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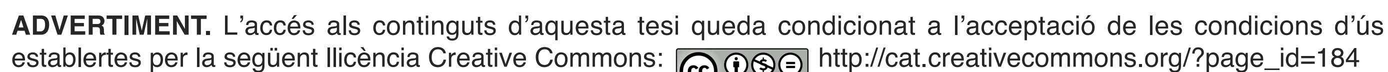
ADVERTIMENT. L'accés als continguts d'aquesta tesi queda condicionat a l'acceptació de les condicions d'ús establertes per la següent llicència Creative Commons:  http://cat.creativecommons.org/?page_id=184

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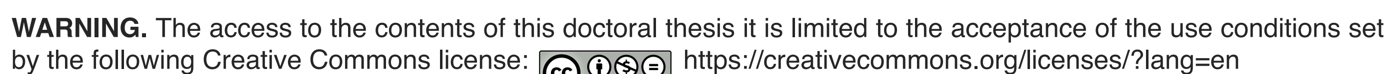
WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license:  https://creativecommons.org/licenses/?lang=en

Facultad de Biociencias

Departamento de Genética y de Microbiología

Unidad de Genética

DAÑO GENÓMICO COMO BIOMARCADOR DE LA ENFERMEDAD RENAL CRÓNICA

Memoria presentada por

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Para optar al grado de Doctor en el Programa de Doctorado en Genética, con mención de Doctorado Internacional

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Trabajo realizado bajo la dirección del Dr. Ricard Marcos Dauder y la Dra. Susana Pastor Benito

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Esta Tesis está inscrita bajo la mención de Doctorado Internacional. Por lo tanto, para obtener la mención de Doctor Internacional, la doctoranda ha realizado una estancia de 3 meses de duración fuera del Estado Español, en el German Cancer Research Center (DKFZ) para realizar parte de los trabajos de investigación. Esta estancia ha sido autorizada por la Comisión Académica responsable del programa de Doctorado de Genética de la UAB, incorporándose así al expediente y al documento de actividades de la doctoranda. Además, se ha redactado en inglés el resumen, las conclusiones y los artículos y se ha presentado parte de la defensa de la tesis doctoral en esta lengua, por ser habitual para la comunicación científica en su campo de conocimiento y por ser diferente a las lenguas oficiales del Estado Español.

A mi familia.

“Nadie sabe lo que hay detrás de una sonrisa” Corredor Z.

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ABREVIATURAS

AGES	*Advanced glycation end products*, productos finales de la glicación avanzada
APO	Apolipoproteínas específicas
BER	*Base excision repair*, reparación por escisión de bases
CBPI	*Cytokinesis-block proliferation index*, índice de proliferación celular
CBPM	Compuestos de bajo peso molecular
CKD	*Chronic kidney disease*
Da	Daltons
DM	*Diabetes mellitus*
DP	Diálisis peritoneal
ECV	Enfermedad cardiovascular
ER	Enfermedad renal
ERC	Enfermedad renal crónica
ERT	Enfermedad renal terminal. ESRD *end-stage renal desease*
EUTox	European Uremic Toxin, grupo de trabajo europeo en toxinas urémicas
FPG	Formamidopirimidina DNA glicosilasa
GPX	*Glutathione Peroxidase*, glutatión peroxidasa
GSH	*Glutathione S-Transferase*, glutatión S-transferasa
GWAS	*Genome wide association studies*, estudios de asociación del genoma completo
HD	Hemodiálisis
HDF	Hemodiafiltración
HDF on-line	Hemodiafiltración on-line
HDL	*High-density lipoprotein*, lipoproteínas plasmáticas de alta densidad
HF	Hemofiltración
HPT	Hormona paratiroidea
HT	Hipertensión arterial
IDL	*Intermediate density lipoprotein*, lipoproteínas de densidad intermedia
IRE	Índice de resistencia a la eritropoyetina.
KDIGO	*Kidney Disease: Improving Global Outcomes*, enfermedad renal: mejorando los resultados globales
Kt/v	Marcador de la eficacia de la diálisis
LDL	*Low-density lipoprotein*, lipoproteínas de baja densidad
NKF KDOQI	*National Kidney Foundation* - Kidney Disease Outcomes Quality Initiative
OR	*Odds ratio*, razón de probabilidades
PCR	Proteína C-reactiva
PD	Prediálisis
ROS	*Reactive oxygen species*, especies reactivas de oxígeno
RNS	*Reactive nitrogen species*, especies reactivas de nitrógeno
SNP	*Single Nucleotide Polymorphism*, polimorfismos de un sólo nucleótido
SOD	Superóxido dismutasa

TEAC	*Trolox equivalent antioxidant capacity*, capacidad antioxidante equivalente
TFG	Tasa de filtración glomerular
TIBC	*Total iron binding capacity,* capacidad total de fijación del hierro
TR	Trasplante renal.
TRS	Tratamiento renal sustitutivo
UGJ	*Unfermented grape juice*, jugo de uva sin fermentar, mosto

RESUMEN

La enfermedad renal crónica (ERC) se define como la pérdida progresiva de la tasa de filtración glomerular, que se traduce en el deterioro de la función renal a largo plazo. Los pacientes con ERC tienen una alta incidencia de enfermedad cardiovascular y cáncer, probablemente originados por la elevada inestabilidad genómica de los propios pacientes. Esta elevada inestabilidad genómica junto con la acumulación de compuestos pro-inflamatorios y pro-oxidantes, originados por la propia enfermedad y por el estrés oxidativo al que están sometidos durante el proceso de diálisis, hace que estos pacientes presenten elevados niveles de daño genómico. Todo ello conduce a los pacientes a un deterioro progresivo y a un progreso de la enfermedad con múltiples complicaciones (hipertensión arterial, hiperglicemia, cáncer, etc).

En este contexto, se planteó como objetivo principal de esta tesis doctoral estudiar el daño genómico de los pacientes con ERC y evaluar el efecto modulador sobre el daño genómico de los suplementos antioxidantes y del cambio de dializador. Para ello, se han realizado los estudios siguientes: 1) Un estudio transversal, evaluando el daño genómico con el ensayo del cometa, en una población de 415 pacientes con ERC y 187 controles; 2) un estudio longitudinal de 25 pacientes con ERC sometidos a HD y suplementados con mosto durante 6 meses, para evaluar el posible efecto antioxidante sobre el daño genómico en estos pacientes; 3) un estudio longitunal, en una población de 34 pacientes, para evaluar los efectos sobre el daño genómico del cambio de terapia de hemodiálisis convencional a hemodiafiltración on-line; 4) un estudio longitudinal de la evolución del daño genómico y oxidativo en pacientes sometidos a un trasplante renal. Los resultados nos indican que los pacientes con ERC tienen altos niveles de daño oxidativo y que el daño genómico es un buen biomarcador del estatus de estos pacientes y a la vez un indicador de un mal pronóstico para los pacientes en hemodiálisis. También hemos podido observar que la suplementación con antioxidantes, concretamente con mosto, reduce el daño oxidativo de los pacientes con ERC, evaluado mediante el ensayo del cometa. A pesar de que la hemodiafiltración on-line es una técnica

mucho más avanzada los pacientes que cambian de hemodiálisis convencional a hemodiafiltración on-line no muestran un descenso significativo en sus niveles de daño genético. Hemos podido comprobar que aunque el trasplante renal es la mejor opción para los pacientes en fase final de ERC, los niveles de daño genómico y oxidativo incrementan significativamente después del trasplante, encontrando las frecuencias más altas de daño en aquellos pacientes que habían recibido el riñón de un donante muerto. Los pacientes después del trasplante mejoran significativamente diversos biomarcadores como glucosa, albumina, calcio, presión arterial o urea. La inestabilidad genómica de los pacientes se mantiene incluso después del trasplante renal.

Dado que esta inestabilidad genómica continúa a pesar de las diferentes alternativas y terapias utilizadas, pensamos que deberían de existir polimorfismos genéticos que nos permitieran explicar estos resultados. Por ello genotipamos 722 individuos, para 38 SNPs. Los resultados sugieren que existen multitud de asociaciones con diferentes genes relacionados directamente con la enfermedad y con patologías afines a la misma, como predisposición al cáncer, hipertensión, diabetes, etc. Otros genes mostraron asociación con los parámetros bioquímicos característicos de la ERC. Se encontraron polimorfismos en genes implicados en el control de la reparación del daño en el DNA, que mostraron asociación con el daño oxidativo y con el daño observado en el ensayo de micronúcleos.

ABSTRACT

Chronic kidney disease (CKD) is defined as the progressive loss of glomerular filtration rate, which results in a deterioration of renal function over time. CKD patients have a high incidence of cardiovascular disease and cancer, probably caused by the high genomic instability characteristic of this type of patients. This high genomic damage, together with the accumulation of pro-oxidant and pro-inflammatory compounds, makes these patients to have high levels of genomic damage. The genotoxic compounds present in CKD patients are originated by the disease itself and by oxidative stress, to which they are subjected during the dialysis process. All the process leads to patients towards a progressive deterioration and a progress of the disease with multiple complications (hypertension, hyperglycemia, cancer, etc.).

In this context, it was proposed as main objective of this thesis to study the levels of genetic damage present in patients with CKD, evaluating the modulatory effect of antioxidant supplements, and changes at the dialyzer. To this end, the following studies have been performed: 1) a cross-sectional study evaluating the levels of genetic damage using the comet assay, in a population of 415 CKD patients and 187 controls. 2) a longitudinal study of 25 patients with CKD undergoing HD and supplemented with grape juice for 6 months to evaluate the possible antioxidant effect on genomic damage levels in these patients. 3) a longitunal study in a population of 34 patients, to evaluate the effects on genomic damage by changing conventional hemodialysis therapy by hemodiafiltration on-line. 4) a longitudinal study of the evolution of genomic and oxidative damage levels in patients undergoing kidney transplantation.

The results confirm that patients with CKD have high levels of oxidative damage and genomic damage. In addition, we stated that genomic damage is a good biomarker of the status of these patients and poor a prognosis for patients on hemodialysis. We have also observed that supplementation with antioxidants (specifically unfermented grape juice), reduces oxidative damage in patients with CKD, as determinated by using the comet assay. Although on-line hemodiafiltration is a more advanced technique patients switching from conventional hemodialysis to on-line

hemodiafiltration not show a significant decrease in their levels of genetic damage. We have found that although the renal transplant is the best option for patients in the final stages of CKD, the levels of genomic and oxidative damage increased significantly after transplantation. The highest levels of damage were observed in those patients who had received the kidney from dead donor. Although patients after transplantation significantly improved various biomarkers such as glucose, albumin, calcium, blood pressure or urea, genomic instability of patients remains even after kidney transplantation. Since genomic instability continues, despite the different alternatives and therapies used, we assumed the existence underlying genetic variants that allow us to explain these results. Therefore we genotyped 722 individuals by using 38 SNPs. The results showed many associations with different genes directly related to the pathologies and/or related to CKD, as predisposition to cancer, hypertension, and diabetes. Other genes showed association with the biochemical parameters characteristic of CKD. Moreover, we detected different polymorphisms in genes involved in controlling the repair of DNA damage, associated with the levels of oxidative damage and chromosome damage presented by CKD patients.

I. INTRODUCCIÓN

1. INTRODUCCIÓN

1.1. Consideraciones generales de la enfermedad renal crónica (ERC)

El riñón es una maquinaria de filtrado altamente especializada cuya función esencial es excretar metabolitos peligrosos desde el torrente sanguíneo, con el objetivo de mantener la homeostasis del fluido corporal, el equilibrio ácido-base y la producción de hormonas que afectan directa o indirectamente a la respuesta inmune. Al equilibrar la composición mineral y el volumen de los líquidos corporales (sangre y orina) colabora con el sistema cardiovascular para controlar la presión arterial (Fridén, 2011). Este intrincado proceso ocurre en un gran número de pequeñas unidades funcionales dentro del riñón, conocidas como nefronas (Figura 1). La nefrona consta de un grupo de capilares glomerulares en forma de bucle, que está rodeado por la cápsula de Bowman (Tanner, 2009).

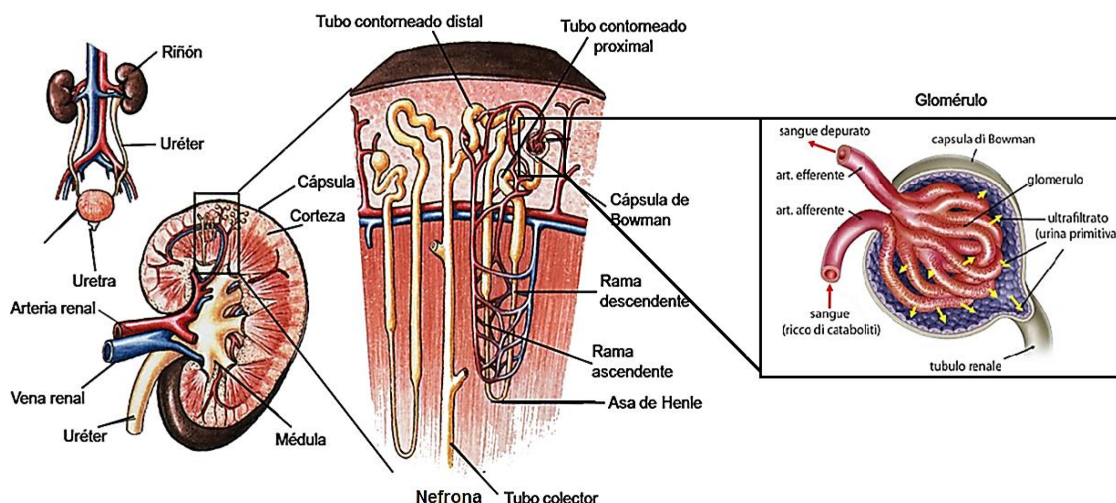

Figura 1. Morfologia del riñón, la nefrona y el glomérulo. Fuente http://docentes.educacion.navarra.es/metayosa/1bach/1nutriani13.html, con modificaciones (último acceso 14/12/2015).

Cuando se presentan cambios en la estructura y/o en la función del riñón éstos conllevan efectos negativos sobre la salud del individuo, pudiendo llegar a ser crónicos, y es en este momento cuando se habla de enfermedad renal crónica (Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group, 2013).

Dentro de la amplia gama de patologías renales se conocen muchos síndromes hereditarios (Vasyutina et al., 2010), por ejemplo el síndrome nefrótico congénito, el síndrome de Denys-Drash, el síndrome de Pierson y el de Alport's (Hildebrandt, 2010), así como distintos tipos de cánceres hereditarios (Choyke et al., 2003). Por otro lado están los trastornos metabólicos, como son una elevada presión arterial, niveles altos de glucosa y de triglicéridos en plasma, bajos niveles de colesterol (HDL), y obesidad abdominal (Chen et al., 2004) que conllevan a la diabetes. La aterosclerosis también puede dar lugar a una disminución irreversible de la función renal con el desarrollo posterior de la enfermedad renal crónica (ERC) (Haroun et al., 2003).

La ERC se está convirtiendo en un importante problema de salud pública en todo el mundo. La nefropatía diabética, seguida de las patologías vasculares nefrológicas, la glomerulonefritis, la poliquistosis renal y la nefritis intersticial, son las principales causas de enfermedad renal desde hace varias décadas (Kälble et al., 2005). Dada la patogénesis de la enfermedad, los pacientes tienen un alto riesgo de progresión a enfermedad renal terminal (ERT), una condición que requiere terapias de reemplazo como la diálisis o el trasplante de riñón para mantener la supervivencia de los pacientes (Barsoum et al., 2006).

El deterioro del riñón abarca un amplio rango de anormalidades observables durante la evaluación clínica, que pueden ser invisibles e inespecíficas como causa de la enfermedad, pero pueden preceder a la reducción de la función renal, como pueden ser la albuminuria, anomalías de sedimento de orina y electrolitos, además de otras anomalías debidas a trastornos tubulares (Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group, 2013).

Actualmente, se han hecho grandes esfuerzos para lograr una mejor detección de la enfermedad renal progresiva. En 2002, en la guía de práctica K/DOQI publicada por la Fundación Nacional del Riñón (NKF por sus siglas en inglés) *(National Kidney Foundation*, 2002) establece la ERC cuando la tasa de filtración glomerular (TFG) se mantiene por debajo de 60 mL/min/1,73 m^2, durante tres o más meses con o sin evidencia de daño renal, e independiente de la causa (Levey et al., 2003). La TFG es el producto de la tasa de filtración en nefronas individuales y del número de nefronas en ambos riñones. La reducción en la tasa de filtrado se debe a la

disminución en el número de nefronas funcionales, ya sea por causas fisiológicas o farmacológicas, que alteran la hemodinámica glomerular (Stevens y Levey, 2005). La ecuación para el cálculo de la TFG más ampliamente desarrollada, y usada en el presente estudio, es la citada en el Estudio de Modificación la Dieta en la Enfermedad Renal (MDRD *the Modification of Diet in Renal Disease*, Levey et al., 2009) como se muestra a continuación.

TFG (mL/min/1,73 m^2) = 186,3 x (creatinina sérica)$^{-1,154}$ x (edad)$^{-0,203}$ x (0,742 si es mujer) x (1,21 si es afroamericano)

Ecuación MDRD.

La TFG es aceptada como el mejor indicador de la función renal (Tabla 1). Nos referimos a una disminución en la tasa de filtrado glomerular cuando la TFG < 60 mL/min/1,73 m^2 y hablamos de insuficiencia renal si la TFG < 15 mL/min/1,73 m^2.

Tabla 1. Estado de la función renal según la tasa de filtrado glomerular.

Categoría TFG	TFG (mL/min/1,73 m^2)	Condiciones
1	≥ 90	Normal o alta
2	60 – 89	Ligeramente disminuido*
3a	45 – 59	De ligera a moderadamente disminuido
3b	30 – 44	De moderada a disminución severa
4	15 – 29	Severamente disminuido
5	<15	Insuficiencia renal

* En relación al nivel de adultos jóvenes. Fuente Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group, (2013), con modificaciones.

Cuando la TFG disminuye, aparecen diferentes complicaciones que se reflejan en la pérdida de la función endocrina o exocrina de los riñones, desarrollando anemia, acidosis, desnutrición, trastornos óseos y minerales, entre otros.

1.2. Etiología de la ERC

A pesar del extenso esfuerzo dedicado a la investigación de la progresión de la ERC para acabar con la enfermedad renal en su etapa terminal, todavía se trabaja para llegar a una plena comprensión de los mecanismos subyacentes de la enfermedad. La progresión (Figura 2) se caracteriza por un continuo e irreversible avance en la erosión de la estructura del riñón debido a la pérdida de nefronas y su posterior sustitución por tejido cicatricial. Además de patologías asociadas como son la arteriosclerosis, la glomeruloesclerosis (Fornoni et al., 2008) y la fibrosis tubulointersticial (c, 2008), la hipertensión (Mahmoodi et al., 2012), la *diabetes mellitus* (Nikolsky et al., 2004) y el envejecimiento (Anderson et al., 2009) son procesos antecedentes o coincidentes que pueden iniciar la lesión de forma independiente y/o potenciar el daño durante la ERC. Estas patologías perturban la hemodinámica renal, imponiendo sobrecargas funcionales y metabólicas en los riñones (Chen et al., 2004), acelerando así la progresión, iniciada por otras enfermedades de origen inmunológico, infeccioso (Kurts et al., 2013) o genético (Köttgen et al., 2009; Chambers et al., 2010; Okada et al., 2012a).

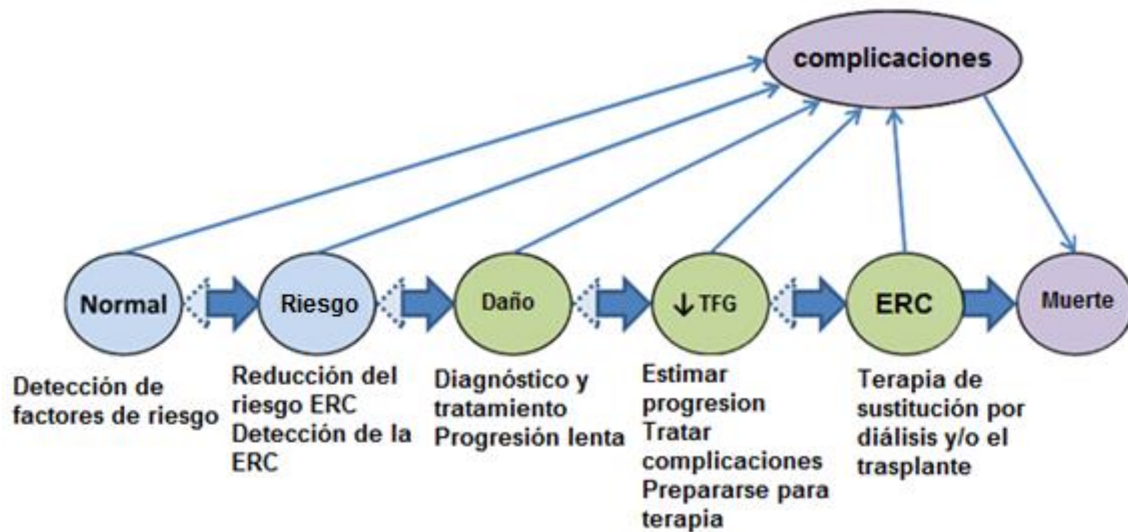

Figura 2. Desarrollo y progresión de la ERC. Las flechas gruesas entre círculos representan el desarrollo, progresión y remisión de la ERC. Las complicaciones, se refiere a todas las complicaciones de la ERC, por ejemplo la disminución de la TFG y la enfermedad cardiovascular. Las flechas horizontales apuntando de izquierda a derecha representan la naturaleza progresiva de la enfermedad renal crónica, las flechas con punta discontinua simbolizan que la remisión es menos frecuente que la progresión. Fuente Levey et al., (2012) con modificaciones.

