J. P.; Broers, C. J.; Gemke, R. J. Prevalence, neonatal characteristics, and first-year mortality of Down syndrome: a national study. *J Pediatr* **152**:15-19; 2008.

[7] Egan, J. F.; Benn, P. A.; Zelop, C. M.; Bolnick, A.; Gianferrari, E.; Borgida, A. F. Down syndrome births in the United States from 1989 to 2001. *Am J Obstet Gynecol* **191**:1044-1048; 2004.

[8] Khoshnood, B.; De Vigan, C.; Vodovar, V.; Goujard, J.; Goffinet, F. A population-based evaluation of the impact of antenatal screening for Down's syndrome in France, 1981-2000. *BJOG* **111**:485-490; 2004.

[9] Jou, H. J.; Kuo, Y. S.; Hsu, J. J.; Shyu, M. K.; Hsieh, T. T.; Hsieh, F. J. The evolving national birth prevalence of Down syndrome in Taiwan. A study on the impact of second-trimester maternal serum screening. *Prenat Diagn* **25**:665-670; 2005.

[10] Lai, F. M.; Woo, B. H.; Tan, K. H.; *et al.* Birth prevalence of Down syndrome in Singapore from 1993 to 1998. *Singapore Med J* **43**:070-076; 2002.

[11] Cheffins, T.; Chan, A.; Haan, E. A.; *et al.* The impact of maternal serum screening on the birth prevalence of Down's syndrome and the use of amniocentesis and chorionic villus sampling in South Australia. *BJOG* **107**:1453-1459; 2000.

[12] Pueschel, S. M. Clinical aspects of Down syndrome from infancy to adulthood. *Am J Med Genet Suppl* **7**:52-56; 1990.

[13] Hasle, H.; Clemmensen, I. H.; Mikkelsen, M. Risks of leukaemia and solid tumours in individuals with Down's syndrome. *Lancet* **355**:165-169; 2000.

[14] Zigman, W. B.; Lott, I. T. Alzheimer's disease in Down syndrome: neurobiology and risk. *Ment Retard Dev Disabil Res Rev* **13**:237-246; 2007.

[15] Roizen, N. J.; Patterson, D. Down's syndrome. *Lancet* **361**:1281-1289; 2003.

[16] Diamandopoulos, A. A.; Rakatsanis, K. G.; Diamantopoulos, N. A neolithic case of Down syndrome. *J Hist Neurosci* **6**:86-89; 1997.

[17] Pueschel, S. M. Do Olmec figurines resemble children with specific dysmorphology syndromes? *J Hist Med Allied Sci* **53**:407-415; 1998.

[18] Bernal, J. E.; Briceno, I. Genetic and other diseases in the pottery of Tumaco-La Tolita culture in Colombia-Ecuador. *Clin Genet* **70**:188-191; 2006.

[19] Martinez-Frias, M. L. The real earliest historical evidence of Down syndrome. *Am J Med Genet A* **132A**:231; 2005.

[20] Ward, O. C. Further early historical evidence of Down syndrome. *Am J Med Genet A* **126A**:220; 2004.

[21] Esquirol, J. E. D. Des maladies mentales considérées sous les rapports médical, hygiénique et médico-légal. Paris, France: J. -B. Baillière; 1838.

[22] Seguin, E. Traitement moral, hygiène et l'éducation des idiots et des autres enfants arriérés. Paris, Baillière.; 1846.

[23] Duncan, P. M. A manual for the classification, training and education of the feeble-minded, imbecile and idiotic. London, UK: Longmans. Green and Co.; 1866.

[24] Fenton, H. J. H. Oxidation of tartaric acid in presence of iron. *J Chem Soc Trans* **65**:899-910; 1894.

[25] Haber, F.; Weiss, J. Über die katalyse des hydroperoxydes. *Naturwiss* **20**:948-950; 1932.

[26] Sobel, A. E.; Strazzulla, M.; Sherman, B. S.; *et al.* Vitamin A absorption and other blood composition studies in mongolism. *Am J Ment Defic* **62**:642-656; 1958.

[27] McCord, J. M.; Fridovich, I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem* **244**:6049-6055; 1969.

[28] Aula, P.; Leisti, J.; von Koskull, H. Partial trisomy 21. *Clin Genet* **4**:241-251; 1973.

[29] Sinet, P. M.; Allard, D.; Lejeune, J.; Jerome, H. Augmentation d'activité de la superoxyde dismutase érythrocytaire dans la trisomie pour le chromosome 21. *CR Acad Sci (Paris)* **278**:3267-3270; 1974.

[30] Sinet, P. M.; Couturier, J.; Dutrillaux, B.; *et al.* Trisomie 21 et superoxyde dismutase-1 (IPO-A): Tentative de localisation sur la sous bande 21 q 22.1 *Exp Cell Res* **97**:47-55; 1976.

[31] Brooksbank, B. W.; Balazs, R. Superoxide dismutase, glutathione peroxidase and lipoperoxidation in Down's syndrome fetal brain. *Brain Res* **318**:37-44; 1984.

[32] Sies, H. Oxidative stress. Introductory remarks. In: Sies, H. (ed.). Oxidative stress. London, UK: Academinc Press; p. 1-8; 1985.

[33] Kedziora, J.; Bartosz, G.; Gromadzinska, J.; Sklodowska, M.; Wesowicz, W.; Scianowski, J. Lipid peroxides in blood plasma and enzymatic antioxidative defence of erythrocytes in Down's syndrome. *Clin Chim Acta* **154**:191-194; 1986.

[34] Anneren, K. G.; Epstein, C. J. Lipid peroxidation and superoxide dismutase-1 and glutathione peroxidase activities in trisomy 16 fetal mice and human trisomy 21 fibroblasts. *Pediatr Res* **21**:88-92; 1987.

[35] De La Torre, R.; Casado, A.; Lopez-Fernandez, E.; Carrascosa, D.; Ramirez, V.; Saez, J. Overexpression of copper-zinc superoxide dismutase in trisomy 21. *Experientia* **52**:871-873; 1996.

[36] Hayn, M.; Kremser, K.; Singewald, N.; *et al.* Evidence against the involvement of reactive oxygen species in the pathogenesis of neuronal death in Down's syndrome and Alzheimer's disease. *Life Sci* **59**:537-544; 1996.

[37] Seidl, R.; Greber, S.; Schuller, E.; Bernert, G.; Cairns, N.; Lubec, G. Evidence against increased oxidative DNA-damage in Down syndrome. *Neurosci Lett* **235**:137-140; 1997.

[38] Monteiro, C. P.; Varela, A.; Pinto, M.; *et al.* Effect of an aerobic training on magnesium, trace elements and antioxidant systems in a Down syndrome population. *Magnes Res* **10**:65-71; 1997.

[39] Pastor, M. C.; Sierra, C.; Dolade, M.; *et al.* Antioxidant enzymes and fatty acid status in erythrocytes of Down's syndrome patients. *Clin Chem* **44**:924-929; 1998.

[40] Pincheira, J.; Navarrete, M. H.; de la Torre, C.; Tapia, G.; Santos, M. J. Effect of vitamin E on chromosomal aberrations in lymphocytes from patients with Down's syndrome. *Clin Genet* **55**:192-197; 1999.

[41] Hattori, M.; Fujiyama, A.; Taylor, T. D.; *et al.* The DNA sequence of human chromosome 21. *Nature* **405**:311-319; 2000.

[42] Kanavin, Ø. J.; Aaseth, J.; Birketvedt, G. S. Thyroid hypofunction in Down's syndrome. Is it related to oxidative stress? *Biol Trace Elem Res* **78**:35-42; 2000.

[43] Carratelli, M.; Porcaro, L.; Ruscica, M.; De Simone, E.; Bertelli, A. A.; Corsi, M. M. Reactive oxygen metabolites and prooxidant status in children with Down´s syndrome. *Int J Clin Pharmacol Res* **21**:79-84; 2001.

[44] Ellis, J. M.; Tan, H. K.; Gilbert, R. E.; *et al.* Supplementation with antioxidants and folinic acid for children with Down's syndrome: randomised controlled trial. *BMJ* **336**:594-597; 2008.

[45] Campos, C.; Guzmán, R.; López-Fernández, E.; Casado, A. Evaluation of urinary biomarkers of oxidative/nitrosative stress in children with Down syndrome. *Waiting to be published.*

[46] Campos, C.; Guzmán, R.; López-Fernández, E.; Casado, A. Evaluation of urinary biomarkers of oxidative/nitrosative stress in adolescents and adults with Down syndrome. *BBA-Mol Basis Dis*; (In Press). DOI: 10.1016/j.bbadis.2011.03.013.

[47] Halliwell, B.; Gutteridge, J. M. C. Free radicals in biology and medicine. 4nd ed. New York, USA: Oxford University Press Inc.; 2007.

[48] Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M. T.; Mazur, M.; Telser, J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* **39**:44-84; 2007.

[49] Sies, H. Oxidative stress: from basic research to clinical application. *Am J Med* **91**:31S-38S; 1991.

[50] Dalle-Donne, I.; Rossi, R.; Colombo, R.; Giustarini, D.; Milzani, A. Biomarkers of oxidative damage in human disease. *Clin Chem* **52**:601-623; 2006.

[51] de Haan, J. B.; Wolvetang, E. J.; Cristiano, F.; *et al.* Reactive oxygen species and their contribution to pathology in Down syndrome. *Adv Pharmacol* **38**:379-402; 1997.

[52] Anneren, G.; Edman, B. Down syndrome - a gene dosage disease caused by trisomy of genes within a small segment of the long arm of chromosome 21, exemplified by the study of effects from the superoxide-dismutase type 1 (SOD-1) gene. *APMIS Suppl* **40**:71-79; 1993.

[53] Liochev, S. I.; Fridovich, I. Mechanism of the peroxidase activity of Cu, Zn superoxide dismutase. *Free Radic Biol Med* **48**:1565-1569; 2010.

[54] Okado-Matsumoto, A.; Fridovich, I. Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu,Zn-SOD in mitochondria. *J Biol Chem* **276**:38388-38393; 2001.

[55] Sinet, P. M.; Lavelle, F.; Michelson, A. M.; Jerome, H. Superoxide dismutase activities of blood platelets in trisomy 21. *Biochem Biophys Res Commun* **67**:904-909; 1975.

[56] Feaster, W. W.; Kwok, L. W.; Epstein, C. J. Dosage effects for superoxide dismutase-1 in nucleated cells aneuploid for chromosome 21. *Am J Hum Genet* **29**:563-570; 1977.

[57] Sebastià, J.; Cristòfol, R.; Pertusa, M.; *et al.* Down's syndrome astrocytes have greater antioxidant capacity than euploid astrocytes. *Eur J Neurosci* **20**:2355-2366; 2004.

[58] Coyle, C. H.; Martinez, L. J.; Coleman, M. C.; Spitz, D. R.; Weintraub, N. L.; Kader, K. N. Mechanisms of H2O2-induced oxidative stress in endothelial cells. *Free Radic Biol Med* **40**:2206-2213; 2006.

[59] Ait Yahya-Graison, E.; Aubert, J.; Dauphinot, L.; *et al.* Classification of human chromosome 21 gene-expression variations in Down syndrome: impact on disease phenotypes. *Am J Hum Genet* **81**:475-491; 2007.

[60] Medvedev, Z. A. An attempt at a rational classification of theories of ageing. *Biol Rev Camb Philos Soc* **65**:375-398; 1990.

[61] Rattan, S. I. Theories of biological aging: genes, proteins, and free radicals. *Free Radic Res* **40**:1230-1238; 2006.

[62] Muravchick, S. Theories of aging. In: Silverstein, J. H.; Rooke, G. A.; Reves, J. G.; McLeskey, C. H. (eds.). Geriatric anesthesiology. 2nd ed. New York, USA: Springer; p. 29-37; 2008.

[63] Harman, D. Aging: a theory based on free radical and radiation chemistry. *J Gerontol* **11**:298-300; 1956.

[64] Martin, G. M. Genetic Syndrome in Man with Potential Relevance to the Pathology of Aging. In: Bergsma, D.; Harrison, D. E. (eds.). Genetic Effects on Aging. New York, USA: Liss; p. 3-39; 1978.

[65] Bokov, A.; Chaudhuri, A.; Richardson, A. The role of oxidative damage and stress in aging. *Mech Ageing Dev* **125**:811-826; 2004.

[66] de Haan, J. B.; Newman, J. D.; Kola, I. Cu/Zn superoxide dismutase mRNA and enzyme activity, and susceptibility to lipid peroxidation, increases with aging in murine brains. *Brain Res Mol Brain Res* **13**:179-187; 1992.

[67] Cristiano, F.; de Haan, J. B.; Iannello, R. C.; Kola, I. Changes in the levels of enzymes which modulate the antioxidant balance occur during aging and correlate with cellular damage. *Mech Ageing Dev* **80**:93-105; 1995.

[68] de Haan, J. B.; Cristiano, F.; Iannello, R. C.; Kola, I. Cu/Zn-superoxide dismutase and glutathione peroxidase during aging. *Biochem Mol Biol Int* **35**:1281-1297; 1995.

[69] Salmon, A. B.; Richardson, A.; Pérez, V. I. Update on the oxidative stress theory of aging: Does oxidative stress play a role in aging or healthy aging? *Free Radic Biol Med* **48**:642-655; 2010.

[70] Lapointe, J.; KHekini, S. When a theory of aging ages badly. *Cell Mol Life Sci* **67**:1-8; 2010.

[71] Lott, I. T.; Head, E.; Doran, E.; Busciglio, J. Beta-amyloid, oxidative stress and down syndrome. *Curr Alzheimer Res* **3**:521-528; 2006.

[72] de Lange, T. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev* **19**:2100-2110; 2005.

[73] Vaziri, H.; Schächter, F.; Uchida, I.; *et al.* Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. . *Am J Hum Genet* **52**:661-667; 1993.

[74] von Zglinicki, T. Role of oxidative stress in telomere length regulation and replicative senescence. *Ann NY Acad Sci* **908**:99-110; 2000.

[75] Jenkins, E. C.; Velinov, M. T.; Ye, L.; *et al.* Telomere shortening in T lymphocytes of older individuals with Down syndrome and dementia. *Neurobiol Aging* **27**:941-945; 2006.

[76] Jenkins, E. C.; Ye, L.; Gu, H.; *et al.* Shorter telomeres may indicate dementia status in older individuals with Down syndrome. *Neurobiol Aging* **31**:765-771; 2010.

[77] Sebastiani, P.; Montano, M.; Puca, A.; *et al.* RNA editing genes associated with extreme old age in humans and with lifespan in C. elegans. *PLoS One* **4**:e8210; 2009.

[78] Lott, I. T.; Dierssen, M. Cognitive deficits and associated neurological complications in individuals with Down's syndrome. *Lancet Neurol* **9**:623-633; 2010.

[79] Brown, F. R., 3rd; Greer, M. K.; Aylward, E. H.; Hunt, H. H. Intellectual and adaptive functioning in individuals with Down syndrome in relation to age and environmental placement. *Pediatrics* **85**:450-452; 1990.

[80] Lai, F.; Williams, R. S. A prospective study of Alzheimer disease in Down syndrome. *Arch Neurol* **46**:849-853; 1989.

[81] Holland, A. J.; Hon, J.; Huppert, F. A.; Stevens, F.; Watson, P. Population-based study of the prevalence and presentation of dementia in adults with Down's syndrome. *Br J Psychiatry* **172**:493-498; 1998.

[82] Roubertoux, P. L.; Carlier, M. Mouse models of cognitive disabilities in trisomy 21 (Down syndrome). *Am J Med Genet C Semin Med Genet* **154C**:400-416; 2010.

[83] Behl, C.; Davis, J. B.; Lesley, R.; Schubert, D. Hydrogen peroxide mediates amyloid beta protein toxicity. *Cell* **77**:817-827; 1994.

[84] Butterfield, D. A.; Drake, J.; Pocernich, C.; Castegna, A. Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid beta-peptide. *Trends Mol Med* **7**:548-554; 2001.

[85] Rovelet-Lecrux, A.; Hannequin, D.; Raux, G.; *et al.* APP locus duplication causes autosomal dominant early-onset Alzheimer disease with cerebral amyloid angiopathy. *Nat Genet* **38**:24-26; 2006.

[86] Sleegers, K.; Brouwers, N.; Gijselinck, I.; *et al.* APP duplication is sufficient to cause early onset Alzheimer's dementia with cerebral amyloid angiopathy. *Brain* **129**:2977-2983; 2006.

[87] Sinet, P. M.; Lejeune, J.; Jerome, H. Trisomy 21 (Down's syndrome). Glutathione peroxidase, hexose monophoshate shunt and I.Q. *Life Sci* **24**:29-33; 1979.

[88] Busciglio, J.; Yankner, B. A. Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons in vitro. *Nature* **378**:776-779; 1995.

[89] Odetti, P.; Angelini, G.; Dapino, D.; *et al.* Early glycoxidation damage in brains from Down's syndrome. *Biochem Biophy Res Commun* **243**:849-851; 1998.

[90] Vitek, M. P.; Bhattacharya, K.; Glendening, J. M.; *et al.* Advanced glycation end products contribute to amyloidosis in Alzheimer disease. *Proc Natl Acad Sci USA* **91**:4766-4770; 1994.

[91] Smith, M. A.; Taneda, S.; Richey, P. L.; *et al.* Advanced Maillard reaction end products are associated with Alzheimer disease pathology. *Proc Natl Acad Sci USA* **91**:5710-5714; 1994.

[92] Porta, S.; Serra, S. A.; Huch, M.; *et al.* RCAN1 (DSCR1) increases neuronal susceptibility to oxidative stress: a potential pathogenic process in neurodegeneration. *Hum Mol Genet* **16**:1039-1050; 2007.

[93] Helguera, P.; Pelsman, A.; Pigino, G.; Wolvetang, E.; Head, E.; Busciglio, J. ets-2 promotes the activation of a mitochondrial death pathway in Down's syndrome neurons. *J Neurosci* **25**:2295-2303; 2005.

[94] Kimura, R.; Kamino, K.; Yamamoto, M.; *et al.* The DYRK1A gene, encoded in chromosome 21 Down syndrome critical region, bridges between beta-amyloid production and tau phosphorylation in Alzheimer disease. *Hum Mol Genet* **16**:15-23; 2007.

[95] Griffin, W. S.; Sheng, J. G.; McKenzie, J. E.; *et al.* Life-long overexpression of S100beta in Down's syndrome: implications for Alzheimer pathogenesis. *Neurobiol Aging* **19**:401-405; 1998.

[96] Spector, A. Oxidative stress-induced cataract: mechanism of action. *Faseb J* **9**:1173-1182; 1995.

[97] Berthoud, V. M.; Beyer, E. C. Oxidative stress, lens gap junctions, and cataracts. *Antioxid Redox Signal* **11**:339-353; 2009.

[98] Vinson, J. A. Oxidative stress in cataracts. *Pathophysiology* **13**:151-162; 2006.

[99] Puri, B. K.; Singh, I. Prevalence of cataract in adult Down's syndrome patients aged 28 to 83 years. *Clin Pract Epidemiol Ment Health* **3**:26; 2007.

[100] Bras, A.; Monteiro, C.; Rueff, J. Oxidative stress in trisomy 21. A possible role in cataractogenesis. *Ophthalmic Paediatr Genet* **10**:271-277; 1989.

[101] Frederikse, P. H.; Ren, X. O. Lens defects and age-related fiber cell degeneration in a mouse model of increased AbetaPP gene dosage in Down syndrome. *Am J Pathol* **161**:1985-1990; 2002.

[102] Frederikse, P. H.; Garland, D.; Zigler, J. S., Jr.; Piatigorsky, J. Oxidative stress increases production of beta-amyloid precursor protein and beta-amyloid (Abeta) in mammalian lenses, and Abeta has toxic effects on lens epithelial cells. *J Biol Chem* **271**:10169-10174; 1996.

[103] Gervais, F. G.; Xu, D.; Robertson, G. S.; *et al.* Involvement of caspases in proteolytic cleavage of Alzheimer's amyloid-beta precursor protein and amyloidogenic A beta peptide formation. *Cell* **97**:395-406; 1999.

[104] Lin, D.; Barnett, M.; Grauer, L.; *et al.* Expression of superoxide dismutase in whole lens prevents cataract formation. *Mol Vis* **11**:853-858; 2005.

[105] Delcourt, C.; Cristol, J. P.; Leger, C. L.; Descomps, B.; Papoz, L. Associations of antioxidant enzymes with cataract and age-related macular degeneration. The POLA Study. Pathologies Oculaires Liees a l'Age. *Ophthalmology* **106**:215-222; 1999.

[106] Casado, A.; de La Torre, R.; Lopez-Fernandez, E. Antioxidant enzyme levels in red blood cells from cataract patients. *Gerontology* **47**:186-188; 2001.

[107] Cengiz, M.; Seven, M.; Suyugul, N. Antioxidant system in Down syndrome: a possible role in cataractogenesis. *Genet Couns* **13**:339-342; 2002.

[108] Graw, J. Genetics of crystallins: cataract and beyond. *Exp Eye Res* **88**:173-189; 2009.

[109] Torun, A. N.; Kulaksizoglu, S.; Kulaksizoglu, M.; Pamuk, B. O.; Isbilen, E.; Tutuncu, N. B. Serum total antioxidant status and lipid peroxidation marker malondialdehyde levels in overt and subclinical hypothyroidism. *Clin Endocrinol (Oxf)* **70**:469-474; 2009.

[110] Guerrero, A.; Pamplona, R.; Portero-Otin, M.; Barja, G.; Lopez-Torres, M. Effect of thyroid status on lipid composition and peroxidation in the mouse liver. *Free Radic Biol Med* **26**:73-80; 1999.

[111] Prasher, V. P. Prevalence of thyroid dysfunction and autoimmunity in adults with Down syndrome. *Downs Syndr Res Pract* **2**:67-70; 1994.

[112] Fort, P.; Lifshitz, F.; Bellisario, R.; *et al.* Abnormalities of thyroid function in infants with Down syndrome. *J Pediatr* **104**:545-549; 1984.

[113] Murdoch, J. C.; Ratcliffe, W. A.; McLarty, D. G.; Rodger, J. C.; Ratcliffe, J. G. Thyroid function in adults with Down's syndrome. *J Clin Endocrinol Metab* **44**:453-458; 1977.

[114] Medda, E.; Olivieri, A.; Stazi, M. A.; *et al.* Risk factors for congenital hypothyroidism: results of a population case-control study (1997-2003). *Eur J Endocrinol* **153**:765-773; 2005.

[115] Erdamar, H.; Demirci, H.; Yaman, H.; *et al.* The effect of hypothyroidism, hyperthyroidism, and their treatment on parameters of oxidative stress and antioxidant status. *Clin Chem Lab Med* **46**:1004-1010; 2008.

[116] Dumitriu, L.; Bartoc, R.; Ursu, H.; Purice, M.; Ionescu, V. Significance of high levels of serum malonyl dialdehyde (MDA) and ceruloplasmin (CP) in hyper- and hypothyroidism. *Endocrinologie* **26**:35-38; 1988.

[117] Costantini, F.; Pierdomenico, S. D.; De Cesare, D.; *et al.* Effect of thyroid function on LDL oxidation. *Arterioscler Thromb Vasc Biol* **18**:732-737; 1998.

[118] Yilmaz, S.; Ozan, S.; Benzer, F.; Canatan, H. Oxidative damage and antioxidant enzyme activities in experimental hypothyroidism. *Cell Biochem Funct* **21**:325-330; 2003.

[119] Sarandöl, E.; Taş, S.; Dirican, M.; Serdar, Z. Oxidative stress and serum paraoxonase activity in experimental hypothyroidism: effect of vitamin E supplementation. *Cell Biochem Funct* **23**:1-8; 2005.

[120] Santos, G. M.; Afonso, V.; Barra, G. B.; *et al.* Negative regulation of superoxide dismutase-1 promoter by thyroid hormone. *Mol Pharmacol* **70**:793-800; 2006.

[121] Lafayette, R. A.; Costa, M. E.; King, A. J. Increased serum creatinine in the absence of renal failure in profound hypothyroidism. *Am J Med* **96**:298-299; 1994.

[122] Montenegro, J.; González, O.; Saracho, R.; Aguirre, R.; González, O.; Martínez, I. Changes in renal function in primary hypothyroidism. *Am J Kidney Dis* **27**:195-198; 1996.

[123] Kumar, J.; Gordillo, R.; Kaskel, F. J.; Druschel, C. M.; Woroniecki, R. P. Increased prevalence of renal and urinary tract anomalies in children with congenital hypothyroidism. *J Pediatr* **154**:263-266; 2009.

[124] Coburn, S. P.; Seidenberg, M.; Mertz, E. T. Clearance of uric acid, urea, and creatinine in Down's syndrome. *J Appl Physiol* **23**:579-580; 1967.

[125] Nishida, Y.; Akaoka, I.; Kobayashi, M.; Maruki, K.; Oshima, Y. Renal impairment in urate excretion in patients with Down's syndrome. *J Rheumatol* **6**:103-107; 1979.

[126] Nève, J.; Sinet, P. M.; Molle, L.; Nicole, A. Selenium, zinc and copper in Down's syndrome (trisomy 21): blood levels and relations with glutathione peroxidase and superoxide dismutase. *Clin Chim Acta* **133**:209-214; 1983.

[127] Shaposhnikov, A. M.; Khal'chitskii, S. E.; Shvarts, E. I. Disorders of phenylalanine and tyrosine metabolism in Down's syndrome. *Vopr Med Khim* **25**:15-19; 1979.

[128] Dowjat, W. K.; Adayev, T.; Kuchna, I.; *et al.* Trisomy-driven overexpression of DYRK1A kinase in the brain of subjects with Down syndrome. *Neurosci Lett* **413**:77-81; 2007.

[129] Shaw, C. K.; Thapalial, A.; Nanda, S.; Shaw, P. Thyroid dysfunction in Down syndrome. *Kathmandu Univ Med J (KUMJ)* **4**:182-186; 2006.

[130] van Trotsenburg, A. S.; Vulsma, T.; van Rozenburg-Marres, S. L.; *et al.* The effect of thyroxine treatment started in the neonatal period on development and growth of two-year-old Down syndrome children: a randomized clinical trial. *J Clin Endocrinol Metab* **90**:3304-3311; 2005.

[131] Prasher, V.; Haque, M. S. Misdiagnosis of thyroid disorders in Down syndrome: time to re-examine the myth? *Am J Ment Retard* **110**:23-27; 2005.

[132] Cuadrado, E.; Barrena, M. J. Immune dysfunction in Down's syndrome: primary immune deficiency or early senescence of the immune system? *Clin Immunol Immunopathol* **78**:209-214; 1996.

[133] Park, E.; Alberti, J.; Mehta, P.; Dalton, A.; Sersen, E.; Schuller-Levis, G. Partial impairment of immune functions in peripheral blood leukocytes from aged men with Down's syndrome. *Clin Immunol* **95**:62-69; 2000.

[134] Bloemers, B. L.; van Bleek, G. M.; Kimpen, J. L.; Bont, L. Distinct abnormalities in the innate immune system of children with Down syndrome. *J Pediatr* **156**:804-809; 2010.

[135] Fong, C. T.; Brodeur, G. M. Down's syndrome and leukemia: epidemiology, genetics, cytogenetics and mechanisms of leukemogenesis. *Cancer Genet Cytogenet* **28**:55-76; 1987.

[136] Mirochnitchenko, O.; Inouye, M. Effect of overexpression of human Cu,Zn superoxide dismutase in transgenic mice on macrophage functions. *J Immunol* **156**:1578-1586; 1996.

[137] Anneren, G.; Bjorksten, B. Low superoxide levels in blood phagocytic cells in Down's syndrome. *Acta Paediatr Scand* **73**:345-348; 1984.

[138] Kedziora, J.; Blaszczyk, J.; Sibinska, E.; Bartosz, G. Down's syndrome: increased enzymatic antioxidative defense is accompanied by decreased superoxide anion generation in blood. *Hereditas* **113**:73-75; 1990.

[139] Ugazio, A. G.; Maccario, R.; Notarangelo, L. D.; Burgio, G. R. Immunology of Down syndrome: a review. *Am J Med Genet Suppl* **7**:204-212; 1990.

[140] Angelopoulou, N.; Matziari, C.; Tsimaras, V.; Sakadamis, A.; Souftas, V.; Mandroukas, K. Bone mineral density and muscle strength in young men with mental retardation (with and without Down syndrome). *Calcif Tissue Int* **66**:176-180; 2000.

[141] Srikanth, R.; Cassidy, G.; Joiner, C.; Teeluckdharry, S. Osteoporosis in people with intellectual disabilities: a review and a brief study of risk factors for osteoporosis in a community sample of people with intellectual disabilities. *J Intellect Disabil Res* **55**:53-62; 2011.

[142] Basu, S.; Michaelsson, K.; Olofsson, H.; Johansson, S.; Melhus, H. Association between oxidative stress and bone mineral density. *Biochem Biophys Res Commun* **288**:275-279; 2001.

[143] Smietana, M. J.; Arruda, E. M.; Faulkner, J. A.; Brooks, S. V.; Larkin, L. M. Reactive oxygen species on bone mineral density and mechanics in Cu,Zn superoxide dismutase (Sod1) knockout mice. *Biochem Biophys Res Commun* **403**:149-153; 2010.

[144] Gardiner, K. J. Molecular basis of pharmacotherapies for cognition in Down syndrome. *Trends Pharmacol Sci* **31**:66-73; 2010.

[145] Lintas, C.; Sacco, R.; Persico, A. M. Genome-wide expression studies in Autism spectrum disorder, Rett syndrome, and Down syndrome. *Neurobiol Dis*; In Press.

[146] Antonarakis, S. E.; Lyle, R.; Dermitzakis, E. T.; Reymond, A.; Deutsch, S. Chromosome 21 and down syndrome: from genomics to pathophysiology. *Nat Rev Genet* **5**:725-738; 2004.

[147] Egger, G.; Liang, G.; Aparicio, A.; Jones, P. A. Epigenetics in human disease and prospects for epigenetic therapy. *Nature* **429**:457-463; 2004.

[148] Barros, S. P.; Offenbacher, S. Epigenetics: connecting environment and genotype to phenotype and disease. *J Dent Res* **88**:400-408; 2009.

[149] Bjornsson, H. T.; Fallin, M. D.; Feinberg, A. P. An integrated epigenetic and genetic approach to common human disease. *Trends Genet* **20**:350-358; 2004.

[150] Zhang, T. Y.; Meaney, M. J. Epigenetics and the environmental regulation of the genome and its function. *Annu Rev Psychol* **61**:439-466; 2010.

[151] Morgan, H. D.; Santos, F.; Green, K.; Dean, W.; Reik, W. Epigenetic reprogramming in mammals. *Hum Mol Genet* **14 Spec No 1**:R47-58; 2005.

[152] Jaenisch, R.; Bird, A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet* **33**:245-254; 2003.

[153] Gardiner, K. Transcriptional dysregulation in Down syndrome: predictions for altered protein complex stoichiometries and post-translational modifications, and consequences for learning/behavior genes ELK, CREB, and the estrogen and glucocorticoid receptors. *Behav Genet* **36**:439-453; 2006.

[154] Aït Yahya-Graison, E.; Aubert, J.; Dauphinot, L.; *et al.* Classification of human chromosome 21 gene-expression variations in Down syndrome: impact on disease phenotypes. *Am J Hum Genet* **81**:475-491; 2007.

[155] Bladier, C.; De Haan, J. B.; Kola, I. Antioxidant genes and reactive oxygen species in Down's syndrome. In: Sen, C. K.; Sies, H.; Baeuerly, P. A. (eds.). Antioxidant and redox regulation of genes. Orlando, FL, USA: Academic; p. 425-449; 2000.

[156] Ishii, T.; Itoh, K.; Takahashi, S.; *et al.* Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *J Biol Chem* **275**:16023-16029; 2000.

[157] Lee, H. C.; Wei, Y. H. Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. *Int J Biochem Cell Biol* **37**:822-834; 2005.

[158] Ferrando-Miguel, R.; Cheon, M. S.; Yang, J. W.; Lubec, G. Overexpression of transcription factor BACH1 in fetal Down syndrome brain. *J Neural Transm Suppl*:193-205; 2003.

[159] Dogliotti, G.; Galliera, E.; Licastro, F.; Porcellini, E.; Corsi, M. M. Serum neutrophil gelatinase-B associated lipocalin (NGAL) levels in Down's syndrome patients. *Immun Ageing* **7**:S7; 2010.

[160] Kumagai, Y.; Wakayama, T.; Lib, S.; *et al.* Zeta-crystallin catalyzes the reductive activation of 2,4,6-trinitrotoluene to generate reactive oxygen species: a proposed mechanism for the induction of cataracts. *FEBS Lett* **478**:295-298; 2000.

[161] Oppermann, U. Carbonyl reductases: the complex relationships of mammalian carbonyl- and quinone-reducing enzymes and their role in physiology. *Annu Rev Pharmacol Toxicol* **47**:293-322; 2007.

[162] Rashid, M. A.; Lee, S.; Tak, E.; *et al.* Carbonyl reductase 1 protects pancreatic beta-cells against oxidative stress-induced apoptosis in glucotoxicity and glucolipotoxicity. *Free Radic Biol Med* **49**:1522-1533; 2010.

[163] Choi, J. Y.; Barlow, W. E.; Albain, K. S.; *et al.* Nitric oxide synthase variants and disease-free survival among treated and untreated breast cancer patients in a Southwest Oncology Group clinical trial. *Clin Cancer Res* **15**:5258-5266; 2009.

[164] Sanij, E.; Hatzistavrou, T.; Hertzog, P.; Kola, I.; Wolvetang, E. J. Ets-2 is induced by oxidative stress and sensitizes cells to H(2)O(2)-induced apoptosis: implications for Down's syndrome. *Biochem Biophys Res Commun* **287**:1003-1008; 2001.

[165] Mazzocco, M.; Maffei, M.; Egeo, A.; *et al.* The identification of a novel human homologue of the SH3 binding glutamic acid-rich (SH3BGR) gene establishes a new family of highly conserved small proteins related to Thioredoxin Superfamily. *Gene* **291**:233-239; 2002.

[166] Yvan-Charvet, L.; Pagler, T. A.; Seimon, T. A.; *et al.* ABCA1 and ABCG1 protect against oxidative stress-induced macrophage apoptosis during efferocytosis. *Circ Res* **106**:1861-1869; 2010.

[167] Schuchmann, S.; Heinemann, U. Increased mitochondrial superoxide generation in neurons from trisomy 16 mice: a model of Down's syndrome. *Free Radic Biol Med* **28**:235-250; 2000.

[168] Robert, K.; Nehme, J.; Bourdon, E.; *et al.* Cystathionine beta synthase deficiency promotes oxidative stress, fibrosis, and steatosis in mice liver. *Gastroenterology* **128**:1405-1415; 2005.

[169] Miyara, N.; Shinzato, M.; Yamashiro, Y.; Iwamatsu, A.; Kariya, K.; Sawaguchi, S. Proteomic analysis of rat retina in a steroid-induced ocular hypertension model: potential vulnerability to oxidative stress. *Jpn J Ophthalmol* **52**:84-90; 2008.

[170] Yamamoto, S.; Takahashi, N.; Mori, Y. Chemical physiology of oxidative stress-activated TRPM2 and TRPC5 channels. *Prog Biophys Mol Biol* **103**:18-27; 2010.

[171] Miller, B. A.; Zhang, W. TRP Channels as Mediators of Oxidative Stress. *Adv Exp Med Biol* **704**:531-544; 2011.

[172] Shrivastava, V.; Pekar, M.; Grosser, E.; Im, J.; Vigodner, M. SUMO proteins are involved in the stress response during spermatogenesis and are localized to DNA double-strand breaks in germ cells. *Reproduction* **139**:999-1010; 2010.

[173] Sang, J.; Yang, K.; Sun, Y.; *et al.* SUMO2 and SUMO3 transcription is differentially regulated by oxidative stress in an Sp1-dependent manner. *Biochem J*; In Press.

[174] Adami, C.; Bianchi, R.; Pula, G.; Donato, R. S100B-stimulated NO production by BV-2 microglia is independent of RAGE transducing activity but dependent on RAGE extracellular domain. *Biochim Biophys Acta* **1742**:169-177; 2004.

[175] Yu, T.; Li, Z.; Jia, Z.; *et al.* A mouse model of Down syndrome trisomic for all human chromosome 21 syntenic regions. *Hum Mol Genet* **19**:2780-2791; 2010.

[176] Ishihara, K.; Amano, K.; Takaki, E.; *et al.* Increased lipid peroxidation in Down's syndrome mouse models. *J Neurochem* **110**:1965-1976; 2009.

[177] Cowley, P. M.; DeRuisseau, K. C. Soleus muscle of Down syndrome (DS) mice exhibits elevated markers of oxidative and nitrosative stress. *FASEB J* **24**:989.984; 2010.

[178] Colton, C. A.; Yao, J. B.; Gilbert, D.; Oster-Granite, M. L. Enhanced production of superoxide anion by microglia from trisomy 16 mice. *Brain Res* **519**:236-242; 1990.

[179] Behar, T. N.; Colton, C. A. Redox regulation of neuronal migration in a Down Syndrome model. *Free Radic Biol Med* **35**:566-575; 2003.

[180] Peled-Kamar, M.; Lotem, J.; Wirguin, I.; Weiner, L.; Hermalin, A.; Groner, Y. Oxidative stress mediates impairment of muscle function in transgenic mice with elevated level of wild-type Cu/Zn superoxide dismutase. *Proc Natl Acad Sci USA* **94**:3883-3887; 1997.

[181] Lesko, L. J.; Atkinson, A. J. Use of biomarkers and surrogate endpoints in drug development and regulatory decision making: Criteria, validation, strategies. *Annu Rev Pharmacol Toxicol* **41**:347-366; 2001.

[182] Campos, C.; Guzmán, R.; López-Fernández, E.; Casado, A. Evaluation of the copper(II) reduction assay using bathocuproinedisulfonic acid disodium salt for the total antioxidant capacity assessment: The CUPRAC-BCS assay. *Anal Biochem* **392**:37-44; 2009.

[183] Montine, T. J.; Quinn, J. F.; Montine, K. S.; Kaye, J. A.; Breitner, J. C. Quantitative in vivo biomarkers of oxidative damage and their application to the diagnosis and management of Alzheimer's disease. *J Alzheimers Dis* **8**:359-367; 2005.

[184] Zafrilla, P.; Mulero, J.; Xandri, J. M.; Santo, E.; Caravaca, G.; Morillas, J. M. Oxidative stress in Alzheimer patients in different stages of the disease. *Curr Med Chem* **13**:1075-1083; 2006.

[185] Seet, R. C.; Lee, C. Y.; Lim, E. C.; *et al.* Oxidative damage in Parkinson disease: Measurement using accurate biomarkers. *Free Radic Biol Med* **48**:560-566; 2010.

[186] Campos, C.; Guzman, R.; Lopez-Fernandez, E.; Casado, A. Urinary biomarkers of oxidative/nitrosative stress in healthy smokers. *Inhal Toxicol* **23**:148-156; 2011.

[187] Knez, W. L.; Jenkins, D. G.; Coombes, J. S. Oxidative stress in half and full Ironman triathletes. *Med Sci Sports Exerc* **39**:283-288; 2007.

[188] Jovanovic, S. V.; Clements, D.; MacLeod, K. Biomarkers of oxidative stress are significantly elevated in Down syndrome. *Free Radic Biol Med* **25**:1044-1048; 1998.

[189] Lloret, A.; Calzone, R.; Dunster, C.; *et al.* Different patterns of in vivo pro-oxidant states in a set of cancer- or aging-related genetic diseases. *Free Radic Biol Med* **44**:495-503; 2008.

[190] Muchová, J.; Sustrová, M.; Garaiová, I.; *et al.* Influence of age on activities of antioxidant enzymes and lipid peroxidation products in erythrocytes and neutrophils of Down syndrome patients. *Free Radic Biol Med* **31**:499-508; 2001.

[191] Garaiová, I.; Muchová, J. S., M.; Blazicek, P.; *et al.* The relationship between antioxidant systems and some markers of oxidative stress in persons with Down syndrome. *Biologia* **59**:787-794; 2004.

[192] Pastore, A.; Tozzi, G.; Gaeta, L. M.; *et al.* Glutathione metabolism and antioxidant enzymes in children with Down syndrome. *The Journal of Pediatrics* **142**:583-585; 2003.

[193] Halliwell, B.; Clement, M. V.; Long, L. H. Hydrogen peroxide in the human body. *FEBS Lett* **486**:10-13; 2000.

[194] Pallardó, F. V.; Degan, P.; d'Ischia, M.; Kelly, F. J.; Zatterale, A.; Calzone, R. Multiple evidence for an early age pro-oxidant state in Down syndrome patients. *Biogerontology* **7**:211-220; 2006.

[195] Campos, C.; Guzmán, R.; López-Fernández, E.; Casado, A. Urinary uric acid and antioxidant capacity in children and adults with Down syndrome. *Clin Biochem* **43**:228-233; 2010.

[196] Tiano, L.; Padella, L.; Carnevali, P.; *et al.* Coenzyme Q10 and oxidative imbalance in Down syndrome: biochemical and clinical aspects. *Biofactors* **32**:161-167; 2008.

[197] Nunomura, A.; Perry, G.; Hirai, K.; *et al.* Neuronal RNA oxidation in Alzheimer's disease and Down's syndrome. *Ann NY Acad Sci* **893**:362-364; 1999.

[198] Kantar, A.; Tiano, L.; Falcioni, G.; *et al.* DNA damage in Down syndrome: contribution of reactive oxygen species. *Neoplasma* **46**:117-118; 1999.

[199] Tiano, L.; Littarru, G. P.; Principi, F.; *et al.* Assessment of DNA damage in Down Syndrome patients by means of a new, optimised single cell gel electrophoresis technique. *Biofactors* **25**:187-195; 2005.

[200] Zana, M.; Szécsényi, A.; Czibula, Á.; *et al.* Age-dependent oxidative stress-induced DNA damage in Down's lymphocytes. *Biochem Biophys Res Commun* **345**:726-733; 2006.

[201] Jayaprakash, T.; Ramachandra, R. K.; Vishnu, B. B.; Parkash, C.; Nandha, K. S. DNA damage studies in cases of Trisomy 21 using Comet Assay. *Curr Pediatr Res* **14**:1-4; 2010.

[202] Gromadzinska, J.; Wasowicz, W.; Sklodowska, M.; Strozynski, H. Glutathione peroxidase activity, lipid peroxides and selenium status in blood in patients with Down's syndrome. *J Clin Chem Clin Biochem* **26**:255-258; 1988.

[203] Shah, S. N.; Johnson, R. C. Antioxidant vitamin (A and E) status of Down's syndrome subjects *Nutr Res* **9**:709-715; 1989.

[204] Capone, G.; Kim, P.; Jovanovich, S.; *et al.* Evidence for increased mitochondrial superoxide production in Down syndrome. *Life Sci* **70**:2885-2895; 2002.

[205] Garcez, M. E.; Peres, W.; Salvador, M. Oxidative stress and hematologic and biochemical parameters in individuals with Down syndrome. *Mayo Clin Proc* **80**:1607-1611; 2005.

[206] Meguid, N. A.; Dardir, A. A.; El-Sayed, E. M.; Ahmed, H. H.; Hashish, A. F.; Ezzat, A. Homocysteine and oxidative stress in Egyptian children with Down syndrome. *Clin Biochem* **43**:963-967; 2010.

[207] Casado, A.; López-Fernández, M. E.; Ruiz, R. Lipid peroxidation in Down syndrome caused by regular trisomy 21, trisomy 21 by Robertsonian translocation and mosaic trisomy 21. *Clin Chem Lab Med* **45**:59-62; 2007.

[208] Reynolds, G. P.; Cutts, A. J. Free radical damage in Down's syndrome brain. *Biochem Soc Trans* **21**:221S; 1993.

[209] Praticò, D.; Iuliano, L.; Amerio, G.; *et al.* Down's syndrome is associated with increased 8,12-iso-iPF2alpha-VI levels: evidence for enhanced lipid peroxidation in vivo. *Ann Neurol* **48**:795-798; 2000.

[210] Zitnanová, I.; Korytár, P.; Sobotová, H.; *et al.* Markers of oxidative stress in children with Down syndrome. *Clin Chem Lab Med* **44**:306-310; 2006.

[211] Massaccesi, L.; Corsi, M. M.; Baquero-Herrera, C. J.; *et al.* Erythrocyte glycohydrolases in subjects with trisomy 21: could Down's syndrome be a model of accelerated ageing? *Mech Ageing Dev* **127**:324-331; 2006.

[212] Licastro, F.; Dogliotti, G.; Goi, G.; Malavazos, A. E.; Chiappelli, M.; Corsi, M. M. Oxidated low-density lipoproteins (oxLDL) and peroxides in plasma of Down syndrome patients. *Arch Gerontol Geriatr* **44**:225-232; 2007.

[213] Tanabe, T.; Kawamura, N.; Morinobu, T.; *et al.* Antioxidant enzymes and vitamins in Down's syndrome. *Pathophysiology* **1**:93-97; 1994.

[214] Teksen, F.; Sayli, B. S.; Aydin, A.; Sayal, A.; Isimer, A. Antioxidative metabolism in Down syndrome. *Biol Trace Elem Res* **63**:123-127; 1998.

[215] Casado, A.; López-Fernández, M. E.; Ruíz, R. Marcadores de estrés oxidativo en el síndrome de Down. *Revista Médica Internacional sobre el Síndrome de Down* **9**:18-25; 2005.

[216] Frants, R. R.; Eriksson, A. W.; Jongbloet, P. H.; Hamers, A. J. Letter: Superoxide dismutase in Down syndrome. *Lancet* **2**:42-43; 1975.

[217] Gilles, L.; Ferradini, C.; Foos, J.; *et al.* The estimation of red cell superoxide dismutase activity by pulse radiolysis in normal and trisomic 21 subjects. *FEBS Lett* **69**:55-58; 1976.

[218] Crosti, N.; Serra, A.; Rigo, A.; Viglino, P. Dosage effect of SOD-A gene in 21-trisomic cells. *Hum Genet* **31**:197-202; 1976.

[219] Kedziora, J.; Jeske, J.; Witas, H.; Bartosz, G.; Leyko, W. The level of superoxide dismutase in erythrocytes of children with Down syndrome (trisomy G and unbalanced translocation G 21/22). *Acta Biol Med Ger* **36**:779-782; 1977.

[220] Garber, P.; Sinet, P. M.; Jerome, H.; Lejeune, J. Copper/zinc superoxide dismutase activity in trisomy 21 by translocation. *Lancet* **2**:914-915; 1979.

[221] Frischer, H.; Chu, L. K.; Ahmad, T.; Justice, P.; Smith, G. F. Superoxide dismutase and glutathione peroxidase abnormalities in erythrocytes and lymphoid cells in Down syndrome. *Prog Clin Biol Res* **55**:269-289; 1981.

[222] Jeziorowska, A.; Jakubowski, L.; Armatys, A.; Kaluzewski, B. Copper/zinc superoxide dismutase (SOD-1) activity in regular trisomy 21, trisomy 21 by translocation and mosaic trisomy 21. *Clin Genet* **22**:160-164; 1982.

[223] Jeziorowska, A.; Jakubowski, L.; Lach, J.; Kaluzewski, B. Regular trisomy 21 not accompanied by increased copper-zinc superoxide dismutase (SOD1) activity. *Clin Genet* **33**:11-19; 1988.

[224] Percy, M. E.; Dalton, A. J.; Markovic, V. D.; *et al.* Red cell superoxide dismutase, glutathione peroxidase and catalase in Down syndrome patients with and without manifestations of Alzheimer disease. *Am J Med Genet* **35**:459-467; 1990.

[225] Ibarra, B.; Rivas, F.; Medina, C.; *et al.* Hematological and biochemical studies in children with Down syndrome. *Ann Genet* **33**:84-87; 1990.

[226] Meguid, N. A.; Kholoussi, N. M.; Afifi, H. H. Evaluation of superoxide dismutase and glutathione peroxidase enzymes and their cofactors in Egyptian children with Down's syndrome. *Biol Trace Elem Res* **81**:21-28; 2001.

[227] Torsdottir, G.; Kristinsson, J.; Hreidarsson, S.; Snaedal, J.; Johannesson, T. Copper, ceruloplasmin and superoxide dismutase (SOD1) in patients with Down's syndrome. *Pharmacol Toxicol* **89**:320-325; 2001.

[228] Sinet, P. M.; Michelson, A. M.; Bazin, A.; Lejeune, J.; Jerome, H. Increase in glutathione peroxidase activity in erythrocytes from trisomy 21 subjects. *Biochem Biophys Res Commun* **67**:910-915; 1975.

[229] Agar, N. S.; Hingston, J. Glutathione peroxidase activity in red blood cells from subjects with Down's syndrome and non-mongoloid mental retardation. *Med J Aust* **1**:556-557; 1980.

[230] Pogribna, M.; Melnyk, S.; Pogribny, I.; Chango, A.; Yi, P.; James, S. J. Homocysteine metabolism in children with Down syndrome: in vitro modulation. *Am J Hum Genet* **69**:88-95; 2001.

[231] Pinto, M.; Neves, J.; Palha, M.; Bicho, M. Oxidative stress in Portuguese children with Down syndrome. *Downs Syndr Res Pract* **8**:79-82; 2002.

[232] Howell, A.; Mason, A. S.; Brown, E.; *et al.* Red cell size and uric acid in Down's syndrome. *Scand J Haematol* **11**:140-147; 1973.

[233] Appleton, M. D.; Haab, W.; Burti, U.; Orsulak, P. J. Plasma urate levels in mongolism. *Amer J Ment Deficiency* **74**:196-199; 1969.

[234] Puukka, R.; Puukka, M.; Leppilampi, M.; Linna, S. L.; Kouvalainen, K. Erythrocyte adenosine deaminase, purine nucleotide phosphorylase and phosphoribosyltransferase activity in patients with Down's syndrome. *Clin Chim Acta* **126**:275-281; 1982.

[235] Zitnanová, I.; Korytár, P.; Aruoma, O. I.; *et al.* Uric acid and allantoin levels in Down syndrome: antioxidant and oxidative stress mechanism? *Clin Chim Acta* **341**:139-146; 2004.

[236] Appleton, M. D.; Haab, W.; Casey, P. J.; Castellino, F. J.; Schorr, J. M.; Miraglia, R. J. Role of Vitamin a in Gamma Globulin Biosynthesis and Uric Acid Metabolism of Mongoloids. *Am J Ment Defic* **69**:324-327; 1964.

[237] Kaufman, J. M.; O'Brien, W. M. Hyperuricemia in mongolism. *N Engl J Med* **276**:953-956; 1967.

[238] Pant, S. S.; Moser, H. W.; Krane, S. M. Hyperuricemia in Down's syndrome. *J Clin Endocr* **28**:472-478; 1968.

[239] Hestnes, A.; Stovner, L. J.; Husoy, O.; Folling, I.; Fougner, K. J.; Sjaastad, O. Hormonal and biochemical disturbances in Down's syndrome. *J Ment Defic Res* **35**:179-193; 1991.

[240] Nagyova, A.; Sustrova, M.; Raslova, K. Serum lipid resistance to oxidation and uric acid levels in subjects with Down's syndrome. *Physiol Res* **49**:227-231; 2000.