1.2.1. El daño renal

El proceso por el cual el daño renal conduce progresivamente a la ERC y a la ERT se caracteriza por una pérdida de la función glomerular y de la arquitectura peritubular capilar, por la proliferación de las células tubulares e intersticiales, por procesos fibróticos, por la acumulación difusa de la matriz extracelular (ME) y por la aparición de epitelios indiferenciados que surgen de la desregulación de la transición epitelio-mesénquima (TEM) durante la reparación de una lesión (Grabias y Konstantopoulos, 2014).

Los procesos fibróticos, se manifiestan por una aparición de miofibroblastos en el espacio intersticial, en donde los fibroblastos se diferencian a partir de fibroblastos residentes a través de la expresión combinada de marcadores para los fibroblastos (FSP1), células de músculo liso (α-actina de músculo) y células endoteliales (PECAM, PDGFRβ) (Eyden, 2008). Los altos niveles de matriz extracelular (ME) conducen a la formación de cicatrices patológicas y, a largo plazo, a una insuficiencia orgánica (Figura 3). Según donde se produzca la ME, en el intersticio glomerular o tubular, se pueden generar glomeruloesclerosis o fibrosis tubulointersticial. Esta última implica la acumulación de los tipos de colágeno I, III, y IV, proteoglicanos y fibronectinas. Este tipo de fibrosis parece ser una característica de la ERC (Grande et al., 2009).

La fibrosis tubulointersticial también es un componente estructural clave de la nefropatía obstructiva, que es la principal causa de enfermedad renal crónica en los niños y representa el 22,9% de los casos incidentes en América del Norte (Smith et al., 2007).

Dentro de los factores que pueden inferir susceptibilidad al daño renal se encuentran: la edad avanzada, antecedentes familiares de enfermedad renal crónica, reducción de la masa renal, bajo peso al nacer, y el componente étnico.

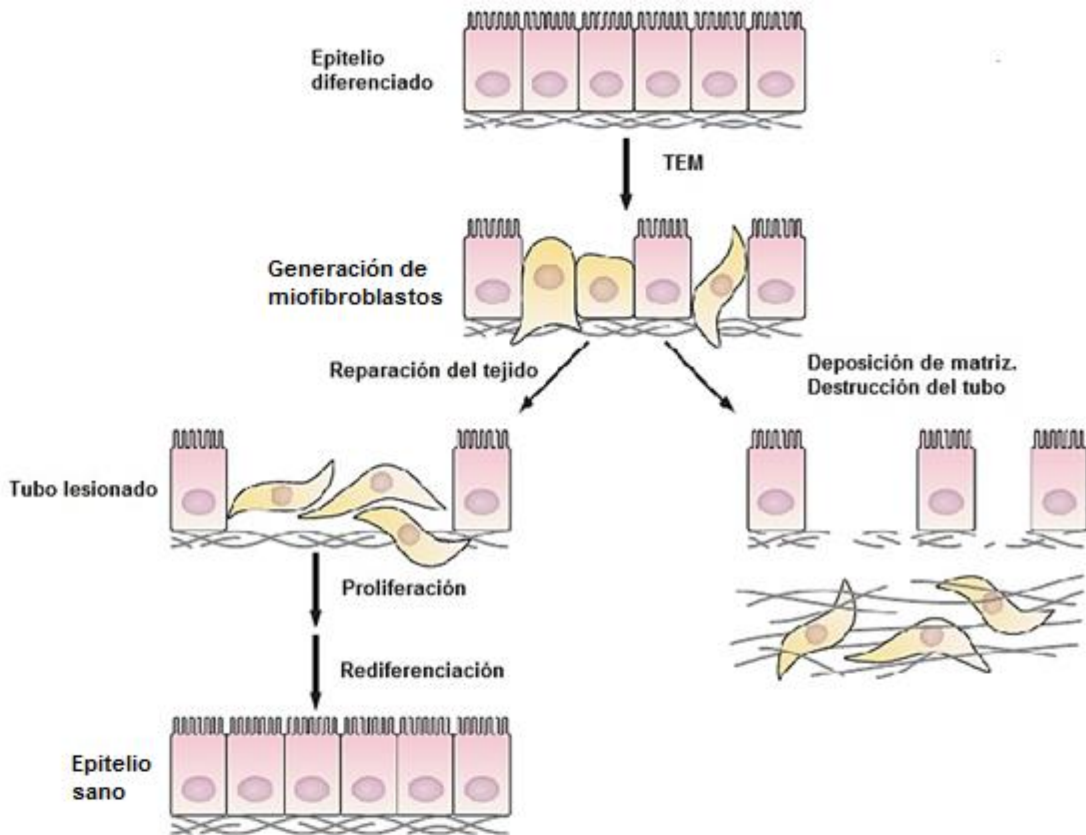

Figura 3. Identificación de la fuente de células productoras de matriz dentro del intersticio renal. La transición epitelio-mesénquima (TEM), desdiferenciación de las células epiteliales en un fenotipo más similar a fibroblastos, en la reparación tubular. Después de una lesión en el túbulo renal, las células epiteliales cercanas se someten a TEM, migran a la zona afectada, y proliferan. Dadas las señales bioquímicas adecuadas, las células mesénquimales podrían recuperar su fenotipo epitelial y restaurar la integridad del túbulo. Fuente Grabias y Konstantopoulos (2014) con modificaciones.

Lo anterior junto con la presencia de diabetes, presión arterial alta, enfermedades autoinmunes, infecciones sistémicas de las vías urinarias, la toxicidad de algunos medicamentos y las enfermedades hereditarias contribuyen al deterioro renal. Una vez en este punto, el avance de la progresión de la enfermedad se hace evidente con los elevados niveles de proteinuria y presión arterial, anemia, micro-albuminuria, mal control glucémico y, posiblemente, dislipidemia (Levey et al., 2005).

1.2.2. La proteinuria

La proteinuria es un término general para indicar la presencia de mayores cantidades de proteína en la orina. Esta puede reflejar la pérdida anormal de proteínas plasmáticas debido a: i) el aumento de la permeabilidad glomerular a las proteínas de peso molecular elevado (albuminuria o proteinuria glomerular), ii) la reabsorción tubular incompleta de las proteínas de peso molecular bajo normalmente filtradas (proteinuria tubular), o iii) al aumento de la concentración plasmática de proteínas de bajo peso molecular (tales como las cadenas ligeras de inmunoglobulina). La proteinuria también puede reflejar la pérdida anormal de proteínas derivadas de los riñones (constituyentes de las células tubulares renales debido a daño tubular) y el tracto urinario inferior (Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group, 2013).

El fuerte valor predictivo de la proteinuria en nefropatías crónicas está firmemente establecido siendo la proteinuria basal un predictor independiente de la disfunción renal (Abbate et al., 2006). Estudios recientes han encontrado que los pacientes tanto con proteinuria, como con reducción de la TFG, tienen mayor riesgo de sufrir enfermedades cardiovasculares que la población general (Johnson et al., 2004; Shimbo et al., 2015).

1.2.3. La albuminuria

La albúmina sérica es el marcador nutricional principal utilizado para identificar la desnutrición en la ERC (Friedman et al., 2010). La albuminuria se refiere a la pérdida anormal de albúmina en la orina. La albúmina es un tipo de proteína plasmática que se encuentra en la orina en sujetos normales y en mayor cantidad en los pacientes con enfermedad renal. Es el marcador más precoz de las enfermedades glomerulares, incluyendo la glomeruloesclerosis diabética, donde generalmente aparece antes que se dé la reducción de la TFG. También es un marcador de la nefroesclerosis hipertensiva, pero puede no aparecer hasta después de la reducción de la tasa de filtración glomerular, y a menudo se asocia con la hipertensión subyacente, la obesidad y la enfermedad vascular (Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group, 2013). Se ha indicado que la micro-albuminuria y el deterioro en la TFG están asociados con un

mayor riesgo de eventos cardiovasculares (Segura et al., 2004; De Jong et al., 2008).

1.2.4. La anemia

La anemia se define como una reducción en una o más mediciones de los glóbulos rojos, como: la concentración de hemoglobina, el hematocrito, o el recuento de glóbulos rojos. La NKF define la anemia como la concentración de hemoglobina inferior a 13,5 g/dL en los hombres y menor a 12,0 g/dL en las mujeres (Malcovati et al., 2011).

La anemia en la ERC aumenta la morbilidad y la mortalidad por complicaciones cardiovasculares (Mehdi et al., 2009), pudiendo llevar a un mayor deterioro de la función renal y al establecimiento de un círculo vicioso llamado el "síndrome de la anemia cardiorrenal". De hecho, los pacientes con enfermedad renal en etapa terminal con hipertrofia ventricular (HTV) tienen una tasa de supervivencia del 30% menor en cinco años, que las personas que carecen de HTV (Muzzarelli et al., 2006). Además, la anemia es un predictor independiente de mortalidad en pacientes con enfermedad arterial coronaria estable con ERC (Vaziri, 2009).

1.2.5. Metabolismo calcio-fósforo

En la ERC (incluso en estadios iniciales) existe disminución de la excreción de fósforo lo que conlleva a su retención y aumento en plasma, provocando disminución de los niveles de calcio, y subsecuentemente al aumento de la secreción de hormona paratiroidea (HPT) para aumentar su excreción. Con la pérdida progresiva de función renal la retención de fósforo continúa a pesar de la concentración elevada de HPT, provocando un estado de paratiroidismo (Zamora y Sabahuja, 2008). Tanto el calcio, como el fósforo son elementos críticos de la calcificación tanto en los huesos y como en los vasos sanguíneos. El depósito de estos elementos influye en la calcificación vascular, común en la ERC y asociada con una mayor morbilidad y mortalidad (Moe y Chen, 2008). Sin embargo, el tratamiento con vitamina D es un factor importante que puede mitigar los efectos de la HPT y la hiperfosfatemia en la mortalidad cardiovascular. El metabolismo de

la vitamina D está íntimamente ligado al riñón, la hidroxilación final a 1,25 hidroxi-vitamina D (calcitriol), que es el metabolito activo de la vitamina D, y se realiza en el túbulo proximal gracias a la enzima 1-hidroxilasa, disminuida en la ERC. El calcitriol aumenta la absorción de calcio y del fósforo en el intestino delgado, aumenta la resorción ósea, ayuda a mantener la homeostasis del calcio y favorece la mineralización ósea (Levin et al., 2007).

1.3. La enfermedad renal crónica y sus complicaciones asociadas

1.3.1. El riesgo cardiovascular

Existen evidencias de que incluso grados leves de insuficiencia renal se asocian con un mayor riesgo cardiovascular (Go et al., 2006). La asociación de la enfermedad renal con la enfermedad cardiovascular (ECV) se debe probablemente a la agrupación de varios factores de riesgo cardiovascular, incluyendo la edad avanzada, la hipertensión, la *diabetes mellitus* y la dislipidemia. Además, otros factores específicos de la ERC como son la anemia, sobrecarga de volumen, alteraciones del metabolismo mineral, la proteinuria, la malnutrición, el estrés oxidativo y la inflamación (Liu et al., 2014) contribuyen a su aparición. El aumento de los niveles de troponina cardíaca (TnTc), y de la proteína C-reactiva (PCR), típicos en la inflamación, predicen eventos cardiovasculares (De Filippi et al., 2003) y se asocian con la enfermedad renal crónica (ERC) y con un mayor riesgo de muerte (Weiner et al., 2008a).

Se ha sugerido que el riesgo cardiovascular está asociado con el deterioro de la función renal durante la progresión de la ERC. Asimismo, existen evidencias de que el nivel de la tasa de filtración glomerular es un factor de riesgo independiente para la enfermedad cardiovascular (Manjunath et al., 2003a,b). Una TFG reducida se asocia con una elevada prevalencia de factores de riesgo cardiovascular y una mayor prevalencia de ECV. Varios estudios (Gerstein et al., 2001, Ruilope et al., 2001, Manjunath et al., 2003a) han demostrado que altos niveles de presión arterial sistólica, elevado colesterol total y niveles bajos de colesterol-HDL son más frecuentes en pacientes con disminución de la TFG.

Finalmente, se sabe que el número de factores de riesgo cardiovascular aumentan con la disfunción renal (Foley et al., 2005). Por lo anterior no se puede desligar la presencia de patologías cardiovasculares en pacientes con enfermedad renal.

1.3.2. La diabetes

La *diabetes mellitus* (DM) es un grupo de enfermedades metabólicas caracterizadas por hiperglicemia, resultado de defectos en la secreción de insulina, acción de la insulina, o de ambos. La hiperglicemia crónica de la diabetes se asocia con daño a largo plazo, disfunción e insuficiencia de diversos órganos, especialmente los ojos, riñones, nervios, corazón y vasos sanguíneos (American Diabetes Association, 2004). Un estudio previo indicó que el 39,7% de los pacientes adultos con *Diabetes mellitus* tipo 2 (DM2) en los Estados Unidos, tuvo algún grado de enfermedad renal crónica (Koro et al., 2009).

Según las estimaciones de la Fundación Nacional del Riñón, alrededor de un tercio de los pacientes con DM2 están en riesgo de desarrollar ERC, y el 45% de todos los casos de insuficiencia renal en los Estados Unidos se asocian con la DM. Esto se debe a que se ve deteriorada la función renal a través de las complicaciones microvasculares causadas por la hiperglicemia crónica.

1.3.3. La hipertensión

Junto con la diabetes, la hipertensión es actualmente la principal causa de insuficiencia renal terminal en todo el mundo (Atkins, 2005). Diferentes estudios han señalado la prevalencia de la hipertensión en pacientes con ERC, dentro de los cuales se tienen cifras que oscilan entre aproximadamente el 22% en la etapa 1 de la ERC a más del 80% en la etapa 4 de la enfermedad (Mahmoodi et al., 2012). Además, otro estudio ha demostrado que la prevalencia fue del 85,7%, en un número elevado de pacientes (N = 3.612) (Muntner et al., 2010).

La hipertensión (HT) es el factor de riesgo cardiovascular más común, donde la disminución de la tasa de filtración glomerular (TFG) y el aumento de la albuminuria,

coexisten frecuentemente, llevando al deterioro progresivo de la función renal y a la disminución de la TFG (Segura et al., 2004) (Figura 4).

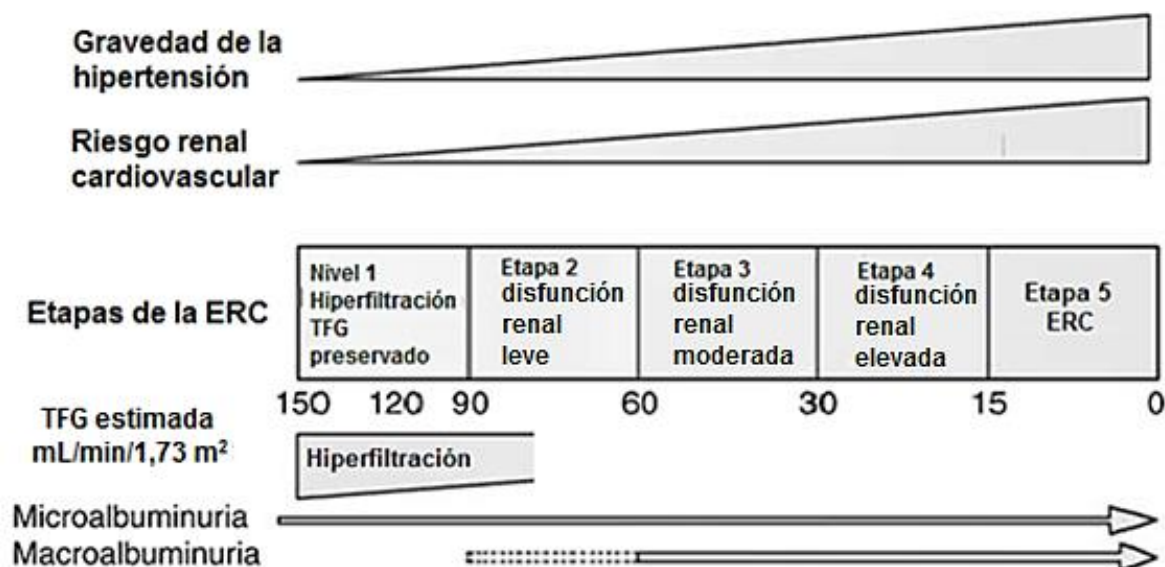

Figura 4. Relación entre la hipertensión, el riesgo cardiovascular y la ERC. Fuente Segura et al., (2004), con modificaciones.

1.3.4. La dislipidemia

Se sabe que además de los factores de riesgo de la ERC previamente mencionados, están los trastornos lipídicos (Tsimihodimos et al., 2011). En los últimos 30 años se han realizado numerosos estudios para discernir las características y los mecanismos de la dislipidemia en la ERC. En el plasma, los lípidos son transportados por partículas solubles en agua conocidos como lipoproteínas, que consisten en un núcleo de lípidos no polares (triglicéridos, ésteres de colesterol) rodeado por una envoltura compuesta de apolipoproteínas específicas (APO), fosfolípidos y otros lípidos polares. Las lipoproteínas plasmáticas son comúnmente clasificadas como de alta densidad (HDL), de densidad intermedia (IDL), de baja densidad (LDL), o de muy baja densidad (VLDL), de acuerdo con sus características de ultracentrifugación. En la ERC es común observar alteraciones del metabolismo de las lipoproteínas. La dislipidemia renal se refleja en un perfil anormal de la apolipoproteína (Apo) y de las concentraciones y composición de la familia de las lipopoteinas. Se caracteriza por una baja

concentración de HDL y el aumento de las concentraciones de triglicéridos intactas o parcialmente metabolizadas, VLDL, IDL y LDL (Attman et al., 2003). Estudios recientes en pacientes con ERC mostraron dislipidemia, ya sea con el colesterol total bajo, el LDL y el HDL alto, asociada con la terapia de reemplazo renal y con la progresión rápida de la disfunción renal. Es por esto que se ha sugerido que la evaluación del perfil de lípidos puede ayudar a identificar los grupos de alto riesgo con resultados renales adversos en pacientes con ERC en las etapas 3 a 5 de la progresión de la enfermedad (Chen et al., 2013).

1.3.5. El cáncer

ERC y cáncer están estrechamente relacionados. El cáncer puede causar ERC ya sea directa o indirectamente a través de los efectos adversos de las terapias sustitutivas; y la ERC puede, ser un factor de riesgo para el cáncer. Esa asociación se puede deber a que comparten factores de riesgo como los compuestos tóxicos a los que estos pacientes están constantemente sometidos. Es así como la ERC se puede producir después de la quimioterapia o radioterapia, o después de la nefrectomía (operación quirúrgica en la que se extirpa total o parcialmente un riñón) para el cáncer de riñón (Choi y Song, 2014). Estudios recopilatorios han observado que en 2.817 pacientes con ERC el 6,7% presentó tumores en diferentes órganos y el 71% en tratamiento renal sustitutivo (TRS) tuvo algún diagnostico tumoral durante el primer año de terapia (Cengiz, 2002). Por otro lado, antiguos estudios recientemente corroborados han mostrado el riesgo incrementado de padecer varios tipos de cáncer en los pacientes bajo TRS, sobre la población en general, tales como el cáncer de riñón (Shebl et al., 2012), carcinoma de células renales (Farivar-Mohseni et al., 2006), cáncer del tracto urinario (Chung et al., 2012), y de vejiga (Stewart et al., 2003); pero, además, se tiene registro de cáncer en otros órganos como hígado (Shebl et al., 2012) y tiroides, incluso después del trasplante renal (Birkeland et al., 2000). El más representativo sigue siendo el de riñón y de las vías urinarias, donde los tumores de vejiga representan el 90-95% de los carcinomas uroteliales, siendo la neoplasia maligna más común del tracto urinario (Rouprê et al., 2013). Es ahí cuando los efectos cancerígenos en la pérdida de la función renal son más evidentes (Stewart et al., 2003).

Patologías como el cáncer y la ERC comparten un factor preponderante en el deterioro del paciente, como es la respuesta inflamatoria que está determinada por la activación de enzimas oxidantes como la NADPH (nicotinamida-adenina-dinucleótido-fosfato) que genera especies reactivas de oxigeno (ROS) además de las especies reactivas de nitrógeno (RNS) (Ohshima et al., 2003); así como proteínas, como las citoquinas, que pueden tener una función tanto protectora como patogénica (neutraliza toxinas, mantiene la homeostasis metabólica y regula la reparación de heridas y la regeneración de tejidos de una infección o lesión (Wynn, 2015), se han encontrado en niveles plasmáticos altos (citoquinas pro-inflamatorias) en pacientes con niveles bajos de la función renal y niveles altos de albuminuria (Gupta et al., 2012). La activación prolongada de las células inflamatorias conlleva a un incremento de radicales libres (cualquier especie molecular con uno o más electrones no apareados "inestables") que son muy reactivos frente al DNA, RNA, proteínas y lípidos (Ziech et al., 2011). Esto conlleva a toxicidad celular, modificaciones en el DNA, mutaciones y aumentos en la inestabilidad genómica (Bartsch y Nair, 2006). Aunque la mayor parte de este daño se repara, sin embargo una pequeña fracción puede fijarse en el tiempo (Stratton et al., 2009).

1.4. Daño genómico en la ERC

Durante la ERC se produce una disminución de la capacidad del riñón para eliminar diversas toxinas tales como moléculas de bajo peso molecular, proteínas como la β_2-microglobulina, y diversos compuestos como guanidinas, índoles y fenoles (Tabla 2) (Meyer y Hostetter, 2007). Hay que señalar que existen más de 90 toxinas urémicas, como ha descrito el grupo de trabajo europeo en toxinas urémicas (EUTox) (http://eutoxdb.odeesoft.com/ soluteList.php último acceso 16/12/2015) (Vanholder et al., 2009). Esta intoxicación hace indispensable la aplicación de tratamientos de remplazo renal.

Se ha descrito que algunas de las toxinas urémicas, como la hidroquinona (McGregor, 2007), el sulfato de indoxilo, la leptina (Horoz et al., 2006), el metilglioxal, el N-ε-carboximetil lisina y el TNF-α (Wheelhouse et al., 2003), poseen

propiedades genotóxicas o mutagénicas. Además, los trihalometanos y las aminas heterocíclicas, no incluidos en la base de datos EUTox, también han mostrado acumulación en los pacientes con ERC (Vanholder et al., 2003). Estas sustancias, junto con otras aún por detectar, pueden contribuir sustancialmente al daño genómico observado en estos pacientes.

Tabla 2. Toxinas urémicas que incrementan el daño genómico en los pacientes con ERC. Fuente Meyer y Hostetter, (2007) con modificaciones.

Soluto	Ejemplo	Fuente	Características
Ácidos dicarboxílicos	Oxalato	Ácido ascórbico	La formación de depósitos de cristales
Aminas alifáticas	Dimetilamina	Colina	Producido por las bacterias del intestino en gran volumen
Carbonilos	Glioxal	Intermediarios glicolíticos	Reacción con proteínas para formar productos finales de glicación avanzada
Fenoles	p-Cresol sulfato	Fenilalanina y tirosina	Proteínas de ligación, producidas por las bacterias del intestino
Furanos	3-Carboxi-4-metil-5-propil-2-ácido furano propiónico.	Desconocido	Estrechamente ligado a proteínas
Guanidinas	Ácido guanidinosuccínico	Arginina	Producción incrementada en uremia
Indoles	3-Indoxil-sulfato	Triptófano	Proteínas de ligación, producidas por las bacterias del intestino
Nucleósidos	Pseudouridina	RNA	El más destacado de varias especies de RNA alterado
Péptidos y proteínas pequeñas	β_2-microglobulina	El complejo mayor de histocompatibilidad	Mal dializada debido al gran tamaño
Polioles	Mioinositol	La ingesta dietética, la síntesis celular de la glucosa.	Normalmente degradado por el riñón en lugar de excretarse

Dentro de la amplia gama de moléculas endógenas, que generan daño genómico en la ERC podemos destacar los productos finales de la glicación avanzada (AGES).

Los AGES son un grupo heterogéneo de compuestos derivados de la glicación no enzimática entre azúcares reductores (glucosa) y proteínas, lípidos o ácidos, a través de una compleja secuencia de reacciones que se conoce como la reacción de Maillard (Stinghen et al., 2015). Por otro lado están los radicales libres (cualquier especie molecular que contenga un electrón desapareado) dentro de los cuales están las especies reactivas de oxigeno (ROS) como por ejemplo, el ion superóxido (O_2^-), e hidroxilo (OH^-); y las especies reactivas de nitrógeno (RNS), tales como peroxilo (ROO^-), el óxido nítrico (NO), el dióxido de nitrógeno (NO_2), el hidroperoxilo (HOO^-), radicales de alcoxilo (RO-) el peroxinitrito ($ONOO^-$), y el nitroxilo (HNO) (Figura 5) (Small et al., 2012). Todos estos radicales interactúan con el DNA, y esta interacción se conoce como **estrés oxidativo**, y puede dejar secuelas, dando origen a patologías como la ERC.

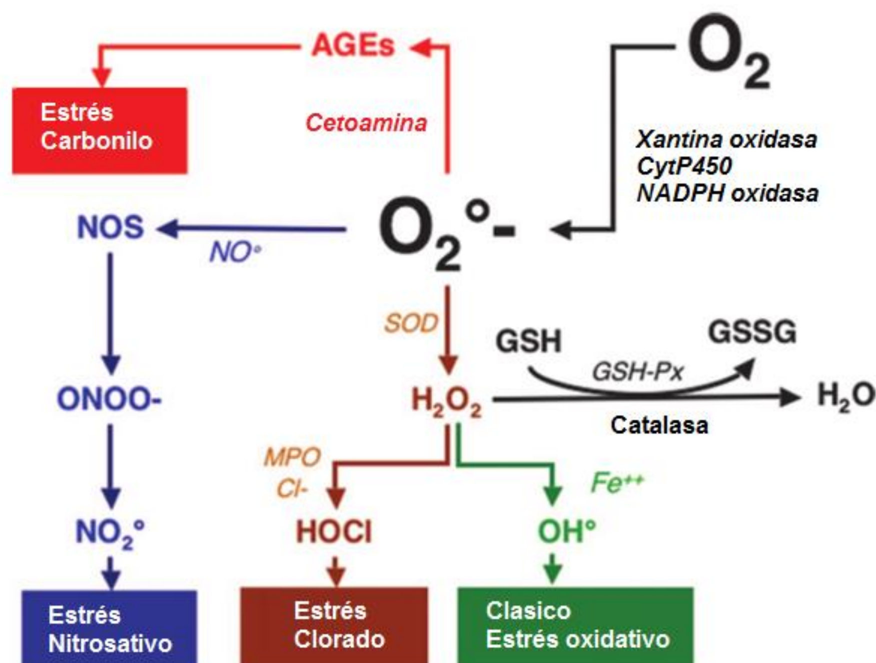

Figura 5. Representación de las rutas oxidantes. Fuente Stenvinkel et al., (2008), con modificaciones

1.4.1. Los productos finales de la glicación avanzada (AGES)

Se ha demostrado que en pacientes con ERC la acumulación de AGES, compuestos pro-inflamatorios y pro-oxidantes, causa una mayor formación de

radicales de oxígeno, con la subsecuente liberación de citoquinas pro-inflamatorias (interleuquina-6, factor de necrosis tumoral-α), factores de crecimiento (factor de crecimiento transformante -β1 [TGF-β1], y moléculas de adherencia de crecimiento similares a la insulina (molécula de adhesión celular vascular-1, molécula de adhesión intercelular-1) (Heidland et al., 2001). Los AGES también juegan un papel importante en la disfunción endotelial y posteriormente en la enfermedad cardiovascular (ECV) (Stinghen et al., 2015). Además, los AGES aumentan los niveles de ROS a través de la activación de la NADPH oxidasa (Wautier et al., 2001) y, recíprocamente, los altos niveles de ROS conducen a mayores niveles de AGES, debido al desequilibrio entre glutatión oxidado y glutatión reducido (Vulcano et al., 2013), así como a cambios en los sistemas antioxidantes, como la superóxido dismutasa (SOD)/peroxidasa (Figura 6) (Mallipattu et al., 2012). Los efectos tóxicos de los AGES son el resultado de alteraciones estructurales y funcionales en plasma y proteínas de la matriz extracelular (PME), en particular del entrecruzamiento de las proteínas y la interacción de AGES con sus receptores y/o proteínas de unión (Heidland et al., 2001).

En la ERC el daño genómico está presente desde los estadios iniciales de la enfermedad, es decir tanto en pacientes que aún conservan un funcionamiento residual del riñón, y por lo tanto no ingresan aún en tratamientos renales sustitutivos (Prediálisis, PD); como en aquellos que requieren de dicho tratamiento. Esto se ha comprobado gracias a varios biomarcadores como la frecuencia de micronúcleos (MNs), el ensayo del cometa y los niveles de 8-hidroxi-2-desoxiguanosina (8-OHdG) en leucocitos, además de deleciones en el DNA mitocondrial, y anormalidades cromosómicas (Domenici et al., 2005).

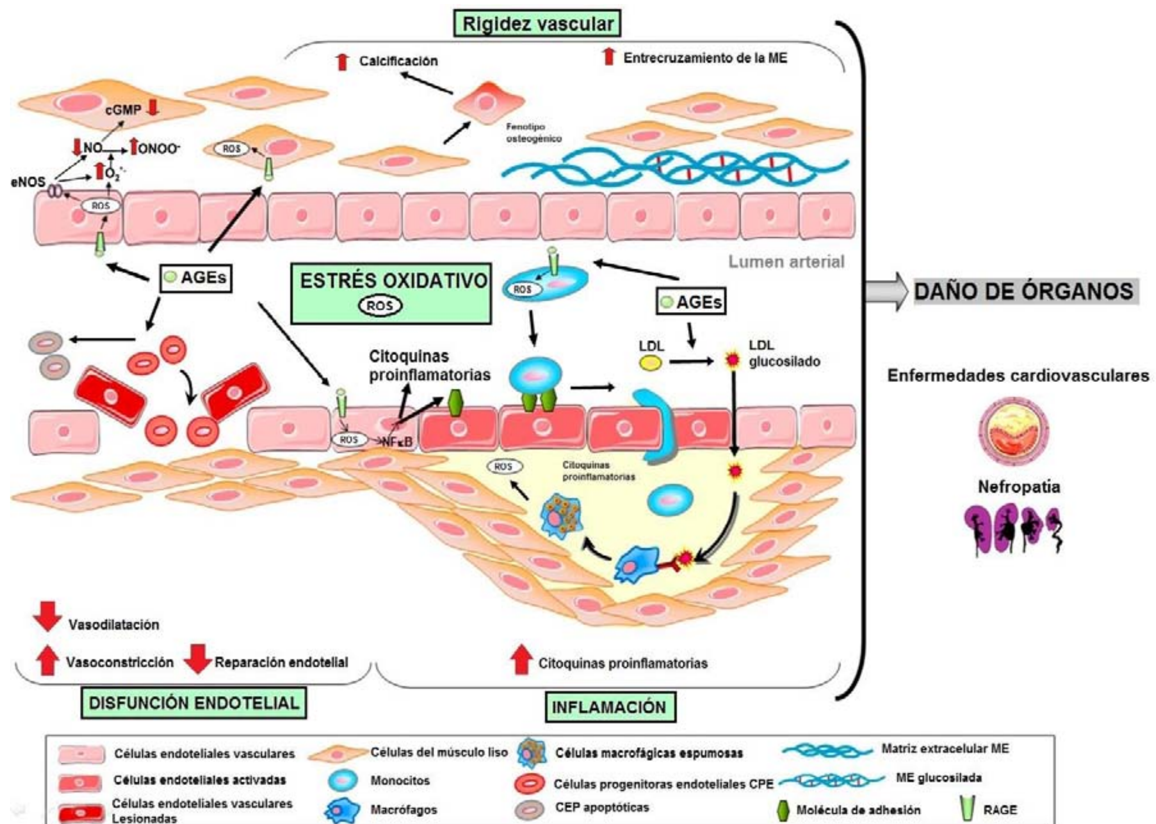

Figura 6. Efectos fisiopatológicos de los AGES. cGMP, monofosfato de guanosina cíclico; ME, matriz extracelular; eNOS, óxido nítrico sintasa endotelial, NO, óxido nítrico; CPE células progenitoras endoteliales; O_2-, anión superóxido; ONOO$^-$, peroxinitrito. Fuente Stinghen et al., (2015), con modificaciones.

El aumento en la frecuencia de los MNs se ha considerado un buen indicador de la inestabilidad genómica y, además, se han propuesto como un biomarcador del riesgo de cáncer (Bonassi et al., 2007). Estudios previos realizados por nuestro grupo de investigación han arrojado resultados que muestran la asociación entre los niveles de creatinina y el daño genómico, lo que confirmaría la relación entre el daño genómico con la progresión de la insuficiencia renal (Sandoval et al., 2010). Más recientemente en pacientes con ERC se ha observado la presencia de inestabilidad genómica, medida como una mayor radiosensibilidad cromosómica frente a la radiación ionizante (Rodríguez-Ribera et al., 2015).

1.4.2. El estrés oxidativo

La oxidación es la transferencia de electrones de un átomo a otro y representa una parte esencial de la vida aeróbica y del metabolismo, ya que el oxígeno es el último receptor de electrones en el sistema de flujo de electrones que produce energía en forma de adenosina trifosfato (ATP). Sin embargo, pueden surgir problemas cuando el flujo de electrones se desacopla (transferencia de electrones no apareados individuales), generando los ya mencionados radicales libres (ROS, RNS) (Pietta, 2000). El efecto de estos radicales libres son la piedra angular del daño genómico por estrés oxidativo. Este “estrés” se define como la alteración en la función celular y molecular causada por un desequilibrio entre la producción de especies reactivas, ya sea de origen endógeno o exógeno, y la capacidad antioxidante intrínseca de las propias células. La existencia de estrés oxidativo ha sido observado en pacientes con patologías urémicas (Sung et al., 2013) (Tabla 3). En presencia de este desequilibrio, el DNA es susceptible de sufrir alteraciones por ROS, producto del metabolismo energético aerobio. Cuando se da una acumulación de estos radicales libres, la tasa de mutación se incrementa, provocando patologías como las enfermedades cardiovasculares y el cáncer (Valko et al., 2004, 2007). Hay que señalar que los mecanismos oxidantes están íntimamente involucrados en la señalización celular y están vinculados con varias vías de señalización redox en la fibrogénesis. Asimismo, la desregulación de los mecanismos antioxidantes y la sobreproducción de ROS promueve el medio fibrótico y la disfunción mitocondrial, lo que agrava aún más la lesión renal (Okamura y Pennathur, 2015).

Tabla 3. Fuentes endógenas y exógenas de especies reactivas.

Factores Endógenos	Factores Exógenos
Angiotensina II	Luz solar, radiación UV, fibroblastos.
Células inflamatorias	Hipoxia
Degradación endosoma/lisosoma	Hiperoxia
Fibroblastos	Infecciones por microorganismos
Fosforilación oxidativa mitocondrial	Contaminantes y toxinas ambientales
La xantina oxidasa, NADPH oxidasa	Humo de cigarrillos
Los peroxisomas	Glucotoxicidad
Retículo endoplasmático	Dieta alta en calorías

Fuente Small et al., (2012), con modificaciones.

De entre las especies reactivas de oxígeno, el radical hidroxilo (OH^-) es altamente reactivo, reaccionando con el DNA por adición de dobles enlaces en las bases nitrogenadas y extracción de un átomo de hidrógeno en los enlaces C-H de la 2´-desoxirribosa (Cooke et al., 2003). El daño oxidativo se evidencia en modificaciones en el azúcar de los nucleótidos, en la inducción de entrecruzamientos covalentes, y de roturas de cadena simple o doble.

El daño oxidativo en el que nos centraremos es el que se da en las purinas. Este ocurre cuando el radical hidroxilo se añade a las posiciones C4, C5, C8 y se generan aductos con radicales OH (Figura 7), provocando roturas en el DNA (Evans et al., 2004). Entre las bases del DNA, la guanina (G) es particularmente susceptible a la oxidación, generando guaninas oxidadas como la 8-hidroxi-2-desoxiguanosina (8-OH-dG) (Sung et al., 2013). La 8-OHdG es una forma importante del daño oxidativo del DNA ya que se ha observado su formación *in vitro* después de la incubación del DNA con mutágenos o carcinógenos que generan radicales libres de oxígeno. Por tal motivo viene utilizando como un marcador sensible del estrés oxidativo durante la carcinogénesis, provocando además, la transversión G:C a T:A (Shinmura et al., 1997; Klaunig et al., 2011).

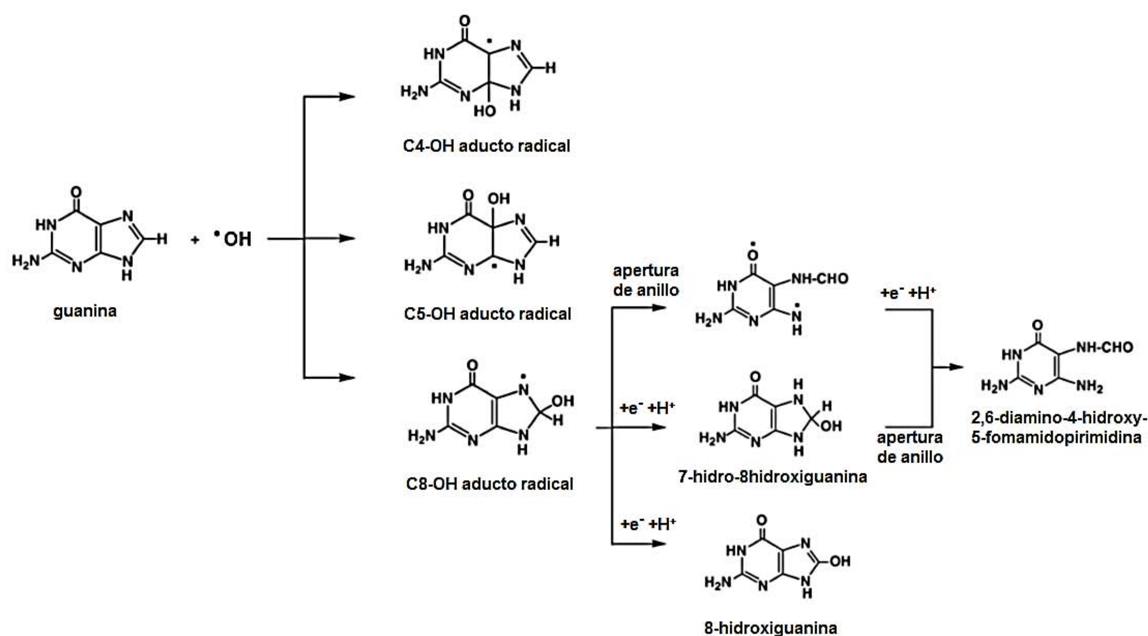

Figura 7. Reacción del radical OH- con las purinas. Producción de aductos de guanina en ausencia de oxígeno. Fuente Evans et al., (2004), con modificaciones.

Las lesiones en el DNA inducidas por oxidaciones no reparadas pueden tener consecuencias graves (apoptosis, mutaciones y cáncer). Por lo tanto, para mantener la estabilidad genómica, las células cuentan con mecanismos para detectar y reparar el daño del DNA. La reparación por escisión de base (BER), una vía de reparación altamente conservada, es responsable de la reparación de la gran mayoría de las lesiones oxidativas (Duclos et al., 2012). La identificación y eliminación de este tipo de lesiones son catalizadas por DNA glicosilasas y se agrupan en dos familias, según su estructura: la familia hélice-horquilla-hélice (HHH), llamado así por el motivo estructural implicado en la unión al DNA, y de la cual hacen parte los miembros de enzimas bacterianas formamidopirimidina DNA glicosilasa (FPG) y endonucleasa ocho (Nei) (Zharkov et al.,2003). Aunque las glicosilasas del DNA, que se especializan en la reparación de las lesiones inducidas por oxidación, se dividen en dos familias estructurales distintas, todas ellas emplean un mecanismo común que implica la escisión de la base de destino en el centro activo de la enzima (Prakash et al., 2012). Estudios bioquímicos muestran que las 8-oxoG y 8-OHdG son sustrato de la FPG (Zhang et al., 2012), lo cual ha permitido el estudio y biomonitoreo de diferentes patologías (como la ERC) utilizando la enzima FPG para detectar la existencia de daño oxidativo en el DNA (Collins, 2009).

1.5. Prevalencia de la enfermedad renal crónica en el mundo

Durante las últimas décadas ha tenido lugar un aumento dramático del número de pacientes con enfermedad renal terminal en Europa y América del Norte. En Estados Unidos (EEUU) la tasa de incidencia de la ERC es mayor en la población caucásica que en la afroamericana (McClellan et al., 2006), y a su vez la tasa de incidencia es tres veces mayor en los EEUU comparado con países europeos como Noruega y Gran Bretaña (Van Dijk et al., 2001; Coresh et al., 2007), lo cual remarca la importancia del componente étnico en el momento de analizar la prevalencia de patologías con componente genético, como la ERC. En América Latina, la prevalencia de la enfermedad renal terminal (ERT) en tratamiento renal sustitutivo (TRS) aumentó de 119 pacientes por millón de población (pmp) en 1991, a 660 pmp

en 2010 (Rosa-Diez et al., 2014). Para diferentes países de América, Europa, Asia y Australia la prevalencia poblacional se muestra resumida en la Tabla 4.

Tabla 4. Prevalencia de la ERC en estudios poblacionales.

Autor	País	Número de participantes	MDRD
Estudios poblacionales en América			
Brown et al. 2003	Estados Unidos	6071	15,6%
Hemmelgarn et al. 2006	Canadá	10184	35,4%
McClellan et al. 2006	Estados Unidos	20667	43,3%
Estudios poblacionales en Europa			
Hallan, 2006	Noruega	65181	4,7%
Cirillo et al. 2006	Italia	4574	6,4%
Otero et al. 2010	España	2746	6,8%
Viktorsdottir et al. 2005	Islandia	19256	7,2%
Nitsch et al. 2006	Suiza	6317	8,1%
Wasén et al. 2004	Finlandia	1246	35,8%
Estudios poblacionales en Asia y Australia			
Li et al. 2006	China	2310	4,9%
Shankar, 2006	Singapur	4898	6,6%
Domrongkitchaiporn et al. 2005	Tailandia	2967	6,8%
Ninomiya et al. 2005	Japón	2634	10,3%
McDonald et al. 2003	Australia	237	12%

MDRD (the Modification of Diet in Renal Disease) Fuente Zhang y Rothenbacher, (2008), con modificaciones.

Según el estudio EPIRCE (Epidemiología de la Insuficiencia Renal Crónica en España) entre las características sociodemográficas y clínicas de los pacientes con ERC, el factor predictor más fuerte es la edad. Las odds ratio (OR) observadas fueron de 34,4 para las personas entre 40-64 años y de 267,5 para las personas por encima de los 64 años. Otros factores predictores fuertes fueron la hipertensión, especialmente cuando se diagnosticó previamente (OR: 5,9), la presión arterial mayor de 60 mmHg (OR: 3,8), antecedentes de eventos cardiovasculares (OR: 4,1 para la enfermedad isquémica del corazón, 3,3 para la enfermedad cerebrovascular y 2,1 para la enfermedad vascular periférica), sobrepeso u obesidad (OR: 2,3-3,5, respectivamente), la diabetes (OR: 2,4 para los pacientes diagnosticados previamente y la dislipidemia (OR: 2,1) (Otero et al., 2010).

1.6. Las diferentes terapias de remplazo para la ERC

El principal reto en los tratamientos de reemplazo renal es lograr un método de purificación de la sangre que elimine la misma gama de toxinas urémicas que el propio riñón, ya que es su acumulación lo que conlleva el deterioro paulatino de la función renal.