[241] Metcalfe, T.; Bowen, D. M.; Muller, D. P. Vitamin E concentrations in human brain of patients with Alzheimer's disease, fetuses with Down's syndrome, centenarians, and controls. *Neurochem Res* **14**:1209-1212; 1989.

[242] Barden, H. S. Vitamin A and carotene values of institutionalised mentally retarded subjects with and without Down's syndrome. *J Ment Defic Res* **21**:63-74; 1977.

[243] Storm, W. Hypercarotenemia in children with Down's syndrome. *J Ment Defic Res* **34**:283-286; 1990.

[244] Ercis, M.; Balci, S.; Atakan, N. Dermatological manifestations of 71 Down syndrome children admitted to a clinical genetics unit. *Clin Genet* **50**:317-320; 1996.

[245] Pueschel, S. M.; Hillemeier, C.; Caldwell, M.; Senft, K.; Mevs, C.; Pezzullo, J. C. Vitamin A gastrointestinal absorption in persons with Down's syndrome. *J Ment Defic Res* **34**:269-275; 1990.

[246] Miles, M. V.; Patterson, B. J.; Chalfonte-Evans, M. L.; *et al.* Coenzyme Q10 (ubiquinol-10) supplementation

improves oxidative imbalance in children with trisomy 21. *Pediatr Neurol* **37**:398-403; 2007.

[247] Chadefaux, B.; Ceballos, I.; Hamet, M.; *et al.* Is absence of atheroma in Down syndrome due to decreased homocysteine levels? *Lancet* **2**:741; 1988.

[248] Fillon-Emery, N.; Chango, A.; Mircher, C.; *et al.* Homocysteine concentrations in adults with trisomy 21: effect of B vitamins and genetic polymorphisms. *Am J Clin Nutr* **80**:1551-1557; 2004.

[249] Noble, R. L.; Warren, R. P. Analysis of blood cell populations, plasma zinc and natural killer cell activity in young children with Down's syndrome. *J Ment Defic Res* **32**:193-201; 1988.

[250] Kadrabova, J.; Madaric, A.; Sustrova, M.; Ginter, E. Changed serum trace element profile in Down's syndrome. *Biol Trace Elem Res* **54**:201-206; 1996.

[251] Anneren, G.; Gebre-Medhin, M.; Gustavson, K. H.; Plantin, L. O. Selenium in plasma and erythrocytes in patients with Down's syndrome and healthy controls. Variation in relation to age, sex and glutathione peroxidase activity in erythrocytes. *Acta Paediatr Scand* **74**:508-514; 1985.

[252] Dillard, C. J.; Litov, R. E.; Savin, W. M.; Dumelin, E. E.; Tappel, A. L. Effects of exercise, vitamin E, and ozone on pulmonary function and lipid peroxidation. *J Appl Physiol* **45**:927-932; 1978.

[253] Bloomer, R. J. Effect of exercise on oxidative stress biomarkers. *Adv Clin Chem* **46**:1-50; 2008.

[254] Fisher-Wellman, K.; Bloomer, R. J. Acute exercise and oxidative stress: a 30 year history. *Dyn Med* **8**:1; 2009.

[255] Ji, L. L. Antioxidants and oxidative stress in exercise. *Proc Soc Exp Biol Med* **222**:283-292; 1999.

[256] Leeuwenburgh, C.; Heinecke, J. W. Oxidative stress and antioxidants in exercise. *Curr Med Chem* **8**:829-838; 2001.

[257] Pallardo, F. V.; Lloret, A.; Lebel, M.; *et al.* Mitochondrial dysfunction in some oxidative stress-related genetic diseases: Ataxia-Telangiectasia, Down Syndrome, Fanconi Anaemia and Werner Syndrome. *Biogerontology* **11**:401-419; 2010.

[258] Cooper, C. E.; Vollaard, N. B.; Choueiri, T.; Wilson, M. T. Exercise, free radicals and oxidative stress. *Biochem Soc Trans* **30**:280-285; 2002.

[259] Ordonez, F. J.; Rosety, M.; Rosety-Rodriguez, M. Regular exercise did not modify significantly superoxide dismutase activity in adolescents with Down's syndrome. *Br J Sports Med* **40**:717-718; 2006.

[260] Ordonez, F. J.; Rosety-Rodriguez, M.; Rosety, M. [A 12-week physical activity program increases glucose-6-phosphate-dehydrogenase activity in Down syndrome adolescents]. *Medicina (B Aires)* **65**:518-520; 2005.

[261] Francisco Javier, O.; Manuel, R.; Manuel, R. R. Regular physical activity increases glutathione peroxidase activity in adolescents with Down syndrome. *Clin J Sport Med* **16**:355-356; 2006.

[262] Ordonez, F. J.; Rosety-Rodríguez, M. Regular exercise as a healthy strategy to reduce oxidative stress in adolescents with Down syndrome. In: Heinz, E. B.; (ed.). Mental retardation. Research advances. Nova Science Publishers, Inc.; p. 35-44.; 2007.

[263] Rosety-Rodriguez, M.; Rosety, M.; Ordonez, F. J. Influence of regular exercise on erythrocyte catalase activity in adolescents with Down syndrome. *Med Clin (Barc)* **127**:533-534; 2006.

[264] Ordonez, F. J.; Rosety-Rodriguez, M. Regular exercise attenuated lipid peroxidation in adolescents with Down syndrome. *Clin Biochem* **40**:141-142; 2007.

[265] Zambrano, J. C.; Marquina, R.; Sulbaran, N.; Rodriguez-Malaver, A. J.; Reyes, R. A. Aerobic exercise reduced oxidative stress in saliva of persons with Down syndrome. *Res Sports Med* **17**:195-203; 2009.

[266] Ordonez, F. J.; Rosety, I.; Rosety, M. A.; *et al.* Aerobic training at moderate intensity reduced protein oxidation in adolescents with Down syndrome. *Scand J Med Sci Sports*; In Press. DOI: 10.1111/j.1600-0838.2010.01153.x.

[267] Rosety-Rodriguez, M.; Rosety, I.; Fornieles-Gonzalez, G.; Diaz, A.; Rosety, M.; Ordonez, F. J. A 12-week aerobic training programme reduced plasmatic allantoin in adolescents with Down syndrome. *Br J Sports Med* **44**:685-687; 2010.

[268] Flore, P.; Bricout, V. A.; van Biesen, D.; *et al.* Oxidative stress and metabolism at rest and during exercise in persons with Down syndrome. *Eur J Cardiovasc Prev Rehabil* **15**:35-42; 2008.

[269] Waring, W. S.; Convery, A.; Mishra, V.; Shenkin, A.; Webb, D. J.; Maxwell, S. R. Uric acid reduces exercise-induced oxidative stress in healthy adults. *Clin Sci (Lond)* **105**:425-430; 2003.

[270] Antunes, F.; Han, D.; Cadenas, E. Relative contributions of heart mitochondria glutathione peroxidase and catalase to H(2)O(2) detoxification in in vivo conditions. *Free Radic Biol Med* **33**:1260-1267; 2002.

[271] Radak, Z.; Chung, H. Y.; Koltai, E.; Taylor, A. W.; Goto, S. Exercise, oxidative stress and hormesis. *Ageing Res Rev* **7**:34-42; 2008.

[272] Song, M.-F.; Li, Y.-S.; Ootsuyama, Y.; *et al.* Urea, the most abundant component in urine, cross-reacts with a commercial 8-OH-dG ELISA kit and contributes to overestimation of urinary 8-OH-dG. *Free Radic Biol Med* **47**:41-46; 2009.

[273] Sacks, B.; Buckley, F. Multi-nutrient formulas and other substances as therapies for Down syndrome: an overview. *Down Syndrome News and Update* **1**:70-83. The Down Syndrome Educational Trust; 1998.

[274] Ani, C.; Grantham-McGregor, S.; Muller, D. Nutritional supplementation in Down syndrome: theoretical considerations and current status. *Dev Med Child Neurol* **42**:207-213; 2000.

[275] Roizen, N. J. Complementary and alternative therapies for Down syndrome. *Ment Retard Dev Disabil Res Rev* **11**:149-155; 2005.

[276] Bumbalo, T. S.; Morelewicz, H. V.; Berens, D. L. Treatment of Down's Syndrome with the "U" Series of Drugs. *JAMA* **187**:361; 1964.

[277] Bittles, A. H.; Bower, C.; Hussain, R.; Glasson, E. J. The four ages of Down syndrome. *Eur J Public Health* **17**:221-225; 2007.

[278] Glasson, E. J.; Sullivan, S. G.; Hussain, R.; Petterson, B. A.; Montgomery, P. D.; Bittles, A. H. Comparative survival advantage of males with Down syndrome. *Am J Hum Biol* **15**:192-195; 2003.

[279] Tiano, L.; Carnevali, P.; Padella, L.; *et al.* Effect of Coenzyme Q(10) in mitigating oxidative DNA

damage in Down syndrome patients, a double blind randomized controlled trial. *Neurobiol Aging;* In Press.

[280] Gualandri, W.; Gualandri, L.; Demartini, G.; *et al.* Redox balance in patients with Down's syndrome before and after dietary supplementation with α-lipoic acid and L-cysteine. *Int J Clin Pharm Res* **23**:23-30; 2003.

[281] Pelsman, A.; Hoyo-Vadillo, C.; Gudasheva, T. A.; Seredenin, S. B.; Ostrovskaya, R. U.; Busciglio, J. GVS-111 prevents oxidative damage and apoptosis in normal and Down's syndrome human cortical neurons. *Int J Dev Neurosci* **21**:117-124; 2003.

[282] Busciglio, J.; Pelsman, A.; Helguera, P.; *et al.* NAP and ADNF-9 protect normal and Down's syndrome cortical neurons from oxidative damage and apoptosis. *Curr Pharm Des* **13**:1091-1098; 2007.

[283] Sinet, P. M. Metabolism of oxygen derivatives in Downs syndrome. In: Sinex, F. M.; Merril, C. R.; (eds.). Alzheimer's Diseases, Downs Syndrome, and Ageing. New York, USA: The New York Academy of Sciences; p. 83-94; 1982.

[284] Antila, E.; Nordberg, U. R.; Syvaoja, E. L.; Westermarck, T. Selenium therapy in Down syndrome (DS): a theory and a clinical trial. *Adv Exp Med Biol* **264**:183-186; 1990.

[285] Anneren, G.; Gebre-Medhin, M.; Gustavson, K. H. Increased plasma and erythrocyte selenium concentrations but decreased erythrocyte glutathione peroxidase activity after selenium supplementation in children with Down syndrome. *Acta Paediatr Scand* **78**:879-884; 1989.

[286] Lima, A. S.; Cardoso, B. R.; Cozzolino, S. F. Nutritional status of zinc in children with Down syndrome. *Biol Trace Elem Res* **133**:20-28; 2010.

[287] Marques, R. C.; de Sousa, A. F.; do Monte, S. J.; Oliveira, F. E.; do Nascimento Nogueira, N.; Marreiro, D. N. Zinc nutritional status in adolescents with Down syndrome. *Biol Trace Elem Res* **120**:11-18; 2007.

[288] Lakshmi, K. T.; Surekha, R. H.; Srikanth, B.; Jyothy, A. Serum cholinesterases in Down syndrome children before and after nutritional supplementation. *Singapore Med J* **49**:561-564; 2008.

[289] Prasad, A. S.; Beck, F. W.; Bao, B.; *et al.* Zinc supplementation decreases incidence of infections in the elderly: effect of zinc on generation of cytokines and oxidative stress. *Am J Clin Nutr* **85**:837-844; 2007.

[290] Cabreiro, F.; Perichon, M.; Jatje, J.; *et al.* Zinc supplementation in the elderly subjects: effect on oxidized protein degradation and repair systems in peripheral blood lymphocytes. *Exp Gerontol* **43**:483-487; 2008.

[291] Beckett, G. J.; Arthur, J. R. Selenium and endocrine systems. *J Endocrinol* **184**:455-465; 2005.

[292] Marreiro Ddo, N.; de Sousa, A. F.; Nogueira Ndo, N.; Oliveira, F. E. Effect of zinc supplementation on thyroid hormone metabolism of adolescents with Down syndrome. *Biol Trace Elem Res* **129**:20-27; 2009.

[293] Licastro, F.; Chiricolo, M.; Mocchegiani, E.; *et al.* Oral zinc supplementation in Down's syndrome subjects decreased infections and normalized some humoral and cellular immune parameters. *J Intellect Disabil Res* **38**:149-162; 1994.

[294] Palmer, S. Influence of vitamin A nutriture on the immune response: findings in children with Down's syndrome. *Int J Vitam Nutr Res* **48**:188-216; 1978.

[295] Park, J. S.; Chyun, J. H.; Kim, Y. K.; Line, L. L.; Chew, B. P. Astaxanthin decreased oxidative stress and inflammation and enhanced immune response in humans. *Nutr Metab* **7**:18; 2010.

[296] Benedetti, S.; Benvenuti, F.; Nappi, G.; *et al.* Antioxidative effects of sulfurous mineral water: protection against lipid and protein oxidation. *Eur J Clin Nutr* **63**:106-112; 2009.

[297] Pritchard, M.; Reeves, R. H.; Dierssen, M.; Patterson, D.; Gardiner, K. J. Down syndrome and the genes of human chromosome 21: current knowledge and future potentials. *Cytogenet Genome Res* **121**:67-77; 2008.

para la valoración de capacidad antioxidante total: El ensayo CUPRAC-BCS

C. Campos, R. Guzmán, E. López-Fernández y Á. Casado

Existe un interés creciente por la determinación del estatus antioxidante en estudios clínicos y experimentales investigando la progresión de enfermedades o diversos aspectos del estrés oxidativo. El objetivo del presente trabajo fue evaluar y optimizar el ensayo de reducción del cobre(II) utilizando sal disódica del ácido 2,9-dimetil-4,7-difenil-1,10-fenantrolindisulfónico (BCS) como agente quelante para la determinación de la capacidad antioxidante total (TAC) en muestras de plasma y orina humanas. A este ensayo lo denominamos CUPRAC-BCS.

Se analizan muestras de plasma heparinizado y orina de 20 individuos mediante el CUPRAC-BCS y otros tres métodos espectrofotométricos de determinación de TAC ampliamente utilizados: el ensayo de capacidad del plasma de reducir el ión férrico (FRAP), el ensayo de capacidad antioxidante equivalente de Trolox (TEAC) y el ensayo 2,2-difenil-1-picrilhidrazil (DPPH), con la finalidad de compararlos.

El método CUPRAC-BCS correlaciona significativamente con los métodos FRAP y TEAC en muestras de plasma y orina ($r>0,5$ y $P<0,05$ en todos los casos) y con el método DPPH en muestras de orina ($r=0,925$ y $P<0,001$), pero no en muestras de plasma ($r=0,366$ y $P=0,112$). Sin embargo, ninguno de los tres métodos concuerda con el CUPRAC-BCS dado que las líneas de igualdad y de regresión no coinciden. La imprecisión del método es menor del 6%, el límite de detección es de 41,8 μmoles equivalentes de Trolox/L, es lineal hasta 2 μmoles/L de Trolox y la sal disódica del ácido etilendiaminotetraacético dihidratada (EDTA) se une al cobre(II) evitando la formación del complejo cobre(I)-BCS, es decir, inhibiendo la reacción.

Se concluye que el ensayo CUPRAC-BCS es una metodología simple, rápida, económica y adecuada para la valoración de TAC en muestras humanas de orina y plasma heparinizado. Además, presenta varias ventajas con respecto a otros ensayos de determinación de TAC, tales como: los reactivos son muy estables, la reacción transcurre a pH fisiológico y a temperatura ambiente, etc. Así, el CUPRAC-BCS fue el método de elección para la determinación de TAC en muestras de orina de individuos con SD en el presente trabajo de Tesis Doctoral.

Analytical Biochemistry 392 (2009) 37–44

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Evaluation of the copper(II) reduction assay using bathocuproinedisulfonic acid disodium salt for the total antioxidant capacity assessment: The CUPRAC–BCS assay

Carlos Campos *, Rodrigo Guzmán, Encarnación López-Fernández, Ángela Casado

Department of Cellular and Molecular Physiopathology, Center for Biological Research, Spanish National Research Council, E-28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 20 February 2009
Available online 21 May 2009

Keywords:
CUPRAC–BCS assay
Total antioxidant capacity
Bathocuproinedisulfonic acid disodium salt
TEAC assay
FRAP assay
DPPH assay

ABSTRACT

There is heightened interest in determining antioxidant status of individuals in experimental and clinical studies investigating progression of diseases or diverse aspects of oxidative stress, among others. The aim of this study was to evaluate the copper(II) reduction assay with bathocuproinedisulfonic acid disodium salt as chelating agent (the CUPRAC–BCS assay) for the total antioxidant capacity (TAC) assessment in human plasma and urine. Samples from 20 individuals were determined with four spectrophotometric assays—CUPRAC–BCS, ferric reducing ability of plasma (FRAP), trolox equivalent antioxidant capacity (TEAC), and 1,1-diphenyl-2-picrylhydrazyl assay (DPPH)—to compare these methods. CUPRAC–BCS was significantly correlated with FRAP and TEAC for plasma and urine samples ($r > 0.5$, $P < 0.05$ for all) and with DPPH for urine samples ($r = 0.925$, $P < 0.001$) but not with DPPH for plasma samples ($r = 0.366$, $P = 0.112$). However, the four methods do not agree given that lines of equality and regression were not matched up. The imprecision of the method is less than 6%, the detection limit is 41.8 μmol trolox equivalents/L, it is linear up to 2 mM trolox, and ethylenediaminetetraacetic acid dihydrate disodium salt (EDTA) binds to Cu(II), avoiding the formation of Cu(I)–BCS complex. This study shows that CUPRAC–BCS is a simple, fast, inexpensive, and suitable method for TAC assessment in human urine and heparinized plasma samples.

It is well known that oxidative stress is involved in the pathogenesis and development of many human diseases such as cardiovascular pathologies, cancer, arteriosclerosis, and other diseases related to aging. Oxidative stress is caused by an excessive bioavailability of reactive oxygen species (ROS)[1] that cause cell damage. Because organisms have mechanisms for preventing and repairing damages caused by ROS, which comprise a variety of antioxidants, there is heightened interest in determining their antioxidants levels and the way in which they are related to pathological states.

In biological terms, it is accepted that an antioxidant could be considered as any molecule that can retard or prevent the action of oxidants [1]. Because of the difficulty in measuring concentrations of individual antioxidants, total antioxidant capacity (TAC) determination can be achieved by simple in vitro methodologies. Moreover, possible interactions among different antioxidants in vivo could also make the measurement of any individual antioxidant less representative of the overall antioxidant status. Therefore, TAC, which is defined as a measure of the amount (in moles) of a given free radical scavenged by the nonenzymatic antioxidants present in an aqueous solution [2], may give more biologically relevant information than that obtained from measuring each antioxidant separately. Thus, TAC is a useful parameter to assess the antioxidant status of an organism as well as to control antioxidant supplementation or to demonstrate the efficacy of a dietary intervention.

Many analytical methods have been developed to determine TAC in a wide range of matrices such as biological fluids, food, beverages, and plant extracts, and several outstanding reviews about

* Corresponding author. Fax: +34 91 536 04 32.
E-mail address: ccampos@cib.csic.es (C. Campos).

[1] *Abbreviations used:* ROS, reactive oxygen species; TAC, total antioxidant capacity; TEAC, trolox equivalent antioxidant capacity; FRAP, ferric reducing ability of plasma; ORAC, the oxygen radical absorbance capacity; TRAP, total radical absorption potential; BC, bathocuproine; BCS, bathocuproinedisulfonic acid disodium salt; NC, neocuproine; Cu, copper; ET, electron transfer; AOP or PAO, antioxidant power assay; CUPRAC, cupric ion reducing antioxidant capacity; DPPH, 1,1-diphenyl-2-picrylhydrazyl; trolox, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; GSH, reduced glutathione; BHA, 2(3)-*t*-butyl-4-hydroxyanisole; BSA, bovine serum albumin (fraction V); NAC, *N*-acetyl-L-cysteine; TPTZ, 2,4,6-tri(2-pyridyl)-*s*-triazine; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (diammonium salt); Tiron, 4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt; EDTA, ethylenediaminetetraacetic acid dihydrate disodium salt; PBS, phosphate-buffered saline; SD, standard deviation; ANOVA, analysis of variance; LOD, limit of detection; LOQ, limit of quantitation.

0003-2697/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2009.05.024

them have been discussed [2–8]. The trolox equivalent antioxidant capacity (TEAC), the ferric reducing ability of plasma (FRAP), the oxygen radical absorbance capacity (ORAC), and the total radical absorption potential (TRAP) assays are some of the most commonly used for TAC determination of biological fluids. Not all of the methods measure the same antioxidants equally, and comparisons between results do not always agree. Therefore, nowadays there is no single widely acceptable specific method for biological samples. Although some comparative studies have been conducted during recent years [9–15], these investigations did not include the copper(II) reduction assay applied to plasma or urine samples.

There are several assays for TAC assessment based on cupric ion reduction that differ in the chelating agent used: bathocuproine (BC), bathocuproinedisulfonic acid disodium salt (BCS), and neocuproine (NC). These chelators form stable complexes with monovalent copper (Cu) that have a typical absorption at 450–490 nm. In these assays, the complex is formed when, in a solution containing Cu(II) and one of these chelators, the nonenzymatic antioxidants present in a sample reduce Cu(II) to Cu(I). Then, as in similar electron transfer (ET) reaction-based assays, the antioxidant capacity is assumed to be equal to the reducing capacity [4]. Although the use of NC has already been studied for TAC determination in biological samples [16] and comparative studies can be found in the literature [17,18], the Cu(II) reduction assay using BC and BCS was patented [19], and nowadays it is commercialized as an "antioxidant power assay" (abbreviated as AOP or PAO). However, this name is very general and could be applied to any TAC assay. Apak and coworkers [16] used CUPRAC (cupric ion reducing antioxidant capacity) to refer to the Cu(II) reducing assay using NC as chelator. Therefore, we established CUPRAC-BCS to name the method for TAC assessment in which BCS is used as chelating agent. Although the CUPRAC-BCS assay presents a wider range of advantages than do other methodologies for TAC assessment of plasma and urine samples, this method has not been well studied, it has not been compared with other TAC assays, and not much information is provided by the manufacturer.

The aim of this study was to evaluate the CUPRAC-BCS assay, based on the commercial AOP kit, for TAC assessment of human plasma and urine samples. This method was compared with three other in vitro antioxidant capacity assays based on ET reactions widely applied and frequently used—FRAP, TEAC, and DPPH (1,1-diphenyl-2-picrylhydrazyl) assays—to assess its use as an applicable methodology for TAC determination.

Materials and methods

Chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), reduced glutathione (GSH), gallic acid, 2(3)-*t*-butyl-4-hydroxyanisole (BHA), bovine serum albumin (fraction V) (BSA), *N*-acetyl-L-cysteine (NAC), bilirubin, DPPH, and methanol were purchased from Sigma-Aldrich (Steinheim, Germany). Uric acid, BCS, Cu(II) sulfate anhydrous, 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ), potassium persulfate, and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (diammonium salt) (ABTS) were obtained from Fluka (Buchs, Switzerland). Ascorbic acid, ethanol, disodium hydrogen phosphate dihydrate, potassium dihydrogen phosphate, and sodium acetate trihydrate were obtained from Merck (Darmstadt, Germany). Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt) and iron(III) chloride anhydrous were obtained from Riedel-de-Haën (Seelze, Germany). Ethylenediaminetetraacetic acid dihydrate disodium salt (EDTA) was obtained from Probus (Barcelona, Spain). Ultrapure water was obtained by a Milli-RO water system (Millipore, Bedford, MA, USA).

Apparatus

Spectrophotometric measurements of FRAP, TEAC, and DPPH were performed with a UVmini-1240 Shimadzu spectrophotometer (Shimadzu, Tokyo, Japan). For the CUPRAC-BCS assay, a microplate reader with a 490-nm filter was used (Bio-Tek ELx808, Bio-Tek Instruments, Winooski, VT, USA). Absorption spectra were measured with a Cary 4000 spectrophotometer (Varian, Palo Alto, CA, USA). A Crison GLP21 pH meter (Crison Instruments, Barcelona, Spain) was used for pH adjustments. Reagents were heated in a water bath (Kowell FB-3-V-B).

Preparation of standards

Trolox, uric acid, GSH, bilirubin, ascorbic acid, tiron, gallic acid, NAC, BHA, and albumin were used for antioxidant studies. Standard stock solutions (2 mM) were prepared for all antioxidants. Trolox was dissolved in phosphate-buffered saline (PBS). Uric acid was prepared in ultrapure water and dissolved with 1 M NaOH. BHA was dissolved in ethanol. Bilirubin was dissolved in 0.1 M NaOH. The rest of the antioxidants were prepared in ultrapure water. Standards were frozen in 1 ml aliquots at −80 °C until analysis.

Plasma and urine sample collection and preparation

Blood from 20 healthy subjects (9 males and 11 females, mean age = 50.5 ± 14.0 years, range = 26–76) was collected into tubes containing heparin. Plasma was obtained by withdrawing supernatants of centrifuged blood at 1200g for 10 min. Collected plasma was immediately stored at −80 °C until analysis. Urine samples were also taken from 20 healthy subjects (6 males and 14 females, mean age = 36.9 ± 20.9 years, range = 14–82), and aliquots were frozen at −20 °C until analysis.

Informed consent was obtained from the participants. The study was approved by the ethics committee of the Spanish National Research Council.

CUPRAC-BCS assay

The CUPRAC-BCS assay was based on the commercial AOP method of Da Cruz (U.S. patent 6,613,577) [19]. For the photometric assay, F16 MaxiSorp microplates (Nunc, Roskilde, Denmark) of 350 µl/well and a microplate reader were used. Thus, 200 µl of each sample diluted 1:40 with 0.25 mM BCS in 10 mM phosphate buffer (pH 7.4) was put into each well in duplicate and a first read at 490 nm was taken. After the addition of 50 µl of 0.5 mM $CuSO_4$ (Cu(II) solution prepared in water), the reaction mixture was incubated 3 min at room temperature. The reaction was stopped by the addition of 50 µl of 0.01 M EDTA, and a second read at 490 nm was taken. The difference between the two readings was used in the calculations. Distilled water was used instead of sample or standard for blanks. Results were compared with a standard curve obtained with trolox and are expressed in mmol trolox equivalents/L.

FRAP assay

The FRAP assay was performed essentially as described by Benzie and Strain [20]. In brief, 30 µl of sample or standard was added to 900 µl of the freshly prepared FRAP reagent (10 volumes of 300 mM acetate buffer [pH 3.6], 1 volume of 10 mM TPTZ [prepared in 40 mM HCl], and 1 volume of 20 mM $FeCl_3$) prewarmed to 37 °C. The reaction mixture was incubated 5 min at 37 °C, and absorbance was read at 593 nm. Results are expressed in mmol trolox equivalents/L.

TEAC assay

The TEAC assay described by Miller and coworkers [21,22] was performed with the modifications of Re and coworkers [23]. Briefly, 1 ml of $ABTS^{\cdot+}$ preheated at 30 °C was added along with 10 µl of sample or standard, and absorbance at 734 nm was measured after 5 s. $ABTS^{\cdot+}$ was prepared by mixing ABTS stock solution (7 mM in water) with 2.45 mM potassium persulfate. This mixture must remain in the dark at room temperature for at least 12 h. Before use, the $ABTS^{\cdot+}$ solution was diluted with PBS (pH 7.4) to an absorbance of 0.700 ± 0.020 at 734 nm. The antioxidant activity of samples was calculated by determining the decrease in absorbance by using the following equation:

$$\text{antioxidant capacity} = (A_0 - A_C)/A_0, \quad (1)$$

where A_0 is the absorbance of the reference sample and A_C is the absorbance of the sample. Results are expressed in mmol trolox equivalents/L.

DPPH assay

The DPPH reduction assay of Blois was performed with the modifications of Janaszewska and Bartosz [12]. Briefly, plasma and urine samples were diluted 1:80 and 1:200, respectively, with 10 mM phosphate buffer (pH 7.4), and 600 µl of 0.2 mM DPPH in methanol was added to an equal volume of the diluted sample. After 30 min at room temperature, samples were centrifuged at 9500*g* for 5 min to remove precipitate. Absorbance was measured at 520 nm and compared with that of a reference sample containing distilled water instead of sample or standard. The antioxidant activity, expressed in mmol trolox equivalents/L, was calculated by using the equation already described for the TEAC assay.

Statistical analysis

Results are expressed as means ± standard deviations ($\bar{X}$ ± SD). Between-group comparisons were performed using an analysis of variance (ANOVA). Correlations are expressed using the Pearson's (product–moment) correlation coefficient. For all tests, $P < 0.05$ was considered as statistically significant. For the statistical analysis, SPSS software (version 17.0, SPSS, Chicago, IL, USA) was used.

Results and discussion

Evaluation of the CUPRAC–BCS assay

Optimization of reagents concentration

Different concentrations of copper sulfate (0.05–0.8 mM) and BCS (0.1–1.6 mM) were investigated by altering one variable at a time to optimize the assay. Final concentrations of 0.2 mM BCS and 0.1 mM $CuSO_4$ gave enough absorption intensity at 490 nm with good linearity up to 2 mM trolox, as is shown below; therefore, they were established as optimal for the assay. EDTA (1.67 mM final concentration) is used as a stop reagent. When EDTA is added at this concentration before the sample, no color is developed (Fig. 1). On the other hand, we noticed that when a concentration of EDTA higher than 1.67 mM is used, a precipitate could appear in plasma samples.

Stability of reagents

All of the reagents are stable for several months at 4 °C. This is an advantage over other methods. In the FRAP assay, the reagent must be used freshly. In the TEAC and DPPH assays, the reagents are unstable.

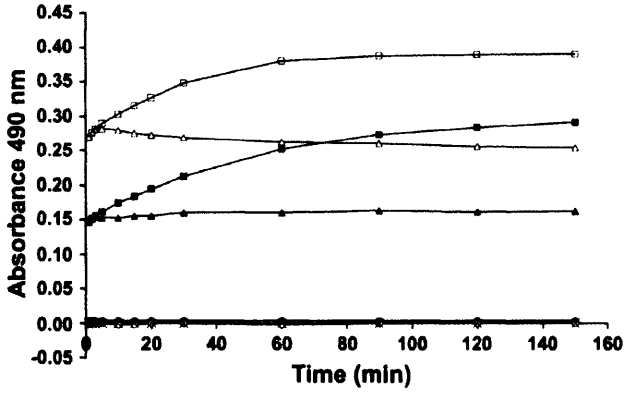


Fig. 1. Kinetics of Cu(II) reduction by plasma adding EDTA before Cu(II) reagent (●), plasma adding EDTA after 3 min of reaction with Cu(II) reagent (▲), plasma without adding EDTA (■), urine diluted 1:8 adding EDTA before Cu(II) reagent (○), urine diluted 1:8 adding EDTA after 3 min of reaction with Cu(II) reagent (△), urine diluted 1:8 without adding EDTA (□), and the reagent alone adding EDTA after 3 min of reaction with Cu(II) reagent (×).

Imprecision

The within- and between-run coefficients of variation (CVs) obtained with the CUPRAC–BCS assay using six different concentrations of uric acid (0.0625, 0.125, 0.25, 0.5, 1, and 2 mM; each batch in triplicate and analyzed on 3 different days to obtain the between-run CV) were 0.9% (mean = 0.995%, SD = 0.009) and 5.6% (mean = 1.008%, SD = 0.055), respectively. Therefore, the commonly acceptability criterion in analytical chemistry for method precision less than 15% was well achieved. All CV estimates for the FRAP, TEAC, and DPPH assays were below 8%.

Limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) of the CUPRAC–BCS assay were calculated from the residual standard deviation of the regression data by the equations $3(S_{xy}/b)$ and $10(S_{xy}/b)$, respectively, where S_{xy} is the residual standard deviation and b is the slope of the linear regression equation [24]. The LOD and LOQ were 12.4 and 41.3 µmol Trolox equivalents/L, respectively. Therefore, the CUPRAC–BCS assay is sensitive enough for TAC assessment in plasma and urine samples.

Linearity

Absorbance at 490 nm was linear from 0.0625 up to 2 mM trolox and uric acid (trolox: $y = -0.002299 + 0.339502x$, $r = 1.000$, $P < 0.001$; uric acid: $y = 0.002657 + 0.334237x$, $r = 0.999$, $P < 0.001$). Therefore, the CUPRAC–BCS assay showed a high linearity.

Absorption spectra

Fig. 2 shows the absorption spectra of the reagent blank with and without EDTA (curves a and b, respectively) and the Cu(I)–BCS complex with and without EDTA (curves c and d, respectively). For Cu(I)–BCS complex formation, 0.5 mM $CuSO_4$ was added to a solution containing 50 µM trolox and 0.2 mM BCS. Then 0.01 M EDTA or distilled water was added after a 3-min reaction. The absorption spectra were recorded from 700 to 300 nm at room temperature using a Cary 4000 double-beam spectrophotometer. An absorbance peak at 482 to 483 nm was obtained for the Cu(I)–BCS complex, agreeing with the results of previous works [25–27]. Comparing curves a and c with curves b and d, respectively, in Fig. 2, we can observe that the Cu(II)–EDTA complex does not absorb at 490 nm. Therefore, its use as a stop reagent is possible.

Microplate readers usually work with 490-nm filters being common. Because the absorption at 490 nm was only 3.4% lower

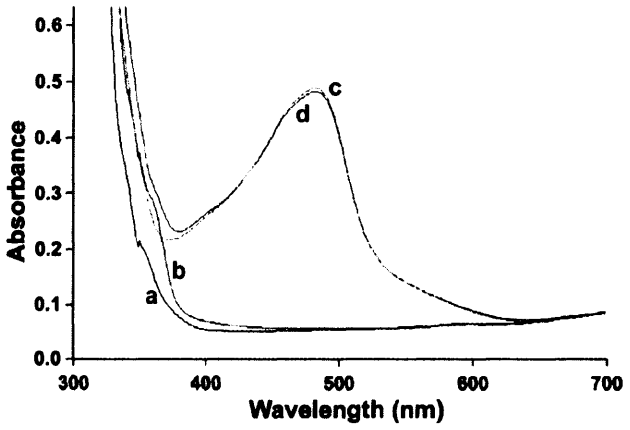


Fig. 2. Absorption spectra of a reagent blank (BCS + $CuSO_4$ + H_2O instead of sample) with EDTA (a) and without EDTA (b) as well as of the Cu(I)–BCS complex with EDTA (c) and without EDTA (d) studied from 700 to 300 nm every 1 nm and recorded by a double-beam spectrophotometer (Cary 4000 spectrophotometer). Spectra were taken after 3 min incubations at room temperature.

than the maximum, microplate reading at 490 nm can be carried out for the CUPRAC–BCS assay.

Stoichiometry of Cu(I)–BCS complex

Stoichiometry of the reaction was studied at pH 7.4 with Job's method of continuous variations. As seen in the Job's plot (Fig. 3), the Cu(I)–BCS complex is formed in a 1:2 ratio because the plot reached a maximum value at a BCS molar fraction (X_m) of 0.67. This result is in accordance with the previous work of Sánchez-Rasero [28], where stoichiometry of the Cu(I)–BC complex was encountered to be in a 1:2 molar ratio. So, stoichiometry of both complexes, Cu(I)–BCS and Cu(I)–BC, is the same.

Kinetics of Cu(II) reduction and effect of EDTA

Reduction of Cu(II) by heparinized plasma and urine samples was studied for 150 min, as is shown in Fig. 1. Individual antioxidant kinetics of Cu(II) reduction was also studied, and results are shown in Fig. 4. Because the SD was less than 0.004 in every point, error bars are not shown in either Fig. 1 or Fig. 4.

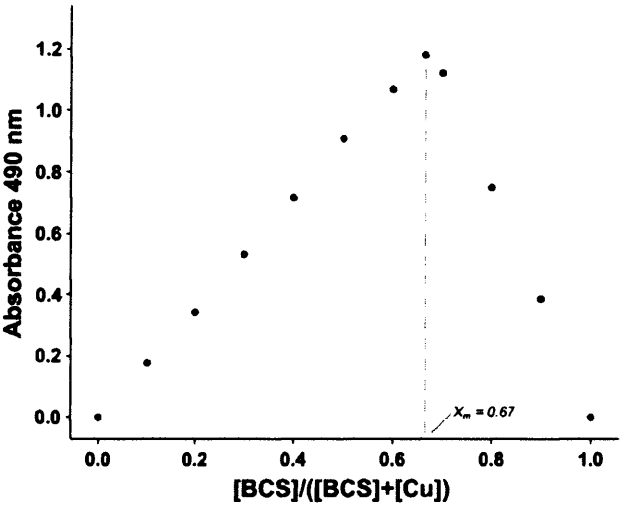


Fig. 3. Job's plot of equimolar solutions for Cu(I)–BCS complex in phosphate-buffered medium at pH 7.4 studied at 490 nm. [BCS] + [Cu(II)] = 0.5 mM and 0.2 mM uric acid was used to reduce Cu(II) to Cu(I). X_m is the molar fraction of BCS.

The CUPRAC–BCS reaction in plasma and urine samples is very fast during the first seconds but too slow afterward (Fig. 1, filled squares for plasma and open squares for urine). That is, Cu(II) reduction by plasma and urine continues up to 150 min, and a possible increase in absorbance caused by some experimental artifact can be rejected because the reagent alone did not show any change in absorbance (Fig. 1, crosses). Although some antioxidants complete the reaction in less than 3 min, Cu(II) reduction by albumin, bilirubin, and uric acid is not finished within 60 min (Fig. 4). Not only CUPRAC–BCS but also TAC assays are, in general, strongly influenced by time measurement [12], making it difficult to do method comparisons when changes in protocols take place. Thus, the incompleteness of the reaction was also found for the FRAP assay in several works [12,29]. Other ET-based reaction assays that measure the absorbance extinction, such as TEAC and DPPH, are also time dependent. This fact has been considered by several authors as an important limitation [14]; nevertheless, this could be the result of possible interactions among different antioxidants present in the sample. Therefore, the CUPRAC and FRAP assays could be used to measure TAC of antioxidants with fast rate of ion reduction as well as to monitor the trend in TAC changes, constituting an important tool for TAC assessment.

We also studied EDTA as a blood anticoagulant and its use as a stop reagent. EDTA, as BCS, is a chelating agent that forms especially strong complex with Cu(II) and can be used as a stop reagent of the reaction because Cu(II)–EDTA complex does not absorb at 490 nm, as we observed (Fig. 2, comparison between curves a and c and between curves b and d). As seen in Fig. 1, when 0.01 M EDTA was added to a BCS phosphate-buffered solution containing Cu(II) and sample (filled triangles for plasma and open triangles for urine), no more color develops after 3 min of reaction. Besides, there was not any change in absorbance with respect to a blank (crosses) when EDTA was added before Cu(II) reagent (filled circles for plasma and open circles for urine) because EDTA binds to Cu(II) ions and Cu(II) reduction by nonenzymatic antioxidants present in the solution is not possible. In brief, the Cu(I)–BCS complex formation is halted by the presence of EDTA. Thus, the value of absorbance at 490 nm is then stable for several hours in plasma samples and for no more than 10 min in urine samples due to a decrease in absorbance from this time (Fig. 1, open triangles). Therefore, in TAC assessment of the urine sample, the second absorbance read should be done within 10 min after the addition of EDTA. These results showed that TAC of EDTA plasma samples cannot be analyzed with the CUPRAC assay because of the quelant property of EDTA. However, its use as a stop reagent allows the

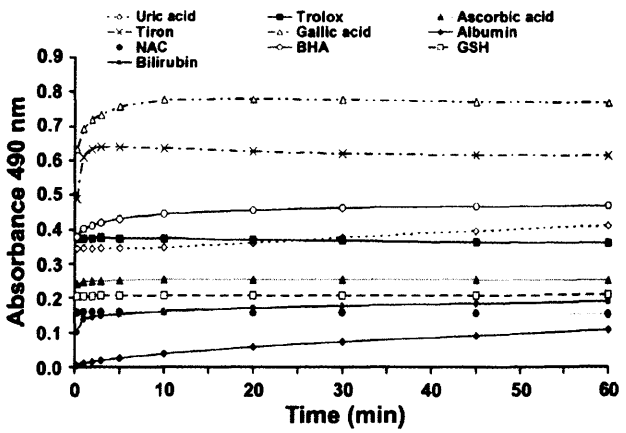


Fig. 4. Kinetics of Cu(II) reaction by individual antioxidants at a concentration of 10 μM for bilirubin and 25 μM for the rest.

analysis of a high number of samples in only one batch, in contrast to other assays such as nonautomatized FRAP and TEAC.

Effect of pH on Cu(II) reduction by samples and uric acid

The reducing capacity of Cu(II) by a plasma sample, a urine sample, and 1 mM uric acid was studied in a phosphate-buffered medium at pHs 3.8, 6.0, 7.4, and 11.4 (Fig. 5). In this pH range, the Cu(I)–BCS complex is stable, as it was found by Blair and Diehl [27]; therefore, changes in TAC must be due only to the effect of pH on antioxidant properties.

Plasma and urine samples showed pH-dependent changes in antioxidant capacity that were similar to those observed for uric acid. This may be due to a high relative contribution of this antioxidant to plasma and urine samples. We found that TAC was the lowest at pH 11.4 and that TAC was slightly higher at pH 3.8 than at pHs 6.0 and 7.4. However, it has been suggested that in acidic conditions the reducing capacity may be suppressed due to protonation on antioxidant compounds, whereas in basic conditions proton dissociation of phenolic compounds would enhance a sample reducing capacity [4]. It is known that at high pH degradation of uric acid occurs [30]. Thus, the reducing capacity of uric acid under alkali conditions may be reduced, as we observed. In any case, antioxidants act at physiological pH in the body, and the CUPRAC–BCS assay has enough sensitivity and precision at pH 7.4, as we showed. Therefore, TAC assessment must give more representative results of in vivo behavior when pH of the method is close to physiological. Thus, the CUPRAC–BCS assay may give more realistic results than other assays such as FRAP, where redox reaction is carried out at acidic conditions (pH 3.6).

Dose–response studies

The dose–response results obtained in the CUPRAC–BCS assay for 10 usual antioxidants (trolox, uric acid, GSH, bilirubin, ascorbic acid, tiron, gallic acid, NAC, BHA, and albumin) are shown in Fig. 6. The SD at every point is less than 0.003; therefore, error bars are not shown. Good linearity was found in the concentration range from 0.0625 to 2 mM for trolox ($y = 0.3452x - 0.0093$, $r = 0.999$, $P < 0.001$), uric acid ($y = 0.3380x + 0.0049$, $r = 1.000$, $P < 0.001$), GSH ($y = 0.1879x + 0.0137$, $r = 0.999$, $P < 0.001$), ascorbic acid ($y = 0.3287x + 0.0033$, $r = 1.000$, $P < 0.001$), and NAC ($y = 0.1607$, $r = 0.999$, $P < 0.001$). Moreover, good linearity was found in the concentration range from 0.0625 to 1 mM for tiron ($y = 0.6004x + 0.0088$, $r = 0.999$, $P < 0.001$), gallic acid ($y = 0.7059x + 0.0135$, $r = 1.000$, $P < 0.001$), and BHA ($y = 0.3772x + 0.0113$, $r = 1.000$, $P < 0.001$), whereas linearity was found in the concentration range from 0.05 to 0.30 mM for bilirubin ($y = 0.9314x + 0.0200$, $r = 0.999$, $P < 0.001$). Albumin did not show linearity within 3 min of reaction. Therefore, trolox, uric acid, ascorbic acid, GSH, and NAC could be used as a standard for the calibration curve in the CUPRAC–BCS assay.

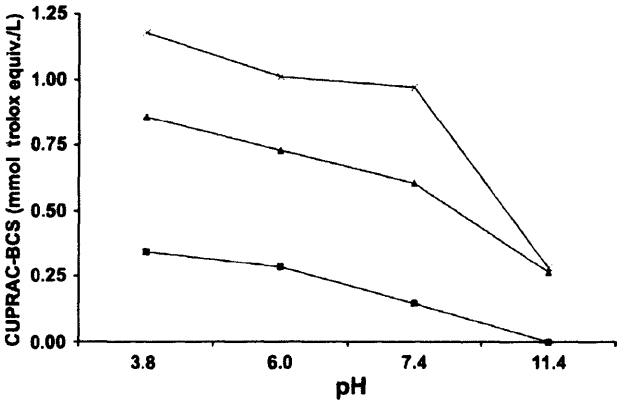


Fig. 5. Antioxidant capacity of uric acid (×), a plasma sample (■), and a diluted 1:10 urine sample (▲) assayed with CUPRAC–BCS at pHs 3.8, 6.0, 7.4, and 11.4.

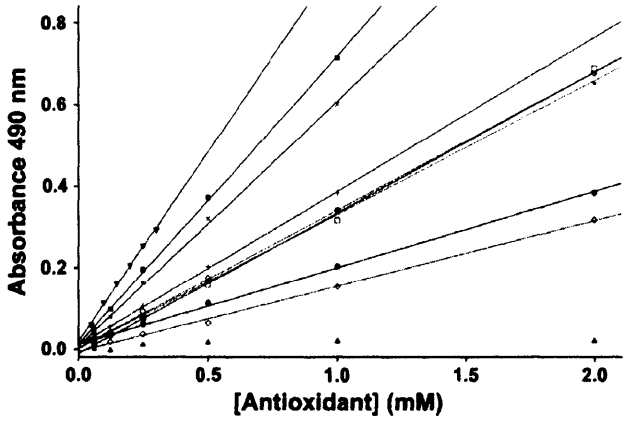


Fig. 6. Dose–response characteristics for the antioxidant capacity of trolox (□), uric acid (○), GSH (●), bilirubin (▼), ascorbic acid (★), tiron (×), gallic acid (■), BHA (+), NAC (◇), and albumin (▲) in the CUPRAC–BCS assay.

In the commercial AOP kit, only uric acid is provided as a standard and the kit offers the possibility of dissolving it using NaOH. As was mentioned above, uric acid degradation to allantoin takes place under alkaline conditions [30]. Besides, trolox is the most common standard in TAC assays. Therefore, trolox instead of uric acid is preferable as a standard to carry out the CUPRAC–BCS assay.

Comparison of CUPRAC–BCS, FRAP, TEAC, and DPPH assays

In vitro TAC assays can be divided primarily into two categories according to the type of reaction in which they are based: (i) assays based on hydrogen atom transfer reactions and (ii) assays based on ET reaction. In the current study, we compare four TAC assays based on ET reactions (CUPRAC–BCS, FRAP, TEAC, and DPPH) to evaluate these assays applied to human plasma and urine samples.

Regression analysis between the TAC assays for plasma and urine samples

As is shown in Table 1, plasma and urine TAC determined in 20 individuals by the CUPRAC–BCS assay was significantly correlated with the FRAP and TEAC assays. Moreover, FRAP and TEAC were significantly correlated for both types of samples as well, whereas a significant correlation was found only in urine samples between DPPH and the rest of the methods.

The DPPH assay is not a common method for TAC determination in body fluids because of the reaction taking place in an organic solvent like methanol being a more specific assay for TAC assessment of hydrophilic antioxidants. Therefore, the lack of correlation between DPPH and the rest of assays in plasma samples is understandable. However, high correlations were found in urine samples that may be due to the high presence of uric acid. On the other hand, FRAP and TEAC are highly correlated in plasma samples, a result in agreement with the study of Erel [31], as well as in urine samples.

Because CUPRAC–BCS correlates well with FRAP and TEAC, the CUPRAC–BCS assay is a suitable method for TAC assessment in human plasma and also in urine. Moreover, CUPRAC–BCS was correlated with DPPH in urine samples. However, it should be noted that, attending to the lines of equality (Fig. 7, dotted lines), results from all of these methods are not interchangeable in spite of the good correlation between them. The lines of equality on which

Table 1
Relationships between TAC assays (CUPRAC-BCS, FRAP, TEAC, and DPPH) for plasma and urine samples ($n = 20$).

	FRAP		TEAC		DPPH	
	Plasma	Urine	Plasma	Urine	Plasma	Urine
CUPRAC-BCS	$y = 1.0473x - 0.1245$, $r = 0.924$, $P < 0.001$	$y = 1.1823x + 0.0140$, $r = 0.982$, $P < 0.001$	$y = 0.3377x + 0.4951$, $r = 0.513$, $P < 0.05$	$y = 1.0818x + 0.3772$, $r = 0.934$, $P < 0.001$	$r = 0.366$, $P = 0.112$	$y = 1.4385x + 0.0636$, $r = 0.925$, $P < 0.001$
FRAP			$y = 0.3893x + 0.5008$, $r = 0.670$, $P < 0.001$	$y = 0.9131x + 0.3725$, $r = 0.949$, $P < 0.001$	$r = 0.381$, $P = 0.097$	$y = 1.2198x + 0.0323$, $r = 0.945$, $P < 0.001$
TEAC					$r = 0.168$, $P = 0.479$	$y = 1.2162x + 0.0728$, $r = 0.906$, $P < 0.001$

r = correlation coefficient; P = significancy.

points would lie if the two measurements were the same do not match up with any regression lines. As is shown in the scatterplots in Figs. 7D, E, and F, all methods overestimate TAC slightly with respect to CUPRAC-BCS in urine samples given that all but one of the points lie to the left of the lines of equality in these three scatterplots. In plasma samples, CUPRAC-BCS overestimates TAC with respect to FRAP (Fig. 7A). This may be due to the fact that this assay considers -SH groups in contrast to FRAP. Besides, the CUPRAC-BCS and TEAC methods are in total disagreement in plasma samples (Fig. 7B); in fact, correlation between them is weak.

TAC of plasma and urine samples

The TAC of plasma and urine samples was assayed for 20 individuals (Fig. 8). TAC values for plasma samples were 0.611 ± 0.119, 0.516 ± 0.134, 0.702 ± 0.078 and 2.670 ± 0.186 mmol trolox equivalents/L in the CUPRAC-BCS, FRAP, TEAC, and DPPH assays, respectively. TAC values for urine samples were 3.807 ± 1.084, 4.515 ± 1.305, 4.495 ± 1.256, and 5.539 ± 1.686 mmol trolox equivalents/L in the CUPRAC-BCS, FRAP, TEAC, and DPPH assays, respectively. TAC values are significantly different for plasma samples between all methods ($P < 0.05$), and for urine samples significant differences were obtained only between DPPH and the rest of methods ($P < 0.05$). Therefore, our results showed that TAC results in urine samples obtained with the CUPRAC-BCS, FRAP, and TEAC assays could be comparable despite the lack of agreement shown by comparison between lines of equality and regression.

Urine samples were found to have 6.2, 8.7, 6.4, and 2.1 times more TAC than plasma in the CUPRAC-BCS, FRAP, TEAC, and DPPH assays, respectively. These results may correspond to the higher content of uric acid in urine than in plasma, and differences between methods may be due to a different relative contribution of uric acid to TAC in each method. Thus, the rank order based on contribution of uric acid to TAC results should be FRAP > TEAC > CUPRAC-BCS > DPPH. In clinical conditions, where uric acid is increased and oxidative stress is also involved (e.g., kidney failure, metabolic disorders), a significant increase in TAC could occur, although the opposite effect might have been expected [32]. Therefore, CUPRAC-BCS seems better than FRAP assay for TAC assessment in these cases. However, the possible reductions of Cu(II) by lipoproteins [33], β-amyloid peptide [34], and prion proteins [35] are other factors to take into account when CUPRAC-BCS is used, although more studies are needed to understand the way in which they affect the CUPRAC assay.