En 2003, el grupo de trabajo europeo en toxinas urémicas (Eutox; http://EUTox.info) enumeró 90 solutos urémicos diferentes (Vanholder et al., 2001). En la última revisión sobre este tipo de toxinas realizada en el 2011, las clasificaron según su tamaño y propiedades de unión, por lo que ahora se sabe que del total de toxinas urémicas conocidas, los compuestos de bajo peso molecular (CBPM) (< 0,5 kD) representaban el 46%, las moléculas medianas (0,5-50 kD) el 28%, y los compuestos de unión a proteínas el 24% (Duranton et al., 2012). La eliminación de solutos debe cubrir toxinas con un peso molecular de aproximadamente 65 kD, porque éste es el punto de corte del riñón natural (Gondouin y Hutchison, 2011) y para ello existen básicamente dos tipos de terapias de remplazo, la diálisis y el trasplante.

La diálisis y el trasplante de riñón son los tratamientos utilizados para la preservación de la vida del paciente con ERT. Existen diferentes tipos de diálisis: la diálisis peritoneal (DP), la hemodiálisis (HD), la hemofiltración (HF) y la hemodiafiltración (HDF), entre otros (Lucas y Briones, 2012). Estos son métodos de tratamiento para filtrar los desechos del cuerpo. La DP utiliza el revestimiento de la cavidad abdominal (membrana peritoneal) y una solución (dializado) para eliminar los desechos y el exceso de líquido del cuerpo (Heras y Díaz, 2012), mientras que la HD y sus derivadas, utilizan una membrana artificial (dializador) para filtrar los desechos y eliminar el exceso de líquido de la sangre. Cada modalidad de diálisis tiene sus ventajas y desventajas, de ahí que a pesar de la mejora continua del tratamiento, la mortalidad anual en estos pacientes oscila entre el 15% y el 27% según informe de 2006 de diálisis y trasplante renal en España (Registro Español de Enfermos Renales, 2009).

A continuación se describirán exclusivamente los tipos de diálisis utilizadas en esta investigación (HD y HDF on-line).

1.6.1. La hemodiálisis (HD)

Desde la década de los sesenta se ha trabajado con la hemofiltración (HF), para mejorar la eliminación de moléculas medianas y mejorar la tolerancia hemodinámica. En la hemofiltración no hay flujo del líquido dializado, la eliminación de solutos se basa exclusivamente en la convección (20 - 25 L/sesión) (Quellhorst et al., 1983). Posteriormente a ello, se desarrolló la hemodiálisis (HD), que en las últimas décadas se ha convertido en un estándar de atención para los pacientes con ERC. Esta técnica de difusión, se basa en el flujo a través de los dializadores del líquido de diálisis en contracorriente a la sangre, y la fuerza motriz para el transporte a través de la membrana viene dada por la diferencia de concentración entre la sangre y el líquido de diálisis para cada soluto en particular (Ledebo et al., 2010). Existen diversas modalidades de HD en función de la eficiencia, permeabilidad y biocompatibilidad del dializador utilizado. La eficiencia está determinada por la capacidad de eliminación de pequeñas moléculas y se mide por el coeficiente de transferencia de masas para la urea (K_oA). La permeabilidad del dializador se mide por su capacidad para la transferencia de agua (coeficiente de ultrafiltración, K_{UF}) y para la depuración de β_2-microglobulina. Según el K_{UF} la hemodiálisis puede ser de bajo flujo (K_{UF} < 10 mL/h/mm Hg) o alto flujo (K_{UF} > 20 - 40 mL/h/mm Hg). Junto a lo anterior está la biocompatibilidad de las membranas de los dializadores, que pueden ser celulósicas, de baja biocompatibilidad, o sintéticas, biocompatibles (Lucas y Briones, 2012). En la presente tesis se hará referencia a la hemodiálisis por difusión y de bajo flujo, como HD convencional.

En la década de 1970, el desarrollo de nuevas estrategias en las terapias renales combinó las ventajas de la eliminación de solutos pequeños por difusión o hemodiálisis (HD) (Maduell et al., 2013), junto a la eliminación de solutos grandes por convección o hemofiltración (HF), dando origen a la hemodiafiltracion (HDF) (Ledebo et al., 2010).

1.6.2. Hemodiafiltración (HDF) y hemodiafiltración on-line (HDF on-line)

La hemodiafiltración (HDF) permite la extracción óptima de moléculas desde pequeñas a grandes (Henderson et al., 1975). Para ofrecer una mayor eliminación de varios tipos de toxinas urémicas se utilizan membranas sintéticas con alta permeabilidad al agua o membranas de alto o bajo flujo (Van Laecke et al., 2006). Las membranas de bajo flujo, un claro ejemplo de difusión, se caracterizan por eliminar solutos con pesos moleculares menores de 5.000 Da y con una alta permeabilidad difusiva, lo que significa que los pequeños solutos se mueven fácilmente a través de la membrana a lo largo de sus respectivos gradientes de concentración (Ledebo et al., 2010). El aumento de la eficiencia, logrado a través del aumento de las tasas de flujo para la sangre, el líquido de diálisis, y dializadores con un área de superficie más grande, han supuesto una mayor eficacia en la eliminación de pequeños solutos como la urea (60 Da) o la creatinina (113 Da) (Duranton et al., 2012), mientras que tiene poco efecto sobre los solutos más grandes tales como $ß_2$-microglobulina (11.800 Da) (Eknoyan et al., 2002). Por otro lado se disponen de membranas de alto flujo, que usan como método de eliminación de toxinas, la convección. Esto implica la eliminación de cantidades sustanciales de plasma a través de membranas con poros relativamente grandes, por ultrafiltración y su sustitución por solución salina (Van Laecke et al., 2006). Dicha membrana ha mostrado una mayor capacidad de eliminar la β_2-microglobulina (principal componente de proteína amiloide relacionada con la diálisis) (Vanholder et al., 2001), y una menor incidencia de la amiloidosis (Vilar et al., 2009). Además, estudios recientes han sugerido un efecto beneficioso de la HDF sobre los parámetros inflamatorios tales como la proteína C-reactiva (PCR) y la interleuquina-6 (IL-6) (Den Hoedt et al., 2014).

Siguiendo con las mejoras en las terapias de remplazo renal, en 1985 se introdujo la hemodiafiltración on-line (HDF on-line) (Figura 8) (Canaud et al., 1985) usando como base, el dializador ultra puro como fuente del líquido de sustitución. El líquido de sustitución usado durante la HDF on-line se mezcla con la sangre y, por lo tanto, debe ser estéril y con una composición similar a la del agua del plasma. El fluido puede ser administrado antes del filtro (predilución), después del filtro (postdilución), o dentro del filtro (diálisis de alto flujo y mediados de dilución) (Ledebo et al., 2010). Estudios aleatorios con tamaños de muestra limitados y

estudios no aleatorizados han mostrado que la HDF on-line mejora la estabilidad hemodinámica, reduce la actividad inflamatoria (Den Hoedt et al., 2014) y la mortalidad por eventos cardiovasculares y enfermedades infecciosas (Maduell et al., 2013).

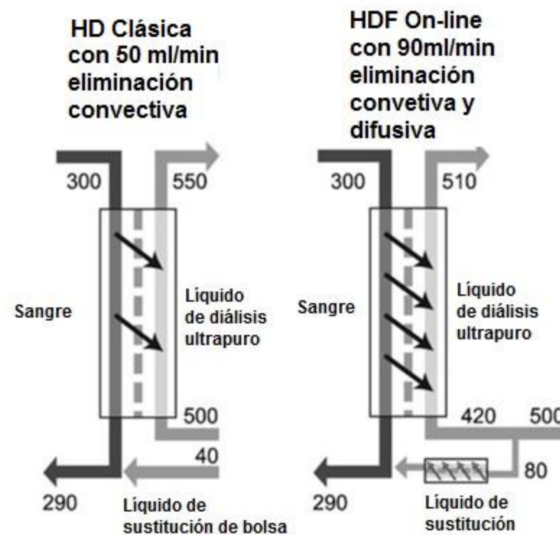

Figura 8. Diagramas del flujo en HD y HDF on-line. Fuente Ledebo et al., (2010), con modificaciones.

De todo lo anterior se puede deducir que la eliminación de toxinas urémicas en pacientes con ERC es el blanco de las terapias de remplazo mencionadas. La eficacia dialítica y la cuantificación de la eliminación de estas toxinas es una herramienta útil para evaluar la idoneidad del tratamiento dado. Para ello se han creado sistemas de control que cuantifican en cada sesión y en tiempo real, la dosis que el paciente recibe. Tradicionalmente, Kt/v_{urea} o simplemente Kt/v es el marcador de la eficacia de la diálisis más ampliamente utilizado (Eloot et al., 2013). El Kt/v tiene en cuenta la eliminación fraccionada de urea por tratamiento de diálisis, expresada como la eliminación (K), multiplicado por el tiempo de tratamiento (t) dividido por el volumen de distribución de urea (v). Durante tres tratamientos por semana, en un estudio con más de 3.000 pacientes se observó que un mayor Kt/v se acompañaba de una mayor supervivencia (Chertow et al., 1999). En 2006, en la guía de práctica K/DOQI publicada por la Fundación Nacional del Riñón, se recomienda un Kt/v mínimo de 1,2 (Hemodialysis Adequacy 2006 Work Group, 2006).

1.6.3. El trasplante renal

El trasplante renal (TR) fue el primer trasplante de órganos exitoso, el cual fue realizado entre gemelos idénticos en la ciudad de Boston en Estados Unidos el 23 de diciembre de 1954. Este hito anunció el inicio de una nueva era para los pacientes con ERC (Murray, 2011). El TR es el tratamiento de elección para el estadio 5 de la enfermedad y, ya desde sus inicios, se demostró que el riesgo de muerte para los receptores del trasplante es menos de la mitad que el de los pacientes en diálisis (Wolfe et al., 1999). El trasplante renal prolonga la vida, reduce la morbilidad, mejora la calidad de vida, permite la rehabilitación social y médica, y reduce los costos asociados con la atención médica de los pacientes con ERC (Kälble et al., 2010).

El trasplante es un procedimiento quirúrgico, con riesgos inherentes. La evaluación pre-trasplante tiene en cuenta posibles contraindicaciones y factores de riesgo para el trasplante (por ejemplo, tumor maligno, o infección en curso) (Karam et al., 2014). Además, la necesidad de terapia inmunosupresora continua puede dar lugar a efectos secundarios. Los fármacos inmunosupresores causan toxicidades significativas, tales como nefrotoxicidad, neurotoxicidad, toxicidad cardíaca, hematotoxicidad, diabetes post-trasplante, disfunción endotelial, y gastrotoxicidad (Masuda y Inui, 2006). Con el objeto de mejorar la eficiencia y la seguridad del tratamiento clínico en los receptores de trasplante, es una práctica habitual el uso combinado de diferentes medicamentos inmunosupresores a dosis más bajas. Los más comúnmente utilizados son el tacrolimus, inhibidor de la activación de las células T, y el micofenolato de mofetilo, que bloquea de *novo* la síntesis de purinas y, a pesar de que inhibe selectivamente la proliferación de linfocitos B y T, también es capaz de reducir significativamente los efectos secundarios de tacrolimus en el sistema renal (Allison y Eugui, 2000). En este sentido, un reciente estudio de genotoxicidad *in vitro*, demostró que el nivel de fragmentación del DNA medido con el ensayo de cometa fue significativamente mayor en células tratadas con estos fármacos; además, mostraron inhibición de la proliferación celular, e incremento de los radicales libres, y de la peroxidación lipídica, además de reducción del potencial de membrana mitocondrial (Ferjani et al., 2015).

A pesar de los efectos secundarios, el trasplante renal sigue siendo la terapia de remplazo renal con mejor pronóstico, como se ha mostrado en un estudio reciente en Europa y en los Estados Unidos con tasas de supervivencia a los 5 años post-trasplante del 77% y 56%, respectivamente (Gondos et al., 2013).

Más específicamente en España, durante el año 2006 se realizaron 2.157 trasplantes renales, de los cuales la comunidad autónoma de Cataluña realizó 408, siendo ésta la región más representativa en el informe de la Sociedad Española de Nefrología y la Organización Nacional de Trasplantes (Sociedad Española de Nefrología, 2009).

1.7. Mecanismos de defensa antioxidante y suplementos

Como ya se ha mencionado anteriormente los pacientes con ERC, y especialmente aquellos pacientes sometidos a diálisis, ven incrementados sus niveles de substancias oxidantes que pueden dañan al DNA. Para hacer frente a los ROS, además de la presencia de antioxidantes (Matés, 2000), la célula está equipada con un sistema de defensa que incluye diversas familias enzimáticas como: las superóxido dismutasas (SOD), que catalizan la descomposición del anión superóxido en peróxido de oxígeno e hidrógeno; las catalasas (CAT), que catalizan la descomposición del peróxido de hidrógeno en agua y oxígeno; las glutatión peroxidasas (GPX) y las glutatión S-transferasas (GSH), que catalizan la descomposición de peróxido de hidrógeno e hidroperóxidos orgánicos (Lobo et al., 2010). En los pacientes con ERC se ha visto que este sistema está desequilibrado y no es del todo eficiente, por lo que dentro de las diferentes alternativas terapéuticas para mejorar la calidad de vida de estos pacientes, además de las terapias de remplazo previamente mencionadas, se ha sugerido el uso de suplementos ricos en antioxidantes para contrarrestar el estrés oxidativo y reducir el daño genómico.

Los antioxidantes son moléculas suficientemente estables como para neutralizar los radicales libres mediante la donación de uno de sus propios electrones, éstos actúan como captadores desempeñando un papel protector por eliminación de

dichos radicales, antes de que tengan la oportunidad de generar lesiones a nivel molecular. Estos compuestos se conocen por su capacidad de detener o estabilizar los radicales libres (Kaur y Kapoor, 2001). Diversos estudios han demostrado la presencia sobretodo en frutas, verduras, cereales, leguminosas, té y vino, de ciertos compuestos como son los polifenoles, flavonoides, isómeros conjugados del ácido linoleico, galato, proteína de soja, isoflanonas, vitaminas A, B, C y E, indoles, tiocianatos y los inhibidores de proteasas (Weisel et al., 2006; Stratil et al., 2007) que pueden actuar independientemente o en combinación, como agentes antioxidantes, anticancerosos o cardioprotectores mediante una gran variedad de mecanismos (Spormann et al., 2008). Dentro de los compuestos flavonoides encontramos seis subclases: flavonoles, flavonas, flavanonas, flavonoides, antocianinas (pigmentos en la piel de la uva) e isoflavonas. Los flavonoides se encuentran ampliamente distribuidos en las uvas, especialmente en semillas y tallos, y contienen principalmente (+) catequinas, (-) epicatequina y polímeros de procianidina. Las antocianinas son los principales polifenoles en las uvas rojas (Xia et al., 2010). Este grupo tiene una estructura básica común que consta de dos anillos aromáticos unidos por tres átomos de carbono que forman un heterociclo oxigenado (Figura 7) (Pandey y Rizvi, 2009).

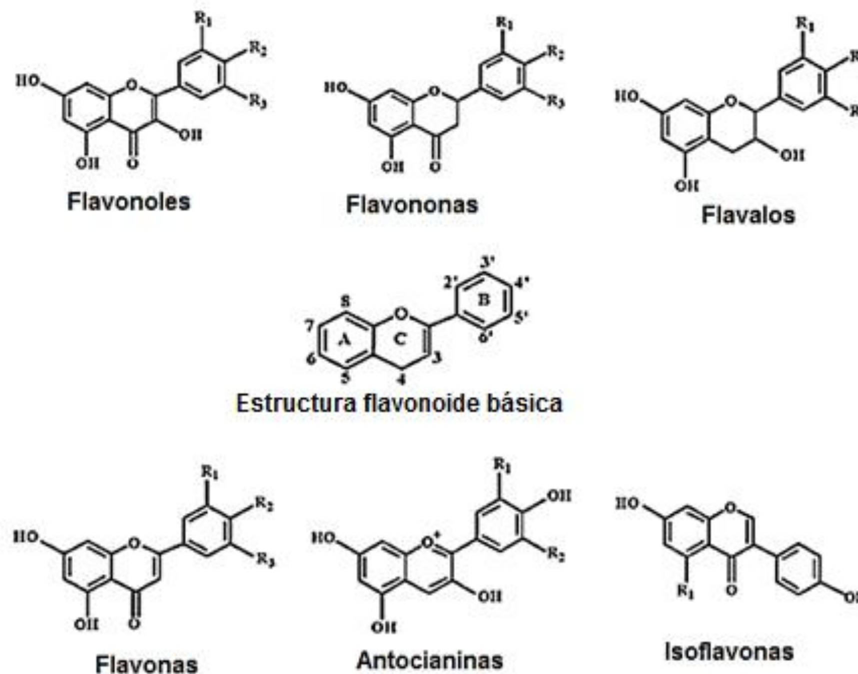

Figura 7. Estructuras químicas de las subclases de flavonoides. Fuente Pandey y Rizvi (2009), con modificaciones.

La mayoría de estas sustancias antioxidantes para ser funcionales deben ser hidrolizadas por enzimas intestinales (Pandey y Rizvi, 2009) y posteriormente absorbidas. Aunque el mecanismo no se ha dilucidado completamente, se sugiere que el transportador de glucosa dependiente de sodio (SGLT1) puede ser capaz de transportar algunos glucósidos (Day y Williamson, 2001). Los polifenoles son potentes inhibidores de la oxidación de LDL y este tipo de oxidación se considera que es un mecanismo clave en el desarrollo de la aterosclerosis (Pandey y Rizvi, 2009).

En pacientes con ERC se ha demostrado que el consumo de 500 mg/día de polifenoles de uva en polvo aumenta la actividad del GSH y evita la progresión de la inflamación en pacientes en HD (Janiques et al., 2014). Por otro lado, se ha visto que el extracto de semilla de uva es capaz de disminuir el daño en el DNA de linfocitos humanos (Szeto et al., 2013). Además, en pacientes con enfermedad renal terminal (ERT) el efecto de la suplementación oral con jugo de uva roja concentrada, disminuyó los niveles de producción de radicales superóxido, al reducir la sobreproducción de ROS, lo cual serviría para mejorar el estado vascular de los pacientes en HD (Castilla et al. 2008).

Otro antioxidante interesante es la vitamina E. Los estudios *in vitro* demuestran las propiedades antioxidantes de esta vitamina, siendo el principal y más potente antioxidante soluble en lípidos, por lo que puede prevenir de la peroxidación lipídica (Singh et al., 2005). A pesar de que hay resultados contradictorios en cuanto a su efecto sobre la inflamación y el estrés oxidativo en pacientes sometidos a HD se ha podido ver que, independientemente del modo de administración, ya sea por dializadores con membranas recubiertas de vitamina E o por vía oral, se da una disminución en los niveles de daño en el DNA medidos tanto mediante el ensayo del cometa como por la cuantificación de 8-oxodG (Tarng et al., 2000a; Müller et al., 2004)

1.8. Predisposición a la ERC

Ya se han mencionado previamente los distintos factores asociados a la ERC, como la diabetes, la hipertensión y enfermedades cardiovasculares, que contribuyen a las diferencias observadas en la progresión de la enfermedad. Sin embargo, la variabilidad de la ERC en pacientes con o sin hipertensión y/o diabetes, señala la importancia de factores adicionales, como las características genéticas individuales. La contribución genética a la función y patologías renales se ve apoyada por la presencia de enfermedades mendelianas como la poliquistosis renal autosómica dominante (Chow y Ong, 2009). Estudios de heredabilidad basados en la medida de la función renal, y estudios de agregación familiar de enfermedades complejas, como la ERC, indican que entre el 33% y el 82% de la variación interindividual en la TFG podría explicarse por los efectos genéticos aditivos (Fox et al., 2004).

A diferencia de las enfermedades monogénicas, las enfermedades complejas, como la ERC, surgen de una combinación de variables entre las cuales está la exposición a múltiples factores ambientales perjudiciales que actúan sobre individuos genéticamente susceptibles (Manolio et al., 2008). Esto ha suscitado una intensa investigación para identificar factores genéticos determinantes y mecanismos fisiopatológicos subyacentes en enfermedades complejas como la ERC. Actualmente se cuenta con estudios de asociación del genoma completo (*genome wide association studies,* GWAS) que han identificados múltiples *loci* en relación con la TFG y la ERC, tanto en poblaciones europeas como no europeas (Köttgen et al., 2009; Gorski et al., 2015).

Estudios en poblaciones europeas han observado riesgo genético asociado a polimorfismos de un solo nucleótido (SNP, *single nucleotide polymorphism*) para la hipertensión (International Consortium for Blood Pressure Genome-Wide Association Studies, 2011), la enfermedad arterial coronaria, la enfermedad vascular subclínica (Schunkert et al., 2011), así como para rasgos funcionales renales y la ERC (Köttgen et al., 2010a).

Recientemente, los estudios de GWAS han identificado con éxito SNPs en los genes *UMOD* y *PRKAG2* que se asocian con el riesgo de la enfermedad renal crónica; así como en *CLDN14* asociado con el riesgo de presentar cálculos renales; y variantes en, o cerca de *SHROOM3, STC1, LASS2, GCKR, NAT8/ALMS1, TFDP2, DAB2, SLC34A1, VEGFA, FAM122A/ PIP5K1B, ATXN2, DACH1, UBE2Q2/FBXO22 y SLC7A9*, asociados con diferencias en la tasa de filtración glomerular (Köttgen et al., 2010b). Por otro lado, el grupo de Böger et al., 2011b encontró que 11 genes (*UMOD, PRKAG2, ANXA9, DAB2, SHROOM3, DACH1, STC1, SLC34A1, ALMS 1/NAT8, UBE2Q2 y GCKR)* asociados con la incidencia de la ERC.