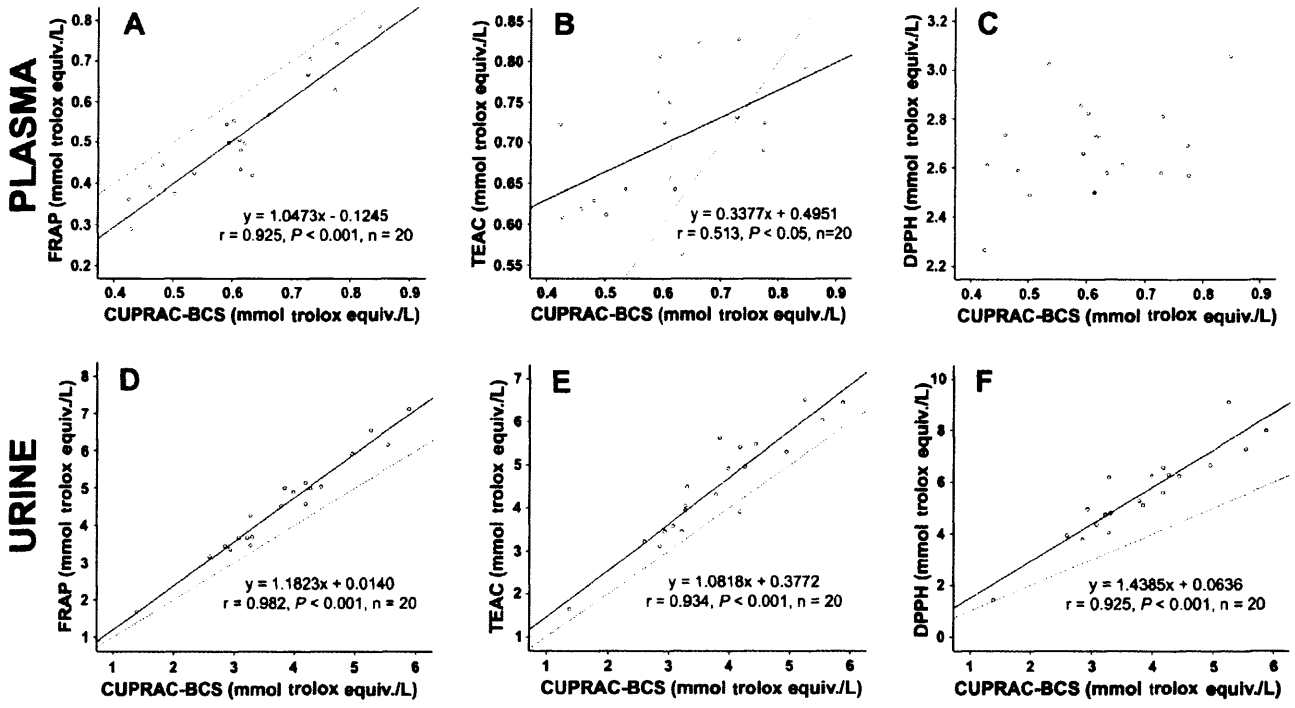


Fig. 7. Scatterplots of plasma CUPRAC-BCS and plasma FRAP (A), plasma CUPRAC-BCS and plasma TEAC (B), plasma CUPRAC-BCS and plasma DPPH (C), urine CUPRAC-BCS and urine FRAP (D), urine CUPRAC-BCS and urine TEAC (E), and urine CUPRAC-BCS and urine DPPH (F). Lines of equality (dotted lines) and correlation (solid lines) are shown.

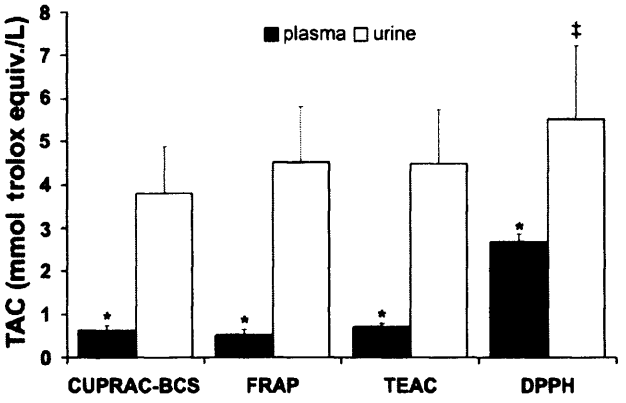


Fig. 8. TAC values of plasma and urine samples of 20 individuals using CUPRAC-BCS, FRAP, TEAC, and DPPH assays. Data are means ± SD (error bars). *$P < 0.05$ (significantly different).

Individual antioxidant studies

The relative antioxidant capacity for some important antioxidants was determined with the CUPRAC-BCS, FRAP, TEAC, and DPPH assays (Table 2). When TAC values of antioxidants measured at the same molar concentration were compared, a similar relative antioxidant capacity was found for uric acid and ascorbic acid in all four assays. On the other hand, the CUPRAC-BCS and FRAP assays showed an inability to detect the antioxidant capacity of albumin nearly within the time established in each protocol. The same occurred for GSH in the FRAP assay, as was also reported by several authors [9,16]. In contrast, the CUPRAC-BCS assay is fast enough to oxidize GSH (Fig. 4), presenting an important advantage over the FRAP assay.

Furthermore, a significant positive linear correlation was found between the number of phenolic groups and TAC of individual antioxidants for the FRAP assay ($r = 0.664$, $P < 0.05$, $y = 0.5332x + 0.7618$,) as well as for the DPPH assay ($r = 0.665$, $P < 0.05$, $y = 0.2847x + 0.7557$,) (Fig. 9). In contrast, no correlation was found either for CUPRAC-BCS ($r = 0.416$, $P = 0.232$) or for TEAC ($r = 0.404$, $P = 0.247$) (Fig. 9). However, when bilirubin is not considered in this study, a significant linear correlation is found for CUPRAC-BCS ($r = 0.882$, $P < 0.01$, $y = 0.5434x + 0.6585$), and similar results are obtained for the rest of assays. That is, TAC increases as a function of the number of phenolic groups of antioxidants for the CUPRAC-BCS, FRAP, and DPPH assays, and it could represent an important advantage for TAC assessment of some types of foods or beverages in which phenolic antioxidants are abundant.

Table 2
Relative antioxidant activity of individual antioxidants in CUPRAC-BCS, FRAP, TEAC, and DPPH assays.

Antioxidant	Relative activity for each TAC assay				
	Number of phenolic groups	CUPRAC-BCS	FRAP	TEAC	DPPH
Uric acid	0	1.08	1.09	1.00	1.21
Ascorbic acid	0	1.09	0.77	0.92	0.91
GSH	0	0.64	0.03	1.13	0.79
Bilirubin	0	3.23	2.27	1.69	0.01
Tiron	2	1.90	1.64	1.04	1.46
NAC	0	0.49	0.52	1.70	0.45
Gallic acid	3	2.25	2.54	2.06	1.46
BHA	1	1.21	1.43	0.93	1.26
Albumin	0	0.07	0.06	0.22	1.00
Trolox	1	1.00	1.00	1.00	1.00

Note: Activity is expressed as mol trolox equivalents/mol.

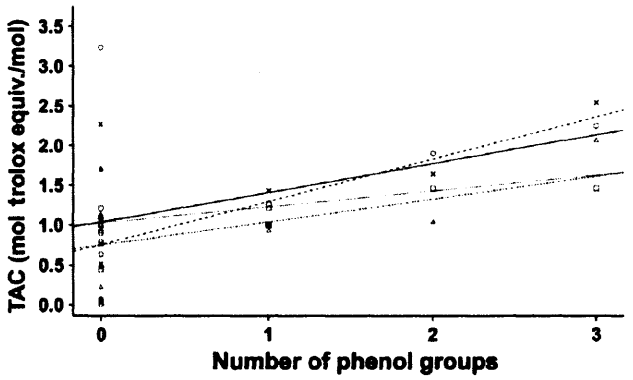


Fig. 9. Relationship between the number of phenol groups of antioxidants studied (trolox, uric acid, bilirubin, ascorbic acid, tiron, GSH, gallic acid, NAC, BHA, and albumin) and TAC determined with CUPRAC-BCS (○, solid line), FRAP (×, dashed line), TEAC (△, dotted line), and DPPH (□, dashed and dotted line).

Conclusions

This study has reported that the CUPRAC-BCS assay is a suitable method to assess TAC in human plasma and urine as well as in individual antioxidants. It is fast, simple, sensitive, linear, and precise enough. The commercial AOP kit for the CUPRAC assay is expensive; the cost per biochemical determination with the commercial kit is approximately 500 times higher than if reagents are prepared at the concentrations described above. Besides, in the commercial kit only uric acid is provided instead of trolox, and the amount of reagent supply is fixed. Although there is not much published about TAC assays based on the Cu(II) reduction, these methods are simple and inexpensive, do not require sophisticated equipment, and can be carried out with a microplate reader involving enough speed to constitute a good election for population studies. Moreover, only very few microliters of sample is necessary, and reagents are easily prepared, inexpensive, and stable at 4 °C for several months and can be used without previous incubation times. The CUPRAC-BCS assay is well correlated with the FRAP and TEAC assays in plasma samples and with the FRAP, TEAC, and DPPH assays in urine samples, although there is no agreement between any of them given that lines of equality and regression do not match up. Therefore, several antioxidant capacity assays should be used jointly for TAC assessment of biological samples, especially in clinical studies.

Acknowledgments

The authors thank the Inocente-Inocente Foundation (Spain) for its funding support of this work. The authors also thank Sergio González for English correction of the manuscript.

References

[1] G. Scott (Ed.), Antioxidants in Science, Technology, Medicine, and Nutrition, Albion, Chichester, UK, 1997.
[2] L.K. MacDonald-Wicks, L.G. Wood, M.L. Garg, Methodology for the determination of biological antioxidant capacity in vitro: a review, J. Sci. Food Agric. 86 (2006) 2046–2056.
[3] G. Bartosz, Total antioxidant capacity, Adv. Clin. Chem. 37 (2003) 219–292.
[4] D. Huang, B. Ou, R.L. Prior, The chemistry behind antioxidant capacity assays, J. Agric. Food Chem. 53 (2005) 1841–1856.
[5] I.P. Kaur, T. Geetha, Screening methods for antioxidants: a review, Mini Rev. Med. Chem. 6 (2006) 305–312.
[6] A. Somogyi, K. Rosta, P. Pusztai, Z. Tulassay, G. Nagy, Antioxidant measurements, Physiol. Meas. 28 (2007) R41–R55.
[7] L.M. Magalhaes, M.A. Segundo, S. Reis, J.L.F.C. Lima, Methodological aspects about in vitro evaluation of antioxidant properties, Anal. Chim. Acta 613 (2008) 1–19.
[8] A. Karadag, B. Ozcelik, S. Saner, Review of methods to determine antioxidant capacities, Food Anal. Methods 2 (2009) 41–60.

[9] G. Cao, R.L. Prior, Comparison of different analytical methods for assessing total antioxidant capacity of human serum, Clin. Chem. 44 (1998) 1309–1315.
[10] R.L. Prior, G. Cao, In vivo total antioxidant capacity: Comparison of different analytical methods, Free Radic. Biol. Med. 27 (1999) 1173–1181.
[11] C.A. Rice-Evans, Measurement of total antioxidant activity as a marker of antioxidant status in vivo: procedures and limitations, Free Radic. Res. 33 (2000) S59–S66.
[12] A. Janaszewska, G. Bartosz, Assay of total antioxidant capacity: Comparison of four methods as applied to human blood plasma, Scand. J. Clin. Lab. Invest. 62 (2002) 231–236.
[13] K. Schlesier, M. Harwat, V. Böhm, R. Bitsch, Assessment of antioxidant activity by using different in vitro methods, Free Radic. Res. 36 (2002) 177–187.
[14] B. Ou, D. Huang, M. Hampsch-Woodill, J.A. Flanagan, E. Deemer, Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study, J. Agric. Food Chem. 50 (2002) 3122–3128.
[15] M. Ozgen, R.N. Reese, A.Z.J. Tulio, J.C. Scheerens, A.R. Miller, Modified 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method to measure antioxidant capacity of selected small fruits and comparison to ferric reducing antioxidant power (FRAP) and 2, 2′-diphenyl-1-picrylhydrazyl (DPPH) methods, J. Agric. Food Chem. 54 (2006) 1151–1157.
[16] R. Apak, K. Güçlü, M. Özyürek, S.E. Karademir, M. Altun, Total antioxidant capacity assay of human serum using copper(II)–neocuproine as chromogenic oxidant: the CUPRAC method, Free Radic. Res. 39 (2005) 949–961.
[17] R. Apak, K. Güçlü, B. Demirata, M. Özyürek, S.E. Çelik, B. Bektaşoğlu, K.I. Berker, D. Özyurt, Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay, Molecules 12 (2007) 1496–1547.
[18] R. Apak, K. Güçlü, M. Özyürek, S.E. Çelik, Mechanism of antioxidant capacity assays and the CUPRAC (cupric ion reducing antioxidant capacity) assay, Microchim. Acta 160 (2008) 413–419.
[19] G. Da Cruz, Use of bathocuproine for the evaluation of the antioxidant power in liquids and solutions, U.S. patent 6613577, 2003.
[20] I.F.F. Benzie, J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay, Anal. Biochem. 239 (1996) 70–76.
[21] N.J. Miller, C. Rice-Evans, M.J. Davies, V. Gopinathan, A. Milner, A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates, Clin. Sci. 84 (1993) 407–412.
[22] C. Rice-Evans, N.J. Miller, Total antioxidant status in plasma and body fluids, Methods Enzymol. 234 (1994) 279–293.
[23] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Radic. Biol. Med. 26 (1999) 1231–1237.
[24] J.C. Miller, J.N. Miller, Statistics for Analytical Chemistry, third ed., Ellis Horwood, Chichester, UK, 1993.
[25] B. Zak, Simple procedure for the single sample determination of serum copper and iron, Clin. Chim. Acta 3 (1958) 328–334.
[26] R. Watkins, L.M. Weiner, B. Zak, Determination of copper, iron, and zinc from a single small sample, Microchem. J. 16 (1971) 14–23.
[27] D. Blair, H. Diehl, Bathophenanthrolinedisulphonic acid and bathocuproinedisulphonic acid, water soluble reagents for iron and copper, Talanta 7 (1961) 163–174.
[28] F. Sánchez-Rasero, Stoichiometry, Ringbom optimal range, and other parameters for the copper(I)–bathocuproine complex, Microchem. J. 26 (1981) 418–425.
[29] R. Pulido, L. Bravo, F. Saura-Calixto, Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay, J. Agric. Food Chem. 48 (2000) 3396–3402.
[30] M. Grootveld, B. Halliwell, Measurement of allantoin and uric acid in human body fluids: a potential index of free-radical reactions in vivo?, Biochem J. 243 (1987) 803–808.
[31] O. Erel, A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation, Clin. Biochem. 37 (2004) 277–285.
[32] A. Ghiselli, M. Serafini, F. Natella, C. Scaccini, Total antioxidant capacity as a tool to assess redox status: critical view and experimental data, Free Radic. Biol. Med. 29 (2000) 1106–1114.
[33] S.M. Lynch, B. Frei, Reduction of copper, but not iron, by human low density lipoprotein (LDL): implications for metal ion-dependent oxidative modification of LDL, J. Biol. Chem. 270 (1995) 5158–5163.
[34] C. Opazo, F.H. Ruiz, N.C. Inestrosa, Amyloid–β-peptide reduces copper(II) to copper(I) independent of its aggregation state, Biol. Res. 33 (2000) 125–131.
[35] F.H. Ruiz, E. Silva, N.C. Inestrosa, The N-terminal tandem repeat region of human prion protein reduces copper: role of tryptophan residues, Biochem. Biophys. Res. Commun. 269 (2000) 491–495.

Adultos con síndrome de Down

C. Campos, R. Guzmán, E. López-Fernández y Á. Casado

Los objetivos del presente trabajo fueron evaluar los niveles urinarios de ácido úrico (AU), capacidad antioxidante total (TAC) y capacidad antioxidante total sin la contribución relativa del ácido úrico (TAC^{-AU}) en niños y adultos con síndrome de Down (SD).

Se analizaron 32 individuos con SD y 29 controles. Se establecieron dos grupos de edad: niños, de entre 1 y 13 años (19 SD y 14 controles), y adultos, de entre 43 y 61 años (13 SD y 15 controles). La determinación de AU se realizó por el método espectrofotométrico de la uricasa, la de TAC por el ensayo de reducción del cobre(II) utilizando sal disódica del ácido 2,9-dimetil-4,7-difenil-1,10-fenantrolindisulfónico como agente quelante (CUPRAC-BCS) y la de TAC^{-AU} de forma indirecta, es decir, restando el valor de TAC equivalente a la concentración de AU de cada muestra al valor de TAC. Todos los parámetros se ajustaron a creatinina.

Los niños con SD mostraron mayores niveles de AU y TAC que los controles, mientras que los niveles de TAC^{-AU} fueron menores en adultos con SD que en controles ($P<0,05$ en todos los casos). En SD, los niveles de AU, TAC y TAC^{-AU} fueron mayores en niños que en adultos ($P<0,05$ en todos los casos). Se obtuvieron correlaciones significativamente positivas entre AU y TAC en todos los grupos estudiados, mientras que las correlaciones fueron negativas con la edad para los niveles urinarios de AU y TAC en niños de ambos grupos.

Los resultados muestran que los niños con SD tienen una mayor TAC que los controles, debido seguramente a los mayores niveles de AU. Además, la TAC esta reducida en adultos con SD, pero solo cuando no se considera la contribución relativa del AU, sugiriendo un estrés oxidativo incrementado en estos individuos. Así, la TAC^{-AU} parece proporcionar una información más fiable sobre el estatus antioxidante, al menos en individuos con SD.

Available online at www.sciencedirect.com
ScienceDirect
Clinical Biochemistry 43 (2010) 228–233
CLINICAL BIOCHEMISTRY

Urinary uric acid and antioxidant capacity in children and adults with Down syndrome

Carlos Campos *, Rodrigo Guzmán, Encarnación López-Fernández, Ángela Casado

Departamento de Medicina Celular y Molecular, Centro de Investigaciones Biológicas, CSIC, C/ Ramiro de Maeztu, 9. E-28040 Madrid, Spain

Received 22 June 2009; received in revised form 15 September 2009; accepted 17 September 2009
Available online 26 September 2009

Abstract

Objectives: To evaluate the urinary levels of uric acid (UA) and total antioxidant capacity (TAC) with and without UA relative contribution (TAC^{-UA}) in children and adults with Down syndrome (DS) and to prove the clinical use of TAC.

Design and methods: Urine samples were obtained from 32 individuals with DS and 29 controls. Two age groups were established (children and adults). Spectrophotometric methods were used for biochemical determinations.

Results: Children with DS had significantly higher UA/Cr and TAC/Cr levels than controls, whereas levels of TAC^{-UA}/Cr were lower in adults with DS than in controls ($P<0.05$ for all). In DS, levels of UA/Cr, TAC/Cr and TAC^{-UA}/Cr were higher in children than in adults ($P<0.05$ for all). Positive correlations between UA/Cr and TAC/Cr were found for all groups studied. Negative correlations with age were found for UA/Cr and TAC/Cr in children of both groups.

Conclusions: Our results proved that TAC is decreased in adults with DS. Besides, TAC^{-UA} seems to provide more reliable information about the antioxidant status, at least in DS.

Keywords: Down syndrome; Oxidative stress; Total antioxidant capacity; Uric acid; Urine; Aging

Introduction

Down syndrome (DS) or trisomy 21 is the most common chromosomal abnormality that comes to term in humans [1] occurring in about one in every 700-1000 live births [2]. It is associated with a wide variety of clinical features, including mental retardation, congenital heart disease, digestive problems, endocrine system deficits, cataracts, immune system disorders and increased risks of leukaemia and Alzheimer disease. Additionally, individuals with DS suffer from premature dementia and accelerated aging, and several studies have shown an increased oxidative stress in individuals with this pathology [3,4].

Antioxidant enzymatic alterations had been found in DS. The gene for Cu/Zn superoxide dismutase (SOD1) is coded on chromosome 21 and it is overexpressed (~50%) in DS [5] resulting in an increase of reactive oxygen species (ROS) due to an overproduction of hydrogen peroxide. ROS lead to oxidative damage of DNA, proteins and lipids. Moreover, the increase of enzymatic antioxidant defences such as SOD1 and catalase seems to be insufficient to prevent the exercise-induced oxidative damage in DS subjects which could be probably associated to a pro-oxidant status in this pathology [6]. Therefore, oxidative stress may play an important role in the pathogenesis of DS.

Oxidative damage can be monitored by the determination of different oxidative stress biomarkers. Some studies have shown higher levels of protein carbonyls, malondialdehyde, allantoin or 8-hydroxydeoxyguanosine in DS than in normal population [7–11]. Due to the ROS overproduction, a diminished

Abbreviations: ANOVA, analysis of variance; BCS, bathocuproinedisulfonic acid disodium salt; Cr, creatinine; Cu, copper; CUPRAC-BCS, copper(II) reduction assay with BCS as chelating agent; DS, Down syndrome; NS, not significant; ROS, reactive oxygen species; SD, standard deviation; SOD1, Cu/Zn superoxide dismutase; TAC, total antioxidant capacity; TAC^{-UA}, total antioxidant capacity without relative contribution of uric acid; TCA, trichloroacetic acid; UA, uric acid.

* Corresponding author.Fax: +34 91 536 04 32.
E-mail address: ccampos@cib.csic.es (C. Campos).

0009-9120/$ - see front matter © 2009 The Canadian Society of Clinical Chemists. Published by Elsevier Inc. All rights reserved.
doi:10.1016/j.clinbiochem.2009.09.017

antioxidants level may be found in DS because of their exhaustion. Separate measurement of different antioxidant molecules is not practical and their antioxidant effects are additive. Therefore, the total antioxidant capacity (TAC) is a useful measurement. Furthermore, the determination of any individual antioxidant could be less representative of the overall antioxidant capacity due to the possible *in vivo* interaction among different antioxidants. TAC is a measure of the amount (expressed in moles) of a given free radical scavenged by the non-enzymatic antioxidants which are present in a sample and in DS this parameter has not been studied in detail. Moreover, the studies are performed only with plasma or serum samples and in a reduced age range (usually only in children).

Uric acid (UA) is a powerful antioxidant and represents a high relative contribution of TAC in biological samples [12,13]. Several studies have reported elevated plasmatic levels of UA in DS, but urinary concentration of this antioxidant is not well known. UA has been associated to Alzheimer disease, cognitive decline, autism and sleep apnea [14–19], and these are pathologies related to DS [20–23]. Therefore, more studies are necessary on UA in DS to try to establish possible relations between this parameter and the pathogenesis of DS.

The present study was performed with urine samples due to the fact that they have several advantages over plasma, serum, saliva or cerebrospinal fluid spots for the determination of biochemical parameters. Urine collection is non-invasive, poses minimal infectious disease risk to participants and researchers and provides sufficient volume for multiple assays and future research. Furthermore, urine specimens are ideally suited for large studies because they can be collected and stored by participants, and compliance is high. Moreover, this sample is not usually used for the study of biochemical parameters in DS, being the first time that TAC had been evaluated in urine samples in this pathology. In addition, the study has been performed in children and also in adults. Since DS individuals suffer from accelerated aging, the age group of adults could be considered as a senescent group, as it was previously established by Bittles et al. [24].

The aim of this work was to compare the urinary levels of UA and antioxidant capacity, with and without UA contribution, in a sample of children and adults with DS with those of healthy age-matched controls in order to assess the role of oxidative stress in these subjects. Moreover, we evaluate the clinical use of urine TAC in a pathology with increased oxidative stress.

Materials and methods

Subjects

The study was performed in 32 individuals with Down syndrome and 29 healthy controls. Two age groups were established: group 1 (children group) consisted of 19 children with DS (13 male and 6 female, mean age=7.6±3.3 years ranging from 1 to 12) and 14 healthy age-matched controls (6 male and 8 female, mean age=9.1±3.0 years ranging from 5 to 13); group 2 (adult group) consisted of 13 adults with DS (7 male and 6 female, mean age=48.8±4.4 years ranging from 43 to 57) and 15 healthy age-matched controls (5 male and 10 female, mean age=52.7±5.3 years ranging from 43 to 61). All participants were non-smokers. Diagnosis of DS was confirmed by karyotyping. Informed consent was obtained from the participants or was given by parents. The study was approved by The Ethics Committee of the Spanish National Research Council.

Urine sample collection and preparation

First morning urine samples on an empty stomach were collected in a sterile flask without any preservative since, according to NCCLS approved guidelines on urinalysis and collection, transportation and preservation of urine specimens; chemical preservatives should be avoid for urinalysis [25] and in the same way Yilmaz et al. [26] showed that addition of preservatives (such as 6N HCl) is not necessary for measurement of uric acid in promptly assayed urine samples. The sterile flasks were covered with aluminium foil to keep out stray light.

All participants were appointed within the same timetable everyday and the group of samples collected each day was processed within 2 h of the collection. Samples were frozen at −20 °C until UA and TAC analysis. For creatinine (Cr) determination, samples were analyzed immediately, without previous freezing.

Reagents

Uric acid, creatinine, bathocuproinedisulfonic acid disodium salt (BCS) and copper(II) sulfate anhydrous were from Fluka (Buchs, Switzerland). *Candida* sp. uricase (EC 1.7.3.3) was from Sigma chemical Co. (St. Louis, MO) (ref. U0880). 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris) was from Boehringer Mannheim GmbH (Mannheim, Germany). Trichloroacetic acid (TCA) was from Riedel-de Haën (Seelze, Germany). Other chemicals were from Panreac (Barcelona, Spain) and Probus (Barcelona, Spain). Deionized water was obtained from a Milli-RO water system (Millipore, Bedford, MA).

Biochemical determinations

UA was measured by the uricase spectrophotometric method of Duncan et al. [27] with modifications. The modifications consisted of the use of *Candida* sp. uricase, incubating the uricase solution mixture at 25 °C (maximum activity temperature of this enzyme) instead of 37 °C. Samples were preheated in a water bath at 60 °C during 5 min in order to dissolve the possible urates precipitates and were then diluted 1/10 with distilled water prior to analysis.

TAC was assayed by a quantitative colorimetric assay using the copper(II) reduction assay with BCS as chelating agent (CUPRAC-BCS) described by Campos et al. [28]. CUPRAC-BCS assay is based on the reduction of Cu(II) into Cu(I) by the action of the non-enzymatic antioxidants which are present in the sample. The chromogenic reagent BCS forms a stable complex with Cu(I) which has a maximum absorbance at

490 nm. The results are compared with a standard curve obtained with UA.

TAC^{-UA} is a novel indirect measurement for the antioxidant capacity without the relative contribution of UA. It has been firstly used in this work in order to study the effect of UA in the TAC assessment. TAC^{-UA} was calculated as follows: (i) urinary UA was determined by uricase method; (ii) TAC was assayed with the CUPRAC-BCS assay; (iii) UA relative antioxidant capacity was calculated by determining the absorbance of the UA concentration (previously determinate for each sample) by using the equation already obtained in the CUPRAC-BCS assay (using UA as standard). The UA absorbance obtained was subtracted from the absorbance found in the CUPRAC-BCS assay; and (iv) the resulting absorbance was used for the TAC^{-UA} determination by intrapolation in the CUPRAC-BCS calibration curve.

Cr was determined according to the spectrophotometric Jaffé method [29] described by Varley and Gowenlock [30].

Statistical analysis

Results are expressed as mean ± standard deviation ($\bar{X} \pm SD$). Between-group comparisons were performed using the analysis of variance (ANOVA). Correlations were expressed using the Pearson's (product-moment) correlation coefficient. Results were considered significantly different at $P<0.05$. Data were processed using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL).

Results

Urinary biochemical parameters studied in individuals with DS and controls are listed in Table 1 for both age groups. Besides, no statistically significant differences were found in Cr levels between DS and controls in any age group and any of the parameters studied differ significantly with gender.

As seen in Table 1, levels of UA/Cr and TAC/Cr were significantly higher in children with DS than in the age-matched controls. In adults with DS, only levels of TAC^{-UA}/Cr were significantly decreased than those of the control group 2. Between age groups, we found significantly higher levels of UA/Cr, TAC/Cr and TAC^{-UA}/Cr in children than in adults with DS, whereas in the control group differences were not significant for any of these parameters (Fig. 1).

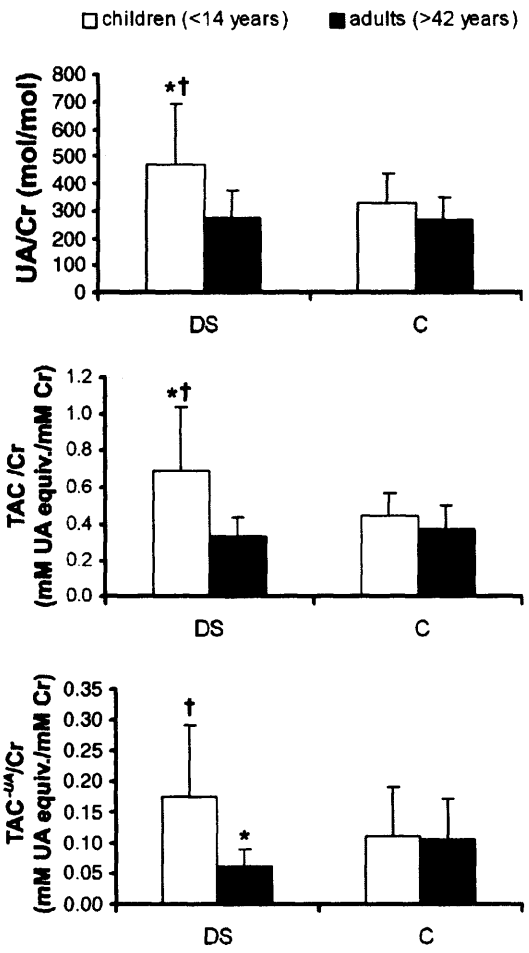


Fig. 1. Urinary UA/Cr (top), TAC/Cr (middle) and TAC^{-UA}/Cr (bottom) levels in children and adults with Down syndrome (DS) and the age-matched controls (C). Data are means±SD (error bars). †,*Indicates $P<0.05$ (significantly different) from the age group and the control group, respectively.

Significant positive correlations were found between UA/Cr and TAC/Cr in children with and without DS (Figs. 2A and 2B, respectively) and also in adults with and without DS (Figs. 2C and 2D, respectively), as it is shown in Fig. 2. The relationship

Table 1
Urinary levels of Cr and UA, TAC and TAC^{-UA} to Cr ratios in subjects with DS and their age-matched controls[a].

Status and age groups	(*n*)	Cr (mg/dL)	*P*	UA/Cr (mol/mol)	*P*	TAC/Cr (mM UA equiv./mM Cr)	*P*	TAC^{-UA}/Cr (mM UA equiv./mM Cr)	*P*
Group 1 (<14 years)									
DS	(19)	99.6±42.1	NS	469±226	0.045	0.692±0.343	0.015	0.174±0.118	NS
Controls	(14)	133.4±65.1		333±105		0.445±0.119		0.111±0.081	
Group 2 (>42 years)									
DS	(13)	129.4±71.0	NS	270±103	NS	0.332±0.107	NS	0.061±0.028	0.033
Controls	(15)	160.0±77.4		265±82		0.372±0.125		0.105±0.067	

Cr=creatinine; DS=Down syndrome; TAC=total antioxidant capacity; TAC^{-UA}=total antioxidant capacity without relative contribution of uric acid; UA=uric acid; NS=not significant.

[a] Data are expressed as mean±SD.

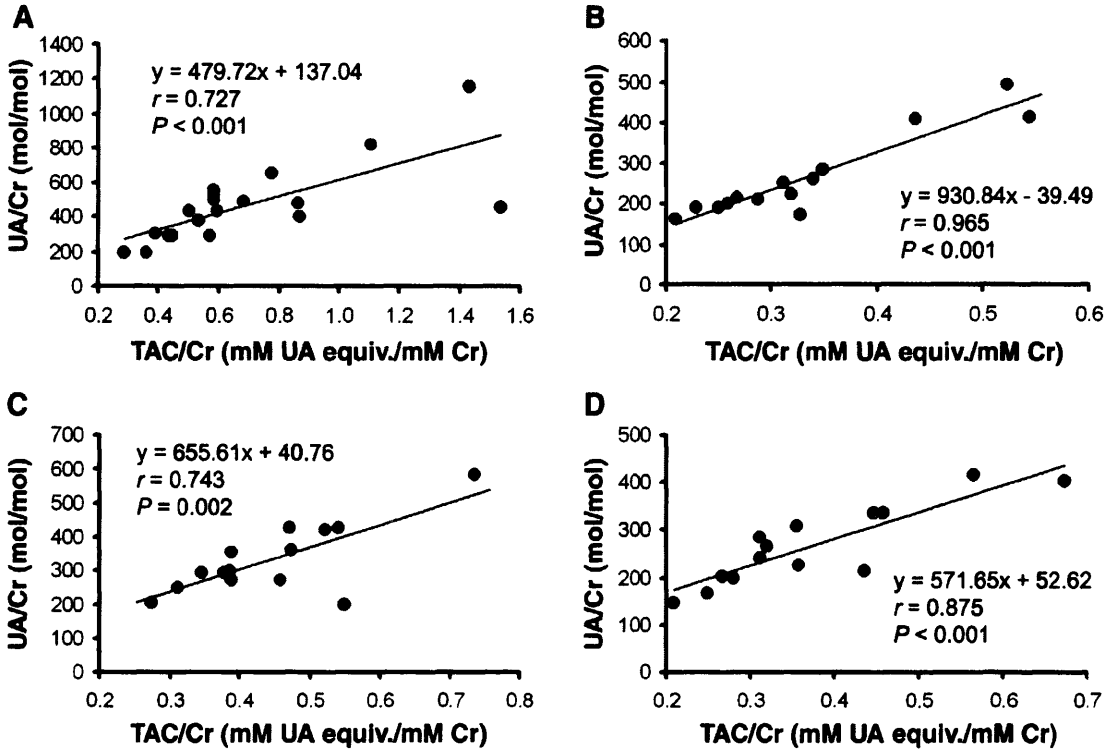


Fig. 2. Scatter plots of UA/Cr vs. TAC/Cr in individuals with DS (children (A) and adults (B)) and without DS (children (C) and adults (D)).

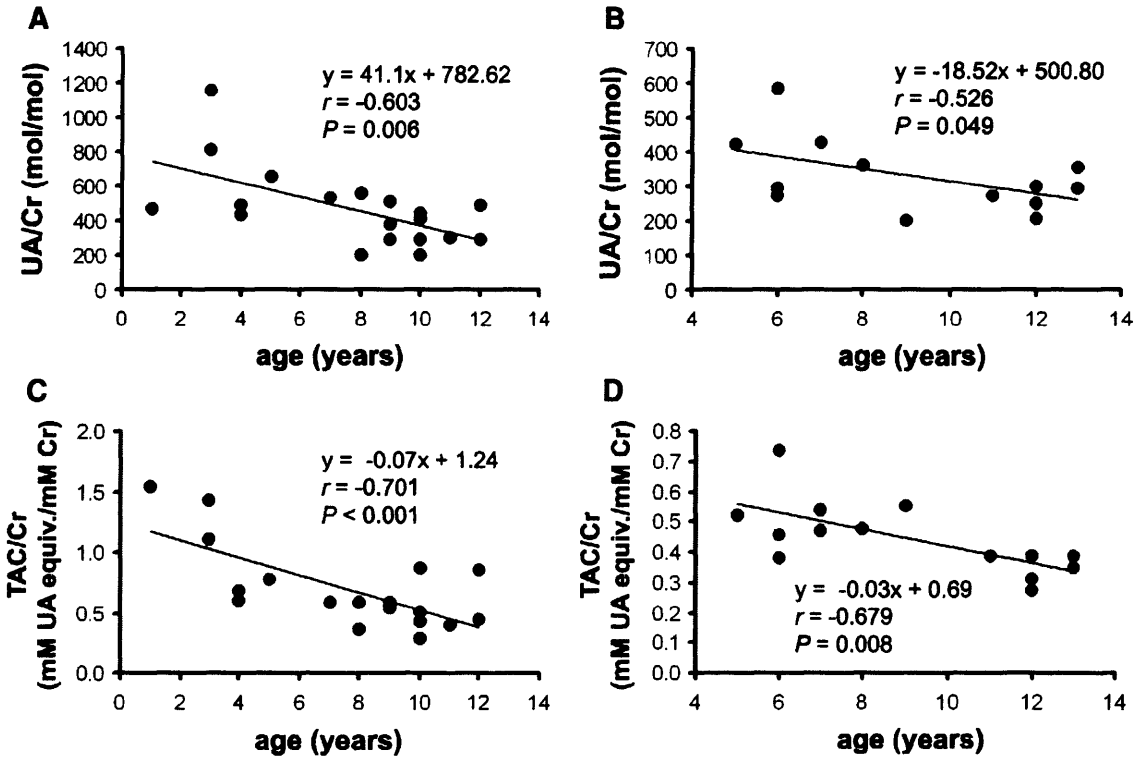


Fig. 3. Scatter plots of UA/Cr vs. age in children with DS (A) and without DS (B) and TAC/Cr vs. age in children with DS (C) and without DS (D).

between age and UA/Cr, TAC/Cr and TAC^{-UA}/Cr was also studied. As shown in Fig. 3, levels of UA/Cr and TAC/Cr were negatively correlated with age in children with DS (Figs. 3A and 3C, respectively) and without DS (Figs. 3B and 3D, respectively).

Discussion

The present study shows that children with DS have higher levels of UA/Cr and TAC/Cr than the control group (Fig. 1), whereas no differences were found in adults for any of these parameters. Previous works have reported that UA levels in serum and plasma samples of DS subjects are increased [7,10,31–35]. Overproduction of UA has been suggested as a possible explanation for these results in several works [35,36]. Thus, increased activities of erythrocyte adenosine deaminase and adenine phosphoribosyl trasferase reported by Puukka et al. [36] could have an influence on the hyperuricemia of DS. Our results support the overproduction hypothesis since excretion of UA was higher in children with DS than in the age-matched controls. However, we found no differences between the adults groups for this parameter. Coburn et al. [37] postulated that the increased blood UA levels observed in DS result from decreased efficiency of UA excretion rather than increased synthesis, and Nishida et al. [38] suggest that glomerular dysfunction could also contribute to hyperuricemia in DS. Therefore, urinary UA/Cr levels were not increased in adults with DS possibly due to a renal dysfunction in DS. However, urinary levels of Cr, which is a marker of renal function [39], were not significantly different comparing DS with controls in any age group. Therefore, UA overproduction in adults with DS could be compensated by its exhaustion due to the increased oxidative stress, being levels of UA/Cr similar in both adults groups.

It is known that UA represents an important percentage of the TAC in biological samples, in fact both variables are highly correlated in DS ($r=0.727$ and $r=0.965$ for children and adult groups, respectively; Figs. 2A and 2B) and also in controls ($r=0.743$ and $r=0.875$ for children and adult groups, respectively; Figs. 2C and 2D). Therefore, similar results were obtained for both parameters. As we shown above, UA levels are increased in DS but also under intense physical exercise and in other pathologies where oxidative stress is also involved, such as kidney disease [40] or metabolic syndrome [41]. In these situations, where UA increases in clinical conditions, high TAC levels are found although the opposite result might have been anticipated [42]. Therefore, we established a novel parameter for the TAC assessment, in which relative contribution of UA to TAC was subtracted in each sample, in order to avoid a possible interpretation error.

The study of oxidative stress in DS has been focused so far on markers, such as malondialdehyde, 8-hydroxydeoxyguanosine and antioxidants enzymes, but TAC is not well studied. There are few works about this parameter in DS subjects and none of them was performed with urine samples. Moreover, these studies were performed only in children or young people. Thus, Carratelli et al. [43] and Garaiová et al. [33] found significantly lower TAC in DS compared with controls, whereas Zitnanová et al. [8] did not obtain differences between the two groups. In contrast with these previous works, we found higher TAC/Cr in children with DS. Since we analyzed urine samples, where UA concentration is ~10 times higher than in plasma or serum samples, relative contribution of UA to TAC is very high and differences obtained for TAC/Cr must be due to increased levels found for UA/Cr in the children group. When TAC^{-UA}/Cr was compared, no differences were found in children. Therefore, results are in agreement with Zitnanová.

It was the first time that TAC has been assessed in adults with DS. In this group, no differences were found for TAC/Cr although TAC^{-UA}/Cr has been found decreased in DS compared with the age-matched controls (Fig. 1). DS subjects have increased oxidative stress and diminished TAC by antioxidant exhaustion is expected. Therefore, since UA levels are altered in DS, our novel TAC^{-UA} parameter seems to be more reliable than TAC at least in clinical studies where UA levels are altered in clinical conditions, such as DS, avoiding possible interpretation errors.

We found a negative correlation between UA/Cr and TAC/Cr with age in children with and without DS (Fig. 3). These results agree with the report of Stapleton et al. [44] and could be due to the greater relative weight of the internal organs, the higher DNA to protein ratio and the accelerated organ growth speed in young children.

More important, when comparison between children and adults was analyzed, levels of UA/Cr, TAC/Cr and TAC^{-UA}/Cr were lower only in adults than in children with DS (Fig. 1). Therefore, our results suggest that non-enzymatic antioxidants decreased with aging in DS probably due to their exhaustion and caused by the increased oxidative stress, and it could contribute to the accelerate aging observed in these subjects. Although in most of cases the age at onset of senescence is after 60 years, it occurs much earlier in DS individuals. Our results evidenced symptoms of premature ageing and support the age proposed by Bittles et al. [24] who consider DS individuals having >40 years as the life stage where senescence begins.

In conclusion, our study shows a lower antioxidant status in adults with DS when relative contribution of UA was subtracted from TAC measured by copper(II) reduction assay, being the first time that TAC is evaluated in urine samples of children and also in adults with DS. Our results indicate that the increased oxidative stress present in DS causes the exhaustion of non-enzymatic antioxidants in adults with DS. Moreover, this study may indicate that the use of TAC as oxidative stress biomarker must be performed using the novel proposed parameter TAC^{-UA}, at least in clinical studies in which UA levels are increased in clinical conditions where oxidative stress is also involved.

Acknowledgments

The authors would like to thank all the people with Down syndrome, families and volunteers which cooperated and made the achievement of this work possible. The authors express their gratitude to Fundación Síndrome de Down de Madrid, Centro Maria Corredentora, Asociación de Empleados de IBERIA Padres de Minusválidos, Fundación Nuestra Señora del

Camino, C.P.E.E. Príncipe de Asturias (Aranjuez), C.P.E.E. Francisco del Pozo de Madrid, C.C.E.E. Buenafuente de Madrid and to Hospital Niño Jesús de Madrid, for their valuable contributions to this work. This study was supported by Fundación Inocente, Inocente.

References

[1] Patterson D. The causes of Down syndrome. Sci Am 1987;257:52–60.
[2] Hook E. Down's syndrome-frequency in human populations and factors pertinent to variation in rates. In: de la Cruzz FF, Gerald PS, editors. Trisomy 21 (Down syndrome): Research Perspectives. Baltimore: University Park Press; 1981. p. 3–68.
[3] de Haan JB, Wolvetang EJ, Cristiano F, et al. Reactive oxygen species and their contribution to pathology in Down syndrome. Adv Pharmacol 1997;38:379–85.
[4] Sinet PM. Metabolism of oxygen derivatives in Down's syndrome. Ann NY Acad Sci 1982;396:83–94.
[5] Annerén G, Edman B. Down syndrome—a gene dosage disease caused by trisomy of genes within a small segment of the long arm of chromosome 21, exemplified by the study of effects from the superoxide-dismutase type 1 (SOD-1) gene. APMIS Suppl 1993;40:71–9.
[6] Aguiar AS, Tuon T, Albuquerque MM, et al. The exercise redox paradigm in the Down's syndrome: improvements in motor function and increases in blood oxidative status in young adults. J Neural Transm 2008;115: 1643–50.
[7] Zitnanová I, Korytár P, Aruoma OI, et al. Uric acid and allantoin levels in Down syndrome: antioxidant and oxidative stress mechanism? Clin Chim Acta 2004;341:139–46.
[8] Zitnanová I, Korytár P, Sobotová H, et al. Markers of oxidative stress in children with Down syndrome. Clin Chem Lab Med 2006;44:306–10.
[9] Casado A, López-Fernández ME, Ruiz R. Lipid peroxidation in Down syndrome caused by regular trisomy 21, trisomy 21 by Robertsonian translocation and mosaic trisomy 21. Clin Chem Lab Med 2007;45: 59–62.
[10] Garcez ME, Peres W, Salvador M. Oxidative stress and hematologic and biochemical parameters in individuals with Down syndrome. Mayo Clin Proc 2005;80:1607–11.
[11] Jovanovic SV, Clements D, MacLeod K. Biomarkers of oxidative stress are significantly elevated in Down syndrome. Free Radic Biol Med 1998;25:1044–8.
[12] Wayner DD, Burton GW, Ingold KU, Barclay LR, Locke SJ. The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. Biochim Biophys Acta 1987;924:408–19.
[13] Somogyi A, Rosta K, Pusztai P, Tulassay Z, Nagy G. Antioxidants measurements. Physiol Meas 2007;28:R41–55.
[14] Rinaldi P, Polidori MC, Metastasio A, et al. Plasma antioxidants are similarly depleted in mild cognitive impairment and in Alzheimer's disease. Neurobiol Aging 2003;24:915–9.
[15] Schretlen DJ, Inscore AB, Jinnah HA, Rao V, Gordon B, Pearlson GD. Serum uric acid and cognitive function in community-dwelling older adults. Neuropsychology 2007;21:136–40.
[16] Euser S, Hofman A, Westendorp R, Breteler M. Serum uric acid and cognitive function and dementia. Brain 2009;132:377–82.
[17] Ruggiero C, Cherubini A, Lauretani F, et al. Uric acid and dementia in community-dwelling older persons. Dement Geriatr Cogn Disord 2009;27: 382–9.
[18] Page T, Coleman M. Purine metabolism abnormalities in a hyperuricosuric subclass of autism. Biochim Biophys Acta 2000;1500:291–6.
[19] Sahebjami H. Changes in urinary uric acid excretion in obstructive sleep apnea before and after therapy with nasal continuous positive airway pressure. Chest 1998;113:1604–8.
[20] Zana M, Janka Z, Kálmán J. Oxidative stress: a bridge between Down's syndrome and Alzheimer's disease. Neurobiol Aging 2007;28:648–76.
[21] Devenny DA, Krinsky-McHale SJ, Sersen G, Silverman WP. Sequence of cognitive decline in dementia in adults with Down's syndrome. J Intell Disabil Res 2000;44:654–65.
[22] Rasmussen P, Börjesson O, Wentz E, Gillberg C. Autistic disorders in Down syndrome: background factors and clinical correlates. Dev Med Child Neurol 2001;43:750–4.
[23] Marcus CL, Keens TG, Bautista DB, von Pechmann WS, Ward SLD. Obstructive sleep apnea in children with Down syndrome. Pediatrics 1991;88:132–9.
[24] Bittles AH, Bower C, Hussain R, Glasson EJ. The four ages of Down syndrome. Eur J Public Health 2007;17:221–5.
[25] The National Committee for Clinical Laboratory Standars (NCCLS): Urinalysis and collection, transportation and preservation of urine specimens; approved guideline-2nd ed. NCCLS Document GP16-A2. Vol. 21. NO. 19 (2001).
[26] Yilmaz G, Yilmaz FM, Hakligör A, Yücel D. Are preservatives necessary in 24-hour urine measurements? Clin Biochem 2008;41:899–901.
[27] Duncan PH, Gochman N, Cooper T, Smith E, Bayse DA. candidate reference method for uric acid in serum. I. Optimization and evaluation. Clin Chem 1982;28:284–90.
[28] Campos C, Guzmán R, López-Fernández E, Casado A. Evaluation of the copper(II) reduction assay using bathocuproinedisulfonic acid disodium salt for the total antioxidant capacity assessment: the CUPRAC-BCS assay. Anal Biochem 2009;392:37–44.
[29] Jaffé M. Über den Niederschlag, welchen Pikrinsäure in normalen Harn erzeugt und über eine neue Reaktion des Kreatinins. Z Physiol Chem 1886;10:391–400.
[30] Varley H, Gowenlock AH, Bell M. Practical Clinical Biochemistry5th ed. . London: Heinemann Medical Books; 1980.
[31] Nagyova A, Sustrova M, Raslova K. Serum lipid resistance to oxidation and uric acid levels in subjects with Down's syndrome. Physiol Res 2000;49:227–31.
[32] Pallardó FV, Degan P, d'Ischia M, Kelly FJ, Zatterale A, Calzone R. Multiple evidence for an early age pro-oxidant state in Down syndrome patients. Biogerontology 2006;7:211–20.
[33] Garaiová I, Muchová JS, Šustrová M, Blazicek P, et al. The relationship between antioxidant systems and some markers of oxidative stress in persons with Down syndrome. Biologia 2004;59:787–94.
[34] Appleton MD, Haab W, Burti U, Orsulak PJ. Plasma urate levels in mongolism. Amer J Ment Deficiency 1969;74:196–9.
[35] Pant SS, Moser HW, Krane SM. Hyperuricemia in Down's syndrome. J Clin Endocr 1968;28:472–8.
[36] Puukka R, Puukka M, Leppilampi M, Linna SL, Kouvalainen K. Erythrocyte adenosine deaminase, purine nucleotide phosphorylase and phosphoribosyltransferase activity in patients with Down's syndrome. Clin Chim Acta 1982;126:275–81.
[37] Coburn SP, Sirlin EM, Mertz ET. Metabolism of N^{15} labeled uric acid in Down's syndrome. Metabolism 1968;17:560–2.
[38] Nishida Y, Akaoka I, Kobayashi M, Maruki K, Oshima Y. Renal impairment in urate excretion in patients with Down's syndrome. J Rheumatol 1979;6:103–7.
[39] Roos JF, Doust J, Tett SE, Kirkpatrick CMJ. Diagnostic accuracy of cystatin C compared to serum creatinine for the estimation of renal dysfunction in adults and children—a meta-analysis. Clin Biochem 2007;40:383–91.
[40] Talaat KM, el-Sheikh AR. The effect of mild hyperuricemia on urinary transforming growth factor beta and the progression of chronic kidney disease. Am J Nephrol 2007;27:435–40.
[41] Ford ES, Li C, Cook S, Choi HK. Serum concentrations of uric acid and the metabolic syndrome among US children and adolescents. Circulation 2007;115:2526–32.
[42] MacKinnon KL, Molnar Z, Lowe D, Watson ID, Shearer E. Measures of total free radical activity in critically ill patients. Clin Biochem 1999;32: 263–8.
[43] Carratelli M, Porcaro L, Ruscica M, De Simone E, Bertelli AA, Corsi MM. Reactive oxygen metabolites and prooxidant status in children with Down's syndrome. Int J Clin Pharmacol Res 2001;21:79–84.
[44] Stapleton FB, Linshaw MA, Hassanein K, Gruskin AB. Uric acid excretion in normal children. J Pediatr 1978;92:911–4.

oxidativo/nitrosativo en niños con síndrome de Down

C. Campos, R. Guzmán, E. López-Fernández y Á. Casado

Se ha sugerido que el estrés oxidativo tiene un papel fundamental en la patogénesis del síndrome de Down (SD). Sin embargo, los biomarcadores de estrés oxidativo han sido poco estudiados en muestras de orina en estos individuos. Por ello, el objetivo del presente trabajo fue valorar un conjunto de biomarcadores urinarios de estrés oxidativo y/o nitrosativo en niños con SD, que comprende: 8-hidroxi-2'-deoxiguanosina (8-OHdG), isoprostano 15-F_{2t}-IsoP, sustancias reactivas del ácido tiobarbitúrico (TBARS), productos finales de glicación avanzada (AGEs), ditirosina (diTyr), peróxido de hidrógeno (H_2O_2) y nitritos y nitratos (NOx).

Los biomarcadores se determinaron empleando técnicas espectrofotométricas y fluorimétricas en muestras de orina de 26 niños con SD, 7 de ellos recibiendo levotiroxina para el tratamiento del hipotiroidismo, y 19 hermanos de los niños con SD sin trisomía 21 (grupo control). Todos los parámetros fueron ajustados a creatinina (Cr).

Solo los niveles de diTyr fueron significativamente mayores en SD que en controles, aunque no se encontraron diferencias significativas entre ambos grupos cuando los niños con SD recibiendo levotiroxina fueron excluidos. Los niveles de 8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs, H_2O_2 y NOx no difirieron ni entre SD y controles ni entre niños con SD recibiendo levotiroxina y aquellos sin hipotiroidismo diagnosticado. Sin embargo, se encontraron menores niveles de Cr y mayores niveles de diTyr en niños con SD recibiendo levotiroxina que en sus hermanos no trisómicos. Se obtuvieron correlaciones negativas con la edad para 8-OHdG, diTyr y NOx en controles y para 8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs y diTyr en DS.

De los resultados de este estudio se puede concluir que el estrés oxidativo incrementado en SD no puede ser explicado por los niveles urinarios de 8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs, diTyr, H_2O_2 y NOx, al menos con las condiciones experimentales del presente trabajo. Aún así, la diTyr urinaria podría ser utilizada como biomarcador de estrés oxidativo/nitrosativo en niños hipotiroideos con SD, si bien estos resultados podrían estar influidos por una posible disfunción renal en base a los menores niveles de Cr obtenidos para estos individuos.

Evaluation of urinary biomarkers of oxidative/nitrosative stress in children with Down syndrome

Carlos Campos*, Rodrigo Guzmán, Encarnación López-Fernández, Ángela Casado

Department of Cellular and Molecular Medicine, Centre for Biological Research - Spanish National Research Council (CIB-CSIC). Madrid, Spain.

Abstract

Background: It has been suggested that oxidative stress plays a key role in the pathogenesis of Down syndrome (DS). However, urinary biomarkers of oxidative stress have been little studied in this condition. Thus, we aimed to assess a set of urinary oxidative/nitrosative stress biomarkers in children with DS, with and without hypothyroidism, which comprise: 8-hydroxy-2'-deoxyguanosine (8-OHdG), isoprostane 15-F_{2t}-IsoP, thiobarbituric acid-reacting substances (TBARS), advanced glycation end products (AGEs), dityrosine (diTyr), hydrogen peroxide (H_2O_2) and nitrite/nitrate (NOx).

Methods: Fluorimetric and spectrophotometric assays were performed in children with DS (n=26), some of them taking levothyroxine for hypothyroidism (n = 7), and their non-Down siblings (n = 19).

Results: We found that only levels of diTyr were increased in DS, although no differences were obtained when hypothyroid DS children were excluded. Levels of 8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs, H_2O_2 and NOx did not differ neither between DS and controls nor between hypothyroid DS children and DS without hypothyroidism diagnosed. However, diTyr is increased in hypothyroid DS children compared with controls. Negative correlations with age were obtained for 8-OHdG, diTyr and NOx in controls and for 8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs and diTyr in DS.

Conclusions: Increased oxidative stress in children with DS cannot be explained by the urinary levels of 8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs, diTyr, H_2O_2 and NOx, at least with the assays used. Nonetheless, urinary diTyr could be used as oxidative/nitrosative stress biomarker in hypothyroid DS children. The present work presents evidence of a probable renal impairment in children with DS and results should be cautiously interpreted.

Keywords: Down syndrome; oxidative stress; nitrosative stress; biomarker; urine; hypothyroidism.

Introduction

Down syndrome (DS), the most frequent genetic disorder occurring in 1 out of every 700 to 1000 live births, is caused by trisomy of all or part of human chromosome 21 (HSA21) and is characterized by a variety, chronicity and severity of abnormalities that include intellectual disability, dysmorphic features and immunological, haematological and endocrinal defects. Thus, children with DS often have thyroid dysfunction, congenital heart disease, diabetes, obstructive sleep apnea, gastrointestinal anomalies, visual impairment and auditory dysfunction [1].

Several lines of study have suggested that individuals with DS are under unusual oxidative stress, which has been proposed to be caused by an excess of Cu/Zn superoxide dismutase (SOD1) activity, an enzyme coded on HSA21 (21q22.1). SOD1 promotes the production of hydrogen peroxide (H_2O_2), an important precursor of hydroxyl radical, being one of the at least 16 genes or predicted genes on HSA21 with a role in mitochondrial energy generation and reactive oxygen species (ROS) metabolism [2]). H_2O_2 is then neutralized to water and oxygen through the actions of either glutathione peroxidase and/or catalase. Hence, the increased ratio of SOD1 to catalase plus glutathione peroxidase can lead to increased oxidative stress in DS [3].

Numerous diseases of children seem to be linked to oxidative damage attributable to ROS in their pathogenesis and progression [4]. Since the half-lives of ROS are usually short and ROS detection methods

Abbreviations: 8-OHdG, 8-hydroxy-2'-deoxyguanosine; 15-F_{2t}-IsoP, isoprostane 15-F_{2t}-IsoP; AFU, arbitrary fluorescence units; AGEs, advanced glycation end-products; BHT, butylated hydroxytoluene; Cr, creatinine; DiTyr, dityrosine; DS, Down syndrome; ELISA, enzyme-linked immunosorbent assay; FOX-2, ferrous ion oxidation xylenol orange version-2; GFR, glomerular filtration rate; H_2O_2, hydrogen peroxide; HPLC, high-performance liquid chromatography; HSA21, chromosome 21; JaICA, Japan Institute for the Control of Aging; MDA, 1,1,3,3-tetraethoxypropane; NO•, nitric oxide; NOx, total nitrite and nitrate; NS, not significant; ROS, reactive oxygen species; SOD1, Cu/Zn superoxide dismutase; TBA, 2-thiobarbituric acid; TBARS, thiobarbituric acid-reacting substances; VCl_3, vanadium(III) chloride.

*Corresponding author: Carlos Campos Vaquero. Department of Cellular and Molecular Medicine, Centre for Biological Research, CSIC. C/ Ramiro de Maeztu, 9. E-28040 Madrid, Spain. Phone: +34 91 837 31 12. Fax: +34 91 536 04 32. E-mail: ccampos@cib.csic.es.

require expensive equipment, oxidative stress biomarkers are desirable to assess oxidative damage. Some of them directly measure the results of oxidative stress, such as isoprostanes, while other methods measure the defence capacity against oxidation, such as total antioxidant capacity [5].

Oxidative stress in children with DS has been investigated in a number of in vivo studies [3,6-10], however urinary biomarkers of oxidative stress have been little studied and the set of markers measured in each work very limited. In addition, several oxidative stress biomarkers such as H_2O_2, advanced glycation end products (AGEs) or dityrosine (diTyr), as well as nitrosative stress biomarkers such as total nitrite and nitrate (NOx), have not been studied yet in urine samples of children with DS. The use of urine samples provides many advantages over plasma or serum in population studies as their collection does not require any invasive technique. Moreover, urine is the preferred body fluid for the measurement of isoprostanes and diTyr. For all these reasons, we chose urine as the specimen of choice in this study.

The aim of this work was to evaluate a comprehensive set of urinary biomarkers of oxidative/nitrosative stress using fast, simple and inexpensive methodologies widely used, which comprise: 8-hydroxy-2'-deoxyguanosine (8-OHdG), isoprostane 15-F_{2t}-IsoP (also 8-*epi*-$PGF_{2\alpha}$, 8-*iso*-$PGF_{2\alpha}$ or $iPF_{2\alpha}$-III), thiobarbituric acid-reacting substances (TBARS), AGEs, diTyr, H_2O_2 and NOx in a sample of children with DS and in their healthy age-matched controls, being the largest set of oxidative stress biomarkers appraised in children with DS. Moreover, we compared these parameters in children with DS who were receiving levothyroxine for hypothyroidism with those without thyroid dysfunction diagnosed as well as with their sibling controls to find out whether hypothyroidism is related to oxidative stress in DS.

Materials and methods

Subjects

The study was performed in 26 children with DS and 19 control subjects who were biological non-Down siblings living in the same household. Clinical characteristics of participants are shown in Table 1. Two age groups were established: <10 years (age group 1) and ≥10 years (age group 2), which represent infancy and adolescence pre-menarche, respectively. Seven subjects with DS were taking levothyroxine (synthetic T_4 hormone) for hypothyroidism treatment. Parental written informed consent was obtained for all subjects. The study was approved by The Ethics Committee of the Spanish National Research Council.

First morning urine samples on an empty stomach were collected in a sterile flask covered with aluminium foil to keep out stray light and processed within 2 hours of the collection. Samples were stored at -80 °C until analysis. Creatinine (Cr) and NOx determinations were performed the day of collection, without previous freezing of the sample.

Table 1 Clinical characteristics of subjects.

	Controls	Down syndrome
Age (years)*		
All ages	9.6±3.3 (5-14), *n* = 19	9.0±3.7 (3-14), *n* = 26
Age group 1	6.6±1.3 (5-9), *n* = 9	6.0±2.5 (3-9), *n* = 13
Age group 2	12.4±1.3 (10-14), *n* = 10	11.9±1.9 (10-14), *n* = 13
Sex (male/female)	8/11	13/13
Hypothyroidism	0	7
Karyotype		
Primary trisomy		24
Translocation		1
Mosaicism		1

*Data are mean±SD (range) and number of subjects (*n*).

Biochemical determinations

Cr was determined according to the spectrophotometric Jaffé method [11].

8-OHdG was determined by a competitive enzyme-linked immunosorbent assay (ELISA) kit from the Japan Institute for the Control of Aging (JaICA, Shizuoka, Japan).

Free isoprostane 15-F_{2t}-IsoP was analyzed using a commercially ELISA kit (Oxford Biomedical Research, Oxford, MI, USA).

TBARS were measured by the method of Uchiyama and Mihara [12] with modifications. Briefly, 33 µL of 0.01% butylated hydroxytoluene (BHT) (in absolute ethanol), 1 mL of 1% phosphoric acid and 300 µL of 42 mmol/L 2-thiobarbituric acid (TBA) were added to 140 µL of urine, mixing in vortex. The mixtures were incubated in boiling water for 45 min and, after cooling tubes on ice, 1.4 mL of 1-butanol was added in each tube. Following a 15 min centrifugation (2,000 *g*), the absorbance of supernatant was read at 535 nm. The standard absorption curve of 1,1,3,3-tetraethoxypropane (MDA) was prepared by dissolving it in phosphate buffer (20 mmol/L, pH 7.0).

AGEs was estimated essentially as described by Yanagisawa *et al.* [13]. Briefly, fluorescence intensity of urine diluted 20-fold in phosphate buffered saline was measured at 440 nm after excitation at 370 nm.

For diTyr measurement the assay was performed essentially as described by Witko-Sarsat *et al.* [14]. In brief, urine was diluted 20-fold in 50 mmol/L phosphate buffer, pH 7.4, containing 6 mol/L urea. Fluorescence intensity was measured at excitation and emission wavelengths of 315 and 410 nm respectively, after 30 min at room temperature. AGEs and diTyr results were reported as arbitrary fluorescence units (AFU).

H_2O_2 was measured using the ferrous ion oxidation xylenol orange version-2 (FOX-2) method [15]. In brief, 90 µL of urine was mixed with 10 µL of methanol and 900 µL of FOX-2 reagent (100 µmol/L xylenol orange, 250 µmol/L ammonium ferrous sulfate, 90% methanol, 4 mmol/L BHT and 25 mmol/L sulfuric acid). Tubes were vortexed and kept at room temperature for 30 min. Following a 10 min centrifugation (15,000 *g*) absorbance of supernatant was read at 560 nm against a methanol blank. Then, the same procedure was followed except for 10 µL of methanol that were replaced by 10 µL of catalase solution (2,200 U/mL in 25 mmol/L phosphate buffer, pH 7.0). Urinary H_2O_2 concentrations were calculated from the absorbance difference (with and without catalase) at 560 nm using a standard curve prepared with H_2O_2. The stability of H_2O_2 in frozen urine was satisfactory, with no significant difference between the H_2O_2 immediately after collection and after storage at -80 °C, at least for up to one month (data not shown).

NOx was measured by the acidic Griess reaction according to the method of Miranda *et al.* [16]. In brief, 100 µL of sample were applied to a microplate well. Following the addition of 100 µL VCl_3 (8 mg/mL in 1 mol/L HCl) to each well 100 µL of the Griess reagent (premixed 50 µL sulfanilamide (2% in 5% HCl) and 50 µL N-(1-Naphthyl) ethylendiamine dihydrochloride (0.1%)) were added immediately. Microplate was then incubated at 37 °C for 30 min and absorbance was read at 540 nm. Nitrate was determined from a linear standard curve established with sodium nitrate. Nitrite was measured in a similar manner except for the fact that samples and nitrite standards were only exposed to Griess reagent.

Spectrophotometric measurements of TBARS and H_2O_2 were performed with a UVmini-1240 Shimadzu spectrophotometer (Shimadzu, Tokyo, Japan). A microplate reader (ELx808, Bio-Tek Instruments, Winooski, VT, USA) was used for the rest of the spectrophotometric measurements. Fluorescence intensity was measured using a Varioskan Flash microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) operating at room temperature.

The levels of all biomarkers were corrected for urinary Cr concentrations.

Statistical analysis

Data are presented as mean ± standard deviation (SD) and range. Normal distribution and homogeneity of variance of the data were tested by the Shapiro-Wilk's and Levene's tests, respectively. For parametric variables Student's unpaired *t* test was used whereas Mann-Whitney U test was used for nonparametric data. Correlation analyses were performed using Pearson's or Spearman's correlation tests when appropriate. Statistical significance was set at $p<0.05$. Data were processed using IBM SPSS Statistics 18.0 (SPSS Inc., Chicago, IL, USA).

Results

Clinical characteristics of subjects are shown in Table 1. No significant differences in age were observed between children with DS and sibling controls ($p=0.531$). When the influence of gender was analyzed in DS and control groups, no significant differences were found for any parameter in any age group ($p>0.05$ for all).

In Table 2 urinary levels of the biochemical parameters studied in children with DS and sibling controls are listed for all ages. We found higher levels of diTyr in DS than in controls whereas no statistically significant differences were found in any of the other parameters. As shown in Figure 1 for the age groups 1 and 2, significant differences were also found in diTyr levels between DS and controls in the age group 1. Moreover, lower levels of Cr and higher levels of 8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs and diTyr were observed in the younger age group of DS subjects. In the control group, the younger children showed only lower levels of Cr and higher levels of diTyr with respect to the age group 2. It must be noted that no particular subject with several extreme marker concentrations biasing these results was found.

No significant differences were found in any parameter between children with DS who were receiving levothyroxine for hypothyroidism and those without thyroid dysfunction diagnosed (Table 3). Significantly lower levels of Cr and higher levels of diTyr were found in DS children treated for hypothyroidism when compared with their sibling controls ($p=0.019$ and $p=0.011$, respectively) and no differences were found for the rest of biomarkers. When subjects with hypothyroidism were excluded no differences were found in diTyr levels between DS and controls ($p>0.05$) and similar results were obtained for the rest of variables.

Correlations between biochemical parameters and age in DS and sibling controls were showed in Table 4. Significant negative correlations were observed between age and 8-OHdG, diTyr and NOx in controls and also between age and 8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs and diTyr in DS. Besides, a significant positive correlation was found between Cr and age in DS and controls.

Table 2 Urinary levels of Cr and biomarkers of oxidative and nitrosative stress (8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs, diTyr, H_2O_2 and NOx) in children with DS and their age-matched controls.

Urinary biomarker	Controls (n=19)	DS (n=26)	p-Value
Cr (mg/dL)	137±62 [59-308]	111±65 [29-366]	0.185[a]
8-OHdG (ng/mg Cr)	11.3±7.3 [3.4-35.4]	11.3±5.4 [3.8-27.5]	0.989[a]
15-F_{2t}-IsoP (ng/mg Cr)	1.20±0.53 [0.37-2.30]	1.15±0.62 [0.46-2.73]	0.787[a]
TBARS (μg/mg Cr)	1.25±0.55 [0.54-2.41]	1.20±0.58 [0.38-2.62]	0.783[a]
AGEs (AFU/mg Cr)	196±61 [102-340]	249±131 [116-672]	0.244[b]
DiTyr (AFU/mg Cr)	755±254 [305-1202]	1064±514 [425-2774]	0.029[b]
H_2O_2 (μg/mg Cr)	0.77±0.49 [0.14-1.65]	1.15±1.24 [0.08-6.26]	0.384[b]
NOx (μg/mg Cr)	83±47 [35-198]	121±80 [23-377]	0.073[b]

The data are expressed as mean±SD. In square brackets are reported ranges.

[a]by Student's unpaired t test, [b]by Mann-Whitney U test.

8-OHdG, 8-hydroxy-2'-deoxyguanosine; 15-F_{2t}-IsoP, isoprostane 15-F_{2t}-IsoP; AFU, arbitrary fluorescence units; AGEs, advanced glycation end products; Cr, creatinine; DiTyr, dityrosine; DS, Down syndrome; H_2O_2, hydrogen peroxide; NOx, total nitrite and nitrate; SD, standard deviation; TBARS, thiobarbituric acid-reacting substances.

Table 3 Urinary levels of Cr and biomarkers of oxidative and nitrosative stress (8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs, diTyr, H_2O_2 and NOx) in children with DS who were receiving levothyroxine for hypothyroidism and those without hypothyroidism diagnosed.

Urinary biomarker	DS without hypothyroidism (n=19)	DS with hypothyroidism (n=7)	p-Value[#]
Cr (mg/dL)	122±74 [29-366]	80±21 [49-110]	0.083
8-OHdG (ng/mg Cr)	10.3±4.3 [3.8-22.5]	14.0±7.5 [6.3-27.5]	0.259
15-F_{2t}-IsoP (ng/mg Cr)	1.06±0.60 [0.50-2.70]	1.39±0.66 [0.69-2.73]	0.234
TBARS (μg/mg Cr)	1.14±0.50 [0.38-2.12]	1.37±0.80 [0.56-2.62]	0.795
AGEs (AFU/mg Cr)	219±86 [132-472]	326±194 [116-672]	0.183
DiTyr (AFU/mg Cr)	951±348 [425-1767]	1500±716 [824-2774]	0.077
H_2O_2 (μg/mg Cr)	1.13±1.37 [0.08-6.26]	1.21±0.85 [0.43-2.92]	0.312
NOx (μg/mg Cr)	109±62 [23-259]	154±118 [45-377]	0.506

The data are mean±SD. In square brackets are reported ranges.

[#]Indicates P-value obtained in the comparisons between DS and controls by Mann-Whitney U test.

8-OHdG, 8-hydroxy-2'-deoxyguanosine; 15-F_{2t}-IsoP, isoprostane 15-F_{2t}-IsoP; AFU, arbitrary fluorescence units; AGEs, advanced glycation end products; Cr, creatinine; DiTyr, dityrosine; DS, Down syndrome; H_2O_2, hydrogen peroxide; NOx, total nitrite and nitrate; SD, standard deviation; TBARS, thiobarbituric acid-reacting substances.

Table 4 Correlations between age and biochemical markers in Down syndrome (DS) and their age-matched controls.

	Controls		DS	
Correlated variables	r	p-Value	r	p-Value
Cr	0.487	0.035[a]	0.481	0.013[a]
8-OHdG	-0.512	0.036[a]	-0.607	0.001[a]
15-F_{2t}-IsoP	0.109	0.687[a]	-0.625	<0.001[a]
TBARS	-0.349	0.143[a]	-0.715	<0.001[a]
AGEs	-0.355	0.163[b]	-0.582	0.002[b]
DiTyr	-0.752	<0.001[b]	-0.644	0.001[b]
H_2O_2	-0.331	0.195[b]	-0.269	0.184[b]
NOx	-0.512	0.025[b]	-0.273	0.178[b]

[a]Compared by Pearson's correlation coefficient analysis, [b]Compared by Spearman's correlation coefficient analysis. 8-OHdG, 8-hydroxy-2'-deoxyguanosine; 15-F_{2t}-IsoP, isoprostane 15-F_{2t}-IsoP; AGEs, advanced glycation end products; Cr, creatinine; DiTyr, dityrosine; DS, Down syndrome; H_2O_2, hydrogen peroxide; NOx, total nitrite and nitrate; TBARS, thiobarbituric acid-reacting substances.

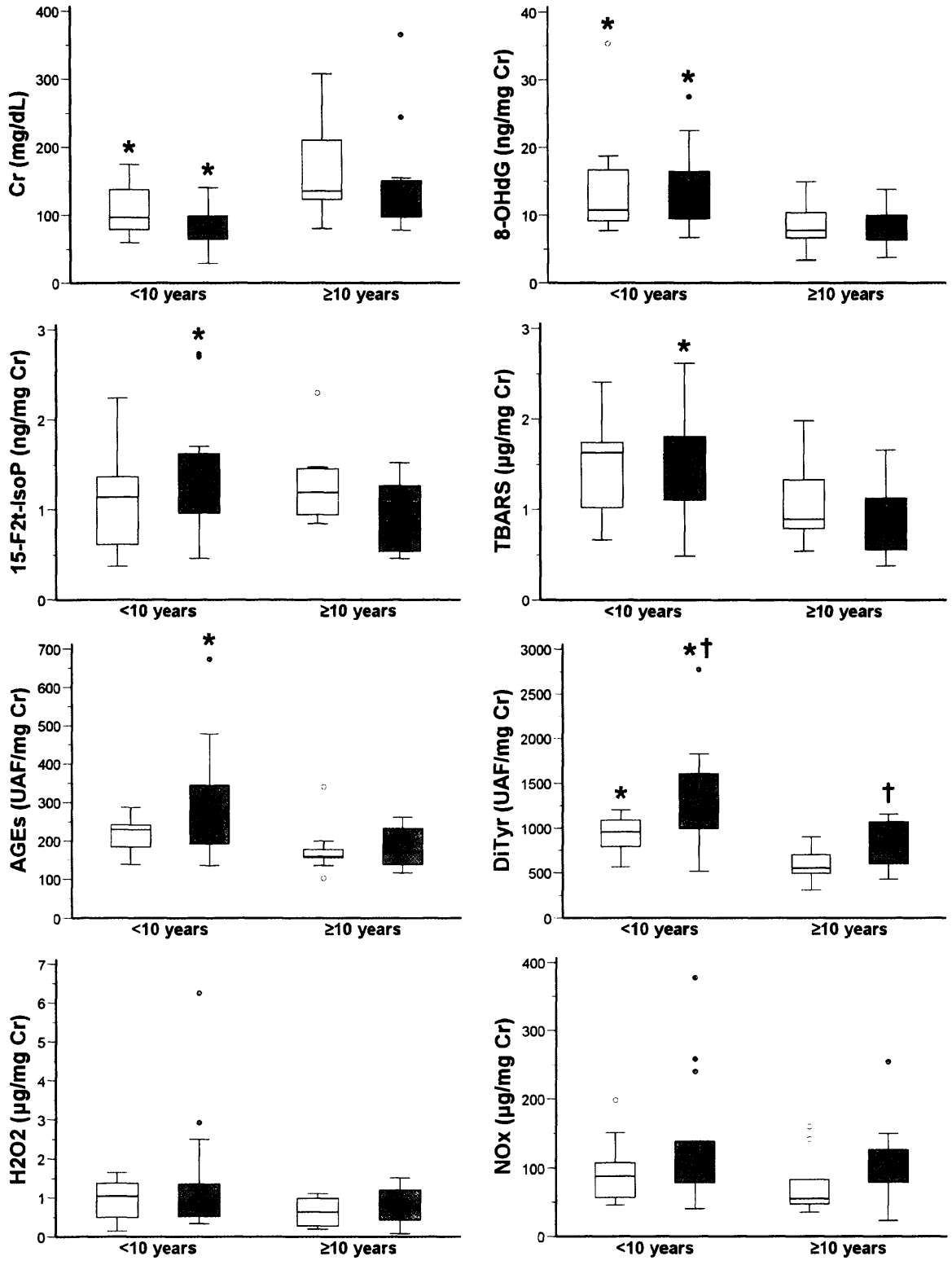


Figure 1 Whisker and box plots of urinary levels of creatinine (Cr), 8-hydroxy-2'-deoxyguanosine (8-OHdG), isoprostane 15-F_{2t}-IsoP, thiobarbituric acid-reacting substances (TBARS), advanced glycation end-products (AGEs), dityrosine (diTyr), hydrogen peroxide (H_2O_2) and total nitrite and nitrate (NOx) in controls (white boxes and dots) and Down syndrome (DS) (grey boxes and dots). The Mann-Whitney U test was used for all between-group comparisons. '*'Indicates $p<0.05$ from the age group and the control group, respectively.

Discussion

It is well established that increased oxidative stress occur in DS, however urinary biomarkers of oxidative stress have been little studied in DS and usually no more than two biomarkers have been measured in the population studied. In the present study, a set of oxidative stress biomarkers in urine samples of a pediatric population of DS, which comprise: 8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs, diTyr, H_2O_2 and NOx, has been investigated using the currently fastest, simplest and more inexpensive methodologies for each biomarker. Controls were siblings of DS subjects living in the same household in order to minimize bias in results caused by environmental factors affecting oxidative stress, such as diet, exposure to environmental tobacco smoke, etc.

DNA damage assessment

8-OHdG is the most commonly used marker of oxidative DNA damage. It has been previously assayed in leukocytes and urine samples of children with DS [3,8] and in both of them higher levels were found in DS subjects compared with those of matched controls. In contrast, we did not find differences in urinary levels of 8-OHdG between DS and controls in any age group.

The ELISA assay for 8-OHdG determination is the currently fastest, simplest and more inexpensive methodology for the determination of this biomarker. However, it has been recently published that urea and other substances contribute to the overestimation of urinary 8-OHdG due to cross-reaction with the commercial ELISA assay of JaICA [17] which we used. Since Jovanovic *et al.* [3] and Pallardó *et al.* [8] used high-performance liquid chromatography (HPLC) methods for the analysis of 8-OHdG in DS children, interferences from urine samples could lead to the unexpected results obtained in the present work. Nonetheless, it has been reported that urea excretion and clearance are not significantly different in DS compared to controls [18], although this study was not performed in children. Therefore, more studies are necessary to elucidate these findings. Moreover, since HPLC and other accurate techniques are currently limited for population studies, novel spectrophotometric assays must be developed for the accurate determination of 8-OHdG.

Lipid peroxidation assessment

With regard to lipid peroxidation biomarkers, 15-F_{2t}-IsoP and TBARS, we found no differences in urinary levels of these biomarkers between DS and controls. Serum lipid resistance to oxidation has been found in subjects with DS [19] and a concomitant increase in serum uric acid was also observed by the same authors. In a previous study of our research group with a representative sample of the population used in this work, significant increase in urinary uric acid levels was found in children with DS [10]. Thus, we proposed that similar levels of lipid oxidation biomarkers (15-F_{2t}-IsoP and TBARS) in DS and controls could be the result of a higher lipid resistance to oxidation in DS, due to higher levels of uric acid.

Notwithstanding, our results studying urinary levels of 15-F_{2t}-IsoP are in contradiction with the results obtained by Praticò *et al.* [6], where children with DS had higher levels of urinary isoprostane compared with those of matched controls. It must be noted that Praticò *et al.* measured 8,12-iso-i$PF_{2\alpha}$-VI and we measured 15-F_{2t}-IsoP. Both are F_2-isoprostanes, however different functions have been attributable to both isoprostanes, being 15-F_{2t}-IsoP the most tested and generally evaluated isomer of the F_2-isoprostanes series. It has been reported that F_2-isoprostanes act as mediators of important biological effects. In addition to being a biomarker of oxidative stress, 15-F_{2t}-IsoP causes vasoconstriction of renal glomerular arterioles with reduction of glomerular filtration rate (GFR) [20], which could result in renal failure. Hence, probable high levels of isoprostanes in blood of DS subjects, caused by increased oxidative stress, could lead to renal impairment and consistently a diminished excretion of metabolites such as oxidative stress biomarkers, included the own 15-F_{2t}-IsoP. Therefore, a comparative study assessing 15-F_{2t}-IsoP in plasma or serum and in urine samples must be done in DS to clarify this hypothesis. On the other hand, it has been reported that DS subjects have a difference in lipoprotein composition and levels [21], which may bias the results, so more studies are clearly needed to further determine the suitability of isoprostanes for the assessment of oxidative stress in DS.

Measurement of MDA levels using TBARS assay is one of the broadly-used to determine urinary lipid peroxidation due to the fact that it is a simple, inexpensive and fast methodology with good results in a number of works studying different pathologies and conditions with increased oxidative stress. However, much controversy has appeared in studies concerning the specificity of TBARS toward composites other than MDA. In fact, results in DS have indicated conflicting results. Jovanovic *et al.* [3] reported higher levels of TBARS in urine samples of children with DS whereas Muchová *et al.* [7] did not find significant differences neither in erythrocytes nor in serum samples of children with DS.

Glycoxidation assessment

Protein glycation and subsequent Maillard or browning reactions of glycated proteins form free carbonyls, which attack the major cellular components represented by proteins resulting in the formation of covalent compounds known as AGEs. Involvement of oxidative stress in the accelerated formation of AGEs has been reported and suggests that AGEs could be considered as an oxidative stress biomarker. A variety of chemical AGEs structures have been elucidated, some of which are fluorescent like pentosidine or the crosslinks.

High levels of AGEs occur in diabetes, cataracts and neurodegenerative diseases, such as Alzheimer disease, and all of them occur more frequently in DS. It has been reported that AGEs depress superoxide production by stimulated polymorphonuclear leukocytes [22] and reduce SOD1 function. Therefore, AGEs could play an important role in the oxidative status of DS subjects. Glyoxal and methylglyoxal have been measured in plasma samples of children with DS being glyoxal levels increased and methylglyoxal levels no significantly different to those obtained in controls [8]. In the present study, we did not find significant differences in urinary fluorescent AGEs between DS and controls, even though variability is higher in DS and we found children with DS having more than double levels than the highest value founded in controls. Therefore, future research is clearly needed to clarify these findings.

Protein oxidation assessment

DiTyr, also evaluated in the present work, is a fluorescent molecule formed as a result of normal posttranslational processes affecting specific structural proteins. Since tyrosine dimerization as well as nitration can be affected by peroxynitrite ($ONOO^-$), a powerful oxidant and nitrating agent, which can be formed by the reaction between active nitric oxide ($NO^\bullet$) metabolites and superoxide radical, diTyr could be considerer as a biomarker of both oxidative and nitrosative stress. Thus, it has been used as an important biomarker for oxidatively modified proteins [23], however it has not been previously measured in urine samples of DS.

We found significantly higher urinary levels of this biomarker in DS subjects than in controls, although no differences were found in diTyr levels between both groups when children with DS receiving levothyroxine were excluded. Comparison between hypothyroid DS children and their non-Down siblings showed significant differences between both groups, being levels of diTyr lower in sibling controls. Therefore, hypothyroidism may increase oxidative damage to proteins in children with DS.

H_2O_2

Urinary H_2O_2 was postulated to be an oxidative stress biomarker [24]. Although an excess of H_2O_2 is expected in DS subjects due to overexpresion of SOD1, no differences in urinary levels of H_2O_2 had been found between DS and controls. In addition, no significant age-related changes in H_2O_2 levels were observed neither in DS nor in controls and it could be attributable to the wide inter-individual variations obtained in H_2O_2 levels adjusted to Cr. Similar variations have been observed in previous works [25] and could be due to the fact that H_2O_2 levels are more determined by metabolic and nutritional influences than by changes in oxidative stress.

Total nitrite and nitrate (NOx)

Nitric oxide ($NO^\bullet$) is a free radical gas which has been implicated in a wide range of physiological functions, including the modulation of renal hemodynamics and excretory function [26]. Since $NO^\bullet$ rapidly degrades to nitrate and nitrite in aqueous solution, the urinary NOx levels were estimated as an index of $NO^\bullet$ production. Although neurodegeneration in DS has been associated with aberrant expression of nitric oxide synthase 3 gene [27] and nitrosative stress is linked to oxidative stress, biomarkers of nitrosative stress have been poorly studied in DS. In the present work we found no differences between children with DS and controls in urinary levels of NOx. However, a negative correlation of NOx with age was found in controls but not in DS. It has been reported that the tendency of urinary nitrate levels to decrease with age in healthy children may be a reflection of an age-related increase in GFR [28], so GFR could be impaired in DS.

Urinary levels of oxidative/nitrosative stress biomarkers in the infancy and in the adolescence pre-menarche

As we found, high levels of urinary oxidative stress biomarkers in younger subjects have been previously reported [4,29]. In the present study, increased levels of 8-OHdG and diTyr were found in the younger groups of DS and controls. In addition, levels of 15-F_{2t}-IsoP, TBARS and AGEs were also increased in the younger group of DS, giving negative correlations with age for some of these biomarkers. The reason why urinary biomarkers of oxidative stress are higher in the younger children is not clear. Some possible explanations given in previous works include the higher frequency of infections in children or the more rapid basal metabolic rates, either of which may yield increased oxidative stress. Alternatively, higher levels of oxidative stress biomarkers observed in the younger groups of children, giving negative correlations with age in some cases, could be the result of the greater relative weight of the internal organs, the higher DNA-to-protein ratio and the accelerated organ growth velocity in young children, similar to what occurs for other analytes such as uric acid [30]. We postulate that an increase of musculature with age in children and a concomitant

increase in urinary excretion of Cr may bias the results of urinary biomarkers. However, the physiological meaning and mechanisms for these results are not clarified yet. In addition, the magnitude of these phenomena may be more pronounced in DS than in non-Down children, possibly due to the growth retardation attributable to growth hormone deficiency in DS. Thus, we found higher levels for more biomarkers in the younger group of DS than in the younger group of controls, which correlate all negatively with age. Supporting our hypothesis, it has been reported that levels of 8-OHdG are possibly affected by the growth and the development of the lung [31]. In addition, positive correlation between Cr and age was found for both DS and control groups, which may be due partly to a developmental growth of muscle mass [32]. Therefore, Cr may bias the results and it could also contribute to the higher levels of oxidative stress biomarkers shown in the younger subjects.

Analysis of hypothyroidism in DS

Hypothyroidism is the most frequent thyroid abnormality in DS [33], being the frequency of children with DS treated with levothyroxine in the present work of 29%. A decrease in free radical production is expected in hypothyroidism due to the metabolic suppression brought about by the decreased levels of thyroid hormone, however data are controversial and several works reported increased oxidative stress in hypothyroidism [34]. In the present study, we found significantly lower levels of Cr and higher levels of diTyr in children with DS receiving levothyroxine for hypothyroidism than in non-Down siblings. Moreover, slightly decreased levels of Cr and slightly increased levels of oxidative stress biomarkers were observed in children with DS receiving levothyroxine for hypothyroidism compared with DS children without hypothyroidism diagnosed, although without significant differences. Impaired renal function has been reported in subjects with hypothyroidism [35]. It enhances serum Cr levels because it reduces GFR and increases production of Cr. Therefore, renal impairment due to hypothyroidism may bias the results in these subjects. Renal impairment has been described in DS based on decreased Cr clearance [18]. In previous studies, included this one, urinary Cr levels in children with DS were slightly lower than in controls, although without statistically significant differences. Thus, Cr used for standardized urinary levels of biomarkers studied may also bias the results, especially in hypothyroid DS subjects.

Conclusions

It can be concluded that the increased oxidative stress in children with DS cannot be explained by the urinary levels of 8-OHdG, isoprostane 15-F_{2t}-IsoP, TBARS, AGEs, diTyr, H_2O_2 and NOx, at least with the assays used in the present work. Nonetheless, levels of diTyr where increased in hypothyroid DS children compared to their non-Down siblings, so urinary diTyr could be used as biomarker of oxidative/nitrosative stress in these subjects. Besides, increased levels of several biomarkers were found in the younger groups of DS and controls, giving negative correlations with age for some of them. Different mechanisms may underlie the oxidative stress in children with DS and their relation with urinary levels of oxidative stress biomarkers. The present work presents evidence of a probable renal impairment in children with DS, which could be raised in those receiving levothyroxine for hypothyroidism. Therefore, results should be cautiously interpreted until they can be confirmed in larger populations and further relevant data can be gathered. Moreover, specific renal function tests, such as Cr clearance or urinary cystatin C, must be performed in DS in order to clarify the results of the present study.

Acknowledgements

The authors would like to thank all the people with Down syndrome, families and volunteers which cooperated and made the achievement of this work possible. The authors express their gratitude to Fundación Síndrome de Down de Madrid, Centro María Corredentora, Asociación de Empleados de IBERIA Padres de Minusválidos, Fundación Nuestra Señora del Camino, C.P.E.E. Príncipe de Asturias (Aranjuez), C.P.E.E. Francisco del Pozo de Madrid, C.C.E.E. Buenafuente de Madrid and to Hospital Niño Jesús de Madrid, for their valuable contributions to this work. This study was supported by Fundación Inocente, Inocente.

References

[1] Pueschel SM. Clinical aspects of Down syndrome from infancy to adulthood. Am J Med Genet Suppl 1990;7:52-6.

[2] Roizen NJ, Patterson D. Down's syndrome. Lancet 2003;361:1281-9.

[3] Jovanovic SV, Clements D, MacLeod K. Biomarkers of oxidative stress are significantly elevated in Down syndrome. Free Radic Biol Med 1998;25:1044-8.

[4] Tsukahara H. Biomarkers for oxidative stress: clinical application in pediatric medicine. Curr Med Chem 2007;14:339-51.

[5] Campos C, Guzmán R, López-Fernández E, Casado A. Evaluation of the copper(II) reduction assay using bathocuproinedisulfonic acid disodium salt for the total antioxidant capacity assessment: The CUPRAC-BCS assay. Anal Biochem 2009;392:37-44.

[6] Praticò D, Iuliano L, Amerio G, Tang LX, Rokach J, Sabatino G, Violi F. Down's syndrome is associated with increased 8,12-iso-iPF2alpha-VI levels: evidence for enhanced lipid peroxidation in vivo. Ann Neurol 2000;48:795-8.

[7] Muchová J, Sustrová M, Garaiová I, Liptáková A, Blazícek P, Kvasnicka P, Pueschel S, Duracková Z. Influence of age on activities of antioxidant enzymes and lipid peroxidation products in erythrocytes and neutrophils of Down syndrome patients. Free Radic Biol Med 2001;31:499-508.

[8] Pallardó FV, Degan P, d'Ischia M, Kelly FJ, Zatterale A, Calzone R. Multiple evidence for an early age pro-oxidant state in Down syndrome patients. Biogerontology 2006;7:211-20.

[9] Casado A, López-Fernández ME, Ruiz R. Lipid peroxidation in Down syndrome caused by regular trisomy 21, trisomy 21 by Robertsonian translocation and mosaic trisomy 21. Clin Chem Lab Med 2007;45:59-62.

[10] Campos C, Guzmán R, López-Fernández E, Casado A. Urinary uric acid and antioxidant capacity in children and adults with Down syndrome. Clin Biochem 2010;43:228-33.

[11] Jaffé M. Über den niederschlag, welchen pikrinsäure in normalen harn erzeugt und über eine neue reaktion des kreatinins. Z Physiol Chem 1886;10:391-400.

[12] Uchiyama M, Mihara M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem 1978;86:271-8.

[13] Yanagisawa K, Makita Z, Shiroshita K, Ueda T, Fusegawa T, Kuwajima S, Takeuchi M, Koike T. Specific fluorescence assay for advanced glycation end products in blood and urine of diabetic patients. Metabolism 1998;47:1348-53.

[14] Witko-Sarsat V, Friedlander M, Capeillere-Blandin C, Nguyen-Khoa T, Nguyen AT, Zingraff J, Jungers P, Descamps-Latscha B. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. Kidney Int 1996;49:1304-13.

[15] Banerjee D, Kumar AP, Kumar B, Madhusoodanan UK, Nayak S, Jacob J. Determination of absolute hydrogen peroxide concentration by spectrophotometric method. Curr Sci 2002;83:1193-4.

[16] Miranda KM, Espey MG, Wink DA. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 2001;5:62-71.

[17] Song M-F, Li Y-S, Ootsuyama Y, Kasai H, Kawai K, Ohta M, Eguchi Y, Yamato H, Matsumoto Y, Yoshida R, Ogawa Y. Urea, the most abundant component in urine, cross-reacts with a commercial 8-OH-dG ELISA kit and contributes to overestimation of urinary 8-OH-dG. Free Radic Biol Med 2009;47:41-6.

[18] Coburn SP, Seidenberg M, Mertz ET. Clearance of uric acid, urea, and creatinine in Down's syndrome. J Appl Physiol 1967;23:579-80.

[19] Nagyova A, Sustrova M, Raslova K. Serum lipid resistance to oxidation and uric acid levels in subjects with Down's syndrome. Physiol Res 2000;49:227-31.

[20] Takahashi K, Nammour TM, Fukunaga M, Ebert J, Morrow JD, Roberts LJ, 2nd, Hoover RL, Badr KF. Glomerular actions of a free radical-generated novel prostaglandin, 8-epi-prostaglandin F2 alpha, in the rat. Evidence for interaction with thromboxane A2 receptors. J Clin Invest 1992;90:136-41.

[21] Murphy EJ, Schapiro MB, Rapoport SI, Shetty HU. Phospholipid composition and levels are altered in Down syndrome brain. Brain Res 2000;867:9-18.

[22] Bernheim J, Rashid G, Gavrieli R, Korzets Z, Wolach B. In vitro effect of advanced glycation end-products on human polymorphonuclear superoxide production. Eur J Clin Invest 2001;31:1064-9.

[23] Giulivi C, Traaseth NJ, Davies KJA. Tyrosine oxidation products: analysis and biological relevance. Amino Acids 2003;25:227-32.

[24] Halliwell B, Clement MV, Long LH. Hydrogen peroxide in the human body. FEBS Lett 2000;486:10-3.

[25] Halliwell B, Long LH, Yee TP, Lim S, Kelly R. Establishing biomarkers of oxidative stress: the measurement of hydrogen peroxide in human urine. Curr Med Chem 2004;11:1085-92.

[26] Mizutani T, Layon AJ. Clinical applications of nitric oxide. Chest 1996;110:506-24.

[27] Sohn YK, Ganju N, Bloch KD, Wands JR, de la Monte SM. Neuritic sprouting with aberrant expression of the nitric oxide synthase III gene in neurodegenerative diseases. J Neurol Sci 1999;162:133-51.

[28] Elli M, Soylemezoglu O, Erbas D, Bakkaloglu SA, Buyan N, Ozkaya O, Hasanoglu E. Plasma and urine nitric oxide levels in healthy Turkish children. Pediatr Nephrol 2005;20:1605-9.

[29] Kauffman LD, Sokol RJ, Jones RH, Awad JA, Rewers MJ, Norris JM. Urinary F2-isoprostanes in young healthy children at risk for type 1 diabetes mellitus. Free Radic Biol Med 2003;35:551-7.

[30] Stapleton FB, Linshaw MA, Hassanein K, Gruskin AB. Uric acid excretion in normal children. J Pediatr 1978;92:911-4.

[31] Svecova V, Rossner Jr P, Dostal M, Topinka J, Solansky I, Sram RJ. Urinary 8-oxodeoxyguanosine levels in children exposed to air pollutants. Mutat Res 2009;662:37-43.

[32] Trowbridge FL, Hiner CD, Robertson AD. Arm muscle indicators and creatinine excretion in children. Am J Clin Nutr 1982;36:691-6.

[33] Fort P, Lifshitz F, Bellisario R, Davis J, Lanes R, Pugliese M, Richman R, Post EM, David R. Abnormalities of thyroid function in infants with Down syndrome. J Pediatr 1984;104:545-9.

[34] Sarandöl E, Taş S, Dirican M, Serdar Z. Oxidative stress and serum paraoxonase activity in experimental hypothyroidism: effect of vitamin E supplementation. Cell Biochem Funct 2005;23:1-8.

[35] Montenegro J, González O, Saracho R, Aguirre R, González O, Martínez I. Changes in renal function in primary hypothyroidism. Am J Kidney Dis 1996;27:195-8.

[redacted] de Down

C. Campos, R. Guzmán, E. López-Fernández y Á. Casado

Los biomarcadores urinarios de estrés oxidativo han sido muy poco estudiados en adultos con SD. Además, los estudios en esta población normalmente valoran pocos biomarcadores y aparecen con frecuencia resultados controvertidos. Así, el objetivo del presente trabajo fue valorar un conjunto de biomarcadores urinarios de estrés oxidativo y/o nitrosativo en adolescentes y adultos con SD, que comprende: 8-hidroxi-2'-deoxiguanosina (8-OHdG), isoprostano 15-F_{2t}-IsoP, sustancias reactivas del ácido tiobarbitúrico (TBARS), productos finales de glicación avanzada (AGEs), ditirosina (diTyr), peróxido de hidrógeno (H_2O_2) y nitritos y nitratos (NOx).

Los biomarcadores se determinaron empleando técnicas espectrofotométricas y fluorimétricas en muestras de orina de 78 individuos con SD, 24 de ellos recibiendo levotiroxina para el tratamiento del hipotiroidismo, y 65 controles. Se analizaron cuatro grupos de edad: 1) todas las edades (15-59 años), 2) adolescencia (15-19 años), 3) adultez (20-40 años) y 4) senescencia (41-59 años); este último grupo basado en una clasificación previa establecida para los individuos con SD. Todos los parámetros fueron ajustados a creatinina (Cr).

Se encontraron niveles incrementados de AGEs, diTyr, H_2O_2 y NOx en alguno o en todos los grupos de edad, mientras que los niveles de Cr fueron menores en SD que en controles en todos los grupos de edad. En individuos con SD recibiendo levotiroxina se encontraron menores niveles de Cr en adolescentes, mayores niveles de TBARS en adultos, mayores niveles de AGEs en adultos y en la senescencia, y mayores niveles de diTyr en adolescentes y adultos que en individuos con SD sin hipotiroidismo diagnosticado. Por otro lado, las correlaciones con la edad en SD fueron positivas para diTyr y negativas para Cr, 15-F_{2t}-IsoP, TBARS y NOx.

De los resultados de este estudio se puede concluir que AGEs, diTyr, H_2O_2 y NOx podrían ser utilizados como biomarcadores de estrés oxidativo y/o nitrosativo en muestras de orina de individuos con SD, al contrario que 8-OHdG, 15-F_{2t}-IsoP y TBARS, al menos en las condiciones experimentales del presente trabajo. Sin embargo, los individuos con SD podrían presentar disfunción renal que podría influir en los resultados, especialmente en los individuos hipotiroideos.

Biochimica et Biophysica Acta xxx (2011) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Evaluation of urinary biomarkers of oxidative/nitrosative stress in adolescents and adults with Down syndrome ☆

Carlos Campos *, Rodrigo Guzmán, Encarnación López-Fernández, Ángela Casado

Department of Cellular and Molecular Medicine, Centre for Biological Research-Spanish National Research Council (CIB-CSIC), C/ Ramiro de Maeztu, 9. E-28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 17 October 2010
Received in revised form 18 March 2011
Accepted 22 March 2011
Available online xxxx

Keywords:
Down syndrome
Oxidative stress
Nitrosative stress
Biomarker
Hypothyroidism
Urine

ABSTRACT

Urinary biomarkers of oxidative stress have been little studied in adults with Down syndrome (DS), usually no more than two biomarkers have been measured in the population studied and controversial results are reported in literature. Thus, we aimed to assess a set of oxidative and nitrosative stress biomarkers in urine samples of adolescents and adults with DS, with and without hypothyroidism, which comprise: 8-hydroxy-2′-deoxyguanosine (8-OHdG), isoprostane 15-F_{2t}-IsoP, thiobarbituric acid-reacting substances (TBARS), advanced glycation end products (AGEs), dityrosine (diTyr), hydrogen peroxide (H_2O_2) and nitrite/nitrate (NOx). Fluorimetric and spectrophotometric assays were performed in DS ($n=78$), some of them taking levothyroxine for hypothyroidism ($n=24$), and in their healthy age-matched controls ($n=65$). We found that levels of AGEs, diTyr, H_2O_2 and NOx are increased in DS patients in any or in all age groups, whereas Cr levels were lower in DS than in controls in all age groups. Besides, correlations with age in DS were positive for diTyr and negative for Cr, TBARS, 15-F_{2t}-IsoP and NOx. We also found lower levels of Cr from 15 to 19 years, higher levels of TBARS and AGEs from 20 to 40 years and higher levels of diTyr from 15 to 40 years in DS patients receiving levothyroxine than in DS without hypothyroidism diagnosed. We conclude that AGEs, diTyr, H_2O_2 and NOx could be used as oxidative stress biomarkers in DS in contrast to 8-OHdG, 15-F_{2t}-IsoP and TBARS, at least with the methods used. However, renal impairment could occur in DS and Cr adjustment may bias the results, particularly in hypothyroid patients.

1. Introduction

Down syndrome (DS), caused by the triplication of chromosome 21 (HSA21) or part of it, is found to be one of the more complex genetic conditions compatible with a large survival. However, individuals with DS suffer from accelerate aging, being DS considered as a progeroid syndrome, which may be the result of an increased oxidative stress observed in these patients. The trisomy, affecting more than 300 genes, is associated with a variety of manifestations, including pathologies which are possibly related to aging such as Alzheimer disease, cataracts, leukemia, diabetes mellitus, hypogonadism, vascular disease, amyloidosis and premature graying or loss of hair.

The amount of studies reporting a number of dysfunctions associating oxidative stress with DS phenotype is increasing. An excess of the enzyme Cu/Zn superoxide dismutase (SOD1) activity has been considered as the main responsible for the increased oxidative stress found in patients with DS [1]. Besides, several abnormalities in mitochondrial function have been found in DS and also in mouse models of this pathology. Indeed, in addition to SOD1, there are at least 15 genes or predicted genes on HSA21 with a role in mitochondrial energy generation and reactive oxygen species metabolism [2].

There are considerable studies providing evidence about the free radical involvement in the pathogenesis of DS based on biomarkers of oxidative stress measurements [3–5], which are stable adducts that are produced as a result of the oxidative processes in vivo. However, urinary biomarkers of oxidative stress have been little studied in this condition, the set of markers measured in each work is very limited and studies are normally performed in a reduced age range. In fact, most works are performed only in children, being the biomarkers of oxidative stress poorly studied in DS adults or in the elderly stage.