La inestabilidad genómica presente en los pacientes con ERC invita a estudiar qué papel pueden jugar variantes genéticas en las vías de reparación del DNA. Asimismo, la asociación entre ERC y mortalidad, calcificación vascular y envejecimiento (Friedman et al., 2009), hace que el estudio de genes implicados en estos procesos también sea de especial interés. Por lo tanto, los estudios de asociación son de vital importancia para determinar no tan sólo la susceptibilidad de la población a padecer ERC, si no que una vez que la enfermedad ha aparecido, saber cuál puede ser su pronóstico.

II. OBJETIVOS

2. OBJETIVOS

Debido a la complejidad de la enfermedad renal crónica se hacen necesarios estudios intensivos para entender las causas, consecuencias, terapias, y predisposición individual a la misma. Si bien no con la finalidad de curarla si, al menos, con el objetivo de mejorar la calidad de vida de los pacientes.

El objetivo principal de esta tesis doctoral ha sido el estudio y caracterización de los niveles de daño genómico presente en los pacientes con enfermedad renal crónica evaluando el efecto modulador, tanto de diferentes técnicas y/o suplementos nutritivos sobre dichos niveles de daño, como el de diferentes polimorfismos genéticos en la incidencia de la patología y el daño genómico.

Para lograr estos objetivos, nos propusimos diferentes estudios:

- Estudiar la asociación entre la ERC y el daño genómico, en una muestra representativa de pacientes con ERC.
- Evaluar el efecto antioxidante del mosto sobre los niveles de daño genómico en pacientes con ERC sometidos a HD mediante el ensayo del cometa, el test de micronúcleos y la capacidad antioxidante equivalente TEAC (Trolox).
- Analizar el efecto que produce el cambio de hemodiálisis convencional a hemodiafiltración on-line sobre los niveles de daño genómico y oxidativo.
- Estudiar la evolución del daño genómico y oxidativo en pacientes sometidos a un trasplante renal, mediante el ensayo del cometa y el test de micronúcleos.
- Determinar la potencial asociación de 38 alelos de riesgo con la enfermedad renal crónica.
- Investigar la asociación entre el daño genómico observado previamente en pacientes con ERC y diversos polimorfismos genéticos.

III. RESULTADOS

3. RESULTADOS

Los resultados de la presente tesis doctoral han dado lugar, hasta el momento, a dos publicaciones, al tiempo que se han generado otros cuatro artículos que se encuentran en distintas fases de su potencial publicación.

Esta actividad se sintetiza en los siguientes artículos:

- Artículo 1: Genomic damage as a biomarker of chronic kidney disease status. *Environmental and Molecular Mutagenesis. 2015; 56: 301-312.*
- Artículo 2: Unfermented grape juice reduces genomic damage on patients undergoing hemodialysis. *Food and Chemical Toxicology. 2016; 92: 1-7.*
- Anexo 1. Artículo 3: Changing to on-line hemodiafiltration affect the levels of genomic damage in patients undergoing hemodialysis.
- Anexo 2. Artículo 4: Genetic damage follow-up in kidney transplant patients with chronic kidney disease.
- Anexo 3. Artículo 5: Genetic variants associated with chronic kidney disease in a Spanish population.
- Anexo 4. Artículo 6: Loci associated with genomic damage levels in Spanish chronic kidney disease patients.

ARTÍCULO 1

Genomic Damage as a Biomarker of Chronic Kidney Disease Status

Zuray Corredor, Elitsa Stoyanova, Lara Rodríguez-Ribera, Elisabet Coll, Irene Silva, Juan Manuel Díaz, José Ballarin, Ricard Marcos, Susana Pastor

Environmental and Molecular Mutagenesis 2015; 56: 301-312

Factor de Impacto: 2,63

3.1. Resumen del artículo 1

Genomic Damage as a Biomarker of Chronic Kidney Disease Status

Los pacientes que sufren enfermedad renal crónica (ERC) se caracterizan por mostrar una alta incidencia de enfermedades cardiovasculares, diabetes y cáncer, junto con ello se ha indicado que, respecto a la población general, estos pacientes presentan elevados niveles de daño genómico.

Para confirmar la asociación de la ERC con el daño genómico se ha llevado a cabo el mayor estudio realizado hasta la fecha con este tipo de pacientes, utilizando un total de 602 sujetos (187 controles, 206 pacientes con ERC pre-hemodiálisis y 209 pacientes con ERC en hemodiálisis). El daño oxidativo del DNA se midió en todos los individuos utilizando el ensayo del cometa complementado con la enzima FPG.

Los resultados obtenidos indican que los pacientes con ERC presentan niveles significativamente más altos de daño en el DNA que los controles, sin observarse diferencias significativas entre los pacientes pre-hemodiálisis (PD) y aquellos en hemodiálisis (HD). Por otro lado, no se observaron diferencias entre los pacientes y controles en cuanto a los niveles de daño oxidativo, mientras que los pacientes en HD si mostraron niveles significativamente más altos de daño oxidativo que los pacientes en PD. Finalmente, se observó que aquellos pacientes que fallecieron durante el estudio fueron los que presentaron, inicialmente, los mayores niveles de daño genómico.

En conclusión, se puede decir que el daño genómico es una característica inherente a la ERC, al tiempo que puede ser un factor de mal pronóstico en pacientes con ERC.

Environmental and Molecular Mutagenesis 56:301–312 (2015)

Research Article

Genomic Damage as a Biomarker of Chronic Kidney Disease Status

Zuray Corredor,[1] Elitsa Stoyanova,[1] Lara Rodríguez-Ribera,[1] Elisabet Coll,[2] Irene Silva,[2] Juan Manuel Diaz,[2] José Ballarin,[2] Ricard Marcos,[1,3]* and Susana Pastor[1,3]*

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Patients suffering from chronic kidney disease (CKD) exhibit a high incidence of cancer and cardiovascular diseases, as well as high levels of genomic damage. To confirm the association of CKD with genomic damage we have carried out the largest study to date addressing this issue, using a total of 602 subjects (187 controls, 206 pre-dialysis CKD patients and 209 CKD patients in hemodialysis). DNA oxidative damage was measured in all individuals using the comet assay. Our results indicate that CKD patients have significantly higher levels of DNA damage than controls, but no significant differences were observed between pre-hemodialysis (pre-HD) and hemodialysis (HD) patients. When oxidative damage was measured, no differences were observed between patients and controls, although HD patients showed significantly higher levels of oxidative damage than pre-HD patients. In addition, a positive relationship was demonstrated between genomic damage and all-cause mortality. Our study confirms that genomic damage can be predictive of prognosis in CKD patients, with high levels of DNA damage indicating a poor prognosis in HD patients. Environ. Mol. Mutagen. 56:301–312, 2015. © 2014 Wiley Periodicals, Inc.

Key words: CKD patients; comet assay; genomic damage

INTRODUCTION

According to the kidney disease outcomes quality initiative (K/DOQI) of the National Kidney Foundation, chronic kidney disease (CKD) is characterized by a progressive loss of kidney function. Thus, CKD patients are defined as those showing either kidney damage or a glomerular filtration rate (GFR) <60 mL min^{-1} per 1.73 m^2 for three or more months [K/DOQI, 2002]. Consequently, a five-stage classification system is used to define the severity of the pathology in adults that, together with persistent proteinuria, are the principal markers of kidney damage [Keane and Eknoyan, 1999]. As renal disease worsens from stage 1 to 5, kidney functions deteriorate and at the end-stage of renal failure, kidney replacement or dialysis therapies are required [Weiner, 2007].

A general trait of CKD patients is that they present an elevated incidence of different types of cancer (mainly cervical, bladder, thyroid and renal cell carcinoma), as well as cardiovascular pathologies [Di Angelantonio et al., 2010; Stengel, 2010]. This increased cancer risk is independent of symptom severity (stages 1 through 4) or whether they are undergoing dialysis. It is not clear whether CKD is the cause or rather a consequence of the increased incidence of cancer; nevertheless, both diseases share common etiological factors [Stengel, 2010]. Either way it appears that increased levels of genomic damage are present in this type of patient [Stopper et al., 2001; Fraderaki et al., 2005; Sandoval et al., 2010]. This genomic damage may be caused by elevated levels of

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TABLE I. Studies Carried out with CRF Patients Using the Comet Assay to Detect DNA Damage

Authors	Year	*P*	Status	*C*	Conclusions (biomarker *vs.* genetic damage)
Stopper et al.	2001	64	CRF:23 HD:26 HDF:15	21	Higher levels in CRF patients than in controls. Tendency to higher values in HD patients.
Kan et al.	2002	36	HD	36	Higher levels in HD patients than in controls. Levels decrease after 14 weeks with vitamin E supplementation.
Muller et al.	2004	21	HD	12	Higher levels in HD patients than in controls. Higher values in patients using cellulose membranes. No differences between PS membranes and those covered with vitamin E.
Domenici et al.	2005	51	PD:22 HD:29	9	Higher levels in HD and PD patients than in controls. Decrease after 4 weeks of supplementation with vitamin E.
Kobras et al.	2006	12	HD:5 HDF:7	4	Higher levels in HD/HDF patients than in controls. No differences between HD and HDF, but there is a tendency to decrease when patients move from HD to HDF
Schupp et al.	2006	12	HD:5 HDF:7	12	Higher levels in HD/HDF patients than in controls. Tendency to decrease when patients move from HD to HDF
Bagatini et al.	2008	25	HD (*T2DM*)	20	Higher levels in patients with T2-diabetes under HD than in controls. Higher values just after HD session.
Stoyanova et al.	2010	141	CRF:64 HD:77	61	Higher levels in CRF/HD patients than in controls; HD shows higher values than CRF. Associations with levels of creatinine and ferritine as well as with time in HD.
Palazzo et al.	2012	22	HD (*T2DM*)	22	Higher levels in patients than in controls.
Zachara et al.	2011	42	HD	30	Higher levels of basal and oxidative damage in HD than in controls that reduce after selenium treatment
Ersson et al.	2012	31	HD	10	Higher levels in HD patients than in controls.
Rangel-López et al.	2013	91	CRF: 23 PD: 33 HD: 35	61	Higher levels in CRF and PD patients than in controls. No differences between HD and controls.

P: number of patients; C: number of controls; HD: hemodialysis; HDF: hemodiafiltration; PD: peritoneal dialysis; T2DM: diabetes mellitus type II.

reactive oxygen species (ROS), or by the increase in circulating advanced glycation end-products (AGEs) that result from oxidative peroxidation. Both endogenous factors are associated with the pathology [Busch et al., 2010; Small et al., 2012].

In addition to the increased cancer incidence, it has been demonstrated that CKD patients show genomic instability [Sandoval et al., 2012]. In an open literature search, we found 41 studies published between 1988 and 2013 showing that CKD patients present higher levels of DNA damage than controls. These findings were generated using different biomarkers such as the presence of 8-OHdG, unscheduled DNA synthesis, telomere length, deletions in mitochondrial DNA, sister-chromatid exchanges, micronuclei and comet assays. Twelve studies used the comet assay [also known as the single-cell gel electrophoresis (SCGE) assay] to measure genomic damage and found higher levels of DNA breaks in patients compared to controls. Two problems are common to most of these studies. The first is that all of them were carried out in hemodialysis patients, while only three also included pre-dialysis CKD patients. The second problem is the low number of patients included in these studies (ranging from 12 to 141). The largest study was performed by our group [Stoyanova et al., 2010], and the next largest study included only 91 patients [Rangel-López et al., 2013]. A description of all 12 studies is provided in Table I.

While there is a wide range of techniques for measuring DNA damage the comet assay has become an increasingly standard tool for assessing DNA damage in the last decade, with applications in genotoxicity testing, human biomonitoring and molecular epidemiology, as well as in fundamental research on DNA damage and repair. The number of publications using this assay rises each year, with the increasing prevalence due to the assay's simplicity, sensitivity, versatility, speed and economy [Collins, 2004; Speit and Hartman, 2006; Burlinson, 2012]. Furthermore, by using proprietary image analysis software, levels of DNA damage are scored objectively and rapidly, with the data immediately accessible for analysis. This methodology therefore offers several advantages, either as a complement of the classical cytogenetic assays, or as a stand-alone biomarker of genetic damage [Møller, 2005]. Furthermore, while the standard comet assay measures only DNA strand breaks and alkali-labile sites, simple modification of the assay allows for the detection of oxidized DNA bases resulting from pro-oxidant factors or different types of exposures [Collins, 2005]. For example, addition of endonuclease III will detect damage in the form of oxidized pyrimidines, and the addition of formamidopyrimidine DNA glycosylase (FPG) detects modified purines (mainly 8-oxodG).

The aim of this study was to confirm the association between CKD and genomic damage, by measuring DNA strand breaks and oxidative DNA damage in a large group

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TABLE II. Description of the Studied Groups

		Predialysis		Hemodialysis
	Control; $N = 187$	Stage 2–3; $N = 105$	Stage 4–5; $N = 101$	$N = 209$
Sex (% men/women)	63.7/36.3	66.7/33.3	56.4/43.6	61.9/38.1
Age (mean ± S.E.)	55.7 ± 1.2	67.5 ± 1.3	66.5 ± 1.3	64.9 ± 1.1
Time in HD (% with 2,4, 6 or >8 years)	ND	ND	ND	63.2 16.5, 10.4, 9.9
RT previous (%)	ND	ND	ND	19.9
Smoking (% yes/no)	26.3/73.8	52/48***	51.5/47.5**	56.7/43.3***
Hypertension (% yes/no)	33.0/67.0	87.4/12.6***	92.1/7.9***	89.5/10.5***
Diabetes mellitus (% yes/no)	10.0/90.0	32/68***	21.8/78.2*	30.4/69.6***
Dyslipidemia (% yes/no)	21.3/78.7	61.2/37.9***	58.4/41.6***	60.8/39.2***
CV pathology (% yes/no)	1.1/98.9	45.6/54.4***	44.6/55.4***	62.6/37.4***
Cancer (% yes/no)	0/100	26.2/73.8***	22.8/77.2***	27.5/72.5***
Familiar CV history (% yes/no)	0/100	24.4/75.5	18.4/81.6	16.4/83.6
Familiar cancer history (% yes/no)	0/100	17.3/82.7	14.4/85.6	10.5/89.5

RT, renal transplant; CV, cardiovascular. Asterisks indicate differences with respect to controls (Mann–Whitney test):*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

of 415 CKD patients. This represents the largest group of CKD patients ever studied for incidence of genomic damage. The study group was composed of a similar number of pre-dialysis patients (206) and patients undergoing dialysis treatment (209), and the comet assay was carried out both with and without the use of FPG to detect oxidized DNA bases. This large sample size permitted us to establish more accurate associations between the levels of DNA damage and different parameters related with the pathology.

SUBJECTS AND METHODS

Study Population

The study involved a total of 602 Caucasian adults, including 415 patients suffering kidney pathologies at different stages and 187 controls. Among the 415 patients, 206 had a reduced glomerular filtration rate (GFR < 60 mL/min/1.73 m^2) and 209 underwent renal substitutive treatment with hemodialysis (HD). The general characteristics of all patients are indicated in Table II. Patients and controls were randomly recruited at the Puigvert Foundation, Barcelona, over a period of 6 years. Controls were selected from urology clinical outpatients suffering from either prostatic pathology, urinary tract infections or kidney stones, and all had normal GFR according to their age. Pre-dialysis patients were recruited from CKD clinical outpatients. All hemodialysis patients were recruited at the Hemodialysis Unit of the Puigvert Foundation and underwent conventional hemodialysis for 3.5–4 h, three times per week, using synthetic low permeability membranes. Patients from our previous work [Stoyanova et al., 2010] were included in the present study.

All individuals participating in the study provided informed consent, and blood samples were collected under protocols approved by the Ethics Committee of the Puigvert Foundation. Hemodialysis patient blood was obtained before the hemodialysis session and in the middle of the week. In addition to the studies carried out to determine the levels of genetic damage in peripheral blood lymphocytes, standard blood analysis was also carried out at the Puigvert Foundation, including the determination of parameters relevant to the illness such as levels of urea, serum creatinine, sodium, potassium, calcium, phosphorus, blood glucose, cholesterol, triglycerides, albumin, hemoglobin and bicarbonate. Moreover ferritin, parathyroid hormone, C-reactive protein, homocysteine, vitamin B12 and folate were also analyzed.

Comet Assay

DNA breaks present in peripheral blood lymphocytes were measured using the comet assay. This assay was performed following the standard protocol used in our lab described elsewhere [Stoyanova et al., 2010], but using Gelbond® film instead of microscopic slides as a support for the agarose gel. The use of hydrophilic films facilitates the rapid processing of numerous samples, increasing the efficiency of the alkaline comet technique, without sacrificing the reliability or sensitivity of the assay [McNamee et al., 2000; Azqueta et al., 2013b]. Thus, lymphocytes were isolated using Ficoll–Paque density gradient from 500 µL of whole blood; cells were adjusted to a concentration of 17,800 cells in 25 µL and carefully re-suspended in 225 µL of 0.75% low melting agarose (LMA) at 37°C and dropped onto a Gelbond® film (10.5 × 7.5 cm^2). Forty-eight drops (7 µL each) were placed on each Gelbond® sheet and samples of eight donors were run simultaneously, so each donor was represented by six drops. Lymphocytes were lysed for a minimum of 1 h at 4°C in a dark chamber containing a cold fresh lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl and 1% Triton X-100, adjusted to pH 10). To allow DNA denaturation, unwinding and exposure of alkali-labile sites, Gelbond® sheets were placed for in a horizontal gel electrophoresis tank filled with freshly cold (4°C) electrophoresis solution (1 mM Na_2EDTA and 300 mM NaOH, adjusted to pH 13) for 35 min. Electrophoresis was performed in the same buffer for 20 min at 20 V and 300 mA. After electrophoresis, Gelbond® sheets were neutralized with two 5-min washes with PBS 1X, followed by 1 min wash with water and then incubated over night in 100% ethanol for fixation. Sheets were then dried and stored in the dark at room temperature until scoring. Just before the microscopic analysis, Gelbond® sheets were stained with 20 µL of SyrbrGold. The images were examined at 20× magnification with a Komet 5.5 Image Analysis System (Kinetic Imaging Ltd, Liverpool, UK) fitted with an Olympus BX50 fluorescence microscope equipped with a 480–550 nm wide band excitation filter and a 590 nm barrier filter. A total of one hundred randomly selected cells were analyzed per patient. The % tail DNA was used as a measure of DNA damage and computed using the Komet version 5.5 Software.

Detection of Induced Oxidative Damage

To determine the levels of oxidized bases present in the lymphocytes, Gelbond® sheets were washed two times (10 and 50 min, 4°C) after cell lysis in an enzyme buffer solution (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg mL^{-1} BSA, pH 8.0) containing FPG enzyme kindly

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donated by A. Collins [Azqueta et al., 2013]. Each sample was also analyzed using two additional Gelbond® sheets. One sheet remained in the cell lysis solution in order to assess basal DNA damage. The second was treated with the enzyme buffer without FPG, to control for any effects of the buffer alone. Gelbond® sheets were incubated in enzyme buffer (with or without FPPG) for 30 min at 37°C, after which samples were processed as in the standard alkaline comet assay procedure. Net oxidative DNA damage was calculated by subtracting the damage scored in the samples incubated with buffer from those incubated with FPG.

Statistical Analysis

There is no general consensus for a standard statistic method in the analysis of comet data [Lovell and Omori, 2008]. Nevertheless, there is an agreement that the mean or the median of the % tail DNA, scored for each sample, are the parameters to be considered; and at least two replicated gels are needed to obtain the mean value for each sample [Dusinska and Collins, 2008].

Data were expressed as mean ± standard error. As we showed that the values of genomic damage did not differ significantly from a normal distribution (Kolmogorov–Smirnov test), parametric tests were used for the statistical analysis. Comparisons between the two groups were analyzed using the Student t-test for continuous variables, and the Chi-square or Mann–Whitney test for discrete variables. Because we observed no effect of variables such as age, gender, smoking, cancer or cardiovascular disease, no further adjustments for such variables were made. Bivariate correlations were made between dependent variables and covariates (vitamins, biochemical parameters, supplements, etc.). Correlation coefficients were assessed by means of the Pearson's correlation test. Outliers were detected using the interquartile range, with extreme outliers defined by the formula Q1±3 (Q3-Q1), and mild outliers defined by the formula Q1±1.5 (Q3-Q1). All data were analyzed using the Statistic Package of Social Sciences (SPSS) software for Windows version 19.0. Statistical significance was defined as a P value lower than 0.05.

RESULTS

Population

Table II shows some general characteristics of the individuals under study. More CKD patients were men than women, reflecting the well-known higher incidence of CKD in males. The majority (63.2%) of patients undergoing HD therapy had been in the HD programme for <2 years. Patients had higher levels of hypertension, diabetes, dyslipidemia, cardiovascular pathologies and cancer compared to controls. Similarly, CKD patients had more familiar history of cardiovascular pathologies and cancer. Patients suffered from renal disease for a variety of reasons including nephroangiosclerosis (17.5%), diabetes mellitus (13.7%), glomerular disease (12.3%), tubulointerstitial nephritis (11.5%), polycystic kidney disease (6.0%), vasculorenal pathologies (3.6%), in addition to other causes with an incidence of <1% (15.2%) and unknown (20.2%).

Blood chemistry data differed between patients and controls, and some parameters also differed according to the stage of the pathology. Figure 1 highlights some of the differences between groups. As indicated, levels of haemoglobin, parathyroid hormone, C-reactive protein, and urea significantly differed between groups. Although the levels of haemoglobin decreased in patients compared to controls, the other parameters increased in patients according to the worsening of the pathology.

DNA Damage

DNA damage measured in the 602 individuals participating in this study is shown in Figure 2. The % tail DNA observed in CKD patients (21.42 ± 0.75) was significantly higher than controls (7.74 ± 0.41) ($P \leq 0.001$, t-test). Given that CKD patients constitute a complex group, we split the overall patient sample into pre-dialysis patients versus those undergoing HD treatment (Fig. 2B). The levels of DNA damage in pre-dialysis patients (22.77 ± 0.75) were similar to those of HD patients (21.18 ± 0.75; P = 0.255, t-test). When pre-dialysis patients were divided according to stage of pathology no significant differences were observed between patients in stages 2–3 compared to those in stages 4–5 ($P = 0.751$; t-test) (Fig. 2C).

Focusing first on pre-dialysis patients, we evaluated whether any of the measured biochemical parameters that are characteristic of CKD patients (creatinine, albumin, PTH, CRP, uric acid, proteinuria, etc.) were associated with DNA damage. Parameters that were significantly correlated with DNA damage in pre-dialysis patients are shown in Figure 3. As observed, the levels of albumin and vitamin B12 were negatively correlated with the levels of DNA damage. In contrast, the levels of creatinine, parathyroid hormone and uric acid levels, which were indicative of impaired renal function, were positively correlated with genomic damage. The correlations for vitamin B12, albumin and PTH remained significant, even following the removal of extreme outliers. No significant associations were found for the other parameters (data not shown). Considering HD patients, the levels of ferritin, vitamin B12 and TSI (transferrin saturation index) correlated positively with DNA damage, whereas negative correlations were observed with HDL (high density lipoprotein) and TIBC (total iron-binding capacity) (Fig. 4). No other significant associations have been found with other biochemical parameters. However, DNA damage in HD patients did correlate positively with the amount of time that patients had been undergoing hemodialysis (Fig. 5).

No association was observed between DNA damage and the presence of cancer or cardiovascular disease (data not shown) among CKD patients. However, there was an association between DNA damage and patient mortality. Specifically, patients who died during the course of the study (mean follow up of 4 years) had an average DNA damage level of 29.08 ± 2.01 ($N = 73$), significantly higher than the surviving patients (12.87 ± 1.42; $N = 99$) Figure 6. The main cause of decease was cardiovascular

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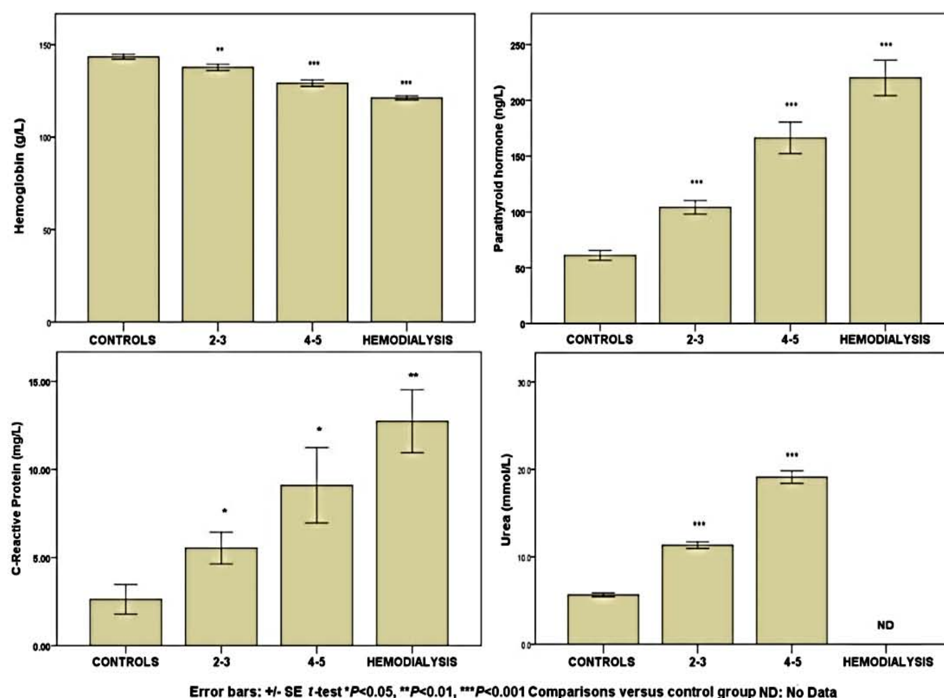

Fig. 1. Biochemical parameters in CKD patients and controls. Only parameters showing significant differences are presented.

disease [Coll et al., 2013]. Levels of DNA damage can therefore be viewed as a biomarker of bad prognosis in those patients.

Oxidative DNA Damage

To determine levels of oxidative DNA damage in CKD patients we carried out the comet assay following incubation with FPG, an enzyme that recognizes oxidized purine bases and excises them to produce DNA breaks. Net measures of oxidized bases are generated by subtracting the values obtained with and without FPG and are indicated in Figure 7. We observed no difference between CKD patients and controls. However, comparing pre-HD patients with HD patients showed that HD patients had significantly higher net oxidative DNA damage values ($P < 0.001$, t-test), despite the lack of significant difference between HD patients and controls ($P = 0.086$). A further split of pre-dialysis patients according to their pathology stage revealed no difference between patients classified as groups 2–3 compared with those classified as 4–5 ($P > 0.05$, t-test).

Significant correlations were observed between oxidative DNA damage and a subset of biochemical measures (Fig. 8). No associations were observed for HD patients. Among pre-dialytic patients, net oxidative damage values were negatively correlated with only the levels of albumin and low density lipoproteins. Similarly, net oxidative DNA damage was not associated with prognosis among HD patients, as damage in HD patients who deceased during the study (15.61 ± 3.07; $N = 73$) was similar to that observed in the HD patients remaining alive (13.44 ± 1.22, $N = 99$; $P > 0.05$, t-test) (Fig. 9).

One limitation of this study is that we did not include reference standard (both positive and negative) to control for possible changes in measurement across the 6 years of sampling. Nevertheless, the same strict protocol was followed for the evaluation of all the samples throughout the study.

DISCUSSION

A key strength of this study is the large sample size. Although previous studies have examined possible associations between CKD pathologies and the levels of DNA damage, as indicated in Table I, all these studies considered only a small numbers of individuals. Thus, our work provides a more robust description of the association between genetic damage and the various parameters related to pathology. In addition our study includes a large number of pre-dialysis patients, whereas other studies mainly included HD patients. This allowed us to make comparisons between pre- and dialysis patients, as well as within pre-dialysis stages.

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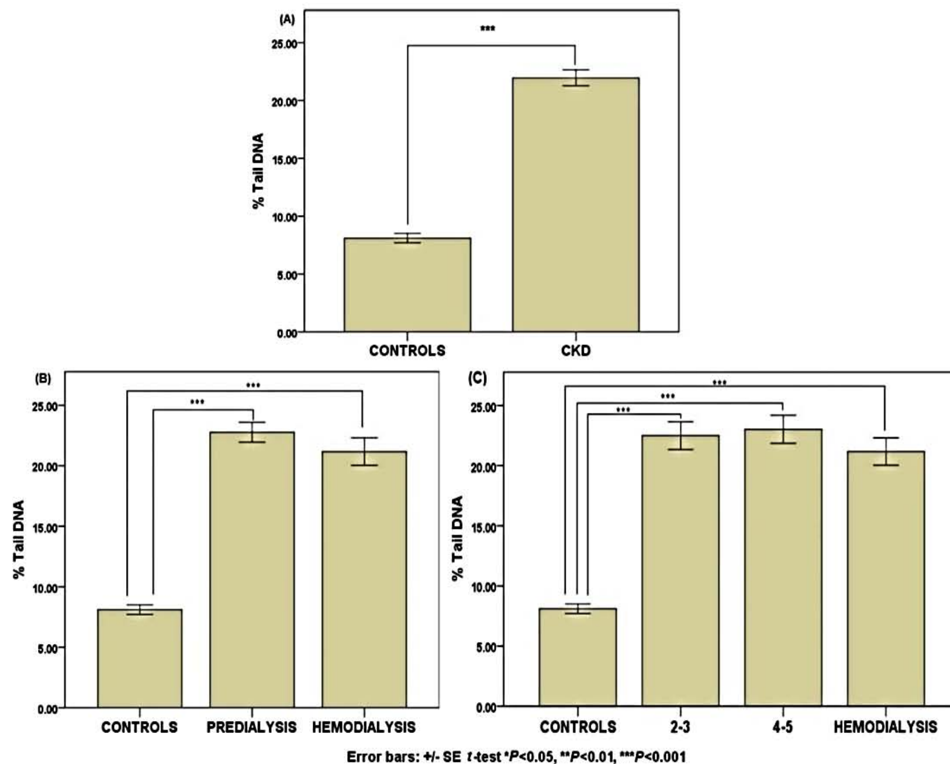

Fig. 2. Levels of genetic damage in CKD patients. (**A**) % tail DNA in controls versus all CKD patients. (**B**) Levels of DNA damage when CKD patients were split as pre-dialysis and hemodialysis. (**C**) Levels of DNA damage when pre-dialysis patients were split according to the stage of the pathology. Statistical significance is based on comparison with controls.

We observed that CKD patients have higher levels of genomic damage than controls. Our results agree with almost all previous studies carried out with similar patients. However, in contrast to most studies we were able to compare between pre-dialysis and HD patients, but found no difference between these groups ($P = 0.255$). This would explain the contradictory results observed in the literature when pre- and HD patients were compared. Thus, although higher values were reported in HD patients [Stopper et al., 2001; Stoyanova et al., 2010], the opposite has recently been observed [Rangel-López et al., 2013] i.e. greater genomic damage was found in pre-dialysis than in HD patients. These differences between studies could be attributed to their small sample size, as well as to differences between patients/groups, such as dietary supplements, drugs prescribed or HD procedures. Therefore, in contrast to previous studies, our results show that HD procedures do not increase DNA damage in CKD patients. Instead, DNA damage seems to be a characteristic of all CKD patients, as opposed to the product of a therapeutic procedure such as HD. The impact of HD on the level of genomic damage has been the focus of various studies, although no definitive conclusions have been reached. Decreased levels of DNA damage have been observed when patients move from standard HD procedures to hemodiafiltration treatment [Schupp et al., 2006; Kobras et al., 2006], or to daily hemodialysis [Fragedaki et al., 2005], suggesting that an amelioration in the uremic state could reduce the degree of genomic damage.

In addition to type of treatment, previous studies have reported contradictory associations between the duration of treatment and amount of DNA damage. Although some studies have found increased levels of DNA damage after long treatment periods [Stopper et al., 1999], others did not find this association [Stopper et al., 2001; Kan et al., 2002]. Our study shows a positive association between time in dialysis and genomic damage ($P < 0.001$). Interestingly, we only observed a significant increase in the levels of genetic damage in patients undergoing hemodialysis treatment for up to 4 years (2–4, $P < 0.05$); after this time no differences were observed (4–6, $P = 0.119$). In contrast, there was no association at all between duration of dialysis and the levels of oxidative DNA damage (2–4, $P = 0.83$; 4–6, $P = 0.941$). The association between DNA damage and treatment duration

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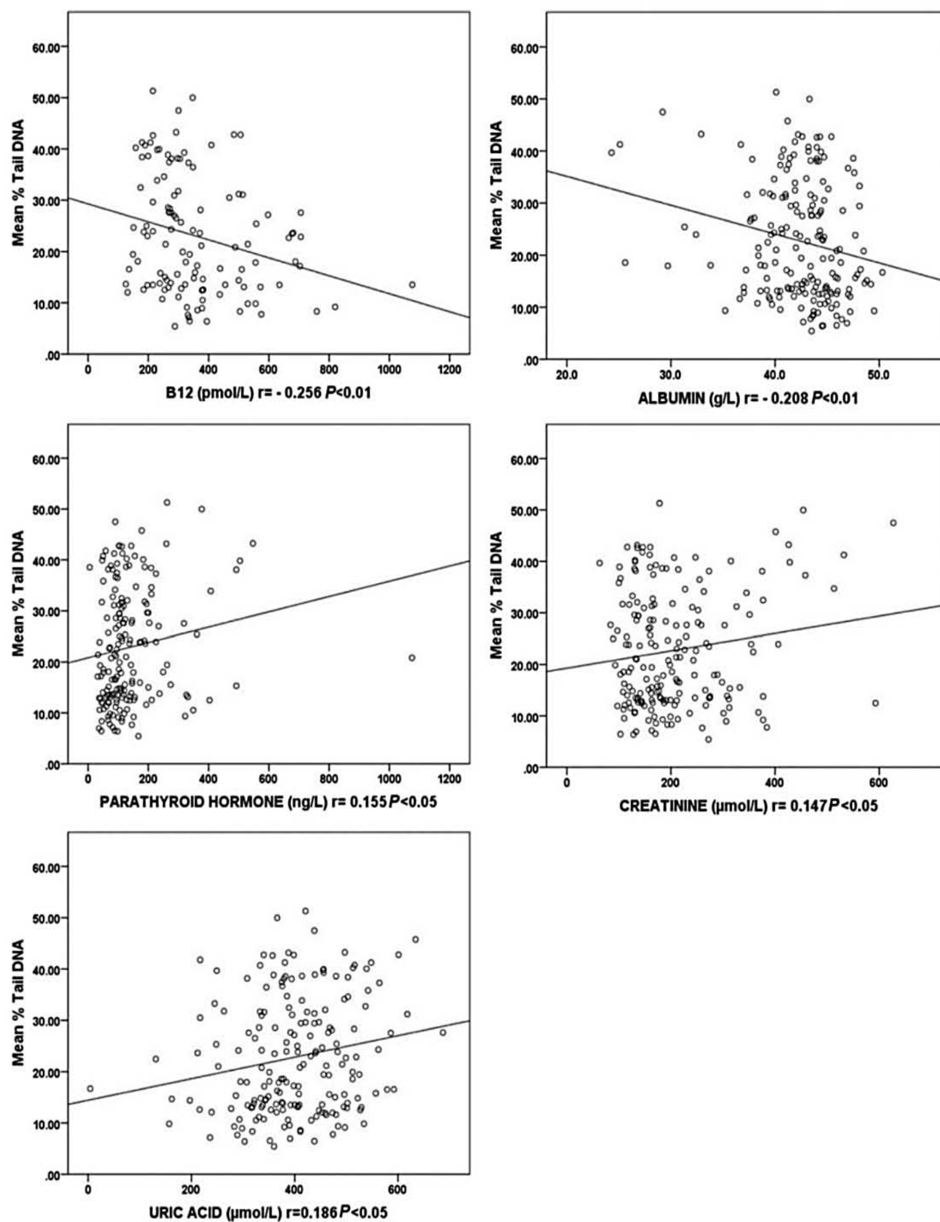

Fig. 3. Correlations between the basal levels ofgenetic damage observed in pre-dialysis CKD patients and different biochemical parameters.

could be due to changes in the antioxidant status of patients. During HD, disequilibrium can be reached between the pro-oxidant nature of the HD procedure and the antioxidant supplements administered to such patients, which may result in increased DNA damage in patients subject to prolonged HD. Alternatively, other factors that accompany the general deterioration of the health status that associates with extended periods on HD may underlie the observed levels of DNA damage.

We did not observe any difference between levels of DNA damage between pre-dialysis and HD patients. This is consistent with the results of Schupp and Kobras [Schuppet al., 2006; Kobras et al., 2006] who reported that the starting of standard dialysis did not induce changes in the levels of genomic damage. However, given that that the studies of Schupp and Kobras were carried out with a small number of patients (5–7), further studies are required focusing on CKD patients in the last stage

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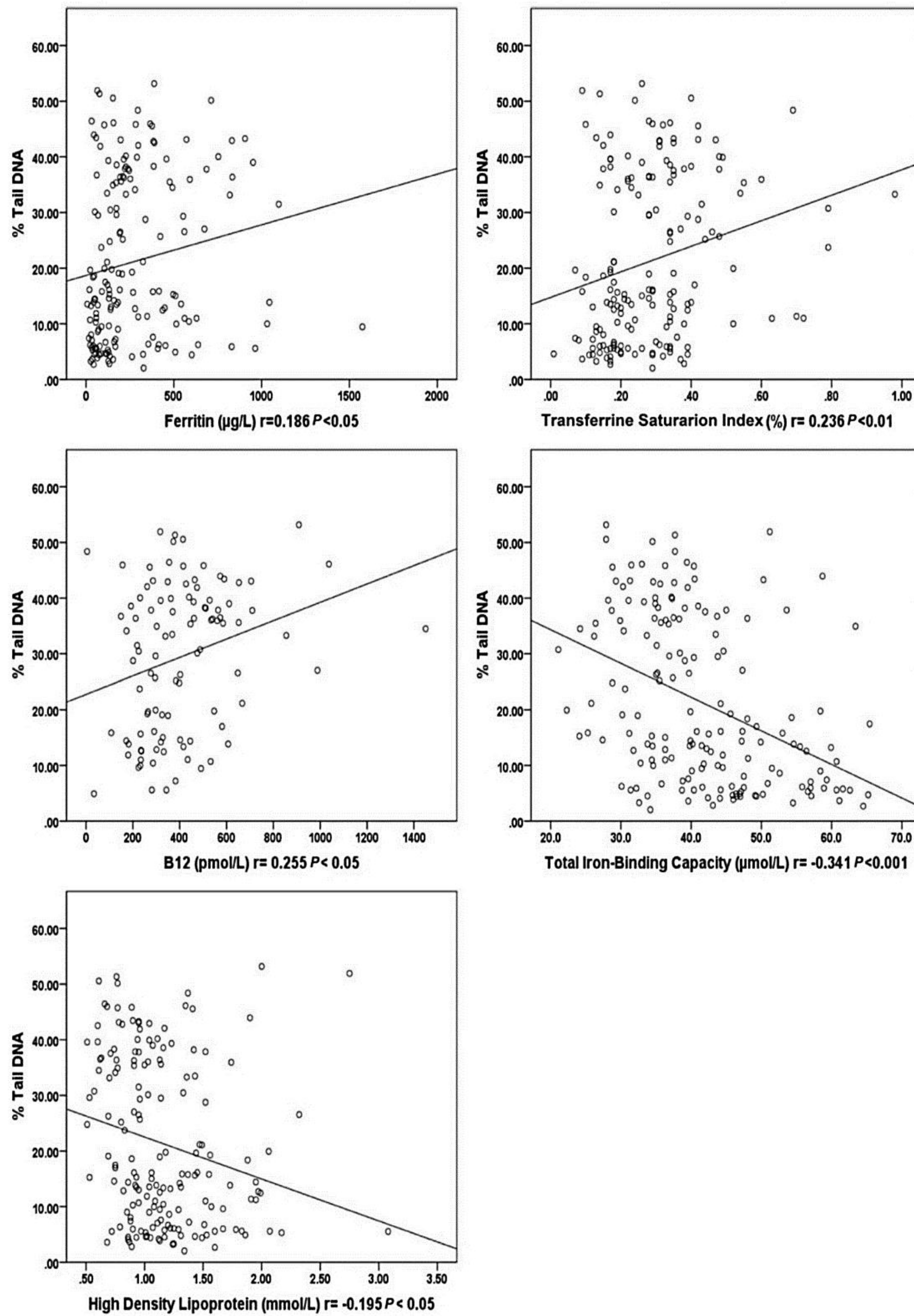

Fig. 4. Correlations between the basal levels of genetic damage observed in HD CKD patients and different biochemical parameters.

(5) moving to dialysis, to determine if changes in the levels of DNA damage are observed. Although longitudinal studies should ideally be pursued to examine changes in DNA damage within the same individuals over time, our results involving a large number of pre-dialysis and HD patients seem to be sufficiently consistent to support the view that no changes in genomic damage levels occur when pre-dialysis patients move into hemodialysis.

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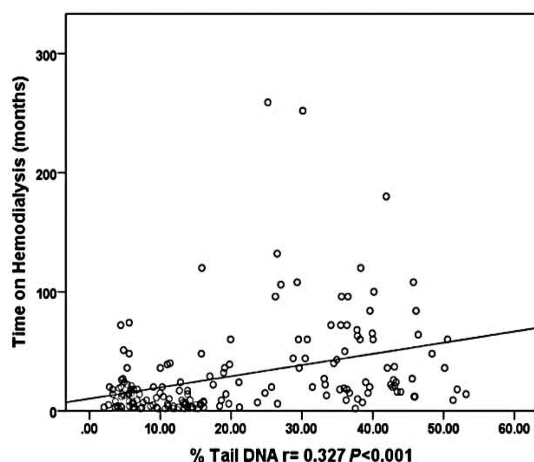

Fig. 5. Correlation between the basal levels of genetic damage observed in HD CKD patients and time on hemodialysis.

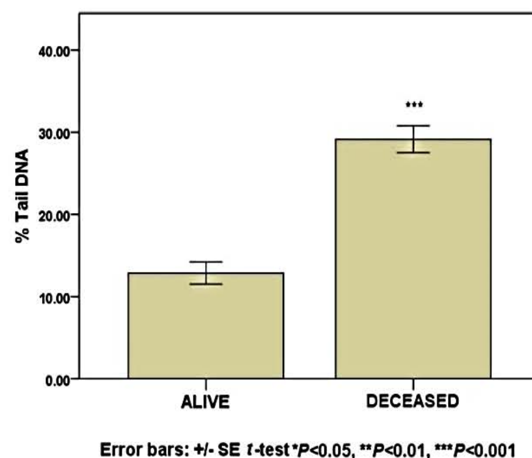

Fig. 6. Basal levels of genetic damage observed in HD patients according to their vital status at the end of the follow up.

Interestingly, no differences were observed in predialysis patients according to their pathological stages, indicating that the level of genomic damage was not related to the severity of the pathology. Because there was no correlation between the glomerular filtration rate and the % tail DNA ($P = 0.381$), and high levels of DNA damage were already observed at the beginning of the pathology (stages 2–3), our study confirms that DNA damage can be considered as a biomarker associated with CKD status. Therefore, increased levels of DNA damage in the general population could be considered as indicative of a potential risk of developing CKD, as well as other pathologies. This may be a consequence of the genomic instability demonstrated in these patients, who presented a poor response to repair damage induced in their DNA [Sandoval et al., 2012]. This genomic instability has also been observed when telomere length, considered a classical biomarker of genomic instability, was used. In this case, a shortening of telomeres was observed in uremic patients compared with controls [Boxal et al., 2006]. Genomic instability has recently been proposed as an important factor in the mechanism of ochratoxin as a renal tumor inducer [Mally, 2012], in addition to its well-known role in cancer development [Pinder et al., 2013]. In the same way that prospective studies have demonstrated that elevated levels of chromosome damage are indicative of a risk to develop tumors [Bonassi et al., 2007, 2008], such studies are needed for CKD patients, although they are complex and require the collaboration of many research groups.