The use of urine samples provides many advantages over plasma or serum in population studies as their collection does not require any invasive technique. Since it is particularly difficult to obtain reliable 24 h urine samples and sampling is often incomplete (which may bias

Abbreviations: 8-OHdG, 8-hydroxy-2′-deoxyguanosine; 15-F_{2t}-IsoP, isoprostane; AFU, arbitrary fluorescence units; AGEs, advanced glycation end-products; BHT, butylated hydroxytoluene; Cr, creatinine; DiTyr, dityrosine; DS, Down syndrome; ELISA, enzyme-linked immunosorbent assay; FOX-2, xylenol orange version-2; H_2O_2, hydrogen peroxide; HCl, hydrochloric acid; HPLC, high-performance liquid chromatography; HSA21, chromosome 21; JaICA, Japan Institute for the Control of Aging; MDA, 1,1,3,3-tetraethoxypropane; NO, nitric oxide; NOx, total nitrite and nitrate; NS, not significant; SOD1, Cu/Zn superoxide dismutase; TBA, 2-thiobarbituric acid; TBARS, thiobarbituric acid-reacting substances; VCl_3, vanadium(III) chloride

☆ This study was supported by Fundación Inocente, Inocente.

* Corresponding author. Tel.: +34 91 8373112; fax: +34 91 5360432.
E-mail address: carloscamposvaquero@gmail.com (C. Campos).

0925-4439/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbadis.2011.03.013

the results), spot urine samples could be employed and compliance is high. Moreover, urine is the preferred body fluid for the measurement of isoprostanes [6]. For all these reasons, we chose urine as the specimen of choice in this study.

In order to assess the reliability of a set of urinary biomarkers of oxidative and nitrosative stress in DS, we studied the urinary levels of: 8-hydroxy-2′-deoxyguanosine (8-OHdG), isoprostane 15-F_{2t}-IsoP (also 8-epi-$PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$ or $iPF_{2\alpha}$-III), thiobarbituric acid-reacting substances (TBARS), advanced glycation end products (AGEs), dityrosine (diTyr), hydrogen peroxide (H_2O_2) and nitrite/nitrate (NOx) in a sample of adolescents, adults and senescent subjects with DS and in those of healthy age-matched controls. Moreover, we compare these parameters in DS treated with levothyroxine with DS without thyroid dysfunction diagnosed to find out whether hypothyroidism is related to oxidative stress in DS.

2. Materials and methods

2.1. Subjects

The study was performed in 78 patients with DS and 65 healthy controls. Clinical characteristics of participants are shown in Table 1. Three age groups were established: from 15 to 19 years (age group 1), from 20 to 40 years (age group 2) and >40 years (age group 3). These groups represent respectively: early adulthood, adulthood and senescence (based on DS individuals according to the classification of Bittles et al. [7]). Participants filled in a comprehensive questionnaire to obtain the following information: a) sociodemographic parameters: age and gender; b) use of drugs or dietary supplements; c) exercise habits; and d) smoking habits. All of them were non-smokers and received no dietary or medicinal vitamin supplementation. Subjects were not affected by diabetes mellitus, myeloproliferative disorders, any type of dementia such as Alzheimer disease, malnutrition or treated with drug possibly interfering with oxidative metabolism, being 24 subjects with DS taking levothyroxine (synthetic T_4 hormone) for hypothyroidism treatment. Informed consent was obtained from the participants or was given by parents. The study was approved by The Ethics Committee of the Spanish National Research Council.

2.2. Urine sample collection and preparation

First morning urine samples on an empty stomach were collected in a sterile flask covered with aluminium foil to keep out stray light and processed within 2 h of the collection. Samples were 1 mL aliquoted and frozen at −80 °C until analysis. Creatinine (Cr) determination was performed the day of collection, without previous freezing of the sample.

Table 1
Clinical characteristics of patients.

	Controls	Down syndrome
Age[a]		
All ages (15–59 years)	32.1 ± 13.1, $n = 65$	28.8 ± 12.4, $n = 78$
Age group 1 (15–19 years)	17.3 ± 1.5, $n = 15$	17.0 ± 1.4, $n = 29$
Age group 2 (20–40 years)	28.7 ± 5.4, $n = 31$	29.6 ± 5.9, $n = 33$
Age group 3 (41–59 years)	49.5 ± 6.5, $n = 19$	48.5 ± 4.6, $n = 16$
Sex (male/female)	26/39	35/43
Hypothyroidism	0	24
Karyotype		
Primary trisomy		72
Robertsonian translocation		5
trisomy der(14;21)(q10;q10)		
Mosaicism (normal cellular line 94%)		1

[a] Data are mean ± SD and number of subjects (n).

2.3. Biochemical determinations

2.3.1. Creatinine (Cr)

Cr was determined according to the spectrophotometric Jaffé method [8] which is based on the reaction of Cr with picric acid in alkaline pH, described by Varley et al. [9].

2.3.2. 8-Hydroxy-2′-deoxyguanosine (8-OHdG)

Urinary 8-OHdG was determined by a competitive enzyme-linked immunosorbent assay (ELISA) kit from the Japan Institute for the Control of Aging (JalCA, Shizuoka, Japan) according to the manufacturer's instruction. Opaque samples were centrifuged at 4000 × g for 10 min. Incubations at 37 °C and absorbance measurements were carried out using a microplate reader (ELx808, Bio-Tek Instruments, Winooski, VT, USA).

2.3.3. Urinary isoprostane (15-F_{2t}-IsoP)

Free isoprostane 15-F_{2t}-IsoP was analyzed using a commercially ELISA kit (Oxford Biomedical Research, Oxford, MI, USA). The procedure was performed following the manufacturer's instruction. Urine samples were diluted 1:4 in the buffer supplied and a microplate reader (ELx808, Bio-Tek Instruments, Winooski, VT, USA) with a 450 nm filter was used for spectrophotometric measurements.

2.3.4. Thiobarbituric acid-reacting substances (TBARS)

TBARS were measured by the method of Uchiyama and Mihara [10] with modifications. Briefly, 33 μL of 0.01% BHT (in absolute ethanol), 1 mL of 1% phosphoric acid and 300 μL of 42 mmol/L TBA (dissolved in water and heating) were added to 140 μL of urine, mixing in vortex. The mixtures were incubated in boiling water for 45 min and, after cooling tubes on ice, 1.4 mL of 1-butanol was added in each tube. Following a 15 min centrifugation (2000 × g), the absorbance of supernatant was read at 535 nm using a UVmini-1240 Shimadzu spectrophotometer (Shimadzu, Tokyo, Japan). The standard absorption curve of MDA was prepared by dissolving it in phosphate buffer (20 mmol/L, pH 7.0).

2.3.5. Advanced glycation end-products (AGEs)

AGEs was estimated essentially as described by Yanagisawa et al. [11]. Fluorescence intensity of urine diluted 20-fold in phosphate buffered saline was measured at 440 nm after excitation at 370 nm, using a Varioskan Flash microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) operating at room temperature. Results were reported as arbitrary fluorescence units (AFU).

2.3.6. Dityrosine (diTyr)

For diTyr measurement the assay was performed essentially as described by Witko-Sarsat et al. [12]. In brief, urine was diluted 20-fold in 50 mmol/L phosphate buffer, pH 7.4, containing 6 mol/L urea. Fluorescence intensity was measured at excitation and emission wavelengths of 315 and 410 nm respectively, using a Varioskan Flash microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) operating at room temperature, after 30 min at room temperature. Results were reported as AFU.

2.3.7. Hydrogen peroxide (H_2O_2)

H_2O_2 was measured using the ferrous ion oxidation xylenol orange version-2 (FOX-2) method of Banerjee et al. [13]. In brief, 90 μL of urine was mixed with 10 μL of methanol and 900 μL of FOX-2 reagent (100 μmol/L xylenol orange, 250 μmol/L ammonium ferrous sulfate, 90% methanol, 4 mmol/L BHT and 25 mmol/L sulfuric acid). Tubes were vortexed and kept at room temperature for 30 min. Following a 10 min centrifugation (15,000 × g) absorbance of supernatant was read at 560 nm against a methanol blank using a UVmini-1240 Shimadzu spectrophotometer (Shimadzu, Tokyo, Japan). Then, the same procedure was followed except for 10 μL of methanol that were

Please cite this article as: C. Campos, et al., Evaluation of urinary biomarkers of oxidative/nitrosative stress in adolescents and adults with Down syndrome, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.03.013

replaced by 10 μL of catalase solution (2200 U/mL in 25 mmol/L phosphate buffer, pH 7.0). Urinary H_2O_2 concentrations were calculated from the absorbance difference (with and without catalase) at 560 nm using a standard curve prepared with H_2O_2.

2.3.8. Total nitrate and nitrite (NOx)

Total nitrate and nitrite concentration (NOx) was measured by the acidic Griess reaction according to the method of Miranda et al. [14]. In brief, 100 μL of sample was applied to a microplate well. Following the addition of 100 μL VCl_3 (8 mg/mL in 1 mol/L HCl) to each well 100 μL of the Griess reagent (premixed 50 μL sulfanilamide (2% in 5% HCl) and 50 μL *N*-(1-Naphthyl) ethylendiamine dihydrochloride (0.1%)) were added immediately. Microplate was then incubated at 37 °C for 30 min and absorbance was read at 540 nm using a microplate reader (ELx808, Bio-Tek Instruments, Winooski, VT, USA). Nitrate was determined from a linear standard curve established with sodium nitrate. Nitrite was measured in a similar manner except for the fact that samples and nitrite standards were only exposed to Griess reagent.

2.4. Statistical analysis

Data are presented as mean ± standard deviation (SD) and range. Normal distribution and homogeneity of variance of the data were tested by the Shapiro–Wilk's and Levene's tests, respectively. For parametric variables Student's unpaired *t*-test was used whereas Mann–Whitney *U*-test was used for nonparametric data. Comparisons between age groups in both DS and control groups were performed by one-way analysis of variance (ANOVA) and Scheffé post-hoc test. Correlation analyses were performed using Pearson's or Spearman's correlation tests when appropriate and then linear regression analyses were carried out. Statistical significance was set at $P<0.05$. Data were processed using IBM SPSS Statistics 18.0 software (SPSS Inc., Chicago, IL, USA).

Table 2
Urinary levels of Cr and biomarkers of oxidative and nitrosative stress (8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs, diTyr, H_2O_2 and NOx) in patients with Down syndrome (DS) and their age-matched controls. The data are expressed as mean ± standard deviation (SD) and ranges.

Urinary biomarker	Controls			DS			*P*
	n	Mean ± SD	Range	*n*	Mean ± SD	Range	
Cr (mg/dL)							
All ages (15–59 years)	65	193 ± 79	37–470	78	127 ± 63***	25–298	<0.001[a]
Age group 1 (15–19 years)	15	198 ± 63	92–334	29	119 ± 55***	25–276	<0.001[b]
Age group 2 (20–40 years)	31	206 ± 86	91–470	33	140 ± 65**	34–298	0.001[b]
Age group 3 (>40 years)	19	169 ± 78	37–308	16	115 ± 72*	27–290	0.045[b]
8-OHdG (ng/mg Cr)							
All ages (15–59 years)	45	8.3 ± 3.5	2.1–17.7	77	8.9 ± 3.5	2.1–24.5	0.181[a]
Age group 1 (15–19 years)	10	8.4 ± 2.2	4.6–12.8	29	9.2 ± 3.2	4.9–19.0	0.623[a]
Age group 2 (20–40 years)	23	8.8 ± 4.1	2.1–17.7	33	8.8 ± 3.9	2.8–24.5	0.809[a]
Age group 3 (>40 years)	12	7.2 ± 3.1	4.3–15.4	15	8.3 ± 2.9	2.1–12.7	0.152[a]
15-F_{2t}-IsoP (ng/mg Cr)							
All ages (15–59 years)	44	0.93 ± 0.44	0.21–2.18	78	0.94 ± 0.57	0.19–2.98	0.924[b]
Age group 1 (15–19 years)	10	0.78 ± 0.48	0.28–1.46	29	1.14 ± 0.65	0.26–2.98	0.127[a]
Age group 2 (20–40 years)	21	1.00 ± 0.40	0.21–1.88	33	0.93 ± 0.49	0.19–2.35	0.608[b]
Age group 3 (>40 years)	13	0.98 ± 0.46	0.41–2.18	16	0.57 ± 0.39*,†,‡	0.22–1.61	0.016[b]
TBARS (μg/mg Cr)							
All ages (15–59 years)	65	0.77 ± 0.32	0.00–1.71	78	0.73 ± 0.35	0.00–2.36	0.457[a]
Age group 1 (15–19 years)	15	0.85 ± 0.37	0.46–1.71	29	0.81 ± 0.41	0.14–2.36	0.990[a]
Age group 2 (20–40 years)	31	0.70 ± 0.26	0.30–1.37	33	0.72 ± 0.26	0.00–1.41	0.861[b]
Age group 3 (>40 years)	19	0.80 ± 0.35	0.00–1.59	16	0.60 ± 0.36*	0.08–1.58	0.029[a]
AGEs (AFU/mg Cr)							
All ages (15–59 years)	61	134 ± 47	76–359	75	150 ± 53	64–324	0.063[a]
Age group 1 (15–19 years)	14	118 ± 33	76–188	29	143 ± 38*	64–234	0.034[a]
Age group 2 (20–40 years)	30	123 ± 25	81–175	31	143 ± 51	86–276	0.215[a]
Age group 3 (>40 years)	17	166 ± 68‡	105–359	15	178 ± 73	99–324	0.653[a]
DiTyr (AFU/mg Cr)							
All ages (15–59 years)	61	494 ± 149	249–1200	75	794 ± 526***	165–3877	<0.001[a]
Age group 1 (15–19 years)	14	512 ± 133	258–731	29	816 ± 479**	165–2473	0.004[a]
Age group 2 (20–40 years)	30	450 ± 106	302–659	31	745 ± 630***	259–3877	<0.001[a]
Age group 3 (>40 years)	17	559 ± 205	249–1200	15	850 ± 377**	349–1858	0.012[a]
H_2O_2 (μg/mg Cr)							
All ages (15–59 years)	58	0.39 ± 0.40	0.00–1.71	72	0.74 ± 0.69***	0.00–3.14	<0.001[a]
Age group 1 (15–19 years)	14	0.59 ± 0.55	0.09–1.71	29	0.91 ± 0.71	0.22–3.14	0.042[a]
Age group 2 (20–40 years)	29	0.33 ± 0.31	0.05–1.56	31	0.51 ± 0.41	0.00–1.39	0.046[a]
Age group 3 (>40 years)	15	0.33 ± 0.35	0.00–1.20	12	0.89 ± 1.03	0.01–2.78	0.152[a]
NOx (μg/mg Cr)							
All ages (15–59 years)	64	46 ± 34	14–186	74	81 ± 79***	18–503	<0.001[a]
Age group 1 (15–19 years)	15	50 ± 36	21–158	29	121 ± 112**	28–503	0.001[a]
Age group 2 (20–40 years)	31	38 ± 24	14–121	31	55 ± 27**,†	18–116	0.005[a]
Age group 3 (>40 years)	18	58 ± 45	16–186	14	58 ± 26†	26–119	0.377[a]

†,‡Indicate $P<0.05$ (significantly different) from the age group 1 and the age group 2, respectively.
*$P<0.05$, **$P<0.01$ and ***$P<0.001$ (significantly different) from the control group.
[a] By Mann–Whitney *U*-test.
[b] By Student's unpaired *t*-test.

Please cite this article as: C. Campos, et al., Evaluation of urinary biomarkers of oxidative/nitrosative stress in adolescents and adults with Down syndrome, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.03.013

3. Results and discussion

In the present study, a set of urinary biomarkers of oxidative and nitrosative stress in a sample of adolescents, adults and senescent subjects with DS, which comprise: 8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs, diTyr, H_2O_2 and NOx, has been analyzed. Clinical characteristics of DS and control subjects are summarized in Table 1. No significant differences in age were observed between patients with DS and controls for any age group ($P=0.369$ for age group 1, $P=0.623$ for age group 2, $P=0.678$ for age group 3 and $P=0.117$ for all ages). When the influence of gender was analyzed in DS and control groups, no significant differences were found for any parameter in any age group ($P>0.05$ for all). Besides, when subjects with Rb translocation or mosaicism were excluded similar results were obtained.

In Table 2 are listed the urinary levels of Cr, 8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs, diTyr, H_2O_2 and NOx in patients with DS and controls for all age groups. Table 3 and Fig. 1 show correlations between these variables and age.

3.1. 8-OHdG

8-OHdG is the most commonly used biomarker of oxidative DNA damage. We did not find differences in urinary levels of 8-OHdG between DS and controls for any age group (Table 2). These results agree with those obtained by Pallardó et al. [15] and Seidl et al. [16], studying 8-OHdG levels in leukocytes and in the cerebral cortex respectively. They found no significant differences between adults with DS and controls in accordance with our results. Moreover, we found no correlation with age neither in DS nor in controls (Table 4 and Fig. 1) as it has been reported in previous works using the ELISA assay of JaICA [17]. However, Jovanovic et al. [1] analyzed 8-OHdG by HPLC obtaining higher levels in urine samples of children with DS than controls. The results obtained in the present work, including the absence of correlation with age in DS and in controls, could be due to cross-reaction of the commercial ELISA assay of JaICA which we used, since urea and other substances contribute to the overestimation of urinary 8-OHdG [18]. Therefore, these results should be taken with caution and more studies are necessary in order to prove the suitability of the ELISA assays used for the DNA damage assessment. Since HPLC and other accurate techniques are currently limited in population studies, novel spectrophotometric assays must be developed for the accurate determination of 8-OHdG.

Table 3
Correlations between age and biochemical parameters in Down syndrome (DS) and their age-matched controls.

	Controls		DS	
Correlated variables with age	*r*	*P*	*r*	*P*
Cr				
All ages (14–59 years)	−0.207	0.098[a]	−0.093	0.418[a]
Age groups 2 and 3 (20–59 years)	−0.265	0.063[a]	−0.300	0.036[a]
8-OHdG				
All ages (14–59 years)	−0.204	0.179[b]	−0.050	0.667[b]
Age groups 2 and 3 (20–59 years)	−0.211	0.223[b]	−0.021	0.886[b]
15-F_{2t}-IsoP				
All ages (14–59 years)	0.147	0.342[a]	−0.328	0.003[a]
Age groups 2 and 3 (20–59 years)	−0.053	0.766[a]	−0.295	0.040[a]
TBARS				
All ages (14–59 years)	0.113	0.368[b]	−0.287	0.011[b]
Age groups 2 and 3 (20–59 years)	0.286	0.044[b]	−0.242	0.044[b]
AGEs				
All ages (14–59 years)	0.297	0.021[b]	0.114	0.339[b]
Age groups 2 and 3 (20–59 years)	0.326	0.025[b]	0.252	0.094[b]
DiTyr				
All ages (14–59 years)	0.106	0.420[b]	0.063	0.594[b]
Age groups 2 and 3 (20–59 years)	0.364	0.013[b]	0.375	0.010[b]
H_2O_2				
All ages (14–59 years)	−0.175	0.190[b]	−0.165	0.167[b]
Age groups 2 and 3 (20–59 years)	0.016	0.918[b]	0.239	0.123[b]
NOx				
All ages (14–59 years)	0.127	0.318[b]	−0.288	0.013[b]
Age groups 2 and 3 (20–59 years)	0.350	0.014[b]	0.171	0.261[b]

[a] Compared by Pearson's correlation coefficient analysis.
[b] Compared by Spearman's correlation coefficient analysis.

3.2. Lipid peroxidation biomarkers (15-F_{2t}-IsoP and TBARS)

Levels of lipid peroxidation biomarkers, 15-F_{2t}-IsoP and TBARS, were significantly lower in DS than in controls for age group 3 (Table 2) and both biomarkers correlated negatively with age in DS (Table 4 and Fig. 1). Possible explanations to these results include that altered levels of lipids and lipoproteins have been reported in DS [19,20]. Moreover, carbonyl reductase 1, which gene (*CBR1*) is located on HSA21, protects cells against lipid peroxidation [21] and is increased in DS individuals [22]. However, our findings will require further investigation. On the other hand, serum lipid resistance to oxidation has been found significantly higher in subjects with DS compared to controls [23] and a concomitant increase in serum uric acid was also observed by the same authors. In fact, Nyyssönen et al. [24] previously reported that ascorbate and urate are the strongest determinants of plasma lipid peroxidation resistance capacity. Although in a previous study of our research group with a representative sample of the population used in this work, no significant increase in urinary uric acid levels was found in adults with DS [4], Pallardó et al. [15] found increased levels of plasmatic uric acid in a group of adolescents and adults (from 15 to 57 years) with DS. Thus, we proposed as another possible explanation for our results that negative correlations with age in DS, resulting in lower levels of both biomarkers in DS than in controls for age group 3, could be the result of an increased lipid resistance to oxidation in DS due to higher levels of uric acid in blood. It has been recently reported, however, that plasma lipid resistance to oxidation is not significantly different in DS than in non-Down subjects [25], although the correlation between uric acid and plasma lipid resistance to oxidation has not been studied in this work and further studies are clearly needed in this area.

One major problem with TBARS assay is the fact that it is performed in numerous variations making comparisons of results between laboratories difficult. Moreover, much controversy has appeared in studies concerning the specificity of TBARS towards composites other than MDA. In fact, results in DS have indicated conflicting results. In a similar manner, commercially available ELISA assays against 15-F_{2t}-IsoP have not been tested for cross reactivity with most isoeicosanoids and most of their metabolites so they are of uncertain quantitative reliability in biological fluids. Hence, although chromatographic techniques are currently limited in population studies, they might provide better results.

3.3. AGEs

High levels of AGEs occur in diabetes, cataracts and neurodegenerative diseases, such as Alzheimer disease, and all of them occur more frequently in DS. It has been reported that oxidative stress is involved in the accelerated formation of AGEs, which contribute to lower retardation, reduced motor activity and decreased immune response, meaning an accelerated aging process [26]. AGEs also depress superoxide production by stimulated polymorphonuclear leukocytes [27] and reduce SOD1 function. Therefore, AGEs could play an important role in the oxidative status of DS patients.

In this study, we found significant differences in urinary levels of AGEs only between adolescents, from 15 to 19 years, with DS and

Please cite this article as: C. Campos, et al., Evaluation of urinary biomarkers of oxidative/nitrosative stress in adolescents and adults with Down syndrome, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.03.013

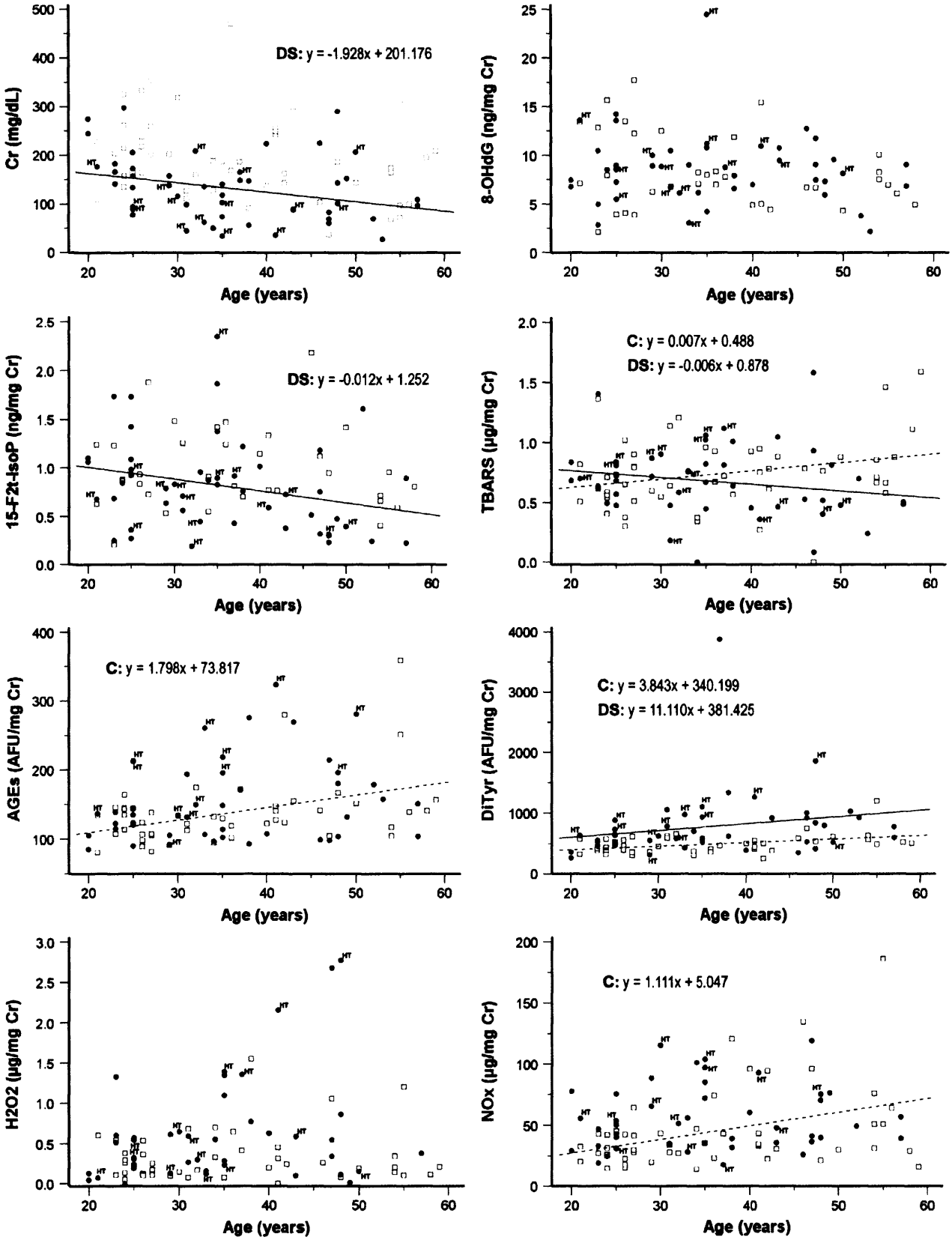


Fig. 1. Scatter plots of Cr, 8-OHdG/Cr, 15-F_{2t}-IsoP/Cr, TBARS/Cr, AGEs/Cr, diTyr/Cr, H_2O_2/Cr and NOx/Cr vs. age in Down syndrome (DS) and controls (C) in the age groups 2 and 3 (from 20 to 59 years). Correlation lines are shown for DS (●, solid lines) and C (□, dashed lines). Individuals with DS receiving levothyroxine for hypothyroidism treatment are identified in each graph as HT.

Please cite this article as: C. Campos, et al., Evaluation of urinary biomarkers of oxidative/nitrosative stress in adolescents and adults with Down syndrome, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.03.013

controls (Table 2). Since differences in Cr between DS and controls are the highest in this age group, Cr adjustment could bias the results. Moreover, positive correlation between AGEs and age was found only in the control group (Table 4 and Fig. 1). By all of them, urinary AGEs measured with the wide used fluorescence assay of Yanagisawa et al. [11] do not seem to be a suitable biomarker of oxidative stress in DS. Pallardó et al. [15] measured glyoxal and methylglyoxal levels in plasma samples of DS patients and found higher levels of glyoxal in DS children than in controls and no significant differences in adolescents and adults group. Opposite to glyoxal, plasma levels of methylglyoxal were significantly lower in adolescents and adults with DS than controls and no significant differences were reported in children. Thus, different types of AGEs instead of the overall AGEs of a sample must be analyzed with more specific techniques in DS patients, in a wider age range and comparing plasma and urine samples in order to clarify the usefulness of a specific AGE as a biomarker of oxidative stress in DS.

3.4. DiTyr

DiTyr, also *o,o'*-dityrosine, is a fluorescent molecule formed as a result of normal posttranslational processes affecting specific structural proteins. Tyrosine is oxidized to diTyr in response to oxidative stress by peroxidase-catalyzed mechanisms, being found in a number of structural (lysozyme, calmodulin, myoglobin, and hemoglobin) and non-structural proteins (insulin, RNase, and chymotrypsin) [28]. Moreover, tyrosine dimerization as well as nitration can be affected by $ONOO^-$ (a powerful oxidant and nitrating agent), which can be formed by the reaction between active NO metabolites and superoxide [29]. Therefore, diTyr could be considered as a biomarker of both oxidative and nitrosative stress. In fact, it has been used as an important biomarker for oxidatively modified proteins during UV and γ-irradiation, aging, and exposure to oxygen free radicals, nitrogen dioxide, peroxynitrite, and lipid hydroperoxides [30], and has been

Table 4
Urinary levels of Cr and biomarkers of oxidative and nitrosative stress (8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs, diTyr, H_2O_2 and NOx) in patients with Down syndrome (DS) who were receiving levothyroxine for hypothyroidism and those without hypothyroidism diagnosed. The data are expressed as mean ± standard deviation (SD) and ranges.

Urinary biomarker	DS without hypothyroidism			DS with treated hypothyroidism			$P^{\#}$
	n	Mean ± SD	Range	*n*	Mean ± SD	Range	
Cr (mg/dL)							
All ages (15–59 years)	54	138 ± 65	27–298	24	102 ± 51*	25–209	0.026
Age group 1 (15–19 years)	20	133 ± 57	45–276	9	88 ± 36*	25–164	0.038
Age group 2 (20–40 years)	22	155 ± 66	50–298	11	112 ± 56	34–209	0.086
Age group 3 (>40 years)	12	118 ± 75	27–290	4	108 ± 72	35–207	0.903
8-OHdG (ng/mg Cr)							
All ages (15–59 years)	54	8.8 ± 3.2	2.1–19.0	23	9.0 ± 4.1	3.0–24.5	0.781
Age group 1 (15–19 years)	20	9.8 ± 3.6	4.9–19.0	9	8.0 ± 1.8	5.7–11.5	0.220
Age group 2 (20–40 years)	22	8.4 ± 2.7	2.8–14.2	11	9.7 ± 5.7	3.0–24.5	0.819
Age group 3 (>40 years)	12	8.0 ± 3.1	2.1–12.7	3	9.5 ± 1.4	8.1–10.9	0.386
15-F_{2t}-IsoP (ng/mg Cr)							
All ages (15–59 years)	54	0.95 ± 0.56	0.22–2.98	24	0.90 ± 0.60	0.19–2.78	0.458
Age group 1 (15–19 years)	20	1.13 ± 0.65	0.26–2.98	9	1.16 ± 0.68	0.46–2.78	0.962
Age group 2 (20–40 years)	22	0.98 ± 0.45	0.25–1.87	11	0.83 ± 0.56	0.19–2.35	0.169
Age group 3 (>40 years)	12	0.60 ± 0.44	0.22–1.61	4	0.50 ± 0.19	0.30–0.73	0.903
TBARS (μg/mg Cr)							
All ages (15–59 years)	54	0.51 ± 0.30	0.00–1.58	24	0.76 ± 0.44	0.14–2.36	0.622
Age group 1 (15–19 years)	20	0.79 ± 0.28	0.34–1.50	9	0.86 ± 0.64	0.14–2.36	0.944
Age group 2 (20–40 years)	22	0.67 ± 0.26	0.00–1.41	11	0.80 ± 0.26*	0.18–1.12	0.047
Age group 3 (>40 years)	12	0.65 ± 0.40	0.08–1.58	4	0.43 ± 0.06	0.36–0.48	0.090
AGEs (AFU/mg Cr)							
All ages (15–59 years)	53	138 ± 45	64–276	22	179 ± 60**	93–324	0.003
Age group 1 (15–19 years)	20	140 ± 39	64–205	9	152 ± 37	116–234	0.696
Age group 2 (20–40 years)	21	129 ± 43	86–276	10	175 ± 53*	93–261	0.014
Age group 3 (>40 years)	12	154 ± 55	99–270	3	267 ± 65*	196–324	0.022
DiTyr (AFU/mg Cr)							
All ages (15–59 years)	53	721 ± 508	165–3877	22	969 ± 538**	309–2473	0.008
Age group 1 (15–19 years)	20	678 ± 267	165–1208	9	1122 ± 694*	557–2473	0.034
Age group 2 (20–40 years)	21	740 ± 756	259–3877	10	757 ± 231*	309–1103	0.043
Age group 3 (>40 years)	12	759 ± 232	349–1032	3	1214 ± 670	520–1858	0.248
H_2O_2 (μg/mg Cr)							
All ages (15–59 years)	48	0.66 ± 0.63	0.00–3.14	24	0.89 ± 0.78	0.07–2.78	0.239
Age group 1 (15–19 years)	20	0.82 ± 0.71	0.22–3.14	9	1.12 ± 0.73	0.44–2.27	0.131
Age group 2 (20–40 years)	20	0.51 ± 0.40	0.00–1.39	11	0.52 ± 0.45	0.07–1.36	0.967
Age group 3 (>40 years)	8	0.63 ± 0.87	0.01–2.68	4	1.42 ± 1.25	0.15–2.78	0.174
NOx (μg/mg Cr)							
All ages (15–59 years)	52	77 ± 81	19–503	22	91 ± 76	18–384	0.119
Age group 1 (15–19 years)	20	117 ± 118	28–503	9	129 ± 104	47–384	0.396
Age group 2 (20–40 years)	21	51 ± 24	19–101	10	62 ± 34	18–116	0.473
Age group 3 (>40 years)	11	54 ± 27	26–119	3	72 ± 23	48–93	0.186

*$P<0.05$ and **$P<0.01$ (significantly different) from the control group.
# Indicates *P*-value obtained in the comparisons between DS and controls by Mann–Whitney *U*-test.

110

Please cite this article as: C. Campos, et al., Evaluation of urinary biomarkers of oxidative/nitrosative stress in adolescents and adults with Down syndrome, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.03.013

implicated in a variety of physiologic and pathologic processes. However, urinary levels of diTyr have been previously measured neither in adolescents nor in adults with DS.

Our results showed significantly higher levels of this biomarker in DS patients than in controls for all age groups. Moreover, positive correlation with age was found in DS and in controls from 20 years old (Table 4 and Fig. 1). Therefore, urinary diTyr could be considered as biomarker of oxidative stress in adolescents and adults with DS and the methodology for its determination is fast, simple and inexpensive to perform.

3.5. H_2O_2

Urinary H_2O_2 was postulated to be a biomarker of oxidative stress [31]. An excess of H_2O_2 is expected in DS patients due to over-expression of SOD1, however urinary levels of this biomolecule have been previously measured neither in adolescents nor in adults with DS. Our results demonstrate that DS patients excreted more H_2O_2 in urine than healthy controls aged from 15 to 40 years. However, we found a large interindividual variation in urine H_2O_2 concentrations, similar to what occurred in previous works [32], and it may bias the results. Thus, a lack of correlation between H_2O_2 and age was found in both DS and control groups (Table 4). Moreover, no significant differences in H_2O_2 levels have been found between both groups in age group 3 (Table 2) probably due to the large biological variation, which limits the usefulness of urinary H_2O_2 as a biomarker of oxidative stress. In addition, it has been reported that drinking coffee, exercise and salt loading affect H_2O_2 excretion [31]. Therefore, urinary levels of H_2O_2 could be more determined by metabolic and nutritional influences than by changes in oxidative stress. In summary, our results suggest that urinary H_2O_2 could be a suitable biomarker of oxidative stress in DS, as it has also been proposed for malignancy [33], although more studies are needed to explore the causes of its biological variation.

3.6. Nitrite and nitrate (NOx)

Nitric oxide (NO) is a free radical gas which has been involved in a wide range of physiological functions, including the modulation of renal hemodynamics and excretory function [34]. Since NO rapidly degrades to nitrite and nitrate (NOx), which are relatively slowly excreted in urine, urinary levels of NOx were estimated as an index of NO production. Although neurodegeneration in DS has been associated with aberrant expression of nitric oxide synthase 3 gene [35] and nitrosative stress is linked to oxidative stress, biomarkers of nitrosative stress have been little studied in DS.

In the present work we found higher levels of NOx in adolescents and adults (from 15 to 40 years) with DS than in the age-matched controls (Table 2). Besides, correlation of NOx with age was found negative in DS from 14 years and positive in controls from 20 years (Table 4). It has been reported that the tendency of urinary nitrate levels to decrease with age in healthy children may be a reflection of an age-related increase in glomerular filtration rate [36]. Therefore, glomerular filtration rate could be impaired in DS as it is also shown by the differences in Cr levels (Table 2), being urinary levels of NOx in DS more influenced by urinary excretion than by oxidative status contrary to what was observed in controls.

It must be taken into account that active NO metabolites can react with superoxide to form peroxynitrite ($ONOO^-$) a powerful oxidant and nitrating agent. Tyrosine dimerization as well as nitration can be affected by $ONOO^-$, so the same oxidant could be responsible for the accumulation of both 3-NO_2-Tyr (3-nitrotyrosine) and diTyr [29]. Therefore, although NOx is a widely used biomarker of nitrosative stress, 3-nitrotyrosine and diTyr would have been better indicators of nitrosative damage.

3.7. Analysis of hypothyroidism in DS

Hypothyroidism is common in adults with DS [37], being the frequency of patients with DS treated with levothyroxine in the present work of 31%. A decrease in free radical production is expected in hypothyroidism due to the metabolic suppression brought about by the decreased levels of thyroid hormone, however data are controversial and several works reported increased oxidative stress in hypothyroidism [38]. In the present study, patients with DS receiving levothyroxine for hypothyroidism treatment showed increased levels of TBARS for age group 2, increased levels of AGEs for age groups 2 and 3 and increased levels of diTyr for age groups 1 and 2 than those without hypothyroidism diagnosed (Table 4). However, impaired renal function has been reported in patients with hypothyroidism [39]. It enhances serum Cr levels because it reduces glomerular filtration rate and increases production of Cr. Our results showed lower levels of urinary Cr in DS patients receiving levothyroxine than DS without hypothyroidism diagnosed for age group 1, being levels in the rest of age groups slightly decreased in DS patients receiving levothyroxine although without significant differences (Table 4). Therefore, renal impairment due to hypothyroidism may bias the results in these patients.

Signs and symptoms of hypothyroidism can be difficult to discriminate from those found in the natural course of DS itself. Moreover, untreated subclinical hypothyroidism is present in DS at birth and persists throughout life [40]. Some abnormalities reported in DS are related to thyroid function: *1*) decreased levels of selenium [41] which acts as an antioxidant protecting the thyrocyte from peroxides, *2*) an impairment in the activity of phenylalanine hydroxylase [42], which converts the phenylalanine in tyrosine, and 3) overexpression of DYRK1A kinase [43], which could reduce the availability of tyrosine. Hence, underlying factors may influence thyroid function in DS. For all these reasons, it could be possible that a group of DS patients without thyroid dysfunction diagnosed but having untreated subclinical hypothyroidism are biasing the results, in the present study and maybe in others researching clinical aspects of DS.

3.8. Analysis of Cr adjustment

Renal impairment has been described in DS based on decreased Cr clearance [44]. Interestingly, lower levels of urinary Cr have been found in DS than in controls in all age groups (Table 2) and similar results were obtained in DS vs. controls comparisons for all the variables studied when patients with hypothyroidism were excluded. Besides, negative correlation between Cr and age was found in DS patients (Table 4 and Fig. 1), so a decline in urinary excretion with aging could occur in DS patients. In fact, with increasing survival, an increasing number of DS patients with renal disease are being reported [45]. In summary, Cr used for standardized urinary levels of biomarkers studied may bias the results, as it has been discussed above for all the biomarkers studied, and it could be due to hypothyroidism in DS.

3.9. Post-hoc comparisons between age groups

Post-hoc comparisons between age groups for both DS and control groups are shown in Table 2. Significant differences were found in urinary AGEs between age groups 2 and 3 ($P=0.032$) in controls. In DS, significant differences were found in urinary 15-F_{2t}-IsoP between age groups 1 and 3 ($P=0.004$) and between age groups 2 and 3 ($P=0.042$), and also in urinary NOx between age groups 1 and 2 ($P=0.005$) and between age groups 1 and 3 ($P=0.035$). These results are in accordance with correlation results. However, we found some correlations between urinary parameters with age, but no significant differences were found in post-hoc comparisons between age groups in both DS and control groups. Further studies must be performed

Please cite this article as: C. Campos, et al., Evaluation of urinary biomarkers of oxidative/nitrosative stress in adolescents and adults with Down syndrome, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.03.013

before age-related changes in urinary biomarkers of oxidative/nitrosative stress can be established.

3.10. Future approaches

Previous studies addressing biomarkers of oxidative stress in DS have shown conflicting results. Methodological aspects are not usually taken into account in the approach to discussion. Moreover, since oxidative conditions have been suggested to be of importance in developmental and maturational processes, biomarkers of oxidative stress may need to be differently interpreted in childhood and adulthood. Nonetheless, we can found several works studying them in disease states, including DS, in which age groups are formed by both children and adolescents or even children and adults, so discrepancies in results are frequently found.

On the other hand, adults with DS over 40 years are under higher risk for dementia. However, the diagnosis of Alzheimer disease in people with DS is far from an easy task and oxidative damage may increase as a consequence of this disease process in addition to aging, biasing the results. Improvements in this area are required before the causes of increased oxidative stress in senescent DS subjects can be well established.

DS subjects have a high prevalence to have or develop hypothyroidism. In our study, we found clinically important changes in hypothyroid DS patients taking levothyroxine compared to those without hypothyroidism diagnosed, suggesting that medical treatment does not normalize alterations caused by thyroid dysfunction in these subjects. Hence, thyroid hormones levels must be assessed together with oxidative stress biomarkers in future works, even more when DS subjects can suffer from untreated subclinical hypothyroidism.

In our study, we obtained *P*-values higher than the conventional cut-off of 0.05 but close to this value in several of the between-group comparisons. Such data are *suggestive* of an effect. Thus, there could be some results of clinical relevance, although no statistical differences have been obtained (type II error). Moreover, high variability in urinary levels of all biomarkers was obtained. Sample size and variability are important primarily because of its effect on statistical power. Hence, statistical power may be limited not only in subgroup analyses, but also by the statistical variability. Therefore, to assess urinary biomarkers of oxidative/nitrosative stress in a large number of adolescent and/or adult individuals with DS must be a major goal in future works.

Individuals with DS could suffer from renal impairment, leading to a diminished excretion of metabolites such as oxidative/nitrosative stress biomarkers. Therefore, a comparative study assessing a comprehensive set of these biomarkers in both urine and plasma or serum samples must be performed in DS to clarify this hypothesis.

Finally, potential biasing factors, such as dietary or exercise habits, exposure to pollutants, etc., must be considered in future works studying oxidative/nitrosative stress biomarkers in DS. In fact, in our study most individuals with DS practiced moderate physical activity weekly. Therefore, improvements in life patterns of DS individuals could contribute to reduce oxidative stress, biasing the results.

In summary, similar studies in a larger number of DS subjects, in which thyroid dysfunctions have been described and performed in various biological matrices, such as serum, plasma and urine, and where potential biasing factors, such as dietary or exercise habits, exposure to pollutants, etc., are considered, would help to clarify the role of oxidative stress in DS phenotype and subsequently devising intervention strategies which improve the quality of life of these patients.

4. Conclusion

This work shows that AGEs, diTyr, H_2O_2 and NOx could be used as biomarkers of oxidative/nitrosative stress in urine samples of adolescents and adults with DS in contrast to 8-OHdG, 15-F_{2t}-IsoP and TBARS, at least with the methods described in the present study. However, intraindividual variability was high for most of the variables studied, mainly in DS patients. Besides, the present work shows evidence of a probable renal impairment in DS and Cr adjustment may bias the results, particularly in hypothyroid patients.

Acknowledgements

The authors would like to thank all the people with Down syndrome, families and volunteers which cooperated and made the achievement of this work possible. The authors express their gratitude to Fundación Síndrome de Down de Madrid, Centro María Corredentora, Asociación de Empleados de IBERIA Padres de Minusválidos, Fundación Nuestra Señora del Camino, C.P.E.E. Príncipe de Asturias (Aranjuez), C.P.E.E. Francisco del Pozo de Madrid, C.C.E.E. Buenafuente de Madrid, and to Hospital Niño Jesús de Madrid, for their valuable contributions to this work.

References

[1] S.V. Jovanovic, D. Clements, K. MacLeod, Biomarkers of oxidative stress are significantly elevated in Down syndrome, Free Radic. Biol. Med. 25 (1998) 1044–1048.
[2] N.J. Roizen, D. Patterson, Down's syndrome, Lancet 361 (2003) 1281–1289.
[3] A. Strydom, M.J. Dickinson, S. Shende, D. Pratico, Z. Walker, Oxidative stress and cognitive ability in adults with Down syndrome, Prog. Neuropsychopharmacol. Biol. Psychiatry 33 (2009) 76–80.
[4] C. Campos, R. Guzmán, E. López-Fernández, A. Casado, Urinary uric acid and antioxidant capacity in children and adults with Down syndrome, Clin. Biochem. 43 (2010) 228–233.
[5] N.A. Meguid, A.A. Dardir, E.M. El-Sayed, H.H. Ahmed, A.F. Hashish, A. Ezzat, Homocysteine and oxidative stress in Egyptian children with Down syndrome, Clin. Biochem. 43 (2010) 963–967.
[6] J.D. Morrow, L.J. Roberts, The isoprostanes: unique bioactive products of lipid peroxidation, Prog. Lipid Res. 36 (1997) 1–21.
[7] A.H. Bittles, C. Bower, R. Hussain, E.J. Glasson, The four ages of Down syndrome, Eur. J. Public Health 17 (2007) 221–225.
[8] M. Jaffé, Über den niederschlag, welchen pikrinsäure in normalen harn erzeugt und über eine neue reaktion des kreatinins, Z. Physiol. Chem. 10 (1886) 391–400.
[9] H. Varley, A.H. Gowenlock, M. Bell, Practical Clinical Biochemistry, 5th ed. Heinemann Medical Books, London, 1980.
[10] M. Uchiyama, M. Mihara, Determination of malonaldehyde precursor in tissues by thiobarbituric acid test, Anal. Biochem. 86 (1978) 271–278.
[11] K. Yanagisawa, Z. Makita, K. Shiroshita, T. Ueda, T. Fusegawa, S. Kuwajima, M. Takeuchi, T. Koike, Specific fluorescence assay for advanced glycation end products in blood and urine of diabetic patients, Metabolism 47 (1998) 1348–1353.
[12] V. Witko-Sarsat, M. Friedlander, C. Capeillere-Blandin, T. Nguyen-Khoa, A.T. Nguyen, J. Zingraff, P. Jungers, B. Descamps-Latscha, Advanced oxidation protein products as a novel marker of oxidative stress in uremia, Kidney Int. 49 (1996) 1304–1313.
[13] D. Banerjee, A.P. Kumar, B. Kumar, U.K. Madhusoodanan, S. Nayak, J. Jacob, Determination of absolute hydrogen peroxide concentration by spectrophotometric method, Curr. Sci. 83 (2002) 1193–1194.
[14] K.M. Miranda, M.G. Espey, D.A. Wink, A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite, Nitric Oxide 5 (2001) 62–71.
[15] F.V. Pallardó, P. Degan, M. d'Ischia, F.J. Kelly, A. Zatterale, R. Calzone, Multiple evidence for an early age pro-oxidant state in Down syndrome patients, Biogerontology 7 (2006) 211–220.
[16] R. Seidl, S. Greber, E. Schuller, G. Bernert, N. Cairns, G. Lubec, Evidence against increased oxidative DNA-damage in Down syndrome, Neurosci. Lett. 235 (1997) 137–140.
[17] M. Honda, Y. Yamada, M. Tomonaga, H. Ichinose, S. Kamihira, Correlation of urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG), a biomarker of oxidative DNA damage, and clinical features of hematological disorders: a pilot study, Leuk. Res. 24 (2000) 461–468.
[18] M.-F. Song, Y.-S. Li, Y. Ootsuyama, H. Kasai, K. Kawai, M. Ohta, Y. Eguchi, H. Yamato, Y. Matsumoto, R. Yoshida, Y. Ogawa, Urea, the most abundant component in urine, cross-reacts with a commercial 8-OH-dG ELISA kit and contributes to overestimation of urinary 8-OH-dG, Free Radic. Biol. Med. 47 (2009) 41–46.
[19] S.M. Pueschel, W.Y. Craig, J.E. Haddow, Lipids and lipoproteins in persons with Down's syndrome, J. Intellect. Disabil. Res. 36 (1992) 365–369.
[20] E.J. Murphy, M.B. Schapiro, S.I. Rapoport, H.U. Shetty, Phospholipid composition and levels are altered in Down syndrome brain, Brain Res. 867 (2000) 9–18.
[21] U. Oppermann, Carbonyl reductases: the complex relationships of mammalian carbonyl- and quinone-reducing enzymes and their role in physiology, Annu. Rev. Pharmacol. Toxicol. 47 (2007) 293–322.
[22] B. Balcz, L. Kirchner, N. Cairns, M. Fountoulakis, G. Lubec, Increased brain protein levels of carbonyl reductase and alcohol dehydrogenase in Down syndrome and Alzheimer's disease, J. Neural Transm. Suppl. 61 (2001) 193–201.
[23] A. Nagyova, M. Sustrova, K. Raslova, Serum lipid resistance to oxidation and uric acid levels in subjects with Down's syndrome, Physiol. Res. 49 (2000) 227–231.

Please cite this article as: C. Campos, et al., Evaluation of urinary biomarkers of oxidative/nitrosative stress in adolescents and adults with Down syndrome, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.03.013

[24] K. Nyyssonen, E. Porkkala-Sarataho, J. Kaikkonen, J.T. Salonen, Ascorbate and urate are the strongest determinants of plasma antioxidative capacity and serum lipid resistance to oxidation in Finnish men, Atherosclerosis 130 (1997) 223–233.
[25] L. Tiano, L. Padella, P. Carnevali, O. Gabrielli, F. Bruge, F. Principi, G.P. Littarru, Coenzyme Q10 and oxidative imbalance in Down syndrome: biochemical and clinical aspects, Biofactors 32 (2008) 161–167.
[26] X. Song, M. Bao, D. Li, Y.M. Li, Advanced glycation in d-galactose induced mouse aging model, Mech. Ageing Dev. 108 (1999) 239–251.
[27] J. Bernheim, G. Rashid, R. Gavrieli, Z. Korzets, B. Wolach, In vitro effect of advanced glycation end-products on human polymorphonuclear superoxide production, Eur J Clin Invest 31 (2001) 1064–1069.
[28] T. DiMarco, C. Giulivi, Current analytical methods for the detection of dityrosine, a biomarker of oxidative stress, in biological samples, Mass Spectrom. Rev. 26 (2007) 108–120.
[29] K. Hensley, M.L. Maidt, Z. Yu, H. Sang, W.R. Markesbery, R.A. Floyd, Electrochemical analysis of protein nitrotyrosine and dityrosine in the Alzheimer brain indicates region-specific accumulation, J. Neurosci. 18 (1998) 8126–8132.
[30] C. Giulivi, N.J. Traaseth, K.J.A. Davies, Tyrosine oxidation products: analysis and biological relevance, Amino Acids 25 (2003) 227–232.
[31] J.W. Yuen, I.F. Benzie, Hydrogen peroxide in urine as a potential biomarker of whole body oxidative stress, Free Radic. Res. 37 (2003) 1209–1213.
[32] B. Halliwell, L.H. Long, T.P. Yee, S. Lim, R. Kelly, Establishing biomarkers of oxidative stress: the measurement of hydrogen peroxide in human urine, Curr. Med. Chem. 11 (2004) 1085–1092.
[33] D. Banerjee, U.K. Madhusoodanan, S. Nayak, J. Jacob, Urinary hydrogen peroxide: a probable marker of oxidative stress in malignancy, Clin. Chim. Acta 334 (2003) 205–209.
[34] T. Mizutani, A.J. Layon, Clinical applications of nitric oxide, Chest 110 (1996) 506–524.
[35] Y.K. Sohn, N. Ganju, K.D. Bloch, J.R. Wands, S.M. de la Monte, Neuritic sprouting with aberrant expression of the nitric oxide synthase III gene in neurodegenerative diseases, J. Neurol. Sci. 162 (1999) 133–151.
[36] M. Elli, O. Soylemezoglu, D. Erbas, S.A. Bakkaloglu, N. Buyan, O. Ozkaya, E. Hasanoglu, Plasma and urine nitric oxide levels in healthy Turkish children, Pediatr. Nephrol. 20 (2005) 1605–1609.
[37] J.C. Murdoch, W.A. Ratcliffe, D.G. McLarty, J.C. Rodger, J.G. Ratcliffe, Thyroid function in adults with Down's syndrome, J. Clin. Endocrinol. Metab. 44 (1977) 453–458.
[38] E. Sarandöl, S. Taş, M. Dirican, Z. Serdar, Oxidative stress and serum paraoxonase activity in experimental hypothyroidism: effect of vitamin E supplementation, Cell Biochem. Funct. 23 (2005) 1–8.
[39] J. Montenegro, O. González, R. Saracho, R. Aguirre, O. González, I. Martínez, Changes in renal function in primary hypothyroidism, Am. J. Kidney Dis. 27 (1996) 195–198.
[40] V. Prasher, M.S. Haque, Misdiagnosis of thyroid disorders in Down syndrome: time to re-examine the myth? Am. J. Ment. Retard. 110 (2005) 23–27.
[41] J. Nève, P.M. Sinet, L. Molle, A. Nicole, Selenium, zinc and copper in Down's syndrome (trisomy 21): blood levels and relations with glutathione peroxidase and superoxide dismutase, Clin. Chim. Acta 133 (1983) 209–214.
[42] A.M. Shaposhnikov, S.E. Khal'chitskii, E.I. Shvarts, Disorders of phenylalanine and tyrosine metabolism in Down's syndrome, Vopr. Med. Khim. 25 (1979) 15–19.
[43] W.K. Dowjat, T. Adayev, I. Kuchna, K. Nowicki, S. Palminiello, Y.W. Hwang, J. Wegiel, Trisomy-driven overexpression of DYRK1A kinase in the brain of subjects with Down syndrome, Neurosci. Lett. 413 (2007) 77–81.
[44] S.P. Coburn, M. Seidenberg, E.T. Mertz, Clearance of uric acid, urea, and creatinine in Down's syndrome, J. Appl. Physiol. 23 (1967) 579–580.
[45] A. Lo, H.G. Brown, B.A. Fivush, A.M. Neu, L.C. Racusen, Renal disease in Down syndrome: autopsy study with emphasis on glomerular lesions, Am. J. Kidney Dis. 31 (1998) 329–335.

Please cite this article as: C. Campos, et al., Evaluation of urinary biomarkers of oxidative/nitrosative stress in adolescents and adults with Down syndrome, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.03.013

antioxidante del ácido úrico

C. Campos, R. Guzmán, E. López-Fernández y Á. Casado

La presente invención se refiere a un método para valorar la concentración y la capacidad antioxidante del ácido úrico (AU) en muestras ya sean biológicas, procedentes de la industria alimentaria, etc. Además mediante la presente invención se permite determinar simultáneamente la capacidad antioxidante total (TAC) y la capacidad antioxidante total sin la contribución relativa del ácido úrico (TAC^{-AU}) de una muestra. El desarrollo de esta metodología surge tras el estudio del AU y la TAC urinarios en individuos con SD, cuyo trabajo se presenta como parte de esta Tesis Doctoral.

El conocimiento de los efectos nocivos de los radicales libres y especies reactivas de oxígeno ha propiciado un interés creciente en la valoración del estatus antioxidante de los individuos en estudios experimentales, epidemiológicos y clínicos, así como de la capacidad antioxidante de determinadas sustancias, bebidas y alimentos. Por ello, se han desarrollado multitud de metodologías para la determinación de la TAC, tales como el FRAP (ensayo de capacidad del plasma de reducir el ión férrico), el TEAC (ensayo de capacidad antioxidante equivalente de Trolox) o el CUPRAC (ensayo de reducción del cobre(II) o capacidad antioxidante de reducción del ión cúprico) entre otros.

Debido a la dificultad de medir todos los antioxidantes de una muestra por separado, esta determinación es muy útil por ser los resultados obtenidos más representativos de la TAC que la determinación de todos los antioxidantes de la muestra de forma individual, ya que considera las posibles interacciones entre los distintos antioxidantes presentes. Además, la determinación de la TAC es mucho más sencilla que la determinación de todos los antioxidantes de la muestra por separado.

Sin embargo, el AU, principal antioxidante presente en muestras biológicas, suele estar en una concentración muy superior a la del resto de antioxidantes y presenta una contribución relativa muy elevada en la TAC. De hecho, la TAC correlaciona altamente con los niveles de AU presentes en las muestras biológicas (plasma, orina, etc.). Ya que los niveles de AU están alterados en multitud de patologías, también después de realizar ejercicio físico o durante la isquemia, los resultados de TAC obtenidos con los métodos actuales pueden llevar a resultados inesperados y a errores de interpretación. Por ello, la determinación de la TAC^{-AU} es una determinación mucho más relevante para el estudio del estatus antioxidante y el estrés oxidativo en estudios experimentales, epidemiológicos o clínicos que la proporcionada por los métodos actuales de determinación de TAC, al menos en individuos con SD.

MINISTERIO
DE INDUSTRIA, TURISMO
Y COMERCIO

Oficina Española
de Patentes y Marcas

Justificante de presentación electrónica de solicitud de patente

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica, utilizando la conexión segura de la O.E.P.M. Asimismo, se le ha asignado de forma automática un número de solicitud y una fecha de recepción, conforme al artículo 14.3 del Reglamento para la ejecución de la Ley 11/1986, de 20 de marzo, de Patentes. La fecha de presentación de la solicitud de acuerdo con el art. 22 de la Ley de Patentes, le será comunicada posteriormente.

Número de solicitud:	P201030674	
Fecha de recepción:	05 mayo 2010, 16:21 (CEST)	
Oficina receptora:	OEPM Madrid	
Su referencia:	ES1641.757	
Solicitante:	CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS (CSIC)	
Número de solicitantes:	1	
País:	ES	
Título:	MÉTODO PARA VALORAR LA CONCENTRACIÓN Y LA CAPACIDAD ANTIOXIDANTE DEL ÁCIDO ÚRICO	
Documentos enviados:	Descripcion.pdf (14 p.) Reivindicaciones-1.pdf (3 p.) Dibujos-1.pdf (2 p.) Resumen-1.pdf (1 p.) FEERCPT-1.pdf (1 p.)	package-data.xml es-request.xml application-body.xml es-fee-sheet.xml feesheet.pdf request.pdf
Enviados por:	CN=ENTIDAD PONS CONSULTORES DE PROPIEDAD INDUSTRIAL SA - CIF A28750891 - NOMBRE PONS ARIÑO ANGEL - NIF 50534279J,OU=703015345,OU=fnmt clase 2 ca,O=FNMT,C=es	
Fecha y hora de recepción:	05 mayo 2010, 16:22 (CEST)	
Codificación del envío:	D9:28:51:FD:9C:22:C1:2A:64:10:39:1C:AC:4F:2D:8C:D8:19:2E:9F	

/Madrid, Oficina Receptora/

RESULTADOS ADICIONALES

En este apartado se comentan resultados que no han sido publicados pero son necesarios para complementar la discusión de los artículos previamente mostrados.

1. Ácido úrico (AU), capacidad antioxidante total (TAC) y capacidad antioxidante total sin la contribución relativa del ácido úrico (TAC^{-AU})

En la Figura 12 se muestran los niveles urinarios de AU, TAC y TAC^{-AU} en SD y controles por grupos de edad. Así, se analizaron cuatro grupos de edad: *1*) 3-14 años (infancia), *2*) 15-19 años (adolescencia), *3*) 20-40 años (adultez) y *4*) 41-59 años (senescencia). El número de individuos en cada grupo fue: 24 SD y 17 controles en el grupo *1*; 29 SD y 15 controles en el grupo *2*; 33 SD y 31 controles en el grupo *3*; y 16 SD y 19 controles en el grupo *4*. Las valoraciones de TAC y de TAC^{-AU} fueron realizadas con el método evaluado y optimizado en el presente trabajo (el CUPRAC-BCS).

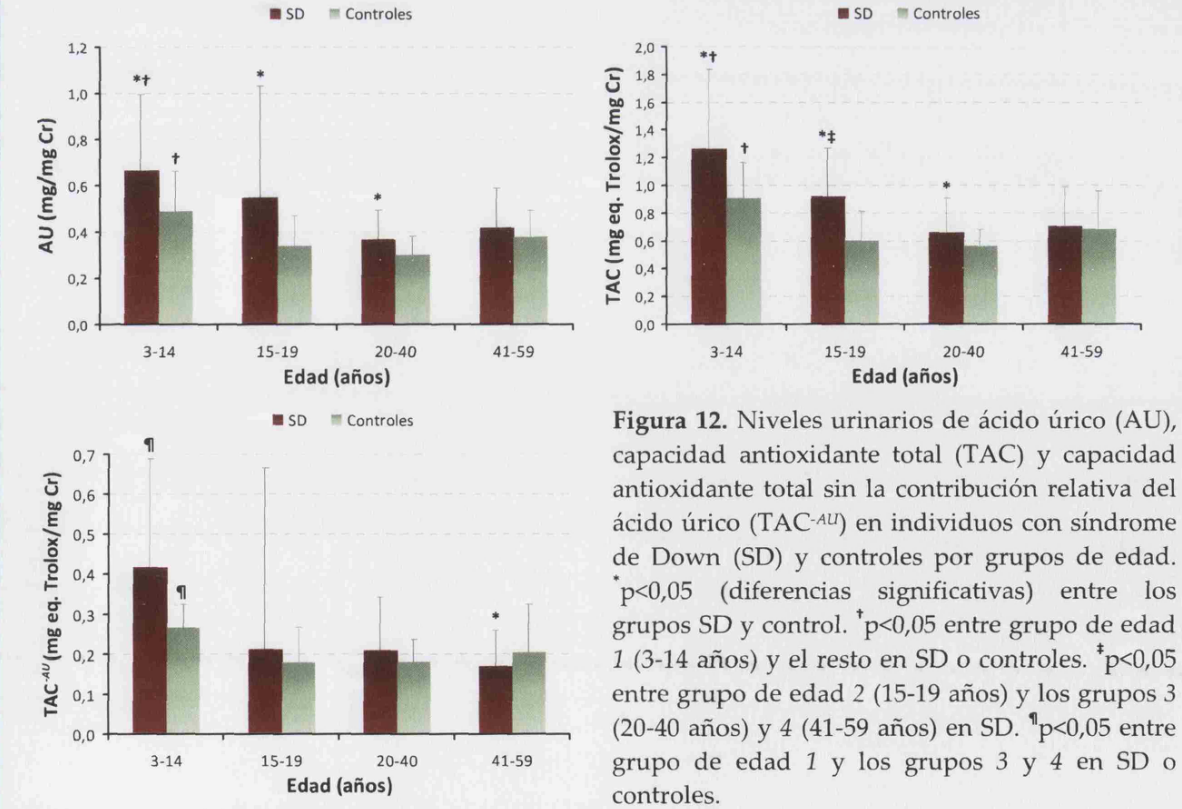


Figura 12. Niveles urinarios de ácido úrico (AU), capacidad antioxidante total (TAC) y capacidad antioxidante total sin la contribución relativa del ácido úrico (TAC^{-AU}) en individuos con síndrome de Down (SD) y controles por grupos de edad. $^{*}p<0,05$ (diferencias significativas) entre los grupos SD y control. $^{\dagger}p<0,05$ entre grupo de edad *1* (3-14 años) y el resto en SD o controles. $^{\ddagger}p<0,05$ entre grupo de edad *2* (15-19 años) y los grupos *3* (20-40 años) y *4* (41-59 años) en SD. $^{\P}p<0,05$ entre grupo de edad *1* y los grupos *3* y *4* en SD o controles.

2. AU, TAC y TAC^{-AU} en individuos con SD recibiendo tratamiento con levotiroxina para el hipotiroidismo

En la Figura 13 se muestran los niveles urinarios de AU, TAC y TAC^{-AU} en individuos con SD recibiendo tratamiento con levotiroxina para el hipotiroidismo e individuos con SD sin hipotiroidismo diagnosticado por grupos de edad. Se analizaron cuatro grupos de edad: *1*) 3-14 años (infancia), *2*) 15-19 años (adolescencia), *3*) 20-40 años (adultez) y *4*) 41-59 años (senescencia). El número de individuos en cada grupo fue: 7 SD con tratamiento y 17 SD sin hipotiroidismo diagnosticado en el grupo *1*; 9 SD con tratamiento y 20 SD sin hipotiroidismo diagnosticado en el grupo *2*; 11 SD con tratamiento y 22 SD sin hipotiroidismo diagnosticado en el grupo *3*; y 12 SD con tratamiento y 19 SD sin hipotiroidismo diagnosticado en el grupo *4*. Como se puede observar, no se obtuvieron diferencias significativas entre ambos grupos en ningún grupo de edad y para ninguno de los parámetros analizados.

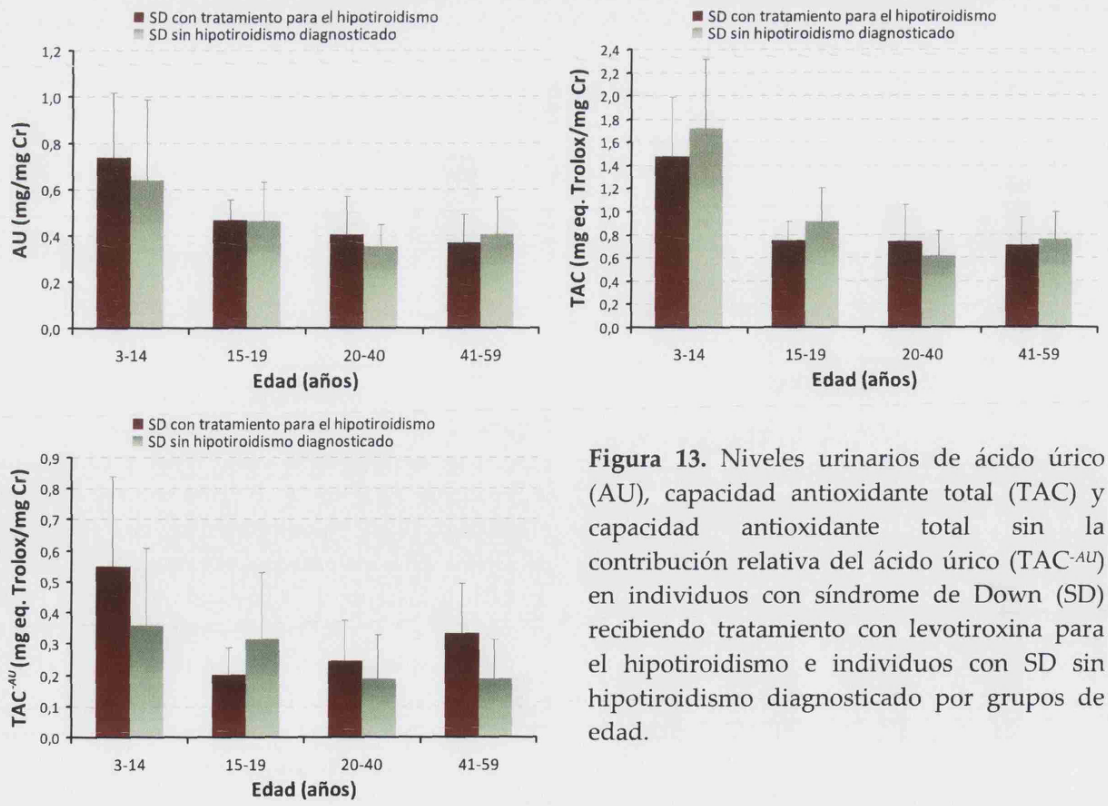


Figura 13. Niveles urinarios de ácido úrico (AU), capacidad antioxidante total (TAC) y capacidad antioxidante total sin la contribución relativa del ácido úrico (TAC^{-AU}) en individuos con síndrome de Down (SD) recibiendo tratamiento con levotiroxina para el hipotiroidismo e individuos con SD sin hipotiroidismo diagnosticado por grupos de edad.

Cabe señalar que cuando los individuos con SD que recibían tratamiento para el hipotiroidismo fueron excluidos del estudio, los resultados obtenidos fueron similares para todos los parámetros estudiados, excepto para los niveles de diTyr en niños, que pasaron de ser significativamente mayores en SD que en controles a no mostrar diferencias significativas entre ambos grupos.

3. Valoración de TAC^{-AU} en individuos con SD empleando el método directo descrito en la patente presentada en la Oficina Española de Patentes y Marcas (OEPM)

Los resultados obtenidos con el método directo de determinación de TAC^{-AU} desarrollado en el presente trabajo de Tesis doctoral fueron similares a los obtenidos mediante el método indirecto descrito en la publicación "*Campos C, et al. Clin Biochem. 2010; 43:228-233*" presentada como parte de esta Tesis, y ambos métodos mostraron un alto grado de correlación (r>0,8).

4. Variaciones con la edad de los niveles urinarios de creatinina (Cr) y de los distintos biomarcadores de estrés oxidativo analizados en síndrome de Down (SD) y controles

La Figura 14 muestra las variaciones con la edad para los distintos parámetros analizados en diagramas de dispersión. La relación entre las variables (parámetros urinarios vs. edad) se estudió mediante regresión polinomial local por el método de LOESS (*locally weighted scatter plot smooth*).

Por otra parte, la correlación entre la Cr y la edad en el grupo control no fue significativa en el grupo de edad analizado (20-59 años), pero a partir de los 22 años (22-59 años) dicha correlación fue significativamente negativa (r=-0,299; p=0,039), siendo la ecuación de la recta de regresión: y = -2,2x + 274,8.

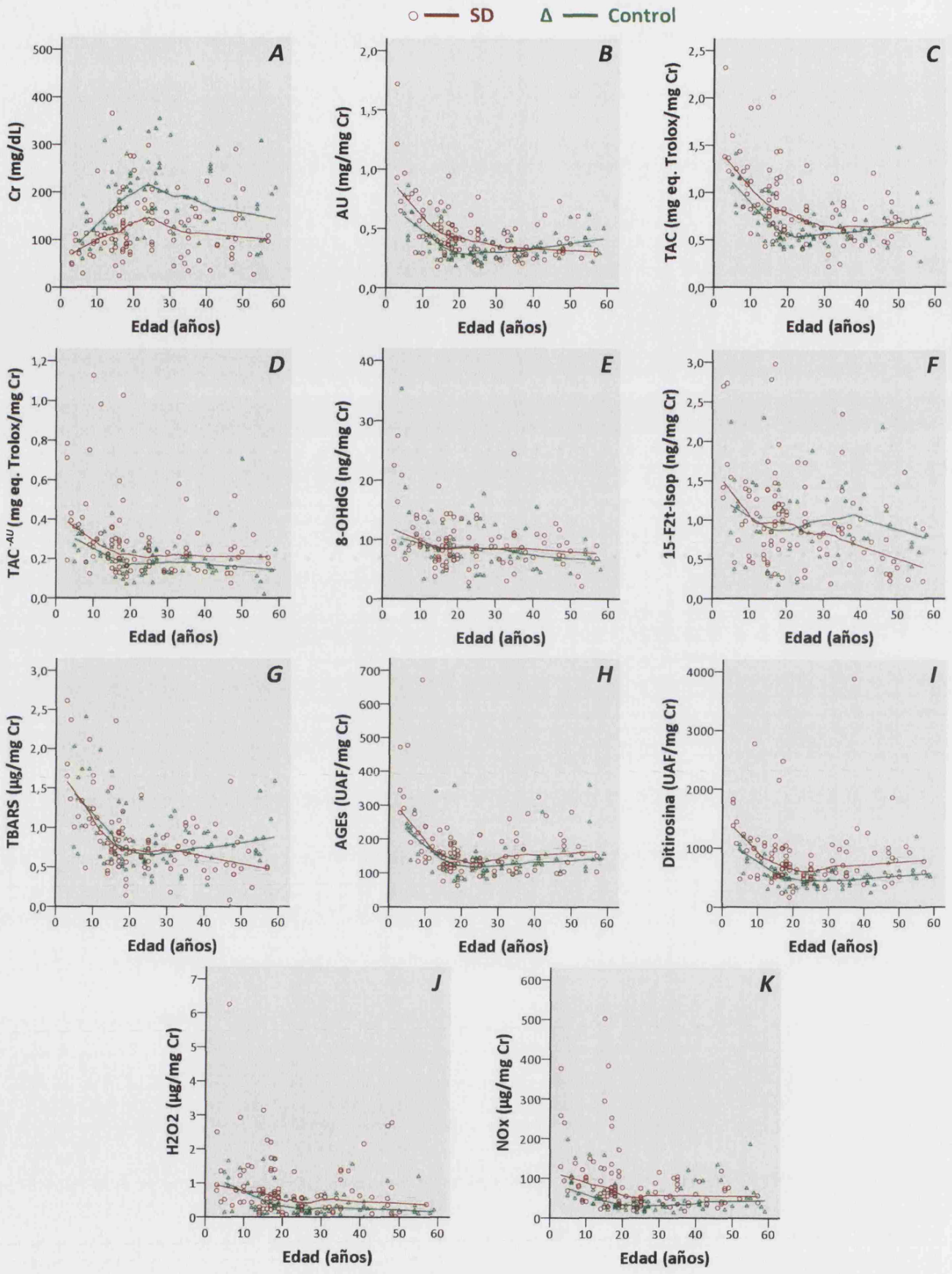


Figura 14. Diagramas de dispersión de la edad y los niveles urinarios de: *A*: creatinina (Cr), *B*: ácido úrico (AU), *C*: capacidad antioxidante total (TAC), *D*: capacidad antioxidante total sin la contribución relativa del ácido úrico (TAC^{-AU}), *E*: 8-hidroxi-2'-deoxiguanosina (8-OHdG), *F*: isoprotano 15-F_{2t}-Isop, *G*: sustancias reactivas del ácido tiobarbitúrico (TBARS), *H*: productos finales de glicación avanzada (AGEs), *I*: ditirosina, *J*: peróxido de hidrógeno (H_2O_2) y *K*: nitritos y nitratos (NOx) en síndrome de Down (SD) y controles. Las líneas de LOESS se muestran en el gráfico para el grupo con SD (círculos y línea roja) y control (triángulos y línea verde). UAF= unidades arbitrarias de fluorescencia.

Discusión

DISCUSIÓN

El estudio del síndrome de Down (SD) está íntimamente ligado a la historia de la genética. En 1865, Gregor Mendel publicó sus leyes de la genética (Mendel, 1865, 1866), y es en éste contexto que John Langdon H. Down publicó la primera descripción clínica del SD (Down, 1866). Sin embargo, la causa del síndrome no fue conocida hasta 1959 (Lejeune *et al.*, 1959), una vez descubierto el cariotipo humano. Desde entonces, los avances en la patogénesis y etiología del SD no han parado de sucederse.

El término "estrés oxidativo" fue definido por Sies en 1985 (Sies, 1985). Sin embargo, fue a partir del descubrimiento de la SOD1 en 1969 (McCord y Fridovich, 1969) y de la posterior observación del incremento en la actividad de esta enzima en individuos con trisomía 21 (Sinet *et al.*, 1974), cuando el interés por el estudio del estrés oxidativo en el SD comienza a aumentar. Así, en los años 70 los estudios se centran principalmente en la determinación de enzimas antioxidantes, pero no es hasta mediados de los 80 cuando se empiezan a investigar los niveles de marcadores de daño oxidativo, pudiendo destacarse los trabajos de Brooksbank y Balazs (1984), por ser el primero en determinar los niveles de lipoperoxidación *in vitro*, y de Kedziora y colaboradores (1986), por hacer lo propio *in vivo*.

Desde entonces, los estudios sobre el estrés oxidativo en el SD se centran fundamentalmente en el estudio de biomarcadores de estrés oxidativo, bien en modelos animales de SD o bien en estudios *in vivo* o *in vitro*. Así, por ejemplo, se ha observado una elevada peroxidación lipídica en modelos de ratón de SD (Ishihara *et al.*, 2009), un aumento en la generación de ROS en neuronas de individuos con SD (Busciglio y Yanker, 1995) y mayores niveles de biomarcadores de daño oxidativo al ADN y de lipoperoxidación en niños con SD comparado con sus hermanos no trisómicos (Jovanovic *et al.*, 1998).

La determinación de biomarcadores de estrés oxidativo en el SD se ha realizado principalmente en muestras de sangre, bien en células sanguíneas bien en plasma o suero, siendo los estudios que emplean muestras de orina para tal fin muy escasos. Sin embargo, la orina presenta grandes ventajas frente a otros fluidos biológicos, ya que permite su obtención de forma no invasiva, el riesgo de infección es mínimo para los

individuos sometidos a examen y para los profesionales que las analizan, proporciona un gran volumen de muestra permitiendo multitud de determinaciones (Barr *et al.*, 2005; Miller *et al.*, 2004) y es la muestra idónea para la determinación de isoprostanos, debido a que en la orina no se produce autoxidación de lípidos (Morrow y Roberts, 1997), y de diTyr, ya que su determinación es mucho más sencilla que si es realizada en plasma debido a que no requiere precipitar proteínas, hidrolizar ni realizar una pre-purificación (Bucknall, 2006). Además, el hecho de que las muestras de orina puedan obtenerse por los propios participantes hace de la orina la muestra idónea para estudios poblacionales. Por todo ello, en el presente trabajo se ha evaluado un conjunto de biomarcadores de estrés oxidativo en un grupo de individuos con SD de un amplio rango de edad, siendo uno de los estudios realizados en este campo con un mayor número de individuos.

Sin embargo, las muestras de orina no están exentas de inconvenientes. Las muestras de orina idóneas son, *a priori*, las de 24 horas, pero su obtención resulta especialmente complicada debido a la incomodidad que esta obtención supone para los participantes, causando frecuentemente una recogida incompleta o inadecuada, por lo que normalmente solo son viables en individuos hospitalizados. Además, ciertos metabolitos no son estables en este tipo de muestras, ya que se degradan rápidamente. Por todo ello, las muestras puntuales, normalmente de primera hora de la mañana, son generalmente las más utilizadas en estudios bioquímicos y clínicos. La principal desventaja de las muestras de orina puntuales es la variación en la concentración de los analitos urinarios por los cambios en el contenido en agua de la orina. Esta variación se suele corregir mediante el ajuste a creatinina (Cr), que consiste en dividir la concentración del analito por la concentración de Cr (Barr *et al.*, 2005). De hecho, una de las mayores dificultades encontradas en el presente trabajo esta relacionada con el ajuste a Cr de los biomarcadores estudiados.

1. Excreción urinaria de creatinina

La Cr es un producto final endógeno del metabolismo normal de los músculos. Es un compuesto orgánico nitrogenado que se genera a partir de la creatina y la fosfocreatina musculares, fuentes de energía para las células musculares, en una tasa

muy constante (aproximadamente el 2% de la Cr corporal se convierte en Cr cada día). La Cr es eliminada del cuerpo a través de los riñones por filtración glomerular y, en menor medida, por secreción activa a través de los túbulos renales (Boeniger *et al.*, 1993; Wyss y Kaddurah-Daouk, 2000).

En el presente estudio, los niveles urinarios de Cr fueron significativamente menores en el grupo de individuos con SD con edades comprendidas entre 15 y 59 años que en el grupo control en el mismo rango de edad, lo que podría ser debido a una alteración en el proceso de excreción renal, mientras que en niños no se encontraron diferencias significativas entre ambos grupos.

La valoración de los niveles de Cr en sangre también se utiliza para determinar la función renal, más concretamente la tasa de filtración glomerular[1] (Eriksen *et al.*, 2010; Haycock, 1981; Nyman *et al.*, 2006). Por tanto, de acuerdo con los resultados obtenidos, cabría pensar que en el SD la tasa de filtración glomerular podría estar disminuida debido a una disfunción renal, al menos a partir de la pubertad. Así, en individuos con "bajos" niveles de Cr, el analito urinario corregido aparecerá falsamente alto debido a que sus valores han sido divididos por un número "bajo", y lo contrario para individuos con niveles "altos" de Cr, dificultando la interpretación de los resultados obtenidos para los biomarcadores de estrés oxidativo.

La bibliografía referente a la función renal en el SD es muy escasa, y se refiere principalmente a dos estudios realizados entre los años 60 y 70, donde se hace referencia a una posible alteración de la filtración glomerular en base a un menor aclaramiento de Cr[2] (Coburn *et al.*, 1967; Nishida *et al.*, 1979). Se ha sugerido que una posible explicación estaría en una deficiencia de vitamina A, que provocaría una queratinización del tejido epitelial y una función defectuosa de los túbulos renales (Appleton *et al.*, 1969). Teniendo en cuenta que un 15-20% de la Cr se excreta por secreción activa a través de los túbulos renales (Boeniger *et al.*, 1993), su disfunción

[1] La tasa de filtración glomerular es el volumen de plasma filtrado del que es eliminada una sustancia o marcador (por ejemplo, la creatinina o la inulina) desde los capilares glomerulares renales hacia el interior de la cápsula de Bowman por unidad de tiempo (normalmente expresado en mL/min).

[2] El aclaramiento de creatinina se define como la velocidad a la cual es eliminada la creatinina del plasma sanguíneo a través de los riñones, y se calcula como el ratio entre la concentración urinaria y plasmática de creatinina multiplicado por la tasa de flujo urinario (normalmente expresado en mL/min), si bien hay diversas fórmulas para su cálculo que dependen, en algunos casos, de la edad, la raza o el sexo de los individuos.

haría disminuir la excreción de Cr a través de la orina, justificando, al menos en parte, los resultados obtenidos. Sin embargo, estudios más recientes no encuentran que exista deficiencia de vitamina A o problemas en su absorción en individuos con SD (Ercis *et al.*, 1996; Pueschel *et al.*, 1990).

Se han detectado diferentes anomalías renales en el SD (Gupta *et al.*, 1991; Málaga *et al.*, 2005; Mercer *et al.*, 2004) que pueden dar lugar a un fallo renal crónico (Málaga *et al.*, 2005), el cual está asociado a una disminución de los niveles de Cr (Kuroda, 1993; Levillain *et al.*, 1995). Sin embargo, la incidencia de estas anomalías renales en el SD no parece que sea distinta a la de la población general (Málaga *et al.*, 2005). Por tanto, parece necesario realizar más estudios sobre la función renal en el SD.

En el presente trabajo se observa que las diferencias en los niveles de Cr urinaria que se encuentran entre el grupo con SD y el grupo control son debidas principalmente a las diferencias que se dan a partir de la pubertad, mientras que en niños las diferencias no son significativas. Esto podría ser debido a que en la edad infantil la musculatura es menor que en adultos. Así, en los niños, tanto controles como con SD, la excreción de Cr se vería muy influenciada por la menor musculatura, haciendo que una posible disfunción renal no sea suficiente como para que se den diferencias significativas entre controles y SD.

Por otra parte, los niveles de Cr correlacionaron positivamente con la edad hasta los 14 años, tanto en el grupo con SD como en el grupo control, y negativamente a partir de los 20 años de edad en el grupo con SD y de los 22 años en el grupo control. Se ha visto que la concentración de Cr urinaria disminuye en adultos a medida que aumenta la edad (Barr *et al.*, 2005), probablemente debido a un deterioro de la musculatura y de la filtración glomerular (Alessio *et al.*, 1985; Driver y McAlevy, 1980). Así, cabría pensar que este deterioro sucede con anterioridad y tiene lugar de forma más acentuada en los individuos con SD.

Una explicación alternativa a los resultados obtenidos sería que los niveles urinarios de Cr no estuvieran reducidos en SD por su menor excreción sino por su menor producción. En primer lugar, podría ser que las personas con SD tuvieran una masa magra corporal menor que la de la población general. Y, en segundo lugar, la tasa metabólica podría estar reducida en estos individuos. En ambos casos podría tener

lugar una menor producción de Cr (Boeniger *et al.*, 1993; Forbes y Bruining, 1976; Wyss y Kaddurah-Daouk, 2000). Sin embargo, no se han encontrado diferencias significativas para la masa magra corporal entre SD y controles, ni en niños (Luke *et al.*, 1996) ni en adultos (Allison *et al.*, 1995), aunque más recientemente se ha visto que la masa muscular es menor en individuos con SD en el rango de edad de entre 14 y 40 años (Baptista *et al.*, 2005) y que la masa magra en adultos con SD también es menor que en controles (Guijarro *et al.*, 2008). Por otro lado, resultados controvertidos también aparecen en estudios sobre la tasa metabólica en SD (Allison *et al.*, 1995; Fernhall *et al.*, 2005), donde los niveles de hormonas tiroideas podrían tener un papel importante.

2. Biomarcadores de estrés oxidativo y/o nitrosativo en el SD

El status oxidativo varía durante el proceso de desarrollo del individuo, por lo que los resultados obtenidos para los biomarcadores de estrés oxidativo deben ser interpretados de forma diferente según sea la edad de los individuos analizados. Por ello, en el presente trabajo se establecieron 5 grupos de edad: 1) menores de 10 años, 2) entre 11 y 14 años, 3) entre 15 y 19 años, 4) entre 20 y 40 años, y 5) mayores de 40 años. Estos grupos representan respectivamente: infancia, adolescencia pre-menarquia, edad adulta temprana o adolescencia propiamente dicha, edad adulta y senescencia (basado en los individuos con SD de acuerdo con la clasificación de Bittles y colaboradores (2007)), habiéndose considerado el conjunto de los dos primeros grupos (<15 años) como el grupo representativo de los niños.

2.1. Valoración del ácido úrico (AU), la capacidad antioxidante total (TAC) y la capacidad antioxidante total sin la contribución del ácido úrico (TAC^{-AU}) en el SD

Debido a la dificultad que entraña medir todos los antioxidantes presentes en una muestra biológica por separado, se han desarrollado métodos para la medida de la TAC. TAC es una medida de los antioxidantes no enzimáticos presentes en una muestra que reaccionan conjuntamente en la eliminación de agentes oxidantes (MacDonald-Wicks *et al.*, 2006), y debe proporcionar una información más relevante que la determinación de antioxidantes individualmente pues considera las posibles interacciones que se dan entre los diferentes antioxidantes presentes en la muestra.

Métodos de estimación de la suma de los antioxidantes no enzimáticos presentes en una muestra son, por ejemplo: TEAC, FRAP, DPPH y CUPRAC. Este último, también llamado ensayo del poder antioxidante (PAO), es el método empleado en este estudio para la determinación de TAC en SD, porque: es sencillo, los reactivos son muy estables, la reacción tiene lugar a pH fisiológico y transcurre a temperatura ambiente, no precisa grandes cantidades de muestra, es fácilmente reproducible y es rápido, adaptándose muy bien a estudios poblacionales. A pesar de sus grandes ventajas frente a otros métodos de determinación de TAC, su uso no es muy habitual debido a que la metodología fue patentada (Da Cruz, 2003) y desde entonces se comercializa en forma de *kit* con el nombre de PAO o AOP. Así, su principal desventaja es el alto coste. Por ello, en el presente trabajo se consiguió realizar esta metodología sin necesidad de adquirir el *kit* comercial, utilizando reactivos de la casa comercial Sigma-Aldrich. Además, se realizaron modificaciones que mejoraron el ensayo, tales como la optimización de las concentraciones de los reactivos o el uso de Trolox como estándar, mucho más habitual y menos problemático a la hora de preparar que el AU aportado por el *kit* comercial. Esta metodología, a la que llamamos CUPRAC-BCS (ensayo de reducción del cobre(II) utilizando sal disódica del ácido 2,9-dimetil-4,7-difenil-1,10-fenantrolindisulfónico) para distinguirla de otras metodologías basadas en la misma reacción de reducción del ión cúprico, fue evaluada en muestras de plasma y de orina de individuos sanos, realizando una comparación con los ensayos TEAC, FRAP y DPPH. De este estudio, se pudo concluir que el ensayo optimizado CUPRAC-BCS es apropiado para la determinación de TAC en muestras de orina y de plasma heparinizado.

Debido al elevado estrés oxidativo en el SD, es lógico pensar que la TAC se encuentre alterada en estos individuos. En el presente trabajo, se obtuvieron niveles de TAC significativamente mayores en niños, adolescentes y adultos con SD que en controles, mientras que las diferencias no fueron significativas en la senescencia. TAC es un parámetro muy poco estudiado en el SD, su determinación ha sido realizada únicamente en muestras de suero o plasma y nunca en individuos adultos, observándose resultados ambiguos. Así, Carratelli y colaboradores (2001) encontraron que la TAC está disminuida en SD mientras que Zitnanová y colaboradores (2006) no

encontraron diferencias con respecto al grupo control, ambos estudios realizados en niños en muestras de suero y plasma respectivamente. Por el contrario, y de acuerdo con los resultados del presente trabajo, Tiano y colaboradores (2008) encontraron niveles plasmáticos de TAC mayores en niños con SD que en controles.

Por otra parte, resultados similares fueron obtenidos para las determinaciones de TAC y de AU. El AU es el producto final del metabolismo de las purinas en humanos y se encuentra en la sangre en forma de urato monosódico, en concentraciones muy superiores a las del resto de primates por carecer el ser humano de la enzima urato oxidasa, también conocida como uricasa. El AU es considerado un potente antioxidante extracelular, ya que elimina especies reactivas solubles en agua tales como radical hidroxilo ($HO^{\bullet}$), radical peroxilo ($ROO^{\bullet}$), peroxinitrito ($ONOO^{-}$), ácido hipocloroso (HOCl) y oxígeno singlete ($^1\Delta gO_2$) (Ames *et al.*, 1981; Glantzounis *et al.*, 2005; Howell y Wyngaarden, 1960; Maples y Mason, 1988; Proctor, 1970). De hecho, hay estudios que demuestran que el AU representa una gran parte (>50%) de la capacidad antioxidante plasmática (Benzie y Strain, 1996; Wayner *et al.*, 1987). Es decir, los niveles de TAC estarán muy influenciados por los de AU. Y así lo demuestra el alto grado de correlación obtenido entre ambos biomarcadores, tanto en SD como en controles, siendo lógico que los resultados obtenidos en las determinaciones de TAC y AU sean similares.

Estudios previos han encontrado mayores niveles de AU en muestras de plasma y suero de individuos con SD que en los controles (Pallardó *et al.*, 2006; Pant *et al.*, 1968; Tiano *et al.*, 2008). También se ha encontrado un aumento en la expresión de la XOR, enzima que cataliza la conversión de hipoxantina a xantina y la de xantina a AU, en SD (Pallardó *et al.*, 2006; Zitnanová *et al.*, 2004), lo cual explicaría los elevados niveles de AU encontrados en muestras tanto de plasma como de suero en estos individuos. Otros autores atribuyen este aumento del AU a una elevada actividad de las enzimas adenosina deaminasa (EC 3.5.4.4) y adenina fosforibosil transferasa (EC 2.4.2.7) eritrocitarias (Puukka *et al.*, 1982), mientras que Nishida y colaboradores (1979) sugieren que la disfunción renal contribuye a la hiperuricemia en SD. A esto podríamos sumarle el hecho de que tanto la sobreexpresión del gen *CBS* (que codifica la enzima cistationina-β-sintasa (EC 4.2.1.22)) como del gen *GART* (que codifica una

enzima trifuncional con las actividades enzimáticas: fosforibosilglicinamida formiltransferasa (EC 2.1.2.2), fosforibosilglicinamida sintetasa (EC 6.3.4.13) y fosforibosilaminoimidazol sintetasa (EC 6.3.3.1)), ambos genes localizados en el cromosoma 21, ha sido asociada con un aumento en la producción de purinas que puede dar lugar a la acumulación de AU (Tiano *et al.*, 2008).

Ya que los niveles de AU están alterados en varias patologías o incluso en situaciones de estrés oxidativo elevado (por ejemplo, tras realizar ejercicio físico intenso debido a estados de hipoxia o de isquemia-reperfusión), las determinaciones de TAC llevadas a cabo con los métodos actuales pueden llevar a resultados inesperados y a errores de interpretación. Por ello, la capacidad antioxidante total sin la contribución relativa del ácido úrico (TAC^{-AU}) es una determinación mucho más relevante para el estudio del estatus antioxidante y el estrés oxidativo en estudios experimentales, epidemiológicos o clínicos que la TAC. En el presente trabajo, la determinación de TAC^{-AU} se analizó en los individuos con SD mediante una determinación indirecta. Como resultado, no se obtuvieron diferencias significativas entre niños con SD y controles, al igual que en trabajo de Tiano y colaboradores (2008), y tampoco en adolescentes y adultos. Sin embargo, los niveles de TAC^{-AU} fueron significativamente menores en los individuos con SD que en los controles en el rango de edad de entre 41 y 59 años. Estos resultados sugieren que los individuos con SD podrían estar bajo condiciones de estrés oxidativo elevado que se reflejaría en una disminución significativa de los antioxidantes no enzimáticos en la senescencia.

Posteriormente, dado que la determinación de TAC^{-AU} fue realizada mediante una determinación indirecta que podría influir en los resultados, el ensayo CUPRAC fue modificado para realizar esta determinación de forma directa. Así, la nueva metodología, cuya solicitud de patente fue presentada el pasado 5 de mayo de 2010 en la OEPM, permite no solo determinar TAC^{-AU} si no también TAC y AU de forma simultánea e independiente. Para verificar los datos obtenidos con la determinación indirecta de TAC^{-AU}, se analizaron las muestras con la nueva metodología. Así, ambos métodos mostraron un alto grado de correlación.

2.2. Valoración del daño oxidativo al ADN en el SD

De entre todos los productos de oxidación del ADN la 8-OHdG es el más estudiado, principalmente debido a que es el más abundante y a que es un mutágeno (Kasai, 1997). La presencia de 8-OHdG en orina fue descrita a finales de los años 80 (Alessio y Cutler, 1987; Ames, 1988; Bergtold *et al.*, 1988; Cundy *et al.*, 1988), siendo el biomarcador de estrés oxidativo empleado en el presente trabajo para la valoración del daño oxidativo al ADN en individuos con SD.

Los resultados obtenidos no muestran diferencias significativas en los niveles de 8-OHdG entre SD y controles en ninguno de los grupos de edad analizados. Sin embargo, en estudios previos realizados en muestras de orina de niños con SD (Jovanovic *et al.*, 1998) y en leucocitos de niños y adolescentes (Pallardó *et al.*, 2006), los niveles de 8-OHdG fueron significativamente mayores en SD que en controles. En ambos trabajos la determinación de 8-OHdG se realizó mediante cromatografía líquida de alta resolución (HPLC), mientras que en el presente estudio se empleó un ELISA de la casa comercial JaICA (*Japan Institute for the Control of Aging*). Por tanto, las discrepancias encontradas en estos trabajos con respecto al presente estudio podrían deberse a diferencias metodológicas. La determinación de 8-OHdG mediante ELISA es actualmente la metodología más rápida, simple y económica que existe, sin embargo ha sido publicado recientemente que la urea y otras sustancias contribuyen a la sobreestimación de 8-OHdG debido a reacciones cruzadas en el ELISA comercial de JaICA (Song *et al.*, 2009).

Por el contrario, cuando los niveles de este biomarcador son analizados en individuos adultos, investigaciones previas no encuentran diferencias significativas entre SD y controles, al igual que en el presente trabajo, ni en leucocitos (Pallardó *et al.*, 2006) ni en muestras de cortex cerebral (Seidl *et al.*, 1997). También cabe mencionar el trabajo de Lloret y colaboradores (2008), donde la 8-OHdG se determina también en muestras de orina de individuos con SD y tampoco se encuentran diferencias entre SD y controles, aunque el número de individuos con SD analizado es muy reducido y el rango de edad de los individuos no es especificado.

En cualquier caso, y aunque tanto la excreción de urea como su aclaramiento en individuos con SD parecen ser similares a los de la población general (Coburn *et al.*,

1967), sería necesario realizar más estudios valorando los niveles de 8-OHdG y otros biomarcadores de daño oxidativo al ADN. Además, ya que la determinación de 8-OHdG por otras técnicas más precisas, como el HPLC, presentan grandes limitaciones en estudios poblacionales, será conveniente desarrollar nuevas metodologías simples, rápidas y económicas para la determinación de este biomarcador. Por otra parte, otros biomarcadores de daño al ADN deberían valorarse en el SD.

2.3. Valoración de la peroxidación lipídica en el SD

En el presente trabajo, se analizaron los niveles urinarios de dos biomarcadores de peroxidación lipídica ampliamente utilizados. Por una parte, el TBARS, representa uno de los biomarcadores más utilizados debido principalmente a que la metodología es sencilla, rápida y muy económica, si bien presenta una serie de problemas que serán comentados más adelante. Y por otra parte, el isoprostano 15-F_{2t}-IsoP, más aceptado en la literatura científica que el TBARS para la determinación de la peroxidación lipídica, aunque su determinación entraña más costes.

Los niveles de ambos biomarcadores fueron significativamente menores en SD que en controles en los individuos mayores de 40 años, no habiendo obtenido diferencias significativas en el resto de grupos de edad analizados. Además, tanto los niveles de 15-F_{2t}-IsoP como de TBARS correlacionaron negativamente con la edad, a partir de los 15 años, en el grupo con SD. Por le contrario, en el grupo control los niveles de TBARS correlacionaron positivamente con la edad a partir de los 20 años de edad.

Como posibles explicaciones a estos resultados encontramos que la acción de la carbonil reductasa 1, cuyo gen (*CBR1*) se encuentra en el cromosoma 21, protege a las células frente a la peroxidación lipídica (Oppermann, 2007) y se ha visto que esta enzima esta incrementada en individuos con SD (Balcz *et al.*, 2001). Además, un aumento en la resistencia a la oxidación de lípidos en suero y un aumento concomitante del AU sérico también han sido descritos en un grupo de niños y adolescentes con SD (Nagyova *et al.*, 2000). De hecho, el AU es uno de los mayores determinantes en la capacidad de resistencia a la peroxidación lipídica plasmática (Nyyssonen *et al.*, 1997). Así, aunque en el presente trabajo no se hayan encontrado

diferencias significativas en los niveles urinarios de AU entre SD y controles en individuos mayores de 40 años, los niveles sanguíneos (plasmáticos o séricos) de este antioxidante aparecen incrementados en SD en multitud de estudios (Pallardó *et al.*, 2006; Tiano *et al.*, 2008). Por tanto, podría ser que niveles similares en los biomarcadores de peroxidación lipídica entre SD y controles en niños, así como los menores niveles encontrados en la senescencia en individuos con SD, sean debidos a una mayor resistencia a la oxidación de lípidos en estos individuos, debido a sus mayores niveles de AU en sangre en comparación con la población general. Sin embargo, hay que tener en cuenta que un trabajo más reciente no encuentra diferencias en la resistencia a la oxidación de lípidos entre SD y controles (Tiano *et al.*, 2008). Además, niveles alterados de lípidos y lipoproteínas han sido encontrados en SD (Murphy *et al.*, 2000; Nagyova *et al.*, 2000; Pueschel *et al.*, 1992) y bien pudieran influir en los niveles de peroxidación lipídica de estos individuos.

Por otro lado, en un estudio previo se encontraron mayores niveles de isoprostano urinario en niños con SD que en controles (Praticò *et al.*, 2000), aunque en dicho trabajo se analizó el isoprostano 8,12-iso-iPF$_{2\alpha}$-VI y no 15-F$_{2t}$-IsoP. Ambos son F$_2$-isoprostanos, pero a cada uno se le atribuyen funciones distintas, siendo el 15-F$_{2t}$-IsoP el isómero más estudiado de la clase F$_2$ de isoprostanos. Los F$_2$-isoprostanos son isómeros químicamente estables de la prostaglandina, formados *in vivo* a través de un mecanismo no enzimático que se inicia por la peroxidación del ácido araquidónico por radicales libres (Roberts y Milne, 2009) y actúan como mediadores de efectos biológicos importantes. Así, además de considerarse un biomarcador de estrés oxidativo, el 15-F$_{2t}$-IsoP causa vasoconstricción de las arteriolas glomerulares renales, dando lugar a una reducción de la tasa de filtración glomerular (Takahashi *et al.*, 1992), pudiendo resultar en fallo renal. Por tanto, niveles elevados de 15-F$_{2t}$-IsoP en sangre de individuos con SD, debidos a un elevado estrés oxidativo, podrían dar lugar a una disfunción renal y consecuentemente a una menor excreción de metabolitos, incluyendo el propio 15-F$_{2t}$-IsoP. Un estudio comparativo en el que se valorasen los niveles de 15-F$_{2t}$-IsoP en muestras orina y de plasma o suero podría aclarar esta hipótesis.

Uno de los principales problemas del ensayo TBARS es el hecho de que existen multitud de variaciones en la metodología a seguir, haciendo que la comparación de

resultados entre laboratorios sea muy complicada. Además, ha aparecido mucha controversia en estudios acerca de la especificidad del TBARS. De igual forma, también se pueden describir problemas en la determinación de isoprostanos. Así, los ELISA comerciales frente a isoprostanos no han sido testados para reactividad cruzada con la mayoría de isoeicosanoides ni para la mayoría de sus metabolitos, por lo que la fiabilidad de esta metodología es incierta.

2.4. Valoración del daño oxidativo a proteínas en el SD

En el presente trabajo, como biomarcador de daño oxidativo a proteínas se analizaron los niveles urinarios de diTyr. Esta, es una molécula fluorescente formada como resultado de modificaciones post-translacionales que afectan a proteínas específicas. La tirosina es oxidada a diTyr en respuesta al estrés oxidativo por mecanismos catalizados por peroxidasas, siendo encontrada en multitud de proteínas tanto estructurales (lisozima, calmodulina, mioglobina o hemoglobina), como no estructurales (insulina, ribonucleasa o quimotripsina) (DiMarco y Giulivi, 2007). Además, la diTyr puede generarse por acción del peroxinitrito ($ONOO^-$), el cual es un potente agente oxidante y nitrante (Hensley *et al.*, 1998). Por tanto, la diTyr puede ser considerada un biomarcador de estrés tanto oxidativo como nitrosativo. Sin embargo, no ha sido analizada previamente en individuos con SD.

Los resultados mostraron que los niveles urinarios de diTyr son significativamente más elevados en los individuos con SD que en los controles en todos los grupos de edad analizados, si bien, en el grupo de niños las diferencias no son significativas cuando los individuos con SD que recibían tratamiento para el hipotiroidismo son excluidos. Además, los niveles de diTyr correlacionaron positivamente con la edad, tanto en SD como en controles, a partir de los 20 años. De esta forma, el conjunto de resultados obtenido indica que la diTyr urinaria podría constituir un buen biomarcador de estrés tanto oxidativo como nitrosativo en individuos con SD, y su determinación puede realizarse siguiendo una metodología rápida, simple y económica.