Another interesting finding from our study is that oxidative damage does not seem to be an intrinsic characteristic of CKD patients, at least as a group overall. Nevertheless, HD procedures have been reported to produce oxidative damage, although this effect may depend on the HD system applied, as well as on the antioxidant supplements administered to patients [Del Vecchio et al., 2011].

Although the levels of oxidative damage did not statistically differ between controls and HD patients ($P = 0.86$), HD patients presented the highest levels, consistent with other studies. Nevertheless, it must be pointed out that these studies have used different approaches to measure oxidative damage, which can be considered a source of variability for further inter-comparisons. Measuring oxidative damage by 8-OHdG levels found higher concentrations in HD patients compared with controls [Rangel-Lopez et al., 2013]. Other authors evaluated intracellular levels of reactive oxygen species (ROS) [Martin-Malo et al., 2012] or the presence of oxidized lipoproteins [Ujhelyi et al., 2006; Kuchta et al., 2011; Samouilidou et al., 2012]. Another indirect approach uses antioxidant supplements to detect an amelioration of the genotoxic status [Kan et al., 2002]. These authors used the comet assay to measure genotoxic damage before and after 14 weeks of antioxidant supplementation in 36 HD patients, showing that DNA damage was reduced after supplementation. Similar results were obtained in another study with 29 HD patients where damage was reduced after oral vitamin E supplementation [Domenici et al., 2005], or in a group of 42 HD patients measured before and after 3 months of selenium supplementation [Zachara et al., 2011]. Our results examining oxidative damage using the comet assay combined with the FPG enzyme do not agree with those reported by Müller et al. [2004]. This pilot study consisting of only 21 hemodialysis patients and 12 controls found that although the differences in genetic damage were marginal (without FPG), these differences were clearly significant after FPG treatment.

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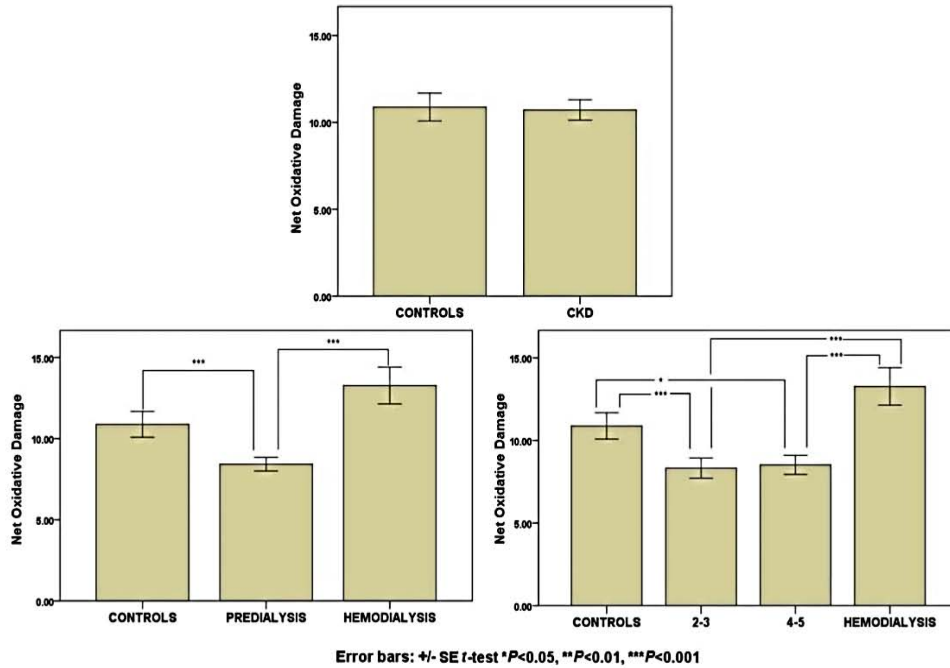

Fig. 7. Levels of net oxidative DNA damage in CKD patients. (A) Control vs. all CKD patients. (B) Control vs. pre-dialysis and hemodialysis. (C) Controls vs. pre-dialysis patients classified according to their stage of the pathology.

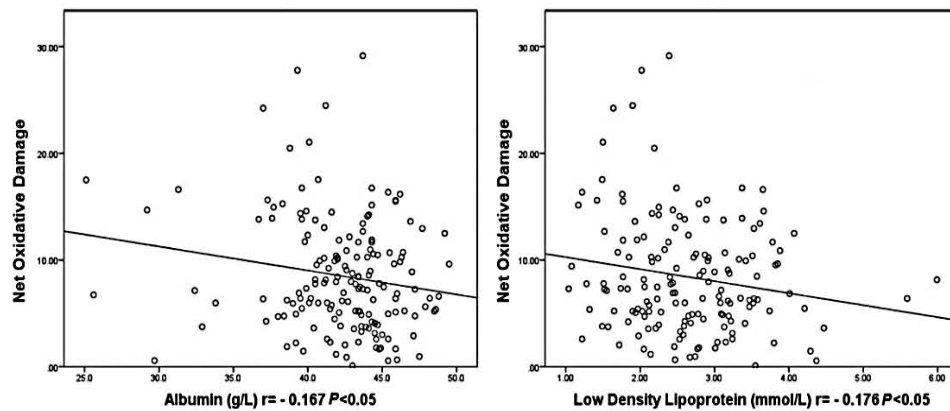

Fig. 8. Correlations between the levels of net oxidative DNA damage of pre-dialysis CKD patients and different biochemical parameters.

Our data indicate that while high levels of genomic damage is a characteristic of CKD patients, oxidative DNA damage may be associated with the HD procedure. This oxidative damage could result from repeated exposure to dialysis membranes and iron infusion or to the reduced antioxidant capacity of these patients. Thus, supplementation with antioxidants seems to be a good strategy to improve the health status of these patients, together with improvements of the HD process itself.

The present study identified a positive correlation between levels of DNA damage and risk of mortality. Interestingly, this correlation was not observed for oxidative DNA damage. This agrees with the results described above, showing that genomic damage is linked to the CKD pathology itself not to the other possible alterations caused by the HD procedure. These results agree with our previous study [Coll et al., 2013] showing for the first time that in HD patients, the presence of high levels of

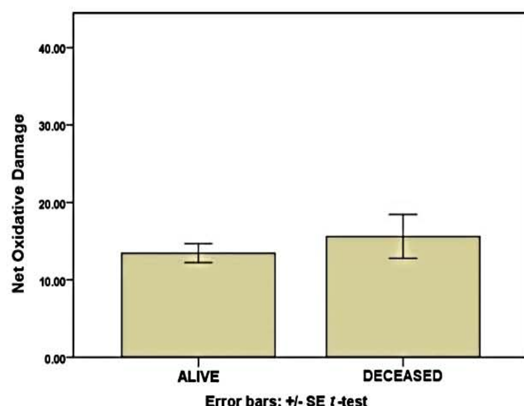

Fig. 9. Net oxidative DNA damage observed in HD patients according to their vital status at the end of the follow up.

genomic damage can be considered as a strong predictor of all-cause mortality.

In conclusion, the results obtained in this large survey of CKD patients confirm that genomic damage is a good biomarker of CKD status, which can also be indicative of a poor prognosis for HD patients. A prospective study remains to be done to show whether genomic instability in the general population is a real prognosis parameter for further CKD development.

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AUTHOR CONTRIBUTIONS

Dr. R. Marcos, Dr. S. Pastor and Dr. E. Coll planned the experiments. Z. Corredor and Dr .E. Stoyanova carried out the experimental part of the work. Dr. E. Coll, I. Silva, Dr. J.M. Diaz and Dr. J. Ballarin supplied all biological samples and information about patients. Z. Corredor and Dr. S. Pastor analyzed the data, carried out the statistical analysis and prepared figures and tables. Dr. S. Pastor, Dr. E. Coll and Dr. R. Marcos wrote the final manuscript.

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ARTÍCULO 2

Unfermented Grape Juice Reduces Genomic Damage on Patients Undergoing Hemodialysis.

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3.2. Resumen del artículo 2

Unfermented Grape Juice Reduces Genomic Damage on Patients Undergoing Hemodialysis

Los pacientes con ERC en hemodiálisis (HD) están sometidos continuamente a estrés oxidativo y este estrés puede causar daño en el DNA, lo que se ha visto reflejado en sus altos niveles de daño genómico respecto a los pacientes pre-diálisis, tal y como describimos en el artículo anterior. Por tal motivo las terapias suplementarias con agentes antioxidantes pueden ser una herramienta de interés ya que pueden atenuar el efecto de dicho estrés. Dentro de los antioxidantes más estudiados, es bien conocido el papel que juegan los polifenoles en reducir los niveles de genotoxicidad y de estrés oxidativo.

De acuerdo con lo anterior se planteó como objetivo evaluar el efecto antioxidante del jugo de uva sin fermentar (*unfermented grape juice, UGJ*) en pacientes sometidos a hemodiálisis.

Para analizar el efecto de la suplementación antioxidante en pacientes en hemodiálisis, se estudiaron 25 pacientes suplementados con mosto durante 6 meses, al tiempo que otros 14 pacientes sirvieron como grupo de referencia, al no suplementarse. En todos los pacientes, se midieron los niveles de daño en el DNA con la ayuda de diferentes biomarcadores: el ensayo del cometa (para medir roturas y bases oxidadas), el ensayo de micronúcleos (para medir daño cromosómico), y la capacidad antioxidante equivalente o TEAC (Trolox).

Los resultados obtenidos mostraron que 6 meses después de la suplementación hay una disminución significativa en los niveles de daño oxidativo en el DNA. En cuanto a los diversos parámetros clínicos evaluados en los pacientes suplementados, hay que mencionar que los niveles de LDL y colesterol se redujeron significativamente.

En conclusión, se puede sugerir que la suplementación con mosto reduce el daño oxidativo del DNA de los pacientes con ERC sometidos a hemodiálisis.

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Unfermented grape juice reduce genomic damage on patients undergoing hemodialysis

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ABSTRACT

Chronic kidney disease (CKD) patients in dialysis (HD) are considered to be submitted to a continuous oxidative stress. This stress can cause damage on DNA and, consequently, contribute to the high levels of DNA damage observed in these patients. Due to the well-known role of polyphenols as antioxidant agents we proposed its use to reduce the levels of genotoxicity present in HD-CKD patients. The objective of this study was to evaluate the antigenotoxic effects of unfermented grape juice (UGJ) on HD-CKD patients. The levels of DNA damage were analyzed using different biomarkers, such as breaks and oxidized DNA bases by the comet assay, chromosome damage by the micronucleus test. In addition, TEAC (Trolox equivalent antioxidant capacity) was also evaluated. Thirty-nine patients were followed for six months, of whom 25 were supplemented by UGJ and 14 were not supplemented. The obtained results showed a significant decrease in the underlying levels of oxidative DNA damage, in the supplemented group. Regarding the clinical parameters, LDL and cholesterol, were significantly reduced in the patients studied after the supplementation period, although cholesterol was also decreased in the non-supplemented patients. In conclusion, in our studied group the supplementation with UGJ reduced the levels of oxidative DNA damage of HD-CKD patients.

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1. Introduction

Oxidative stress is the result of an accumulation of reactive oxygen species (ROS), together with a low antioxidant capacity, that leads to biochemical alterations causing structural and functional modifications of these biomolecules (Massy et al., 2009). Many studies have focused on the detection of oxidative stress in patients with renal alterations to determine whether this is an underlying cause/effect mechanism in chronic kidney disease (CKD). At this point, it must be indicated that CKD patients under dialysis (HD) are submitted to a continuous oxidative stress (McDonald et al., 2014). The HD process contribute to the elimination of plasma antioxidants and together with the dietary restrictions of these patients, the reduced antioxidant enzyme activity, and iron and erythropoietin supplements, these patients became a group with potentially high levels of ROS (Spormann et al., 2008). These reasons support the view that HD process induces oxidative stress in CKD patients and it has been reported that HD-CKD patients show significantly higher levels of oxidative DNA damage than CKD predialysis (PD) patients (Corredor et al., 2015). One of the hypotheses explaining this fact is that, during the dialysis session, the contact blood-membrane induced the activation of macrophages, losing antioxidant capacity and contributing to enhanced oxidative stress in CKD patients. In this scenario the use of antioxidants supplements can be helpful to this type of patients.

It is well known that phenolic compounds act as antioxidant, specially due to their ability to donate hydrogen or electrons and prevent the oxidation of various compounds, particularly fatty

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acids and oils (Gülçin, 2012). Fruits, especially those with red or blue color (such as grapes, plums and cherries), are the most important sources of polyphenols (Stratil et al., 2007). In grapes, flavonoids are primarily located in the epidermal layer of berry skin and in the seeds (Waterhouse, 2002). Flavonoids are the main group of soluble phenolic compounds in grapes, as well as the main contributors of the biological activities in products derived from grapes (Conde et al., 2007). It has been reported that ingestion of unfermented grape juice (UGJ) as a polyphenol-rich dietary supplement exerts hypolipidemic, antioxidant, and anti-inflammatory effects in hemodialysis patients (Castilla et al., 2006). In fact, increased uptake of food-based antioxidants can be a promising alternative measure to reduce oxidative cell damage and stress response (Weisel et al., 2006). In HD patients, few studies (Castilla et al., 2006, 2008; Spormann et al., 2008; Alipour et al., 2012; Janiques et al., 2014) have evaluated the effects of polyphenolic-rich fruit juices on antioxidant capacity, and oxidative stress. No previous studies have been carried out to determine its effect on the levels of genetic damage in such patients. Therefore, the aim of this study was to evaluate the effect of UGJ on the levels of genomic damage in CKD patients under HD by analyzing markers such as genomic/oxidative DNA damage (comet assay) and chromosome damage (micronucleus test). Additionally, variations on TEAC (Trolox equivalent antioxidant capacity) values were also determined.

2. Materials and methods

2.1. Study population

The study involved a total of 39 CKD patients undergoing hemodialysis three times per week, with 3:30–4 h per session. Patients were recruited at the hospital Fundació Puigvert (Barcelona, Spain). Two blood samples were obtained for each patient before the HD session, before and after 6 months. Patients were randomly distributed in 2 groups: UGJ and reference. During that period of time, 25 CKD patients were supplemented, during the last half hour of each dialysis session, with 100 mL of unfermented grape juice (UGJ), assuming that they did not changed their food intake habits during the studied period. A descriptive of the general characteristics of the studied population is indicated on Table 1. Medications and supplements administered to these patients during the follow up are indicated in Table 2. Clinical data was recovered directly from medical history, and clinical parameters (Table 3).

Standard blood analysis included the determination of calcium, phosphorus, glucose, cholesterol, triglycerides, albumin and hemoglobin, among other parameters. Moreover ferritin, iron, transferrin saturation index, parathyroid hormone and C-reactive protein were also analyzed. The erythropoiesis stimulating agents (ESA) resistance index (ERI) was determined as the weekly weight-adjusted ESA dose (IU/week/kg) divided by the product of the patient's weight (Kg) and the hemoglobin level (g/dL). A conversion ratio of 1:200 was used to convert the darbepoetin dose (mcg) to international units (IU) of erythropoietin. All individuals participating in the study provided written informed consent, and blood samples were collected under protocols approved by the Ethics Committee of the Puigvert Foundation. Blood samples were sent to the Universitat Autònoma of Barcelona and immediately processed to analyze the levels of genomic damage and the antioxidant capacity.

2.2. Unfermented grape juice

The UGJ concentrate administered to the patients, was purchased from Concentrados Pallejà S.L. (Riudoms-Tarragona, Spain). The UGJ was unpasteurized, fresh concentrate to avoid losing the antioxidant properties. The polyphenol composition was assessed at the Instituto de Investigación en Ciencias de la Alimentación (CSIC, Madrid). To determine the composition of the UGJ, two different methods were applied (Monagas et al., 2006): (1) the method of total polyphenols, based on oxidation in basic medium of phenol hydroxyl groups fear the Folin-Ciocalteu reagent; and (2) the method of total anthocyanins, based on a colorimetric pH change. The results are expressed in mg of gallic acid/L. From these studies it was concluded that the UGJ contained: total polyphenols 5888 ± 262 mg/L and total anthocyanins 1515 ± 98 mg/L. The levels of potassium were 7.5 mEq/100 mL and correspond to the 6.6% of the estimated daily intake of potassium in hemodialysis patients. The UGJ concentrate was bottled in 1 L-cans, stored in a refrigerator at 4 °C, and administered by the nurses before the end of each hemodialysis session.

2.3. Comet assay

DNA breaks present in peripheral blood lymphocytes were measured using the comet assay performed following the standard protocol, as previously described (Singh et al., 1988; Stoyanova et al., 2010) with minor modifications. Briefly, isolated lymphocytes from 2 mL of blood from each patient were cryopreserved until use, in 500 μL of medium containing 90% serum and 10% DMSO. Comet assay was carried using Gelbond® films (GF) instead of microscopic slides as a support for the agarose gel. The use of hydrophilic films facilitates the rapid processing of numerous samples, increasing the efficiency of the alkaline comet technique, without sacrificing the reliability or sensitivity of the assay (McNamee et al., 2000; Azqueta et al., 2013). Lymphocytes were isolated using Ficoll–Paque density gradient from 500 μL of whole blood; cells were adjusted to a concentration of 17,800 cells in 25 μL

Table 1
General description of the studied groups.

	Supplemented patients (N = 25)	Reference patients (N = 14)
Gender (men/women) (%)	15 (60)/10 (40)	9 (64.3)/5 (35.7)
Age (years) (mean ± SE)	66.16 ± 2.55	59.71 ± 4.61
BMI (mean ± SE)	25.47 ± 1.22	23.93 ± 1.06
Time in HD (months) (mean ± SE)[a]	36.24 ± 5.50	16.57 ± 7.36*
RT previous (% yes/no)	8/92	21.4/78.6
Hypertension (%yes/no)	92/8	92.9/7.1
CV pathology (%yes/no)	56/44	71.4/28.6
Previous cancer (%yes/no)	44/56	35.7/64.3
Diabetes mellitus (%yes/no)	32/68	35.7/64.3
Dyslipidemia (% yes/no)	76/24	64.3/35.7

SE, standard error;; *t*-test, *$P < 0.05$.
[a] HD time before treatment.

Table 2
Medication and supplementation received during the study in the studied groups.

	Supplemented CKD patients (N = 25)[a]		Reference CKD patients (N = 14)[a]	
	Before UGJ supplement	6 months after UGJ supplement[b]	First sample	Second sample
Folic acid	8/92	8/92	28.6/71.4	41.7/58.3*
Vitamin B and C	16/84	16/84	21.4/78.6	8.3/91.7
L-carnitine	52/48	56/44	14.3/85.7	41.7/58.3
ACE inhibitor	36/64	36/64	42.9/57.1	50/50
Statins	68/32	68/32	78.6/21.4	83.3/16.7
Sevelamer	80/20	76/24	42.9/57.1	58.3/41.7
Calcium	12/88	12/88	57.1/42.9	42.9/57.1
Vitamin D	88/12	92/8	50/50	66.7/33.3
Venofer dose (mg/month)	264 ± 28.21	304 ± 45.63	142.85 ± 25.05	191.66 ± 39.32
ESA (µg darbepoetin/mes)	201.20 ± 28.78	187.60 ± 29.86	297.14 ± 63.31	261.66 ± 55.23

*$P < 0.05$.
[a] (% yes/no); ACE inhibitor: angiotensin-converting-enzyme inhibitor; Venofer: iron sucrose injection; ESA: Erythropoiesis stimulating agents; ERI: Erythropoietin resistance index.
[b] No differences were observed between sampling periods (two-tailed Fisher test) or paired-samples t-test; Two-tailed Fisher test.

Table 3
Comparison of blood biochemical data between both periods of sampling.

	Supplement patients (N = 25)		Non-supplement patients (N = 14)	
	Before UGJ supplement	6 months after UGJ supplement	First sample	Second sample
Hemoglobin (120–160 g/L)	119.76 ± 2.56	121.68 ± 2.97	117.58 ± 4.34	118.66 ± 3.38
Glucose (4–5.8 µmol/L)	4,81 ± 0.15	4.91 ± 0.21	5.18 ± 0.35	5.11 ± 0.36
Albumin (37–47 g/L)	41.02 ± 0.55	41.25 ± 0.66	36.69 ± 1.08	39.08 ± 1.33
Calcium (2.1–2.55 mmol/L)	2.24 ± 0.03	2.17 ± 0.02*	2.21 ± 0.04	2.25 ± 0.05
Phosphorus (0.8–1.3 mmol/L)	1.35 ± 0.07	1.52 ± 0.08*	1.81 ± 0.16	1.63 ± 0.13
Ca × P ($mmol^2/L^2$)	3.05 ± 0.19	3.29 ± 0.18	3.98 ± 0.34	3.66 ± 0.30
Parathyroid hormone (7–53 ng/L)	214.95 ± 30.04	307.59 ± 32.36***	149.50 ± 29.43	201.46 ± 46.47
Ferritin (25–250 µg/L)	265.53 ± 94.14	306.26 ± 89.34	175.14 ± 57.71	276.42 ± 58.00
Transferrin saturation (12–44%)	24.25 ± 0.02	21.54 ± 0.02	21.90 ± 0.02	28.10 ± 0.03
Iron (9–27 µmol/L)	10.88 ± 0.97	10.10 ± 0.72	9.80 ± 1.21	11.51 ± 1.31
ERI (IU/week/kg/g/dL > 10)	13.80 ± 2.16	12.60 ± 2.21	20.31 ± 4.13	9.64 ± 4.48[a]
C-reactive protein (<10 mg/L)	9.14 ± 2.21	15.57 ± 5.78	7.98 ± 3.71	10.28 ± 4.65
HDL (>1.40 mmol/L)	1.35 ± 0.09	1.24 ± 0.07	1.14 ± 0.08	1.08 ± 0.08
LDL (<4.13 mmol/L)	2.08 ± 0.15	1.75 ± 0.13*	2.47 ± 0.32	1.85 ± 0.22
Cholesterol (3.20–5.20 mmol/L)	4.01 ± 0.19	3.59 ± 0.17**	4.51 ± 0.36	3.58 ± 0.26*
Triglycerides (0.30–1.40 mmol/L)	1.26 ± 0.13	1.42 ± 0.20	1.68 ± 0.24	1.59 ± 0.20
Kt/V (>1.3)	1.58 ± 0.05	1.61 ± 0.05	1.50 ± 0.08	1.63 ± 0.07
Systolic blood pressure (<140 mmHg)	138.80 ± 3.91	138.00 ± 4.01	124.62 ± 8.52	137.37 ± 5.87
Diastolic blood pressure (<90 mmHg)	72.40 ± 2.00	73.0 ± 2.08	68.37 ± 5.99	81.62 ± 4.97*

Paired-samples t-test: *$P < 0.05$, **$P < 0.05$, ***$P < 0.001$; Ca x P, calcium phosphorus product; ERI, ESA resistance index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PRU, Urea reduction percentage.
[a] Values determined only for 3 patients.