2.5. Valoración de la glicoxidación en el SD

La glicación de proteínas y las subsecuentes reacciones de Maillard dan lugar a carbonilos libres que atacan componentes proteicos celulares originando la formación de compuestos covalentes conocidos como AGEs. El estrés oxidativo esta implicado en la formación acelerada de estos compuestos, por lo que los AGEs son considerados biomarcadores de estrés oxidativo.

Altos niveles de AGEs han sido encontrados en diabetes, cataratas y enfermedades neurodegenerativas, como la enfermedad de Alzheimer, y todas ellas ocurren más frecuentemente en SD que en la población general. Los AGEs contribuyen al retraso mental, reducen la actividad motora y la respuesta inmune, dando lugar a un proceso acelerado de envejecimiento (Song *et al.*, 1999). Además, diversos estudios han encontrado que los AGEs reducen la producción de $O_2^{\bullet-}$ (Bernheim *et al.*, 2001) y la actividad de SOD1 y de CAT (Choudhary *et al.*, 1997; Singh *et al.*, 2001). Por tanto, los AGEs deben tener un papel muy importante en el estatus oxidativo de los individuos con SD.

Se han identificado una gran variedad de AGEs, como la pentosidina, el glioxal o el metilglioxal. Estos dos últimos han sido previamente analizados en muestras de plasma de un grupo de niños y otro de adolescentes y adultos con SD (Pallardó *et al.*, 2006), siendo los niveles de glioxal mayores en niños con SD que en controles y no habiendo diferencias en el grupo formado por adolescentes y adultos. Y al contrario que el glioxal, los niveles plasmáticos de metilglioxal fueron significativamente menores en el grupo formado por adolescentes y adultos con SD que los controles, no habiendo diferencias en niños. Sin embargo, los AGEs totales no han sido analizados en individuos con SD hasta el presente trabajo, donde solo aparecen diferencias significativas en el grupo de adolescentes, siendo los niveles mayores en SD que en controles. Ya que las diferencias en los niveles urinarios de Cr entre SD y controles son las más elevadas en este grupo de edad, el ajuste a Cr podría estar influyendo en los resultados obtenidos. Por otra parte, el metabolismo de carbohidratos parece estar alterado en el SD (Labudova *et al.*, 1999a; Labudova *et al.*, 1999b), lo que podría influir en los niveles de AGEs.

2.6. Valoración de H_2O_2 en el SD

El H_2O_2 urinario puede considerarse un biomarcador de estrés oxidativo (Halliwell *et al.*, 2000). De hecho, se ha propuesto que su concentración urinaria debe ser función de la actividad de la SOD1 (Banerjee *et al.*, 2004; Banerjee *et al.*, 2003). Sin embargo, sus niveles no han sido analizados en individuos con SD hasta el presente trabajo.

Es de esperar que los individuos con SD tengan niveles elevados de H_2O_2 debido a que presentan una actividad incrementada de la SOD1 (De La Torre *et al.*, 1996; Garaiová *et al.*, 2004; Garber *et al.*, 1979; Torsdottir *et al.*, 2001). De hecho, en el presente trabajo se encontraron mayores niveles urinarios de este metabolito en SD que en controles, tanto en adolescentes como en adultos, no siendo así ni en niños ni en la senescencia. Es en estos dos grupos de edad, niñez y senescencia, donde la variabilidad interindividual en individuos con SD es mayor, lo que podría influir en los resultados. Variaciones similares aparecen en trabajos previos (Halliwell *et al.*, 2004), y podrían ser debidas a que los niveles de H_2O_2 estuviesen determinados más por influencias nutricionales y metabólicas que por cambios en el estrés oxidativo. Así, por ejemplo, la práctica de ejercicio físico o el consumo de café y sal afectan a la excreción de H_2O_2 (Yuen y Benzie, 2003), limitando en gran medida la utilidad de este metabolito como biomarcador de estrés oxidativo. Por tanto, nuestros resultados sugieren que el H_2O_2 urinario podría utilizarse como biomarcador de estrés oxidativo en el SD, si bien sería necesario realizar más estudios para dilucidar las causas de su variación biológica.

2.7. Valoración de nitritos y nitratos (NOx) en el SD

El $NO^{\bullet}$ es un radical libre implicado en una gran cantidad de funciones fisiológicas, incluyendo la modulación de la hemodinámica renal y de la función excretora (Mizutani y Layon, 1996). Debido a que $NO^{\bullet}$ se degrada rápidamente a nitrato y nitrito en solución acuosa, los niveles urinarios de NOx fueron analizados como estimador del índice de producción de $NO^{\bullet}$ y, por tanto, del estrés nitrosativo.

A pesar de que la neurodegeneración en el SD ha sido asociada con una expresión aberrante del gen de la óxido nítrico sintasa 3 (Sohn *et al.*, 1999) y de que el

estrés nitrosativo está íntimamente asociado al estrés oxidativo, los biomarcadores de estrés nitrosativo no han sido previamente estudiados en el SD.

En el presente estudio, se encontraron mayores niveles urinarios de NOx en adolescentes y adultos con SD que en controles de las mismas edades, mientras que las diferencias no fueron significativas ni en niños ni en la senescencia. Sin embargo, aunque la variabilidad interindividual también es elevada para los niveles de NOx, ésta es mayor en adolescentes, donde sí se encuentran diferencias significativas. Por tanto, deben existir otras causas para explicar estos resultados.

Ha sido sugerido que los niveles urinarios de NOx tienden a decrecer con la edad en niños sanos debido a un incremento de la tasa de filtración glomerular asociado a la edad (Elli *et al.*, 2005). En el presente trabajo, los niveles de NOx correlacionaron negativamente con la edad solo en los niños del grupo control. Esta correlación negativa se dio también en individuos con SD, pero a partir de los 15 años, siendo positiva en controles a partir de los 20 años. Por tanto, la filtración glomerular debe estar alterada en el SD, de forma que los niveles urinarios de NOx en estos individuos estén más influenciados por la función excretora que por el estatus oxidativo, contrariamente a lo que debe ocurrir en el grupo control. En cualquier caso, la regresión de LOESS muestra una variación en los niveles de NOx con la edad muy similar en SD y controles, por lo que son necesarios más estudios para comprender el significado de estos resultados.

2.8. Correlación de biomarcadores con la edad en niños

Del estudio de las correlaciones entre los distintos biomarcadores y la edad cabe destacar los resultados obtenidos en el grupo de niños. Así, correlaciones negativas con la edad fueron obtenidas para 8-OHdG, diTyr y NOx en controles y para 8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs y diTyr en SD. De hecho, mayores niveles de 8-OHdG y diTyr fueron encontrados en el grupo más joven de niños con SD y controles así como de 15-F_{2t}-IsoP, TBARS y AGEs en SD, lo cual estaría en concordancia con estudios previos realizados en niños no trisómicos (Kauffman *et al.*, 2003; Tsukahara, 2007). Además, los niveles de AU y TAC también correlacionaron negativamente con la edad en niños, tanto en SD como en controles.

La razón de estos resultados aún no esta clara. Algunas posibles explicaciones dadas en trabajos previos incluyen una alta frecuencia de infecciones en los niños más jóvenes o una tasa metabólica basal más rápida. Alternativamente, un mayor peso relativo de los órganos internos, el mayor ratio ADN-proteína y la mayor velocidad de crecimiento de los órganos en los individuos más jóvenes podría dar lugar a mayores niveles de metabolitos excretados, similar a lo obtenido previamente para el AU (Stapleton *et al.*, 1978). Por otro lado, se obtuvo una correlación positiva entre los niveles de Cr y la edad en SD y controles que, como se ha comentado previamente, podría ser debida a la presencia de una menor musculatura en los niños más jóvenes. Así, el grado de musculatura de los niños también podría influir en la obtención de estos resultados.

2.9. Análisis del estrés oxidativo en individuos con SD recibiendo medicación para el tratamiento del hipotiroidismo

La disfunción tiroidea más común en el SD es el hipotiroidismo, que puede ser congénito, con una incidencia en bebés con SD de 1:141 nacimientos vivos (Fort *et al.*, 1984) comparado con una incidencia de entre 1:2500 y <1:5000 entre recién nacidos sin SD (Medda *et al.*, 2005), o adquirido a cualquier edad tras el nacimiento. En el presente trabajo, el 30% de los integrantes del estudio con SD recibían tratamiento con levotiroxina para el hipotiroidismo, bien con Eutirox® bien con Levothroid®, lo que permitió el análisis de este factor.

Las hormonas tiroideas están asociadas con el estrés oxidativo y el estatus antioxidante. Regulan la síntesis y degradación de proteínas, vitaminas y enzimas antioxidantes, así como el consumo de oxígeno y el metabolismo energético mitocondrial. De hecho, se ha sugerido que variaciones en los niveles de hormonas tiroideas pueden ser uno de los principales moduladores fisiológicos del estrés oxidativo (Guerrero *et al.*, 1999).

En el hipotiroidismo, sería de esperar encontrar una menor producción de ROS debido a la supresión metabólica causada por los menores niveles de hormonas tiroideas (Erdamar *et al.*, 2008). Sin embargo, hay estudios que encuentran un estrés oxidativo incrementado en pacientes tanto con hipertiroidismo como con

hipotiroidismo (Costantini *et al.*, 1998; Dumitriu *et al.*, 1988; Erdamar *et al.*, 2008; Sarandöl *et al.*, 2005; Torun *et al.*, 2009; Yilmaz *et al.*, 2003).

En el presente trabajo, los individuos con SD recibiendo tratamiento para el hipotiroidismo presentaron mayores niveles de TBARS en adultos, de diTyr en adolescentes y adultos y de AGEs en adultos y en la senescencia, que aquellos sin tiroidismo diagnosticado. Por otra parte, se observaron mayores niveles de diTyr en niños con SD recibiendo tratamiento para el hipotiroidismo que sus hermanos no trisómicos. Estos resultados indican que el hipotiroidismo en el SD debe incrementar el daño oxidativo, aún siendo los individuos tratados con levotiroxina.

La peroxidación lipídica en el hipotiroidismo se encuentra altamente influenciada por los lípidos séricos (Costantini *et al.*, 1998). Por tanto, la composición lipídica debería ser estudiada en individuos hipotiroideos con SD antes de poder confirmar los resultados obtenidos para el TBARS.

Por otro lado, los individuos con SD recibiendo tratamiento para el hipotiroidismo presentaron menores niveles de Cr que los individuos con SD pero sin tiroidismo diagnosticado en la adolescencia, si bien el análisis de todos los individuos (de entre 15 y 59 años) también mostró diferencias significativas. Además, los niveles de Cr fueron significativamente menores en niños con SD recibiendo tratamiento para el hipotiroidismo que en sus hermanos no trisómicos. Por tanto, una posible disfunción renal debido al hipotiroidismo podría influir en los resultados obtenidos para los biomarcadores de estrés oxidativo.

Esta patología, caracterizada por una disminución de la función de la glándula tiroides que ocasiona un déficit de hormonas tiroideas, está asociada a cambios significativos en la función renal (Montenegro *et al.*, 1996). Estos cambios consisten en una disminución del flujo sanguíneo renal y de la tasa de filtración glomerular (Gabow, 1992; Robertson y Berl, 1991) y llevan asociados una disminución en la concentración de Cr en orina y, en consecuencia, un aumento de ésta en suero (Montenegro *et al.*, 1996). Por otro lado, el hipotiroidismo reduce la tasa metabólica basal (Allison *et al.*, 1995), con lo que la formación de Cr es de esperar que sea menor, contribuyendo así a la disminución de la eliminación urinaria de la misma. Sin embargo, se ha visto que estos defectos son corregidos por el tratamiento con hormona

tiroidea (Montenegro *et al.*, 1996). En nuestro caso, los individuos son tratados con levotiroxina, una hormona tiroidea que se usa para el tratamiento del hipotiroidismo, y, en contra de lo esperado, los niveles de Cr fueron menores a los encontrados en los individuos con SD sin hipotiroidismo diagnosticado. Hay estudios que indican que la levotiroxina no influye en la excreción de Cr (Gullu *et al.*, 2005), por lo que deben existir factores asociados al hipotiroidismo en SD que justifiquen la disminución en la excreción de Cr observada en estos individuos.

La etiología del hipotiroidismo en el SD podría explicar estos resultados. Así, se ha visto que en estos individuos existen diversos factores que causan o agravan el hipotiroidismo:

- la actividad de la fenilalanina hidroxilasa (EC 1.14.16.1) parece ser defectuosa en el hígado en estos individuos (Shaposhnikov *et al.*, 1979). Esta enzima convierte la fenilalanina en tirosina, la cual es esencial para la producción de ciertas hormonas tiroideas (Guyton y Hall, 1996).
- tienen sobreexpresada la DYRK1A quinasa (EC 2.7.12.1) en un 50%, la cual está parcialmente regulada por tirosina (Dowjat *et al.*, 2007; Park *et al.*, 2009), de forma que una mayor actividad de esta enzima podría reducir la disponibilidad de tirosina.
- tienen menores niveles plasmáticos de selenio, el cual puede proteger la biosíntesis de tirosina del ataque de radicales libres (Kanavin *et al.*, 2000; Nève *et al.*, 1983).

Todos estos factores podrían afectar a la producción hormonal del tiroides pudiendo causar de una forma indirecta la disminución de la excreción de Cr a través de la orina, entre otras anomalías.

Por otra parte, los signos y síntomas del hipotiroidismo pueden ser difíciles de discriminar de los encontrados en el curso natural del propio SD. Además, parece ser frecuente encontrar individuos con SD y con hipotiroidismo subclínico sin tratamiento (Prasher y Haque, 2005). Por tanto, individuos con SD y problemas tiroideos deben ser tenidos en cuenta en futuros trabajos sobre el estrés oxidativo en este síndrome, incluso cuando estén recibiendo tratamiento.

3. Consideraciones finales

Todos los resultados obtenidos en el presente trabajo deben ser interpretados teniendo en cuenta las siguientes consideraciones:

1) Existe una gran variabilidad interindividual en el SD, tanto en la presencia de características específicas como en su severidad. Así, por ejemplo, la dieta, la práctica de ejercicio físico, el consumo de alcohol o de tabaco[3], el grado de contaminación ambiental o de exposición a los rayos UV a la que los individuos están expuestos, pueden influir en el estatus oxidativo y deben ser controlados, en la medida de lo posible, en los estudios de valoración del estrés oxidativo. En el presente trabajo se establecieron unos criterios de exclusión. Así, ninguno de los participantes era fumador, ni recibía suplementos antioxidantes ni tratamiento con algún medicamento que pudiera interferir en el metabolismo oxidativo, ni padecía diabetes, ni trastornos mieloproliferativos, ni malnutrición ni ningún tipo de demencia. Sin embargo, la mayoría de los participantes con SD del presente trabajo practicaba deporte moderado habitualmente. Se sabe que la práctica de ejercicio físico moderado previene frente al estrés oxidativo, lo cual podría influir en los resultados del presente trabajo.
2) El diagnóstico de la enfermedad de Alzheimer en el SD es particularmente difícil. Por tanto, es probable que en el presente estudio haya individuos con SD y con demencia tipo Alzheimer no diagnosticada, lo cual podría influir en los resultados.
3) Tanto el hipertiroidismo como el hipotiroidismo podrían influir en el grado de estrés oxidativo de los individuos. Se ha visto que los individuos con SD tienen frecuentemente niveles moderadamente elevados de la hormona estimulante de la tiroides (TSH). Además, es habitual que el hipotiroidismo subclínico no sea tratado en estas personas, lo cual podría influir en los resultados del presente trabajo. Por ello, los niveles de hormonas tiroideas

[3] En el Anexo se muestra un estudio recientemente publicado sobre los niveles urinarios de biomarcadores de estrés oxidativo y nitrosativo en fumadores sanos, el cual ha sido realizado con los datos obtenidos para la realización de la presente Tesis Doctoral. En él se puede ver como el consumo de tabaco, aún en individuos sanos, aumenta los niveles de varios biomarcadores de estrés oxidativo.

deberían ser considerados en futuros estudios valorando biomarcadores de estrés oxidativo en el SD.

4) Teniendo en cuenta que un buen biomarcador de estrés oxidativo debe poder analizarse mediante una metodología rápida, sencilla y económica, más aún en estudios poblacionales con un elevado número de muestras, las determinaciones realizadas en el presente trabajo se realizaron utilizando los ensayos más rápidos y económicos disponibles. Sin embargo, existen otras metodologías que emplean técnicas más sensibles y específicas para la determinación de la mayoría de los biomarcadores analizados en el presente trabajo.
5) Los resultados del presente trabajo sugieren que los individuos con SD podrían tener disfunción renal, especialmente los que recibían tratamiento para el hipotiroidismo, lo cual podría influir en los resultados del presente trabajo así como en estudios previos realizados en muestras tanto de orina como de plasma o suero.

Conclusiones

CONCLUSIONES

Las conclusiones generales que pueden extraerse de los resultados presentados en esta Tesis Doctoral son:

1. De la revisión bibliográfica sobre el estrés oxidativo en el SD:

1.1. El conjunto de estudios realizados a lo largo de la historia de la investigación del SD muestra un estrés oxidativo incrementado en estos individuos, cuya causa debe ser, en un primer término, la sobreexpresión de multitud de genes que afectarían al estatus oxidativo en grado variable según el individuo.

1.2. En el cromosoma 21 hay al menos 18 genes, además de *SOD1*, que podrían estar implicados en el estatus oxidativo de los individuos con SD tanto o más que *SOD1*.

2. De la evaluación del método CUPRAC-BCS:

2.1. El ensayo optimizado CUPRAC-BCS es un método adecuado para la determinación de TAC en muestras de orina y de plasma heparinizado.

3. De la evaluación de biomarcadores de estrés oxidativo y/o nitrosativo en el SD:

3.1. Los niveles urinarios de TAC^{-AU}, 8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs, H_2O_2 y NOx en niños con SD no difieren de los encontrados en controles, mientras que los de diTyr son mayores en los niños con SD, si bien las diferencias no son significativas cuando los individuos tratados para el hipotiroidismo son excluidos del estudio.

3.2. AGEs, diTyr, H_2O_2 y NOx podrían constituir biomarcadores adecuados de daño oxidativo y/o nitrosativo en muestras de orina de adolescentes y adultos con SD, en contraposición a 8-OHdG, 15-F_{2t}-IsoP y TBARS.

3.3. Los niveles urinarios de AU, TAC y TAC^{-AU} son mayores en niños que en la senescencia en SD, además los niveles urinarios de TAC^{-AU} son menores en adultos con SD que en controles, indicando una disminución de los antioxidantes no enzimáticos en la senescencia.

3.4. Los niveles de AU, aumentados en niños, adolescentes y adultos con SD, influyen en gran medida sobre los niveles de TAC. Por tanto, la valoración de TAC^{-AU} debe proporcional resultados más fiables en individuos con SD y puede realizarse de una forma directa y fiable con el método desarrollado en el presente trabajo y descrito en la patente presentada en la OEPM.

3.5. Los parámetros: AU, TAC, 8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs y diTyr en SD, y: AU, TAC, 8-OHdG, diTyr y NOx en el grupo control, correlacionan negativamente con la edad en niños, lo cual podría ser debido a la correlación positiva obtenida entre la Cr y la edad en ambos grupos.

3.6. En el SD, los niveles de AU, TAC, 15-F_{2t}-IsoP, TBARS y NOx correlacionan negativamente con la edad a partir de los 15 años de edad.

3.7. Los individuos con SD podrían presentar una disfunción renal, debido a que presentan menores niveles urinarios de Cr a partir de los 15 años, que podría influir en los resultados obtenidos en el presente trabajo.

3.8. Los individuos con SD que reciben tratamiento para el hipotiroidismo podrían presentar un estrés oxidativo incrementado, por sus mayores niveles de AGEs y diTyr, y/o una disfunción renal, por sus menores niveles de Cr.

Bibliografía

BIBIOGRAFÍA

Alessio, H. y Cutler, R. G. (1987). Average rate of oxyradical oxidation of deoxyguanosine in DNA of mammalian species is proportional of their age rate. *Gerontologist* **27:**151A.

Alessio, L., Berlin, A., Dell'Orto, A., Toffoletto, F. y Ghezzi, I. (1985). Reliability of urinary creatinine as a parameter used to adjust values of urinary biological indicators. *Int Arch Occup Environ Health* **55:**99-106.

Allison, D. B., Gomez, J. E., Heshka, S., Babbitt, R. L., Geliebter, A., Kreibich, K. y Heymsfield, S. B. (1995). Decreased resting metabolic rate among persons with Down Syndrome. *Int J Obes Relat Metab Disord* **19:**858-861.

Ames, B. N. (1988). Measuring oxidative damage in humans: relation to cancer and ageing. *IARC Sci Publ*:407-416.

Ames, B. N., Cathcart, R., Schwiers, E. y Hochstein, P. (1981). Uric acid provides an antioxidant defense in humans against oxidant-and radical-caused aging and cancer: a hypothesis. *Proc Natl Acad Sci USA* **78:**6858-6862.

Anneren, G. y Edman, B. (1993). Down syndrome - a gene dosage disease caused by trisomy of genes within a small segment of the long arm of chromosome 21, exemplified by the study of effects from the superoxide-dismutase type 1 (SOD-1) gene. *APMIS Suppl* **40:**71-79.

Appleton, M. D., Haab, W., Burti, U. y Orsulak, P. J. (1969). Plasma urate levels in mongolism. *Amer J Ment Deficiency* **74:**196-199.

Arbuzova, S., Hutchin, T. y Cuckle, H. (2002). Mitochondrial dysfunction and Down's syndrome. *Bioessays* **24:**681-684.

Auten, R. L. y Davis, J. M. (2009). Oxygen toxicity and reactive oxygen species: the devil is in the details. *Pediatr Res* **66:**121-127.

Balcz, B., Kirchner, L., Cairns, N., Fountoulakis, M. y Lubec, G. (2001). Increased brain protein levels of carbonyl reductase and alcohol dehydrogenase in Down syndrome and Alzheimer's disease. *J Neural Transm Suppl* **61:**193-201.

Banerjee, D., Jacob, J., Kunjamma, G., Madhusoodanan, U. K. y Ghosh, S. (2004). Measurement of urinary hydrogen peroxide by FOX-1 method in conjunction with catalase in diabetes mellitus--a sensitive and specific approach. *Clin Chim Acta* **350:**233-236.

Banerjee, D., Madhusoodanan, U. K., Nayak, S. y Jacob, J. (2003). Urinary hydrogen peroxide: a probable marker of oxidative stress in malignancy. *Clin Chim Acta* **334:**205-209.

Baptista, F., Varela, A. y Sardinha, L. B. (2005). Bone mineral mass in males and females with and without Down syndrome. *Osteoporos Int* **16:**380-388.

Barr, D. B., Wilder, L. C., Caudill, S. P., Gonzalez, A. J., Needham, L. L. y Pikle, J. L. (2005). Urinary creatinine concentrations in the U.S. population: Implications for urinary biologic monitoring measurements. *Environ Health Perspect* **113:**192-200.

Basu, S. (2008). F2-isoprostanes in human health and diseases: from molecular mechanisms to clinical implications. *Antioxid Redox Signal* **10:**1405-1434.

Baynes, J. W. y Thorpe, S. R. (2000). Glycoxidation and lipoxidation in atherogenesis. *Free Radic Biol Med* **28:**1708-1716.

Benzie, I. F. F. y Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem* **239:**70-76.

Bergtold, D. S., Simic, M. G., Alessio, H. y Cutler, R. G. (1988). Urine biomarkers for oxidative DNA damage. *Basic Life Sci* **49:**483-489.

Berlett, B. S. y Stadtman, E. R. (1997). Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem* **272:**20313-20316.

Bernheim, J., Rashid, G., Gavrieli, R., Korzets, Z. y Wolach, B. (2001). In vitro effect of advanced glycation end-products on human polymorphonuclear superoxide production. *Eur J Clin Invest* **31:**1064-1069.

Bittles, A. H., Bower, C., Hussain, R. y Glasson, E. J. (2007). The four ages of Down syndrome. *Eur J Public Health* **17:**221-225.

Boeniger, M. F., Lowry, L. K. y Rosenberg, J. (1993). Interpretation of urine results used to assess chemical exposure with emphasis on creatinine adjustments: A review. *Am Ind Hyg Assoc J* **54:**615-627.

Brooksbank, B. W. y Balazs, R. (1984). Superoxide dismutase, glutathione peroxidase and lipoperoxidation in Down's syndrome fetal brain. *Brain Res* **318:**37-44.

Bucknall, M. P. (2006). Dityrosine as a biomarker of free radical induced oxidative damage in diseases of aging. (Tesis Doctoral - University of New South Wales).

Busciglio, J. y Yankner, B. A. (1995). Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons in vitro. *Nature* **378:**776-779.

Cadet, J., Douki, T. y Ravanat, J. L. (2010). Oxidatively generated base damage to cellular DNA. *Free Radic Biol Med* **49:**9-21.

Carratelli, M., Porcaro, L., Ruscica, M., De Simone, E., Bertelli, A. A. y Corsi, M. M. (2001). Reactive oxygen metabolites and prooxidant status in children with Down's syndrome. *Int J Clin Pharmacol Res* **21:**79-84.

Casado, A., López-Fernández, M. E. y Ruiz, R. (2007). Lipid peroxidation in Down syndrome caused by regular trisomy 21, trisomy 21 by Robertsonian translocation and mosaic trisomy 21. *Clin Chem Lab Med* **45:**59-62.

Coburn, S. P., Seidenberg, M. y Mertz, E. T. (1967). Clearance of uric acid, urea, and creatinine in Down's syndrome. *J Appl Physiol* **23:**579-580.

Costantini, F., Pierdomenico, S. D., De Cesare, D., De Remigis, P., Bucciarelli, T., Bittolo-Bon, G., Cazzolato, G., Nubile, G., Guagnano, M. T., Sensi, S., Cuccurullo, F. y Mezzetti, A. (1998). Effect of thyroid function on LDL oxidation. *Arterioscler Thromb Vasc Biol* **18:**732-737.

Coyle, C. H., Martinez, L. J., Coleman, M. C., Spitz, D. R., Weintraub, N. L. y Kader, K. N. (2006). Mechanisms of H2O2-induced oxidative stress in endothelial cells. *Free Radic Biol Med* **40:**2206-2213.

Cundy, K. C., Kohen, R. y Ames, B. N. (1988). Determination of 8-hydroxydeoxyguanosine in human urine: a possible assay for in vivo oxidative DNA damage. *Basic Life Sci* **49:**479-482.

Choudhary, D., Chandra, D. y Kale, R. K. (1997). Influence of methylglyoxal on antioxidant enzymes and oxidative damage. *Toxicol Lett* **93:**141-152.

Da Cruz, G. (2003). Use of bathocuproine for the evaluation of the antioxidant power in liquids and solutions. Patente nº US 6.613.577.

Dalle-Donne, I., Rossi, R., Colombo, R., Giustarini, D. y Milzani, A. (2006). Biomarkers of oxidative damage in human disease. *Clin Chem* **52:**601-623.

De Haan, J. B., Wolvetang, E. J., Cristiano, F., Iannello, R., Bladier, C., Kelner, M. J. y Kola, I. (1997). Reactive oxygen species and their contribution to pathology in Down syndrome. *Adv Pharmacol* **38:**379-402.

De La Torre, R., Casado, A., Lopez-Fernandez, E., Carrascosa, D., Ramirez, V. y Saez, J. (1996). Overexpression of copper-zinc superoxide dismutase in trisomy 21. *Experientia* **52:**871-873.

DiMarco, T. y Giulivi, C. (2007). Current analytical methods for the detection of dityrosine, a biomarker of oxidative stress, in biological samples. *Mass Spectrom Rev* **26:**108-120.

Dowjat, W. K., Adayev, T., Kuchna, I., Nowicki, K., Palminiello, S., Hwang, Y. W. y Wegiel, J. (2007). Trisomy-driven overexpression of DYRK1A kinase in the brain of subjects with Down syndrome. *Neurosci Lett* **413:**77-81.

Down, J. L. (1866). Observations on an ethnic classification of idiots. *London Hospital Clinical Lectures and Reports* **3:**259-262.

Driver, A. G. y McAlevy, M. T. (1980). Creatinine height index as a function of age. *Am J Clin Nutr* **33:**2057-2062.

Dumitriu, L., Bartoc, R., Ursu, H., Purice, M. y Ionescu, V. (1988). Significance of high levels of serum malonyl dialdehyde (MDA) and ceruloplasmin (CP) in hyper- and hypothyroidism. *Endocrinologie* **26:**35-38.

Elli, M., Soylemezoglu, O., Erbas, D., Bakkaloglu, S. A., Buyan, N., Ozkaya, O. y Hasanoglu, E. (2005). Plasma and urine nitric oxide levels in healthy Turkish children. *Pediatr Nephrol* **20:**1605-1609.

Ercis, M., Balci, S. y Atakan, N. (1996). Dermatological manifestations of 71 Down syndrome children admitted to a clinical genetics unit. *Clin Genet* **50:**317-320.

Erdamar, H., Demirci, H., Yaman, H., Erbil, M. K., Yakar, T., Sancak, B., Elbeg, S., Biberoglu, G. y Yetkin, I. (2008). The effect of hypothyroidism, hyperthyroidism, and their treatment on parameters of oxidative stress and antioxidant status. *Clin Chem Lab Med* **46:**1004-1010.

Eriksen, B. O., Mathisen, U. D., Melsom, T., Ingebretsen, O. C., Jenssen, T. G., Njolstad, I., Solbu, M. D. y Toft, I. (2010). Cystatin C is not a better estimator of GFR than plasma creatinine in the general population. *Kidney Int* **78:**1305-1311.

Evans, M. D., Olinski, R., Loft, S. y Cooke, M. S. (2010). Toward consensus in the analysis of urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine as a noninvasive biomarker of oxidative stress. *FASEB J* **24:**1249-1260.

Fernhall, B., Figueroa, A., Collier, S., Goulopoulou, S., Giannopoulou, I. y Baynard, T. (2005). Resting metabolic rate is not reduced in obese adults with Down syndrome. *Ment Retard* **43:**391-400.

Finkel, T. y Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* **408:**239-247.

Forbes, G. B. y Bruining, G. J. (1976). Urinary creatinine excretion and lean body mass. *Am J Clin Nutr* **29:**1359-1366.

Fort, P., Lifshitz, F., Bellisario, R., Davis, J., Lanes, R., Pugliese, M., Richman, R., Post, E. M. y David, R. (1984). Abnormalities of thyroid function in infants with Down syndrome. *J Pediatr* **104:**545-549.

Gabow, P. A. (1992). Hypo-hypernatraemia. En: Cameron, S., Davison, A. M., Grünfeld, J. P., Kerr, D., Ritz, E., editores. Oxford Textbook of Clinical Nephrology. New York, Oxford University Press. Págs. 869-894.

Garaiová, I., Muchová, J. S., M., Blazicek, P., Sivonová, M., Kvasnicka, P., Pueschel, S. y Duracková, Z. (2004). The relationship between antioxidant systems and some markers of oxidative stress in persons with Down syndrome. *Biologia* **59:**787-794.

Garber, P., Sinet, P. M., Jerome, H. y Lejeune, J. (1979). Copper/zinc superoxide dismutase activity in trisomy 21 by translocation. *Lancet* **2:**914-915.

Glantzounis, G. K., Tsimoyiannis, E. C., Kappas, A. M. y Galaris, D. A. (2005). Uric acid and oxidative stress. *Curr Pharm Des* **11:**4145-4151.

Guerrero, A., Pamplona, R., Portero-Otin, M., Barja, G. y Lopez-Torres, M. (1999). Effect of thyroid status on lipid composition and peroxidation in the mouse liver. *Free Radic Biol Med* **26:**73-80.

Guijarro, M., Valero, C., Paule, B., Gonzalez-Macias, J. y Riancho, J. A. (2008). Bone mass in young adults with Down syndrome. *J Intellect Disabil Res* **52:**182-189.

Gullu, S., Sav, H. y Kamel, N. (2005). Effects of levothyroxine treatment on biochemical and hemostasis parameters in patients with hypothyroidism. *Eur J Endocrinol* **152:**335-361.

Gupta, S. K., Venkataseshan, V. S. y Churg, J. (1991). Mesangiocapillary glomerulonephritis in Down's syndrome. *Am J Nephrol* **11:**112.

Gutteridge, J. M. y Halliwell, B. (1990). The measurement and mechanism of lipid peroxidation in biological systems. *Trends Biochem Sci* **15:**129-135.

Guyton, A. C. y Hall, J. E. (1996). Textbook of medical physiology. Phyladelphia. W. B. Saunders.

Halliwell, B. (1997). Antioxidants: the basics--what they are and how to evaluate them. *Adv Pharmacol* **38:**3-20.

Halliwell, B., Clement, M. V. y Long, L. H. (2000). Hydrogen peroxide in the human body. *FEBS Lett* **486:**10-13.

Halliwell, B. y Gutteridge, J. M. C. (2007). Free radicals in biology and medicine. Cuarta edición. New York. Oxford University Press Inc.

Halliwell, B., Long, L. H., Yee, T. P., Lim, S. y Kelly, R. (2004). Establishing biomarkers of oxidative stress: the measurement of hydrogen peroxide in human urine. *Curr Med Chem* **11:**1085-1092.

Halliwell, B. y Whiteman, M. (2004). Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *Br J Pharmacol* **142:**231-255.

Hasle, H., Clemmensen, I. H. y Mikkelsen, M. (2000). Risks of leukaemia and solid tumours in individuals with Down's syndrome. *Lancet* **355:**165-169.

Haycock, G. B. (1981). Old and new tests of renal function. *J Clin Pathol* **34:**1276-1281.

Hensley, K., Maidt, M. L., Yu, Z., Sang, H., Markesbery, W. R. y Floyd, R. A. (1998). Electrochemical analysis of protein nitrotyrosine and dityrosine in the Alzheimer brain indicates region-specific accumulation. *J Neurosci* **18:**8126-8132.

Hook, E. B. (1981). Down syndrome: Its frequency in human populations and some factors pertinent to variation in rates. En: de la Cruzz, F. F. y Gerald, P. S., editores. Trisomy 21 (Down syndrome): research perspectives. Baltimore. University Park Press. Págs. 3-68.

Howell, R. R. y Wyngaarden, J. B. (1960). On the Mechanism of Peroxidation of Uric Acids by Hemoproteins. *J Biol Chem* **235:**3544-3550.

Ishihara, K., Amano, K., Takaki, E., Ebrahim, A. S., Shimohata, A., Shibazaki, N., Inoue, I., Takaki, M., Ueda, Y., Sago, H., Epstein, C. J. y Yamakawa, K. (2009). Increased lipid peroxidation in Down's syndrome mouse models. *J Neurochem* **110:**1965-1976.

Jones, D. P. (2006). Redefining oxidative stress. *Antioxid Redox Signal* **8:**1865-1879.

Jovanovic, S. V., Clements, D. y MacLeod, K. (1998). Biomarkers of oxidative stress are significantly elevated in Down syndrome. *Free Radic Biol Med* **25:**1044-1048.

Kanavin, Ø. J., Aaseth, J. y Birketvedt, G. S. (2000). Thyroid hypofunction in Down's syndrome. Is it related to oxidative stress? *Biol Trace Elem Res* **78:**35-42.

Karadag, A., Ozcelik, B. y Saner, S. (2009). Review of methods to determine antioxidant capacities. *Food Anal Methods* **2:**41-60.

Kasai, H. (1997). Analysis of a form of oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. *Mutat Res* **387:**147-163.

Kauffman, L. D., Sokol, R. J., Jones, R. H., Awad, J. A., Rewers, M. J. y Norris, J. M. (2003). Urinary F2-isoprostanes in young healthy children at risk for type 1 diabetes mellitus. *Free Radic Biol Med* **35:**551-557.

Kedziora, J., Bartosz, G., Gromadzinska, J., Sklodowska, M., Wesowicz, W. y Scianowski, J. (1986). Lipid peroxides in blood plasma and enzymatic antioxidative defence of erythrocytes in Down's syndrome. *Clin Chim Acta* **154:**191-194.

Kowaltowski, A. J., de Souza-Pinto, N. C., Castilho, R. F. y Vercesi, A. E. (2009). Mitochondria and reactive oxygen species. *Free Radic Biol Med* **47:**333-343.

Kuroda, M. (1993). Study on impaired metabolism of guanidinoacetic acid in chronic renal failure rabbits with special reference to impaired conversion of arginine to guanidinoacetic acid. *Nephron* **65:**605-611.

Labudova, O., Cairns, N., Kitzmuller y E., Lubec, G. (1999a). Impaired brain glucose metabolism in patients with Down syndrome. *J Neural Transm Suppl* **57:**247-256.

Labudova, O., Kitzmueller, E., Rink, H., Cairns, N. y Lubec, G. (1999b). Increased phosphoglycerate kinase in the brains of patients with Down's syndrome but not with Alzheimer's disease. *Clin Sci* **96:**279-285.

Lejeune, J., Gautier, M. y Turpin, R. (1959). Etude des chromosomes somatiques de 9 enfants mongoliens. *C.R. Acad Sci Paris* **248:**1721-1728.

Lesko, L. J. y Atkinson, A. J. (2001). Use of biomarkers and surrogate endpoints in drug development and regulatory decision making: Criteria, validation, strategies. *Annu Rev Pharmacol Toxicol* **41:**347-366.

Levillain, O., Marescau, B. y De Deyn, P. P. (1995). Guanidino compound metabolism in rats subjected to 20% to 90% nephrectomy. *Kidney Int* **47:**464-472.

Loft, S., Vistisen, K., Ewertz, M., Tjonneland, A., Overvad, K. y Poulsen, H. E. (1992). Oxidative DNA damage estimated by 8-hydroxydeoxyguanosine excretion in humans: influence of smoking, gender and body mass index. *Carcinogenesis* **13:**2241-2247.

Luke, A., Sutton, M., Schoeller, D. A. y Roizen, N. J. (1996). Nutrient intake and obesity in prepubescent children with Down syndrome. *J Am Diet Assoc* **96:**1262-1267.

Lloret, A., Calzone, R., Dunster, C., Manini, P., d'Ischia, M., Degan, P., Kelly, F. J., Pallardó, F. V., Zatterale, A. y Pagano, G. (2008). Different patterns of in vivo pro-oxidant states in a set of cancer- or aging-related genetic diseases. *Free Radic Biol Med* **44:**495-503.

MacDonald-Wicks, L. K., Wood, L. G. y Garg, M. L. (2006). Methodology for the determination of biological antioxidant capacity in vitro: a review. *J Sci Food Agric* **86:**2046-2056.

Málaga, S., Pardo, R., Málaga, I., Orejas, G. y Fernández-Toral, J. (2005). Renal involvement in Down syndrome. *Pediatr Nephrol* **20:**614-617.

Maples, K. R. y Mason, R. P. (1988). Free radical metabolite of uric acid. *J Biol Chem* **263:**1709-1712.

McCord, J. M. y Fridovich, I. (1969). Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem* **244:**6049-6055.

Medda, E., Olivieri, A., Stazi, M. A., Grandolfo, M. E., Fazzini, C., Baserga, M., Burroni, M., Cacciari, E., Calaciura, F., Cassio, A., Chiovato, L., Costa, P., Leonardi, D., Martucci, M., Moschini, L., Pagliardini, S., Parlato, G., Pignero, A., Pinchera, A., Sala, D., Sava, L., Stoppioni, V., Tancredi, F., Valentini, F., Vigneri, R. y Sorcini, M. (2005). Risk factors for congenital hypothyroidism: results of a population case-control study (1997-2003). *Eur J Endocrinol* **153:**765-773.

Mendel, G. (1865). Experiments in plant hybridization. Read at the February 8th, and March 8th, 1865, meetings of the Brünn Natural History Society.

Mendel, G. (1866). Versuche über Pflanzenhybriden. *Verh Des Naturf. Vereines in Brünn (Abhandlungen)* **4:**3-47. Traducción al inglés disponible online en <http://www.mendelweb.org/Mendel.html>.

Mercer, E. S., Broecker, B., Smith, E. A., Kirsch, A. J., Scherz, H. C. y Massad, C. A. (2004). Urological manifestations of Down syndrome. *J Urol* **171:**1250-1253.

Meydani, M. (1997). Isoprostanes as oxidant stress markers in coronary reperfusion. *Nutr Rev* **55:**404-407.

Mikkelsen, M. (1977). Down syndrome: cytogenetical epidemiology. *Hereditas* **86:**45-50.

Miller, R. C., Brindle, E., Holman, D. J., Shofer, J., Klein, N. A., Soules, M. R. y O'Connor, K. A. (2004). Comparison of specific gravity and creatinine for normalizing urinary reproductive hormone concentrations. *Clin Chem* **50:**924-932.

Mizutani, T. y Layon, A. J. (1996). Clinical applications of nitric oxide. *Chest* **110:**506-524.

Montenegro, J., González, O., Saracho, R., Aguirre, R., González, O. y Martínez, I. (1996). Changes in renal function in primary hypothyroidism. *Am J Kidney Dis* **27:**195-198.

Montuschi, P., Barnes, P. J. y Roberts, L. J. 2nd. (2004). Isoprostanes: markers and mediators of oxidative stress. *FASEB J* **18:**1791-1800.

Morrow, J. D. y Roberts, L. J. (1997). The isoprostanes: unique bioactive products of lipid peroxidation. *Prog Lipid Res* **36:**1-21.

Muchová, J., Sustrová, M., Garaiová, I., Liptáková, A., Blazícek, P., Kvasnicka, P., Pueschel, S. y Duracková, Z. (2001). Influence of age on activities of antioxidant enzymes and lipid peroxidation products in erythrocytes and neutrophils of Down syndrome patients. *Free Radic Biol Med* **31:**499-508.

Murphy, E. J., Schapiro, M. B., Rapoport, S. I. y Shetty, H. U. (2000). Phospholipid composition and levels are altered in Down syndrome brain. *Brain Res* **867:**9-18.

Nagyova, A., Sustrova, M. y Raslova, K. (2000). Serum lipid resistance to oxidation and uric acid levels in subjects with Down's syndrome. *Physiol Res* **49:**227-231.

Nève, J., Sinet, P. M., Molle, L. y Nicole, A. (1983). Selenium, zinc and copper in Down's syndrome (trisomy 21): blood levels and relations with glutathione peroxidase and superoxide dismutase. *Clin Chim Acta* **133:**209-214.

Niki, E. (2010). Assessment of antioxidant capacity in vitro and in vivo. *Free Radic Biol Med* **49:**503-515.

Nishida, Y., Akaoka, I., Kobayashi, M., Maruki, K. y Oshima, Y. (1979). Renal impairment in urate excretion in patients with Down´s syndrome. *J Rheumatol* **6:**103-107.

Nordberg, J. y Arnér, E. S. (2001). Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic Biol Med* **31:**1287-1312.

Nyman, U., Bjork, J., Sterner, G., Back, S. E., Carlson, J., Lindstrom, V., Bakoush, O. y Grubb, A. (2006). Standardization of p-creatinine assays and use of lean body mass allow improved prediction of calculated glomerular filtration rate in adults: A new equation. *Scand J Clin Lab Invest* **66:**451-468.

Nyyssonen, K., Porkkala-Sarataho, E., Kaikkonen, J. y Salonen, J. T. (1997). Ascorbate and urate are the strongest determinants of plasma antioxidative capacity and serum lipid resistance to oxidation in Finnish men. *Atherosclerosis* **130:**223-233.

Okado-Matsumoto, A. y Fridovich, I. (2001). Subcellular distribution of superoxide dismutases (SOD) in rat liver. Cu,Zn-SOD in mitochondria. *J Biol Chem* **276:**38388-38393.

Oppermann, U. (2007). Carbonyl reductases: the complex relationships of mammalian carbonyl- and quinone-reducing enzymes and their role in physiology. *Annu Rev Pharmacol Toxicol* **47:**293-322.

Pallardó, F. V., Degan, P., d'Ischia, M., Kelly, F. J., Zatterale, A. y Calzone, R. (2006). Multiple evidence for an early age pro-oxidant state in Down syndrome patients. *Biogerontology* **7:**211-220.

Pant, S. S., Moser, H. W. y Krane, S. M. (1968). Hyperuricemia in Down's syndrome. *J Clin Endocr* **28:**472-478.

Park, J., Song, W. J. y Chung, K. C. (2009). Function and regulation of Dyrk1A: towards understanding Down syndrome. *Cell Mol Life Sci* **66:**3235-3240.

Peled-Kamar, M., Lotem, J., Wirguin, I., Weiner, L., Hermalin, A. y Groner, Y. (1997). Oxidative stress mediates impairment of muscle function in transgenic mice with elevated level of wild-type Cu/Zn superoxide dismutase. *Proc Natl Acad Sci USA* **94:**3883-3887.

Prasher, V. y Haque, M. S. (2005). Misdiagnosis of thyroid disorders in Down syndrome: time to re-examine the myth? *Am J Ment Retard* **110:**23-27.

Praticò, D., Iuliano, L., Amerio, G., Tang, L. X., Rokach, J., Sabatino, G. y Violi, F. (2000). Down's syndrome is associated with increased 8,12-iso-iPF2alpha-VI levels: evidence for enhanced lipid peroxidation in vivo. *Ann Neurol* **48:**795-798.

Pratico, D., Lawson, J. A. y Fitzgerald, G. A. (1994). Cyclooxygenase-dependent formation of the isoprostane 8-epi prostaglandin F2 alpha. *Ann NY Acad Sci* **744:**139-145.

Proctor, P. (1970). Similar Functions of Uric Acid and Ascorbate in Man? *Nature* **228:**868-868.

Pryor, W. A. y Godber, S. S. (1991). Noninvasive measures of oxidative stress status in humans. *Free Radic Biol Med* **10:**177-184.

Pueschel, S. M. (1990). Clinical aspects of Down syndrome from infancy to adulthood. *Am J Med Genet Suppl* **7:**52-56.

Pueschel, S. M., Craig, W. Y. y Haddow, J. E. (1992). Lipids and lipoproteins in persons with Down's syndrome. *J Intellect Disabil Res* **36:**365-369.

Pueschel, S. M., Hillemeier, C., Caldwell, M., Senft, K., Mevs, C. y Pezzullo, J. C. (1990). Vitamin A gastrointestinal absorption in persons with Down's syndrome. *J Ment Defic Res* **34:**269-275.

Puukka, R., Puukka, M., Leppilampi, M., Linna, S. L. y Kouvalainen, K. (1982). Erythrocyte adenosine deaminase, purine nucleotide phosphorylase and phosphoribosyltransferase activity in patients with Down's syndrome. *Clin Chim Acta* **126:**275-281.

Ricciarelli, R., Zingg, J. M. y Azzi, A. (2001). Vitamin E: protective role of a Janus molecule. *FASEB J* **15:**2314-2325.

Roberts, L. J. 2nd y Milne, G. L. (2009). Isoprostanes. *J Lipid Res* **50:**S219-223.

Robertson, G. L. y Berl, T. (1991). Pathophysiology of water metabolism. En: Brenner, B. M. y Rector, F. C. Jr., editores. The Kidney. Cuarta edición. Philadelphia. W. B. Saunders. Págs. 677-736.

Sanders, S. A., Eisenthal, R. y Harrison, R. (1997). NADH oxidase activity of human xanthine oxidoreductase--generation of superoxide anion. *Eur J Biochem* **245:**541-548.

Sarandöl, E., Taş, S., Dirican, M. y Serdar, Z. (2005). Oxidative stress and serum paraoxonase activity in experimental hypothyroidism: effect of vitamin E supplementation. *Cell Biochem Funct* **23:**1-8.

Seidl, R., Greber, S., Schuller, E., Bernert, G., Cairns, N. y Lubec, G. (1997). Evidence against increased oxidative DNA-damage in Down syndrome. *Neurosci Lett* **235:**137-140.

Shapiro, B. L. (1983). Down syndrome--a disruption of homeostasis. *Am J Med Genet* **14:**241-269.

Shaposhnikov, A. M., Khal'chitskii, S. E. y Shvarts, E. I. (1979). Disorders of phenylalanine and tyrosine metabolism in Down's syndrome. *Vopr Med Khim* **25:**15-19.

Sherman, S. L., Allen, E. G., Bean, L. H. y Freeman, S. B. (2007). Epidemiology of Down syndrome. *Ment Retard Dev Disabil Res Rev* **13:**221-227.

Sies, H. (1985). Oxidative stress. Introductory remarks. En: Sies, H., editor. Oxidative stress. London. Academinc Press. páps. 1-8.

Sies, H. (1991a). Oxidative stress: oxidants and antioxidants. London. Academic Press.

Sies, H. (1991b). Oxidative stress: from basic research to clinical application. *Am J Med* **91:**31S-38S.

Sinet, P. M. (1982). Metabolism of oxygen derivatives in Down's syndrome. *Ann NY Acad Sci* **185:**83-94.

Sinet, P. M., Allard, D., Lejeune, J. y Jerome, H. (1974). Augmentation d'activité de la superoxyde dismutase érythrocytaire dans la trisomie pour le chromosome 21. *CR Acad Sci (Paris)* **278:**3267-3270.

Singh, R., Barden, A., Mori, T. y Beilin, L. (2001). Advanced glycation end-products: a review. *Diabetologia* **44:**129-146.

Sohn, Y. K., Ganju, N., Bloch, K. D., Wands, J. R. y de la Monte, S. M. (1999). Neuritic sprouting with aberrant expression of the nitric oxide synthase III gene in neurodegenerative diseases. *J Neurol Sci* **162:**133-151.

Song, M. -F., Li, Y. -S., Ootsuyama, Y., Kasai, H., Kawai, K., Ohta, M., Eguchi, Y., Yamato, H., Matsumoto, Y., Yoshida, R. y Ogawa, Y. (2009). Urea, the most abundant component in urine, cross-reacts with a commercial 8-OH-dG ELISA kit and contributes to overestimation of urinary 8-OH-dG. *Free Radic Biol Med* **47:**41-46.

Song, X., Bao, M., Li, D. y Li, Y. M. (1999). Advanced glycation in d-galactose induced mouse aging model. *Mech Ageing Dev* **108:**239-251.

Stapleton, F. B., Linshaw, M. A., Hassanein, K. y Gruskin, A. B. (1978). Uric acid excretion in normal children. *J Pediatr* **92:**911-914.

Takahashi, K., Nammour, T. M., Fukunaga, M., Ebert, J., Morrow, J. D., Roberts, L. J. 2nd, Hoover, R. L. y Badr, K. F. (1992). Glomerular actions of a free radical-generated novel prostaglandin, 8-epi-prostaglandin F2 alpha, in the rat. Evidence for interaction with thromboxane A2 receptors. *J Clin Invest* **90:**136-141.

Tiano, L., Padella, L., Carnevali, P., Gabrielli, O., Bruge, F., Principi, F. y Littarru, G. P. (2008). Coenzyme Q10 and oxidative imbalance in Down syndrome: biochemical and clinical aspects. *Biofactors* **32:**161-167.

Tice, R. R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J. C. y Sasaki, Y. F. (2000). Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen* **35:**206-221.

Torsdottir, G., Kristinsson, J., Hreidarsson, S., Snaedal, J. y Johannesson, T. (2001). Copper, ceruloplasmin and superoxide dismutase (SOD1) in patients with Down's syndrome. *Pharmacol Toxicol* **89:**320-325.

Torun, A. N., Kulaksizoglu, S., Kulaksizoglu, M., Pamuk, B. O., Isbilen, E. y Tutuncu, N. B. (2009). Serum total antioxidant status and lipid peroxidation marker malondialdehyde levels in overt and subclinical hypothyroidism. *Clin Endocrinol (Oxf)* **70:**469-474.

Tsukahara, H. (2007). Biomarkers for oxidative stress: clinical application in pediatric medicine. *Curr Med Chem* **14:**339-351.

Valko, M., Izakovic, M., Mazur, M., Rhodes, C. J. y Telser, J. (2004). Role of oxygen radicals in DNA damage and cancer incidence. *Mol Cell Biochem* **266:**37-56.

Vertuani, S., Angusti, A. y Manfredini, S. (2004). The antioxidants and pro-antioxidants network: an overview. *Curr Pharm Des* **10:**1677-1694.

Wayner, D. D., Burton, G. W., Ingold, K. U., Barclay, L. R. y Locke, S. J. (1987). The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. *Biochim Biophys Acta* **924:**408-419.

Wu, X. W., Muzny, D. M., Lee, C. C. y Caskey, C. T. (1992). Two independent mutational events in the loss of urate oxidase during hominoid evolution. *J Mol Evol* **34:**78-84.

Wyss, M. y Kaddurah-Daouk, R. (2000). Creatine and creatinine metabolism. *Physiol Rev* **80:**1107-1213.

Yilmaz, S., Ozan, S., Benzer, F. y Canatan, H. (2003). Oxidative damage and antioxidant enzyme activities in experimental hypothyroidism. *Cell Biochem Funct* **21:**325-330.

Yuen, J. W. y Benzie, I. F. (2003). Hydrogen peroxide in urine as a potential biomarker of whole body oxidative stress. *Free Radic Res* **37:**1209-1213.

Zitnanová, I., Korytár, P., Aruoma, O. I., Sustrová, M., Garaiová, I., Muchová, J., Kalnovicová, T., Pueschel, S. y Duracková, Z. (2004). Uric acid and allantoin levels in Down syndrome: antioxidant and oxidative stress mechanism? *Clin Chim Acta* **341:**139-146.

Zitnanová, I., Korytár, P., Sobotová, H., Horáková, L., Sustrová, M., Pueschel, S. y Duracková, Z. (2006). Markers of oxidative stress in children with Down syndrome. *Clin Chem Lab Med* **44:**306-310.

Anexo

fumadores sanos

C. Campos, R. Guzmán, E. López-Fernández y Á. Casado

En el presente trabajo de Tesis Doctoral, un número considerable de los voluntarios fumaba. El análisis de biomarcadores de estrés oxidativo/nitrosativo en este grupo de individuos ha dado lugar a la siguiente publicación, cuyo objetivo fue evaluar el efecto de fumar cigarrillos sobre el estrés oxidativo y nitrosativo mediante la valoración de los niveles urinarios de 8-hidroxi-2'-deoxiguanosina (8-OHdG), isoprostano 15-F_{2t}-IsoP, sustancias reactivas del ácido tiobarbitúrico (TBARS), productos finales de glicación avanzada (AGEs), ditirosina (diTyr), peróxido de hidrógeno (H_2O_2), nitritos y nitratos (NOx) y capacidad antioxidante equivalente de Trolox (TEAC) en fumadores sanos.

Los biomarcadores se determinaron empleando técnicas espectrofotométricas y fluorimétricas en muestras de orina de 33 fumadores sanos y 58 controles, con edades comprendidas entre los 19 y los 67 años. El grupo de no fumadores (control) estaba formado por 44 individuos que nunca habían fumado y por 14 ex-fumadores. Todos los parámetros fueron ajustados a creatinina (Cr).

Los niveles de 8-OHdG, 15-F_{2t}-IsoP y AGES fueron significativamente mayores en fumadores que en controles ($P<0,05$ en todos los casos). Se encontraron correlaciones positivas entre la edad y los niveles de AGEs y diTyr en fumadores ($r=0,380$, $P<0,035$ y $r=0,418$, $P<0,019$, respectivamente), así como entre la edad y AGEs, diTyr y TEAC en controles ($r=0,474$, $P<0,001$; $r=0,463$, $P<0,001$; y $r=0,576$, $P<0,001$, respectivamente), sindo esta correlación negativa para la 8-OHdG en controles ($r=-0,295$, $P=0,041$). También se encontro una correlación positiva entre el número de cigarrillos fumados al día y los niveles de AGES ($r=0,355$, $P=0,044$).

De los resultados de este estudio se puede concluir que 8-OHdG, 15-F_{2t}-IsoP y AGEs urinarios deben representar biomarcadores de estrés oxidativo adecuados en fumadores sanos, siendo la valoración de AGEs un posible indicador de exposición al tabaco. Por otra parte, el mayor estrés oxidativo encontrado en fumadores sanos debe ser consecuencia de una excesiva producción de especies reactivas del oxígeno y no de un agotamiento de las defensas antioxidantes no enzimáticas.

Inhalation Toxicology, 2011; 23(3): 148–156

ISSN 0895-8378 print/ISSN 1091-7691 online
DOI: 10.3109/08958378.2011.554460

informa
healthcare

RESEARCH ARTICLE

Urinary biomarkers of oxidative/nitrosative stress in healthy smokers

Carlos Campos, Rodrigo Guzmán, Encarnación López-Fernández, and Ángela Casado

Department of Cellular and Molecular Medicine, Centre for Biological Research—Spanish National Research Council (CIB-CSIC), Madrid, Spain

Abstract

Objective: To evaluate the effect of cigarette smoking on oxidative and nitrosative stress, we assessed urinary levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), isoprostane 15-F_{2t}-IsoP, thiobarbituric acid-reacting substances (TBARS), advanced glycation end-products (AGEs), dityrosine (diTyr), hydrogen peroxide, total nitrite and nitrate and trolox equivalent antioxidant capacity (TEAC) in healthy smokers.

Methods: Fluorimetric and spectrophotometric assays were performed in urine samples of 33 healthy smokers and 58 age-matched controls.

Results: Levels of 8-OHdG, 15-F_{2t}-IsoP and AGES were found significantly higher in smokers than in controls (10.7 ng/mg Cr vs. 8.3 ng/mg Cr, 1.41 ng/mg Cr vs. 1.01 ng/mg Cr and 189 AFU/mg Cr vs. 143 AFU/mg Cr, respectively; $P < 0.05$ for all). Positive correlations were found between age and levels of AGEs and diTyr in smokers ($r = 0.380$, $P < 0.035$ and $r = 0.418$, $P < 0.019$, respectively) and also between age and AGEs, diTyr and TEAC in controls ($r = 0.474$, $P < 0.001$, $r = 0.463$, $P < 0.001$ and $r = 0.576$, $P < 0.001$, respectively), being this correlation negative for 8-OHdG in controls ($r = -0.295$, $P = 0.041$). Positive correlation between the number of cigarettes smoked per day and AGEs was also found ($r = 0.355$, $P = 0.044$).

Conclusion: Urinary 8-OHdG, 15-F_{2t}-IsoP and AGEs may represent a non-invasive quantitative index of oxidant stress in healthy smokers, being AGEs a possible indicator of tobacco toxin exposure. The increased oxidative stress in healthy smokers observed may be generated because of an excessive production of reactive oxygen species and not by exhaustion of antioxidant defenses.

Keywords: Oxidative stress, nitrosative stress, biomarker, cigarette smoking, urine

Introduction

Cigarette smoking is a major risk factor for many forms of cancer, stroke, atherosclerosis, chronic obstructive pulmonary disease and cardiovascular diseases. Tobacco is also linked to susceptibility to infectious diseases and to cognitive dysfunction, which include increased risk of Alzheimer's disease (Cataldo et al., 2010) and, although the adverse health consequences of tobacco smoking have been known for over 50 years, no other product of consumption is so dangerous as tobacco, which causes premature mortality killing >5 million people worldwide each year, as it has been reported in the 2009 World Health Organization Report on the Global Tobacco Epidemic (WHO, 2009).

There are >5000 different chemical constituents in cigarette smoke which include free radicals, carcinogens, heavy metals, organic compounds, gaseous substances, such as carbon monoxide or nitrogen dioxide and other toxic substances which promote reactive oxygen species (ROS) formation and pose a significant oxidant stress *in vivo* (Rodgman and Perfetti, 2009). Free radicals and other ROS and reactive nitrogen species (RNS) are continuously generated in the human body due to endogenous as well as exogenous factors. When the generation of ROS/RNS exceeds the ability of antioxidant defense systems to remove them, such an imbalance can cause oxidative/nitrosative damage to cellular constituents (DNA, proteins, lipids, and carbohydrates), which is

Address for Correspondence: Carlos Campos Vaquero, Department of Cellular and Molecular Medicine, Centre for Biological Research, CSIC. C/ Ramiro de Maeztu, 9. E-28040 Madrid, Spain. Phone: +34-91-8373112; Fax: +34-91-5360432. E-mail: ccampos@cib.csic.es.

(Received 07 December 2010; revised 10 January 2011; accepted 10 January 2011)

Abbreviations

8-OHdG, 8-hydroxy-2′-deoxyguanosine;
15-F_{2t}-IsoP, isoprostane;
ABTS$^{\bullet+}$, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical;
AFU, arbitrary fluorescence units;
AGEs, advanced glycation end-products;
BHT, butylated hydroxytoluene;
Cr, creatinine;
DiTyr, dityrosine;
ELISA, enzyme-linked immunosorbent assay;
FOX-2, xylenol orange version-2;
H_2O_2, hydrogen peroxide;
HCl, hydrochloric acid;
JaICA, Japan Institute for the Control of Aging;
MDA, 1,1,3,3-tetraethoxypropane;
NO, nitric oxide;
NOx, total nitrite and nitrate;
NS, not significant;
RNS, reactive nitrogen species;
ROS, reactive oxygen species;
SD, standard deviation;
TAC, total antioxidant capacity;
TBA, 2-thiobarbituric acid;
TBARS, thiobarbituric acid-reacting substances;
TEAC, Trolox equivalent antioxidant capacity;
VCl_3, vanadium(III) chloride,
WHO, World Health Organization.

defined as oxidative/nitrosative stress (Halliwell and Gutteridge, 1999). Thus, oxidative and nitrosative stress have been involved in the pathology of several human diseases as well as in the reduction of life span.

The main difficulty in measuring oxidative stress *in vivo* is in capturing the very short-lived ROS/RNS. Thus, many biomarkers have been developed for the assessment of oxidative/nitrosative damage in biological samples. Biomarkers of oxidative and nitrosative stress in smokers have been analysed in a number of *in vivo* studies (Loft et al., 1992; Morrow et al., 1995; Nowak et al., 1996; Nicholl et al., 1998; Kovács et al., 2000; Nia et al., 2001; Lykkesfeldt, 2007), however these works are usually performed using invasive procedures which include measurements in tissue biopsies and more frequently in serum or plasma samples, which are often difficult to get and not comfortable for patients. In comparison, little information is available regarding non-invasive techniques for the assessment of oxidative stress in smokers.

The use of urine samples provides many advantages over plasma or serum in population studies as their collection does not require any invasive technique, poses minimal infectious disease risk to participants and researchers and provides enough volume for multiple assays and future research. Urine specimens are ideally suited for large studies because they can be collected by participants and compliance is high, being easier to obtain than other non-invasive samples such as saliva or exhaled breath condensate. Furthermore, urine is the preferred body fluid for the measurement of isoprostanes and dityrosine (diTyr). In spite of these advantages, urinary biomarkers of oxidative stress have been little studied in tobacco smokers and the set of biomarkers measured in each work limited. By all of them, we chose urine as the specimen of choice in this study.

Such advantages of urine samples and limitations in the literature led us to evaluate a comprehensive set of urinary biomarkers of oxidative/nitrosative stress widely used in a sample of healthy smokers and their age-matched non-smoker controls, which comprise: 8-hydroxy-2′-deoxyguanosine (8-OHdG), isoprostane 15-F_{2t}-IsoP (also 8-*epi*-$PGF_{2\alpha}$, 8-*iso*-$PGF_{2\alpha}$ or $iPF_{2\alpha}$-III), thiobarbituric acid-reacting substances (TBARS), advanced glycation end-products (AGEs), diTyr, hydrogen peroxide (H_2O_2) and total nitrite and nitrate (NOx) using fast, simple and inexpensive methodologies as well as urine as non-invasive sample, in order to probe the suitability of these urinary biomarkers in smokers. In addition, we evaluated the trolox equivalent antioxidant capacity (TEAC) in order to determine if the presumable increased oxidative/nitrosative stress in smokers is generated because of an excessive production of ROS/RNS, an exhaustion of antioxidant defenses or both.

Materials and methods

Subjects

The study was performed in 33 healthy current smokers (13 male and 20 female, mean age = 37.0 ± 14.0 years) and 58 healthy non-smoker controls (27 male and 31 female, mean age = 38.7 ± 14.8 years) with ages ranging from 19 to 67 years in both groups. The non-smoker group comprised 44 never-smokers and 14 ex-smokers (who had stopped smoking for at least 2 years before the study). The smokers group smoked a mean of 12 cigarettes per day (SD = 9), ranging from 1 to 40. Participants filled in a comprehensive questionnaire to obtain the following information: (a) sociodemographic parameters: age and gender; (b) use of medicines or dietary supplements; and (c) smoking habits. All of them received no dietary or medicinal vitamin supplementation and were healthy. Informed consent was obtained from the participants. The study was approved by The Ethics Committee of the Spanish National Research Council.

Urine sample collection and preparation

First morning urine samples on an empty stomach were collected in a sterile flask covered with aluminium foil to keep out stray light and processed within 2 h of the collection. Samples were 1 mL-aliquoted and frozen at −80°C until analysis. Creatinine (Cr) and NOx determination

were performed the day of collection, without previous freezing of the sample.

Biochemical determinations

Spectrophotometric methods were used to determine Cr, TBARS, H_2O_2, NOx, TEAC, 8-OHdG and 15-F_{2t}-IsoP. Thus, Cr was determined according to the spectrophotometric Jaffé method (Jaffé, 1886) which is based on the reaction of Cr with picric acid in alkaline pH.

TBARS were measured by the method of Uchiyama and Mihara (1978) with modifications. Briefly, 33 µL of 0.01% BHT (in absolute ethanol), 1 mL of 1% phosphoric acid and 300 µL of 42 mmol/L TBA (dissolved in water and heating) were added to 140 µL of urine, mixed in vortex. The mixtures were incubated in boiling water for 45 min and, after cooling tubes on ice, 1.4 mL of 1-butanol was added in each tube. Following a 15 min centrifugation (2000*g*), the absorbance of supernatant was read at 535 nm. The standard absorption curve of MDA was prepared by dissolving it in phosphate buffer (20 mmol/L, pH 7.0).

H_2O_2 was measured using the ferrous ion oxidation xylenol orange version-2 (FOX-2) method of Banerjee et al. (2002). In brief, 90 µL of urine was mixed with 10 µL of methanol and 900 µL of FOX-2 reagent (100 µmol/L xylenol orange, 250 µmol/L ammonium ferrous sulfate, 90% methanol, 4 mmol/L BHT and 25 mmol/L sulfuric acid). Tubes were vortexed and kept at room temperature for 30 min. Following a 10 min centrifugation (15,000*g*) absorbance of supernatant was read at 560 nm against a methanol blank. Then, the same procedure was followed except for 10 µL of methanol that were replaced by 10 µL of catalase solution (2200 U/mL in 25 mmol/L phosphate buffer, pH 7.0). Urinary H_2O_2 concentrations were calculated from the absorbance difference (with and without catalase) at 560 nm using a standard curve prepared with H_2O_2.

NOx was measured by the acidic Griess reaction according to the method of Miranda et al. (2001). In brief, 100 µL of sample were applied to a microplate well. Following the addition of 100 µL VCl_3 (8 mg/mL in 1 mol/L HCl) to each well 100 µL of the Griess reagent (premixed 50 µL sulfanilamide (2% in 5% HCl) and 50 µL N-(1-Naphthyl) ethylendiamine dihydrochloride (0.1%)) were added immediately. Microplate was then incubated at 37°C for 30 min and absorbance was read at 540 nm. Nitrate was determined from a linear standard curve established with sodium nitrate. Nitrite was measured in a similar manner except for the fact that samples and nitrite standards were only exposed to Griess reagent.

TEAC assay was performed as previously described (Re et al., 1999). Briefly, 1 mL of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical $ABTS^{\cdot+}$ preheated at 30°C was added along with 10 µL of sample or standard (Trolox) and absorbance at 734 nm was measured after 5 s. $ABTS^{\cdot+}$ was prepared by mixing ABTS stock solution (7 mM in water) with 2.45 mM potassium persulfate. This mixture has to remain in the dark at room temperature for at least 12 h. Before use, the $ABTS^{\cdot+}$ solution was diluted with PBS, pH 7.4, to an absorbance of 0.700 ± 0.020 at 734 nm. The antioxidant activity of samples was calculated by determining the decrease in absorbance by using the following equation: Antioxidant capacity = $(A_0-A_C)/A_0$, where A_0 is the absorbance of the reference sample and A_C the absorbance of the sample.

Urinary 8-OHdG and 15-F_{2t}-IsoP determinations were performed by using spectrophotometric enzyme-linked immunosorbent assays (ELISAs) kits from the Japan Institute for the Control of Aging (JaICA, Shizuoka, Japan) and from Oxford Biomedical Research (Oxford Biomedical Research, Oxford, MI), respectively. For ELISA and NOx assays, absorbance measurements were carried out using a microplate reader (ELx808, Bio-Tek Instruments, Winooski, VT). Other spectrophotometric measurements were made in a UVmini-1240 Shimadzu spectrophotometer (Shimadzu, Tokyo, Japan).

Urinary AGEs and diTyr were assayed fluorimetrically using a Varioskan Flash microplate reader (Thermo Fisher Scientific, Waltham, MA) operating at room temperature. Thus, AGEs was estimated essentially as described by Yanagisawa et al. (1998). Briefly, fluorescence intensity of urine diluted 20-fold in phosphate buffered saline was measured at 440 nm after excitation at 370 nm. For diTyr measurement the assay was performed essentially as described by Witko-Sarsat et al. (1996). In brief, urine was diluted 20-fold in 50 mmol/L phosphate buffer, pH 7.4, containing 6 mol/L urea. Fluorescence intensity was measured at excitation and emission wavelengths of 315 and 410 nm, respectively, after 30 min at room temperature. Results were reported as arbitrary fluorescence units (AFU).

Statistical analysis

Data are presented as mean ± standard deviation (SD) and range. Normal distribution and homogeneity of variance of the data were tested by the Shapiro-Wilk's and Levene's tests, respectively. Since data did not satisfy the conditions of normality or homoscedasticity, group differences were analyzed by nonparametric Mann-Whitney *U*-test (or Kruskal-Wallis test for multiple group comparisons) and correlation analyses were performed using Spearman's correlation test. Statistical significance was set at $P < 0.05$. Data were processed using SPSS 17.0 software (SPSS Inc., Chicago, IL).

Results

No significant differences in age were observed between smokers and non-smoker controls ($P = 0.679$). When the influence of gender was analyzed, no significant differences were found for any parameter in any group ($P > 0.05$ for all). With regard to Cr levels, no statistically significant differences were found between healthy smokers and controls (189 ± 82 mg/dL vs. 217 ± 84 mg/dL, respectively; $P = 0.181$).

Table 1. Urinary levels of biomarkers of oxidative and nitrosative stress (8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs, diTyr, H_2O_2, NOx and TEAC) in healthy smokers and their age-matched non-smoker controls. The data are expressed as mean ± standard deviation (SD) and ranges.

	Controls			Smokers			
Urinary biomarker	*n*	Mean ± SD	Range	*n*	Mean ± SD	Range	*P*
8-OHdG (ng/mg Cr)	41	8.3±3.6	2.1-17.7	22	10.7±4.1	5.3-22.2	0.013*
15-F_{2t}-IsoP (ng/mg Cr)	38	1.01±0.40	0.21-2.18	22	1.41±0.82	0.30-3.36	0.041*
TBARS (μg/mg Cr)	58	0.74±0.30	0.00-1.59	33	0.78±0.21	0.50-1.55	0.262
AGEs (AFU/mg Cr)	54	143±58	78-359	31	189±88	106-538	<0.001***
DiTyr (AFU/mg Cr)	54	522±236	249-1713	31	586±300	202-1571	0.268
H_2O_2 (μg/mg Cr)	51	0.36±0.38	0.00-1.89	30	0.32±0.32	0.04-1.27	0.503
NOx (μg/mg Cr)	57	43±32	14-186	33	44±27	10-118	0.766
TEAC (mg Trolox eq./mg Cr)	52	0.86±0.26	0.53-1.75	32	0.88±0.22	0.45-1.53	0.384

*$P<0.05$, ***$P<0.001$.

In Table 1, the urinary levels of 8-OHdG, 15-F_{2t}-IsoP, TBARS, AGEs, diTyr, H_2O_2, NOx and TEAC in healthy smokers and controls are listed. We found higher levels of 8-OHdG, 15-F_{2t}-IsoP and AGEs in smokers than in controls ($P<0.05$ for all). In contrast, no statistically significant differences were found in any of the other parameters.

Figure 1 shows correlations between biochemical parameters and age in smokers and controls. A significant negative linear correlation was found between age and Cr in smokers and non-smokers ($r=-0.328$, $P=0.043$, $y=2.22x+298.78$ and $r=-0.351$, $P=0.007$, $y=1.88x+262.04$, respectively) and between age and 8-OHdG in non-smokers ($r=-0.295$, $P=0.041$, $y=0.07x+10.76$). A significant positive linear correlation was found between age and AGEs in smokers and non-smokers ($r=0.380$, $P=0.035$, $y=4.02x+47.26$ and $r=0.474$, $P<0.001$, $y=2.04x+63.92$, respectively), diTyr in smokers and non-smokers ($r=0.418$, $P=0.019$, $y=12.04x+162.70$ and $r=0.463$, $P<0.001$, $y=7.34x+229.59$, respectively) and TEAC in non-smokers ($r=0.576$, $P<0.001$, $y=0.01x+0.52$). In contrast, no correlation was found between age and 8-OHdG in smokers ($r=-0.251$, $P=0.260$), 15-F_{2t}-IsoP in smokers and non-smokers ($r=-0.031$, $P=0.891$ and $r=-0.008$, $P=0.960$, respectively), TBARS in smokers and non-smokers ($r=-0.073$, $P=0.685$ and $r=0.174$, $P=0.192$, respectively), H_2O_2 in smokers and non-smokers ($r=-0.190$, $P=0.315$ and $r=0.084$, $P=0.557$, respectively), NOx in smokers and non-smokers ($r=0.053$, $P=0.768$ and $r=0.166$, $P=0.216$, respectively) and TEAC in smokers ($r=-0.024$, $P=0.895$).

We also analyzed possible correlations between oxidative and nitrosative stress biomarkers and the number of cigarettes smoked per day in healthy smokers (Table 2). A significant positive correlation was found only for AGEs ($r=0.355$, $P=0.044$).

When ex-smokers were excluded from the non-smoker control group similar results were obtained for all the markers analysed. Besides, when levels of Cr and biomarkers of oxidative/nitrosative stress were compared between smokers and ex-smokers, similar results to those found in the comparison between never-smokers and smokers were obtained.

Discussion

The occurrence of oxidative and nitrosative stress was usually analysed by measuring different biomarkers. Despite substantial evidence linking tobacco smoke and oxidative damage, urinary biomarkers of oxidative stress have been little studied in tobacco smokers and the set of markers measured in each work was limited and usually performed with invasive, high cost and technically difficult procedures. However, available biomarkers are not equivalent indicators at all and, since the process is complex, it has been suggested that several oxidative stress biomarkers should be observed at a time to evaluate the oxidative status of an individual (Dotan et al., 2004). Thus, in this study we evaluate a comprehensive set of urinary biomarkers of oxidative and nitrosative stress in a sample of healthy smokers and their age-matched non-smoker controls using fast, simple and inexpensive methodologies and urine as a non-invasive sample in order to assess their suitability in smokers.

DNA damage assessment

It has been reported that oxidative DNA damage permanently occurs in living cells and it may contribute to carcinogenesis (Loft and Poulsen, 1996), being 8-OHdG the most commonly used biomarker for its assessment. Our results show that urinary levels of 8-OHdG were significantly higher in healthy smokers than in controls, being the level on average 35% higher in smokers, which is in agreement with other studies (Loft et al., 1992; Loft et al., 1994). However, no correlation was found between urinary 8-OHdG and age in smokers as it has also been reported by Feng et al. (2006), being this correlation significantly negative in non-smokers. Conflicting results have been reported in the relationship between age and oxidative DNA damage. Sakano et al. (2009) found a positive correlation between urinary 8-OHdG and age in healthy subjects. In contrast, it has been speculated that the elimination of oxidative DNA damage decreases with age (Loft and Poulsen, 1998), which is in accordance with previous studies (Fraga et al., 1990) and also with our results. Fraga et al. (1990) suggested that the decrease of the urinary excretion of 8-OHdG with age could be a consequence

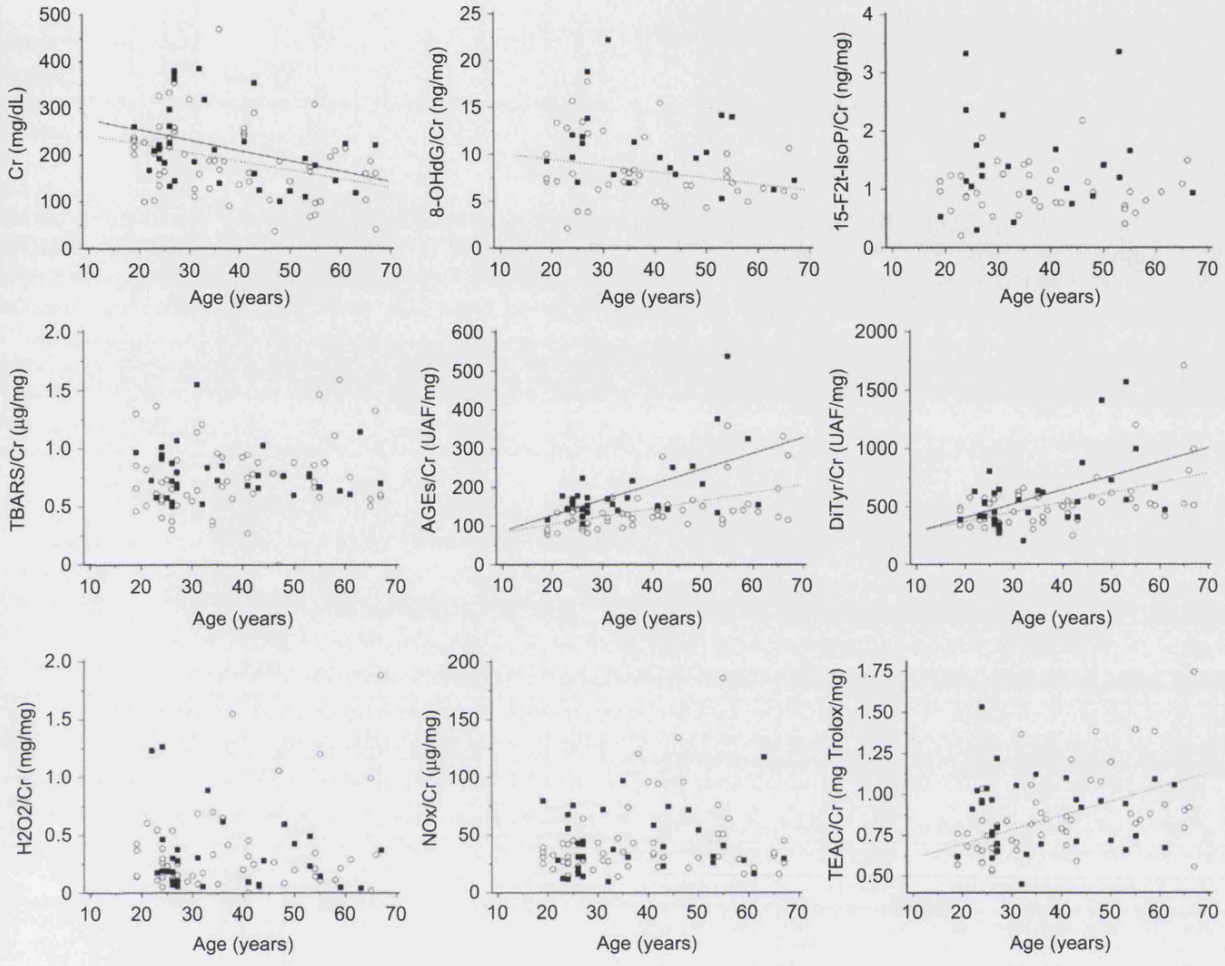


Figure 1. Scatter plots of biochemical parameters vs. age in healthy smokers (■, solid line) and their age-matched non-smoker controls (○, dotted line).

of an age-dependent decrease in the nuclease activities responsible for the repair of 8-OHdG. In our study no correlation between urinary 8-OHdG and age in healthy smokers were found, so the increased DNA damage caused by smoking could be masking the reducing effect of age on the 8-OHdG levels.

On the other hand, since urea and other substances contribute to the overestimation of urinary 8-OHdG due to cross-reaction of the commercial ELISA assay of JaICA which we used, as it has been recently reported (Song et al., 2009), and intra-individual variability is high, these results should be taken with caution and more studies are necessary in order to prove the suitability of the ELISA assay used for the DNA damage assessment.

Lipid peroxidation assessment

With regard to lipid peroxidation biomarkers, 15-F_{2t}-IsoP and TBARS, we found that urinary levels of 15-F_{2t}-IsoP were significantly higher in healthy smokers than in controls, with an increase in smokers of 40% when compared to non-smokers, which is in good accordance with previously published data (Morrow et al., 1995; Basu et al., 2009). However, no significant differences were found between the two groups in TBARS levels.

It has been shown that F_2-isoprostane levels are significantly increased in atherosclerotic plaques compared with normal vascular tissue, suggesting that these compounds may play a role in the pathogenesis of the disease (Gniwotta et al., 1997). The observation of elevated levels of urinary 15-F_{2t}-IsoP in patients with coronary heart disease strengthens this hypothesis (Schwedhelm et al., 2004). Therefore, although the biological role of isoprostanes is not clear yet, they seem to be good biomarkers of oxidative stress in smokers.

On the other hand, measurement of MDA levels using TBARS assay is one of the more broadly-used methods to determine urinary lipid peroxidation due to the fact that it is a simple, inexpensive and fast methodology. Most works studying the effect of smoking on MDA/TBARS levels found significantly increased MDA/TBARS levels in smokers vs. non-smokers (Lykkesfeldt, 2007). However, according to our results, two of the largest studies performed in smokers in which TBARS

Table 2. Spearman's correlations of urinary biomarkers of oxidative and nitrosative stress adjusted to creatinine with the number of cigarettes smoked per day in healthy smokers.

Correlated variables with the number of cigarettes smoked per day	r	P
8-OHdG	−0.380	0.089
15-F_{2t}-IsoP	0.010	0.964
TBARS	0.002	0.990
AGEs	0.355	0.044*
DiTyr	0.086	0.651
H_2O_2	−0.153	0.429
NOx	0.215	0.238
TEAC	−0.073	0.698

*$P < 0.05$.

was assayed, reported non-significant increases of this biomarker in plasma samples of smokers vs. non-smokers (Berr et al., 1998; Marangon et al., 1998). One major problem with TBARS assay is the fact that it is performed in numerous variations making comparisons of results between laboratories difficult. Moreover, much controversy has appeared in studies concerning the specificity of TBARS toward composites other than MDA.

In summary, our study showed that lipid peroxidation in smokers can be assessed by the measurement of 15-F_{2t}-IsoP and not by the TBARS assay, at least with the methods described above.

Glycoxidation assessment

Protein glycation and subsequent Maillard or browning reactions of glycated proteins form free carbonyls, which attack the major cellular components represented by proteins resulting in the formation of covalent compounds known as AGEs. The involvement of oxidative stress in the accelerated formation of AGEs has been reported and suggests that these compounds could be considered as an oxidative stress biomarker.

It has been reported that both aqueous extracts of tobacco and cigarette smoke contain glycotoxins, highly reactive glycation products that can rapidly induce AGE formation on proteins *in vitro* and *in vivo* and cause DNA mutations *in vitro*, being responsible in part for the increased rates of atherosclerotic vascular disease and cancer detected among cigarette smokers (Cerami et al., 1997). Therefore, increased levels of AGEs are expected in smokers as it was found in this study in urine samples of healthy smokers compared with non-smokers, being the level on average 32% higher in smokers. Additional evidence was available supporting the fact that cigarette smoke would increase oxidative stress and the levels of AGEs in serum (Cerami et al., 1997) and in tissues (Nicholl et al., 1998). Altogether, these results suggest that urinary AGEs may be considered as a suitable biomarker of oxidative stress in smokers. Moreover, we found that urinary AGEs correlated positively with age in the two groups studied, so urinary AGEs could also be a useful biomarker of aging.

Protein oxidation assessment

DiTyr, also *o,o′*-diTyr, is a fluorescent molecule formed as a result of normal posttranslational processes affecting specific structural proteins. It has been used as an important biomarker for oxidatively modified proteins (Giulivi et al., 2003).

It has been reported that gas-phase cigarette smoke is capable of converting tyrosine to 3-nitrotyrosine and diTyr, indicating free radical mechanisms of protein damage by nitrogen oxides (Eiserich et al., 1994). However, urinary levels of diTyr in smokers have only been measured by Orhan et al. (2005), but without comparing with a non-smoker group. In this study, urinary levels of diTyr do not differ between smokers and non-smokers as it was suggested by Orhan et al. Therefore, urinary diTyr, which has been considered a promising biomarker of protein oxidation, is not suitable for this purpose in smokers, at least when it is measured using the fluorimetric method described above. However, we also found that urinary levels of diTyr were positively correlated with age in the two groups studied, so urinary diTyr could be a useful biomarker of aging.

H_2O_2

Cigarette smoke can generate H_2O_2, an important ROS, in aqueous solution (Nakayama et al., 1984). Moreover, the cigarette tar semiquinone radical can reduce oxygen to produce superoxide, and hence H_2O_2 and the hydroxyl radical (Pryor, 1997). Since H_2O_2 itself is toxic to the cells and plays a central role in the formation of even more toxic ROS, H_2O_2 may significantly contribute to the pathogenic mechanisms of smoking-related diseases. Besides, urinary H_2O_2 was postulated to be an oxidative stress biomarker (Halliwell et al., 2000). However, urinary levels of H_2O_2 have not been previously assessed in smokers.

Our results show that urinary levels of H_2O_2 are similar in healthy smokers than in controls. Thus, since cigarette smokers had higher H_2O_2 levels in expired breath condensate than non-smokers (Nowak et al., 1996), H_2O_2 generated directly from cigarette smoke could be removed by antioxidants present in the alveolar lining fluid. On the other hand, our results show wide inter-individual variations obtained in H_2O_2 levels adjusted to Cr. Similar variations have been observed in previous works (Halliwell et al., 2004) and could show that H_2O_2 concentration is more determined by metabolic and nutritional influences than by changes in oxidative stress. Therefore, further studies are needed to understand the underlying mechanisms of these findings.

Nitrosative stress assessment

Nitric oxide (NO) is a free radical gas which has been involved in a wide range of physiological functions and plays an important role in pathologic areas. NO is present in cigarette smoke at up to 500 ppm, and probably represents one of the greatest exogenous sources of NO to which humans are exposed (Eiserich et al., 1994).

Since NO rapidly degrades to nitrate and nitrite in aqueous solution, the urinary NOx levels can be estimated as an index of NO production. In smokers, not only endogenous NO production but also degradation of NO inhaled with smoke may cause the increased levels of urinary NOx, therefore urinary NOx could be determined as an index of NO production and inhalation. Nevertheless, the effects of smoking on NO metabolism are complex and controversial results are found in literature. Smoking, particularly heavy smoking, has been paradoxically associated with lower serum NOx (Node et al., 1997). However, the only published work studying urinary levels of NOx in smokers showed that the amount of NOx excreted in 24-h urine of healthy smokers is significantly higher than in non-smokers (Kovács et al., 2000). In contrast, we found no differences in urinary NOx between both groups, being likely that NO coming from cigarette smoke rapidly degrades to inactive metabolites.

TAC assessment

Due to the free radicals present in cigarette smoke and the ROS overproduction, a diminished antioxidants level may be found in smokers because of their exhaustion. Separate measurement of different antioxidant molecules is not practical and their determination separately could be less representative of the overall antioxidant capacity due to the possible *in vivo* interaction among different antioxidants. Therefore, the total antioxidant capacity (TAC) is a useful measurement for the assessment of the antioxidant status of individuals. In this study, we found no differences in TAC measured by TEAC assay between healthy smokers and non-smokers, being these results in agreement with Nia et al. (2001).

Therefore, our results show that the increased oxidative/nitrosative stress in healthy smokers observed in the present work is generated because of an excessive production of ROS/RNS and not by exhaustion of antioxidant defenses. In fact, the higher levels of biomarkers of oxidative stress with no decreased of TAC could also be the reason for the current healthy state of the smokers of this study.

On the other hand, TEAC was positively correlated with age only in non-smokers, as it was also found by Nia et al. (2001) for the overall of smokers and non-smokers. Potential misleading factors, such as dietary habits, could explain these results and must be analyzed in future investigations. Another possible explanation is that smoking really induces the production of antioxidants in the whole-body to try to reduce the increased oxidative stress induced by cigarette smoke.

Analysis of Cr adjustment

Normalization of urinary analyte concentrations to Cr compensates for individual differences in the glomerular filtration rate and is the standard and widely used comparative measure for urinary biomarkers in spot and first-morning specimens. Cr is also used for the renal function determination, specifically the glomerular filtration rate. Thus, in agreement with our results, it has been reported a decreased urinary Cr concentrations in adults with increasing age, probably because of a general decline in muscularity and glomerular filtration rate (Alessio et al., 1985). Therefore, it must be noted that Cr could give rise to errors of interpretation attending to correlations of analysed biomarkers with age. Moreover, it has been suggested that a general increase in oxidative conditions with age is not supported by the decrease in metabolic rate occurring with age (Fraga et al., 1990).

Dose-effect relationship between the number of cigarettes smoked per day and biomarkers of oxidative/nitrosative stress

With regard to smoke exposure, we found a significant positive correlation only between the number of cigarettes smoked per day and the excretion of AGEs in healthy smokers. Since levels of AGEs are also significantly increased in healthy smokers vs. non-smokers, urinary AGEs may be considered not only as a suitable biomarker of oxidative stress in smokers but also as a biomarker of tobacco toxin exposure, at least in the range from 1 to 40 cigarettes smoked per day. However, it must be noted that the correlation found was weak. Besides, although asking the number of cigarettes smoked per day is currently regarded as the gold standard measure of exposure, it must be take into account that it has been reported that this statistic may not be a good indicator of toxin exposure (Joseph et al., 2005).

On the other hand, we also found that cigarette smoking increased the urinary levels of 8-OHdG and 15-F_{2t}-IsoP with independence of the number of cigarettes smoked per day, at least in the range studied (1–40 cigarettes per day). Other studies did not found any association between smoke exposure and biomarkers of oxidative stress (Nia et al., 2001; Feng et al., 2006), and it may show that the negative consequences of smoking to health are notable.

Effect of tobacco smoking on oxidative/nitrosative stress biomarkers in ex-smokers

With regard to the effect of tobacco smoking in ex-smokers, we analyzed oxidative/nitrosative stress biomarkers in 14 ex-smokers who had stopped smoking for at least 2 years before the study. However, when ex-smokers were excluded from the study, similar results were obtained for all the parameters analysed. Moreover, when levels of Cr and biomarkers of oxidative/nitrosative stress were compared between smokers and ex-smokers, similar results to those found in the comparison between never-smokers and smokers were obtained. Therefore, these results may indicate that the effect of cigarette smoking on biomarkers of oxidative/nitrosative stress levels is not sustainable in ex-smokers, at least in those who had stopped smoking >2 years before the study.

Future directions

Other biomarkers must be assessed in non-invasive samples of healthy smokers, such as byopyrrins (bilirubin

oxidative metabolites) or homocysteine, in order to establish suitable biomarkers of oxidative stress which allow achieving a better understanding of the effects of tobacco smoking on health. Homocysteine, a marker of increased risk of cardiovascular disease and atherosclerosis, has been shown to be associated with both primary and second-hand smoke (Sobczak et al., 2004; Kim et al., 2010). However the link between this biomarker and cigarette smoking-induced oxidative damage has not been studied. Moreover, further works must be conducted in order to study the possible effect of duration of tobacco smoking on oxidative stress.

Conclusions

In summary, we found that urinary 8-OHdG, 15-F_{2t}-IsoP and AGEs, assayed with the methods described in this study, may represent a non-invasive quantitative index of oxidant stress *in vivo* in healthy smokers, being excretion of AGEs a possible good indicator of tobacco toxin exposure. Besides, the increased oxidative stress in healthy smokers observed may be generated because of excessive production of ROS/RNS and not by exhaustion of antioxidant defenses. Moreover, positive correlations with age were found for urinary AGEs and diTyr in smokers and in non-smokers and also for TEAC in non-smokers. However, these results could be biased by the positive correlation between age and Cr found in both groups. Besides, different lifestyle patterns, such as dietary habits or exercise routines, may be analyzed in future works in order to confirm the results of the present work.

Quantification of these non-invasive biomarkers of *in vivo* DNA damage, lipid peroxidation and glycoxidation may be especially helpful to test the efficacy of new drugs, to assess the efficacy of a dietary intervention or in early detection and prevention of diseases in an easily accessible and cost-effective manner in smokers.

Acknowledgements

The authors thank all the volunteers which cooperated and made the achievement of this work possible. We apologize to the authors who feel that their work was overlooked and those whose work is not described or referenced here. This study was supported by Fundación Inocente, Inocente.

Declaration of interest

The authors report no declarations of interest.

References

Alessio L, Berlin A, Dell'Orto A, Toffoletto F, Ghezzi I. 1985. Reliability of urinary creatinine as a parameter used to adjust values of urinary biological indicators. Int Arch Occup Environ Health 55:99-106.

Banerjee D, Kumar AP, Kumar B, Madhusoodanan UK, Nayak S, Jacob J. 2002. Determination of absolute hydrogen peroxide concentration by spectrophotometric method. Curr Sci 83:1193-1194.

Basu S, Helmersson J, Jarosinska D, Sällsten G, Mazzolai B, Barregård L. 2009. Regulatory factors of basal F(2)-isoprostane formation: population, age, gender and smoking habits in humans. Free Radic Res 43:85-91.

Berr C, Coudray C, Bonithon-Kopp C, Roussel AM, Mainard F, Alperovitch A. 1998. Demographic and cardiovascular risk factors in relation to antioxidant status: the EVA Study. Int J Vitam Nutr Res 68:26-35.

Cataldo JK, Prochaska JJ, Glantz SA. 2010. Cigarette smoking is a risk factor for Alzheimer's Disease: an analysis controlling for tobacco industry affiliation. J Alzheimers Dis 19:465-480.

Cerami C, Founds H, Nicholl I, Mitsuhashi T, Giordano D, Vanpatten S, Lee A, Al-Abed Y, Vlassara H, Bucala R, Cerami A. 1997. Tobacco smoke is a source of toxic reactive glycation products. Proc Natl Acad Sci USA 94:13915-13920.

Dotan Y, Lichtenberg D, Pinchuk I. 2004. Lipid peroxidation cannot be used as a universal criterion of oxidative stress. Prog Lipid Res 43:200-227.

Eiserich JP, Vossen V, O'Neill CA, Halliwell B, Cross CE, van der Vliet A. 1994. Molecular mechanisms of damage by excess nitrogen oxides: nitration of tyrosine by gas-phase cigarette smoke. FEBS Lett 353:53-56.

Feng S, Roethig HJ, Liang Q, Kinser R, Jin Y, Scherer G, Urban M, Engl J, Riedel K. 2006. Evaluation of urinary 1-hydroxypyrene, S-phenylmercapturic acid, trans,trans-muconic acid, 3-methyladenine, 3-ethyladenine, 8-hydroxy-2'-deoxyguanosine and thioethers as biomarkers of exposure to cigarette smoke. Biomarkers 11:28-52.

Fraga CG, Shigenaga MK, Park JW, Degan P, Ames BN. 1990. Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. Proc Natl Acad Sci USA 87:4533-4537.

Giulivi C, Traaseth NJ, Davies KJ. 2003. Tyrosine oxidation products: analysis and biological relevance. Amino Acids 25:227-232.

Gniwotta C, Morrow JD, Roberts LJ 2nd, Kühn H. 1997. Prostaglandin F2-like compounds, F2-isoprostanes, are present in increased amounts in human atherosclerotic lesions. Arterioscler Thromb Vasc Biol 17:3236-3241.

Halliwell B, Clement MV, Long LH. 2000. Hydrogen peroxide in the human body. FEBS Lett 486:10-13.

Halliwell B, Gutteridge JMC. 1999. Oxidative stress. In: Halliwell B, Gutteridge JMC, eds. Free radicals in biology and medicine. New York: Oxford University Press, 246-350.

Halliwell B, Long LH, Yee TP, Lim S, Kelly R. 2004. Establishing biomarkers of oxidative stress: the measurement of hydrogen peroxide in human urine. Curr Med Chem 11:1085-1092.

Jaffé M. 1886. Über den niederschlag, welchen pikrinsäure in normalen harn erzeugt und über eine neue reaktion des kreatinins. Z Physiol Chem 10:391-400.

Joseph AM, Hecht SS, Murphy SE, Carmella SG, Le CT, Zhang Y, Han S, Hatsukami DK. 2005. Relationships between cigarette consumption and biomarkers of tobacco toxin exposure. Cancer Epidemiol Biomarkers Prev 14:2963-2968.

Kim DB, Oh YS, Yoo KD, Lee JM, Park CS, Ihm SH, Jang SW, Shim BJ, Kim HY, Seung KB, Rho TH, Kim JH. 2010. Passive smoking in never-smokers is associated with increased plasma homocysteine levels. Int Heart J 51:183-187.

Kovács T, Vas T, Wagner L, Schmelczer M, Kocsis B, Nagy J. 2000. Effect of smoking on urinary NOx and cGMP excretion in IgA nephropathy and in health. Contrib Nephrol 130:124-129.

Loft S, Astrup A, Buemann B, Poulsen HE. 1994. Oxidative DNA damage correlates with oxygen consumption in humans. FASEB J 8:534-537.

Loft S, Poulsen HE. 1996. Cancer risk and oxidative DNA damage in man. J Mol Med 74:297-312.

Loft S, Poulsen HE. 1998. Estimation of oxidative DNA damage in man from urinary excretion of repair products. Acta Biochim Pol 45:133-144.

Loft S, Vistisen K, Ewertz M, Tjønneland A, Overvad K, Poulsen HE. 1992. Oxidative DNA damage estimated by 8-hydroxydeoxyguanosine excretion in humans: influence of smoking, gender and body mass index. Carcinogenesis 13:2241-2247.

Lykkesfeldt J. 2007. Malondialdehyde as biomarker of oxidative damage to lipids caused by smoking. Clin Chim Acta 380:50-58.

Marangon K, Herbeth B, Lecomte E, Paul-Dauphin A, Grolier P, Chancerelle Y, Artur Y, Siest G. 1998. Diet, antioxidant status, and smoking habits in French men. Am J Clin Nutr 67:231-239.

Miranda KM, Espey MG, Wink DA. 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 5:62-71.

Morrow JD, Frei B, Longmire AW, Gaziano JM, Lynch SM, Shyr Y, Strauss WE, Oates JA, Roberts LJ 2nd. 1995. Increase in circulating products of lipid peroxidation (F2-isoprostanes) in smokers. Smoking as a cause of oxidative damage. N Engl J Med 332:1198-1203.

Nakayama T, Kodama M, Nagata C. 1984. Generation of hydrogen peroxide and superoxide anion radical from cigarette smoke. Gann 75:95-98.

Nia AB, Van Schooten FJ, Schilderman PA, De Kok TM, Haenen GR, Van Herwijnen MH, Van Agen E, Pachen D, Kleinjans JC. 2001. A multi-biomarker approach to study the effects of smoking on oxidative DNA damage and repair and antioxidative defense mechanisms. Carcinogenesis, 22, 395-401.

Nicholl ID, Stitt AW, Moore JE, Ritchie AJ, Archer DB, Bucala R. 1998. Increased levels of advanced glycation endproducts in the lenses and blood vessels of cigarette smokers. Mol Med 4:594-601.

Node K, Kitakaze M, Yoshikawa H, Kosaka H, Hori M. 1997. Reversible reduction in plasma concentration of nitric oxide induced by cigarette smoking in young adults. Am J Cardiol 79:1538-1541.

Nowak D, Antczak A, Krol M, Pietras T, Shariati B, Bialasiewicz P, Jeczkowski K, Kula P. 1996. Increased content of hydrogen peroxide in the expired breath of cigarette smokers. Eur Respir J 9:652-657.

Orhan H, Coolen S, Meerman JH. 2005. Quantification of urinary o,o'-dityrosine, a biomarker for oxidative damage to proteins, by high performance liquid chromatography with triple quadrupole tandem mass spectrometry. A comparison with ion-trap tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 827:104-108.

Pryor WA. 1997. Cigarette smoke radicals and the role of free radicals in chemical carcinogenicity. Environ Health Perspect 105 Suppl 4:875-882.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 26:1231-1237.

Rodgman A, Perfetti TA. 2009. The chemical components of tobacco and tobacco smoke. Boca Raton, FL, USA: CRC Press, Taylor and Francis Group.

Sakano N, Takahashi N, Wang DH, Sauriasari R, Takemoto K, Kanbara S, Sato Y, Takigawa T, Takaki J, Ogino K. 2009. Plasma 3-nitrotyrosine, urinary 8-isoprostane and 8-OHdG among healthy Japanese people. Free Radic Res 43:183-192.

Schwedhelm E, Bartling A, Lenzen H, Tsikas D, Maas R, Brümmer J, Gutzki FM, Berger J, Frölich JC, Böger RH. 2004. Urinary 8-iso-prostaglandin F2alpha as a risk marker in patients with coronary heart disease: a matched case-control study. Circulation 109:843-848.

Sobczak A, Wardas W, Zielinska-Danch W, Pawlicki K. 2004. The influence of smoking on plasma homocysteine and cysteine levels in passive and active smokers. Clin Chem Lab Med 42:408-414.

Song MF, Li YS, Ootsuyama Y, Kasai H, Kawai K, Ohta M, Eguchi Y, Yamato H, Matsumoto Y, Yoshida R, Ogawa Y. 2009. Urea, the most abundant component in urine, cross-reacts with a commercial 8-OH-dG ELISA kit and contributes to overestimation of urinary 8-OH-dG. Free Radic Biol Med 47:41-46.

Uchiyama M, Mihara M. 1978. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem, 86:271-278.

WHO. 2009. World Health Organization Report on the Global Tobacco Epidemic. Geneva, Switzerland.

Witko-Sarsat V, Friedlander M, Capeillère-Blandin C, Nguyen-Khoa T, Nguyen AT, Zingraff J, Jungers P, Descamps-Latscha B. 1996. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. Kidney Int 49:1304-1313.

Yanagisawa K, Makita Z, Shiroshita K, Ueda T, Fusegawa T, Kuwajima S, Takeuchi M, Koike T. 1998. Specific fluorescence assay for advanced glycation end products in blood and urine of diabetic patients. Metabolism 47:1348-1353.