PBS and carefully re-suspended in 225 µL of 0.75% low melting agarose (LMA) at 37 °C and dropped onto a GF (10.5 × 7.5 cm). Forty-eight drops (7 µL each) were placed on each GF and samples of eight donors were run simultaneously, each donor being represented by six drops. Lymphocytes were lysed for a minimum of 1 h at 4 °C in a dark chamber containing a cold fresh lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl and 1% Triton X-100, adjusted to pH 10). To allow DNA denaturation, unwinding and exposure of alkali-labile sites, GF were placed in a horizontal gel electrophoresis tank filled with freshly cold (4 °C) electrophoresis solution (1 mM Na_2EDTA and 300 mM NaOH, adjusted to pH 13) for 35 min. Electrophoresis was performed in the same buffer for 20 min at 20 V and 300 mA. After electrophoresis, GF were neutralized with two 5-min washes with PBS 1X, followed by 1 min wash with water and then incubated overnight in 100% ethanol for fixation. Sheets were then dried and stored in the dark at room temperature until scoring. Just before the microscopic analysis, GF were stained with 20 µL of SybrGold. The images were examined at 20× magnification with a Komet 5.5 Image Analysis System (Kinetic Imaging Ltd, Liverpool, UK) fitted with an Olympus BX50 fluorescence microscope equipped with a 480–550 nm wide band excitation filter and a 590 nm barrier filter. A total of one hundred randomly selected cells were analyzed per patient and the % tail DNA was used as a measure of DNA damage.

2.4. Detection of oxidative damage

To determine the levels of oxidized bases present in the lymphocytes, GF were washed two times (10 and 50 min, 4 °C) after cell lysis in an enzyme buffer solution (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8.0) containing the enzyme FPG (formamidopyrimidine DNA glycosylase) (Azqueta et al., 2013). FPG enzyme was produced in our laboratory and a concentration of 5.76 µg/µL of enzyme extract was used in each treatment.

Each sample was analyzed using two GF. One GF remained in the cell lysis solution in order to assess basal DNA damage. The second was treated with the enzyme buffer without FPG, to control for any effects of the buffer alone. GF were incubated with enzyme buffer (with and without FPG) for 30 min at 37 °C, after that, samples were processed as in the standard alkaline comet assay procedure. Net oxidative DNA damage was calculated by subtracting the damage scored in the samples incubated with buffer from those incubated

with FPG.

2.5. Lymphocyte culture and micronucleus assay

Blood samples from heparinized vacutainers were processed as described previously using cytochalasin-B to arrest cytokinesis (Rodríguez-Ribera et al., 2014). Two of the four cultures set up were irradiated with 0.5 Gy ^{137}Cs gamma rays in an irradiator IBL 437C, type H, No. 701 (SCHERING CIS Bio International) at the Unitat Tècnica de Protecció Radiològica (UTPR-UAB). To determine the frequency of binucleated cells with micronuclei (BNMN), 1000 binucleated lymphocytes per sample (irradiated and non-irradiated) were blind scored on coded slides, according to standard criteria (Fenech, 2007). In addition, 500 cells with one, two or more nuclei were scored to determine the cytokinesis-block proliferation index (CBPI) (Surrallés et al., 1995). The net effect of irradiation was calculated by subtracting the background BNMN values in the non-irradiated samples from the values obtained in the irradiated samples (Rodríguez-Ribera et al., 2015). The resulting net BNMN value represents the frequency of BNMN induced by IR. In the same way, the net CBPI was also calculated.

2.6. Trolox equivalent antioxidant capacity (TEAC) assay

The plasma antioxidant capacity was measured using the TEAC assay as already described (Kambayashi et al., 2009) with minor modifications. Venous blood samples from EDTA tubes were centrifuged at 170 g during 5 min to obtain plasma and, after that, it was stored at −80 °C. Ten μL of plasma or Trolox standard reacted with 6.20 μM myoglobin solution (20 μL), 183 μM ABTS solution (150 μL) and 10 mM H_2O_2 (25 μL) on a microplate. Reaction was followed at 405 nm with the plate reader Sunrise (Tecan Trading AG, Switzerland). Lag time from kinetic curves and Trolox calibration curve was calculated and plasma TEAC was expressed as Trolox equivalent.

2.7. Statistical analyses

Continuous variables (MN, BNMN, CBPI, TEAC, and comet assay) were assessed for normality using the Kolmogorov–Smirnov test. For the statistical analysis of the continuous data t-test for paired samples was used. For qualitative data, Fisher's test was used (Table 2). *Data is presented as mean* ± standard error of the mean (SEM) *if not* otherwise *indicated*. Pearson and Spearman correlations were used to determine relationship between variables. For the statistical analysis of the genomic and oxidative damage obtained by the comet assay, non-parametric tests were used. Comparisons between the two groups (before and after 6 months supplementation with unfermented grape juice) were analyzed using the Wicolxon test for continuous variables, and Mann–Whitney test for discrete variables (Table 4). All the analyses were done using the Statistic Package of Social Sciences (SPSS) software for Windows version 19.0.

3. Results

All selected patients were included in the HD program of the Fundació Puigvert and underwent conventional hemodialysis for 3.5–4 h, on a thrice weekly dialysis schedule. HD was carried out using synthetic low permeability membranes (ultrafiltration coefficient of 13 mL/h/mm Hg) of polyethersulfone with a surface between 1.8 and 2 m^2, with a bicarbonate dialyzate. The average time they have been subjected to conventional HD previous to the study was 36.24 ± 5.50 months for the treated group, and 16.57 ± 7.36 months for the reference group. Patients included in the study drank 100 mL of UGJ, in the last 30 min of their hemodialysis session for 6 months.

General characteristics of CKD patients are shown in Table 1. The main causes of disease in supplemented and reference patients, respectively were: vascular nephropathy (4%, 14.3%), diabetes mellitus (24%, 14.3%), glomerulonephritis (36%, 43%), polycystic (12%, 7.1%), and others (20%, 14.3%). CKD is more common in male than in female and, consequently, about 60% of the CKD patients were men. Both groups of study are homogenous for most of the parameters tested. The most remarkable difference was in the time that patients have been in hemodialysis. The reference group showed approximately half of the time in HD than the supplemented group. It is interested to remark the high incidence of hypertension, cardiovascular pathologies and dyslipidemia among the participants, something well reported for these types of patients.

Table 2 list the medication and supplements received by the CKD patients previous and during the study. No significant changes were found regarding the treatment received before and at the end of the study for the supplemented group. Slight but significant differences were showed between for periods of time within the reference group regarding the levels of folic acid, vitamin B and C, and L-carnitine. We assume that these differences are mainly due to the sampling size more than changes in the supplements provided to HD patients.

Blood analysis is summarized in Table 3. The only changes attaining statistical significant differences correspond to calcium ($P = 0.021$), phosphorus ($P = 0.029$), parathyroid hormone ($P < 0.001$), LDL ($P = 0.012$), and cholesterol ($P = 0.004$), after 6 months under UGJ supplement. In the reference group, only differences are observed in diastolic blood pressure ($P = 0.014$) and cholesterol ($P = 0.016$), while some parameters showed borderline effect, as albumin ($P = 0.063$), ferritin ($P = 0.077$), LDL ($P = 0.057$), and Kt/V ($P = 0.061$).

The levels of genetic damage before and after 6 months supplemented with UGJ, and for the HD patients without supplementation are described in Table 4. As observed, no statistically

Table 4
Levels of DNA damage and antioxidant capacity before and after 6 months of supplementation with unfermented grape juice.

	Supplement CKD patients (N = 25)			Reference CKD patients (N = 14)		
	Before UGJ supplement	6 months after UGJ supplement	Paired *t*-test *P*	First sample	Second sample	Paired *t*-test *P*
BNMN	11.24 ± 1.28	9.92 ± 1.42	0.374	6.50 ± 1.01	5.91 ± 1.19	0.704
BNMN net After irradiation	39.44 ± 20.54	35.56 ± 14.84	0.334	24.90 ± 5.69	20.72 ± 3.92	0.591
CBPI	1.54 ± 0.15	1.65 ± 0.03	**0.005***	1.45 ± 0.05	1.44 ± 0.05	0.910
% DNA tail Basal damage	16.15 ± 0.96	15,61 ± 1.03	0.594	13.52 ± 1.83	13.13 ± 2.13	0.902
% DNA tail Oxidative damage	26.36 ± 1.30	22.41 ± 1.18	**0.025***	8.12 ± 1.68	14.61 ± 2.80	0.125[a]
TEAC (mMol/L)	0.21 ± 0.02	0.17 ± 0.01	0.072	NDA		

Mean ± SE; NDA: no data available. *$P < 0.05$
[a] Paired Wilcoxon test.

significant differences were observed neither in the basal BNMN frequency nor in the individual radiosensitivity (BNMN net) between sampling times, for both studied groups. In the same way, no differences were observed between sampling periods in the comet assay, used to determine the basal levels of DNA breaks under alkaline conditions, nor in the Trolox equivalent antioxidant capacity (TEAC) assay. Nevertheless, when the comet assay was complemented with FPG enzyme to determine the levels of oxidized DNA bases, a significant decreased was observed when patients were supplemented with UGJ. Regarding the cytokinesis block proliferation index (CBPI), an increase in cell proliferation was also observed when patients were supplemented with UGJ.

When different associations between parameters were determined, a good correlation was observed between the levels of BNMN and net-BNMN before and after the supplementation ($r = 0.426$, $P = 0.034$; $r = 0.417$ $P = 0.038$, respectively). This would confirm the lack of effect of UGJ supplementation on chromosome damage. On the other hand, the levels of CBPI ($r = 0.135$, $P = 0.530$), oxidative DNA damage measured by the comet assay ($r = 0.123$, $P = 0.556$), nor TEAC ($r = 0.303$, $P = 0.141$) did not show any correlation, what would indicate that UGJ supplementation interfere on these parameters. In addition, if we focus in factors modulating genetic damage in patients supplemented with UGJ, a negative correlation was observed between the antioxidant capacity and the oxidative net damage ($r = -0.422$, $P = 0.035$). This would indicate that those patients with initial high damage have less antioxidant capacity.

4. Discussion

In our study we found slight decreases, but not significant changes, in the basal frequencies of primary DNA damage (comet assay) or in chromosomal damage (micronucleus assay) when CKD patients submitted to HD were supplemented with UGJ for six months. Nevertheless, significant decreases in the underlying levels of oxidative DNA damage were obtained (comet assay plus FPG) in such patients after UGJ supplementation. This would suggest an antioxidant role of UGJ at least in this type of patients.

Under normal conditions ROS (which include various compounds such as superoxide anions, hydrogen peroxide, and hydroxyl radical) are generated in the mitochondria of mammalian cells in the course of energy production, by reducing oxygen during aerobic respiration. However, excessive ROS levels can produce cellular damage by interacting with biomolecules (such as proteins, lipids, and nucleic acids), having negative effects on tissue function and structure. This oxidative stress plays an important role in different pathological situations such as cardiovascular diseases (Jeremy et al., 2004), aging (Dugan and Quick, 2005) and Alzheimer (Muhammad et al., 2009), as well as in renal diseases (Dobashi et al., 2000). Another effect associated to oxidative stress is inflammation, also typical of CKD patients.

In CKD patients it has been indicated that the negative effects of oxidative stress are associated with the progression of the disease; finding a correlation with the level of renal function (Dounousi et al., 2006). Our group has found that oxidative DNA damage measured by Comet with FPG is higher in HD patients with respect to pre-dialysis patients (Corredor et al., 2015). Recent studies comparing oxidative stress under various types of dialysis modalities showed increased levels of advanced oxidation protein products in conventional HD patients compared with those submitted to peritoneal dialysis (Zhou et al., 2012; Marques de Mattos et al., 2012). Nevertheless, other studies found similar or lower levels of oxidative stress markers (advanced oxidation protein products, myeloperoxidase, and 8-OHdG levels) in HD patients when compared to patients submitted to peritoneal dialysis (Castoldi et al., 2010; Samouilidou et al., 2012). Thus, the contribution of the oxidative damage to CKD disease and/or vice versa is still controversial.

Dialysis treatment, by itself, appears to contribute to oxidative stress by creating alterations in the balance between free radicals generation and antioxidant protection systems (Sung et al., 2013). In fact, previous studies have shown increased levels of the lipid peroxidation product malondialdehyde and decreased levels of the primary lipid-soluble antioxidant α-tocopherol, suggesting that HD procedure is associated with oxidative stress (Westhuyzen et al., 1997). It should be taken into account, that dialysis procedure does not eliminates efficiently all advanced glycation end-products (AGEs) and non-important changes in total AGEs and lipoperoxide levels are observed after dialysis treatment (Gugliucci et al., 2007). Therefore, it is assumed that alterations of plasma lipoprotein levels and lipid peroxidation of low density lipoproteins (LDL), occurs in HD patients (Kaysen and Eiserich, 2004).

In front of this situation different antioxidant supplementation therapies have been proposed to be used in CKD patients. Among these supplements grape juice has been indicated as a suitable proposal to reduce the levels of oxidative stress in these type of patients (Castilla et al., 2006, 2008), and also to decrease the levels of genetic damage (Weisel et al., 2006). Grapes contain a large number of secondary metabolites such as flavonoids, which constitute a large group of polyphenolic compounds also found in fruits and other foods (Rice-Evans et al., 1996; Georgiev et al., 2014), and the consumption of foods rich in flavonoids is associated with a reduced risk of various chronic diseases (Hertog et al., 1995). The protective benefits of dietary flavonoids may be due in part to their antioxidant properties and their ability to reduce oxidative stress (Rice-Evans et al., 2000). In fact, as reviewed by Kumar and Pandey (2013) significant antioxidant activity of specific dietary flavonoids, and of some of the major metabolites and conjugated derivatives that occur in the circulation after consumption of dietary flavonoids has been reported.

In this context, the aim of this study was to evaluate the effect of supplementation, with unfermented grape juice, in a group of hemodialysis patients over a period of six months. We found that some of the clinical parameters analyzed, such as LDL and cholesterol, were significantly reduced in the patients studied after the supplementation. Nevertheless, since cholesterol levels were also reduced in controls we cannot attribute this decrease uniquely to the ingestion of UGJ.

Several studies have demonstrated that ingestion of polyphenols rich extracts reduced plasma LDL-cholesterol concentrations in humans (O'Byrne et al., 2002; Castilla et al., 2006), and a similar effect was found for red wine polyphenols in hamsters (Vinson et al., 2001). This has been explained because polyphenols may act like a HMG-CoA reductase inhibitor agent, and treatment with these inhibitors usually results in large increases in the mRNA and protein for HMG-CoA reductase, the LDL receptor, as well as in other genes (Endo, 1992). A previous study demonstrated that hyperlipidemic hamsters fed with dealcoholized red wine had a 45% reduction in plasma LDL concentrations relative to control hamsters drinking water (Vinson et al., 2001). Another *in vivo* study suggested that the decrease observed in circulating lipoproteins in hamsters fed dealcoholized red wine may be a result of increased LDL receptor binding activity and a suppression of hepatic lipoprotein production (Pal et al., 2003). These results would agree with the prospective study reporting that anthocyanidins, flavanones, and foods rich in flavonoids were associated with lower cardiovascular disease mortality (Mink et al., 2007). Although we observed a higher decrease in LDL values in UGJ supplemented patients than in controls we failed to find an increase in plasma HDL-cholesterol as described by some other authors who found

that red grape juice improves the lipoprotein profile, reduces plasma concentrations of inflammatory biomarkers and oxidized LDL in both HD patients and controls (Castilla et al., 2006). Differences in the dose of UGJ concentrate could be an explanation of this discrepancy: 1766 mg/week with respect to the 4508 mg/week administered by Castilla et al. (2006). Supplements with fruit juices in HD patients have already proved its protective effects. HD patients supplemented for one year with pomegranate juice showed significant reduction of protein oxidation, lipid oxidation, and inflammation biomarkers (Shema-Didi et al., 2012). In spite of those reported studies our results did not support the view that lipidic profile changes are uniquely attributed to the ingestion of UGJ. This view would agree with the meta-analysis results reported by Liu et al. (2013) who did not found a direct effect of fruit juices with antioxidant properties on cholesterol levels and LDL values.

Interestingly, in our studied group of HD patients the intake of UGJ significantly reduced the levels of oxidative DNA damage, while these levels remained stable in the reference group. Although the antioxidant effect of grape juice on HD patients has been previously demonstrated (Castilla et al., 2006, 2008) no previous studies have evaluated the effect of UGJ on the levels of genomic damage in HD patients. Others studies have already observed changes in the levels of genomic damage on HD patients after supplementation with vitamin E (Kan et al., 2002), vitamin C (Tarng et al., 2004) or selenium (Zachara et al., 2011). Even, it has been observed that in healthy people, grape juice continuously exerted persistent antioxidant activity up to 2 h after supplementation (Ko et al., 2005). These suggest that the consumption of fruit juice rich in polyphenols can reduce the oxidative stress, causing DNA damage, and that this effect may be the result of theirs antioxidant activity of the over the reactive oxygen species generated in human plasma. Overall, these studies show that supplementation with antioxidants reduced genomic damage levels.

5. Conclusions

In our study we have detected a slight but non-statistically significant reduction in the levels of the biomarkers used (MN, comet, and TEAC assays) after 6 month of UGJ supplementation. Nevertheless, a significant decrease in the levels of oxidative DNA damage was observed, what would support the antioxidant role of UGJ and suggest its potential benefits in reducing in some extension the levels of genomic damage and, consequently, their potential hazardous effects.

Conflict of interest

There are no conflicts of interest, and the results presented in this paper have not been published previously in whole or part.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fct.2016.03.016

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ANEXO 1

ARTÍCULO 3

Changing to On-Line Hemodiafiltration Affect the Levels of Genomic Damage in Patients Undergoing Hemodialysis

Zuray Corredor, Lara Rodríguez-Ribera, Irene Silva, Juan Manuel Díaz, José Ballarin, Ricard Marcos, Elisabet Coll, Susana Pastor

3.3. Resumen del artículo 3

Changing to On-Line Hemodiafiltration Affect the Levels of Genomic Damage in Patients Undergoing Hemodialysis

La enfermedad renal crónica (ERC) supone una compleja disfunción orgánica, lo que se atribuye a la retención de una gran variedad de toxinas urémicas, que en condiciones normales serían excretadas. Este problema ha dado lugar a avances en la eficiencia del tratamiento renal sustitutivo. Entre las terapias de reemplazo renal se encuentra la hemodiafiltración on-line (HDF on-line), ésta toma lo mejor de la eliminación de toxinas por difusión y convección, con el factor aditivo de infusión del dializador (líquido de sustitución) en modo posdilucional. Diversos estudios han demostrado que este procedimiento tiene un efecto positivo en la reducción de la mortalidad en pacientes con ERC.

Teniendo en cuenta lo anterior, el propósito de este estudio fue evaluar el efecto del cambio a HDF on-line sobre el daño genómico.

Para responder a dicha pregunta se analizaron 34 pacientes que cambiaron de HD convencional a HDF on-line durante un periodo de 6 meses. Se estableció como grupo control a 15 pacientes que continuaron en HD convencional. El daño genómico se estudió para todos los pacientes mediante del ensayo del cometa, que detecta roturas en el DNA y bases oxidadas (con la adición de FPG). La capacidad antioxidante equivalente TEAC (Trolox) también se evaluó, para determinar cambios en el equilibrio pro-/antioxidante.

Los resultados obtenidos muestran una ligera disminución de los niveles de daño en el DNA de aquellos pacientes que cambiaron a HDF on-line (16,44 ± 2,46 *versus* 11,14 ± 0,98, P=0,048), y una ligera tendencia a incrementar la capacidad antioxidante TEAC (0,11 ± 0,01 *versus* 0,18 ± 0,03, P=0,058). De estos resultados se concluye que el cambio de la terapia a HDF on-line produce una leve reducción en los niveles de daño genómico basal. Además, también se detectó una mejora en los niveles de triglicéridos (P=0,038). Por otra parte, podemos ver que el tratamiento con HDF on-line tiene un beneficio más notable en pacientes con

mayores niveles de daño genómico. Esto confirmaría la utilidad de la técnica HDF on-line frente al procedimiento de HD estándar.

ANEXO 2

ARTÍCULO 4

Genetic Damage Follow-up in Kidney Transplant Patients with Chronic Kidney Disease

Zuray Corredor, Lara Rodríguez-Ribera, Elisabet Coll, Irene Silva, Juan Manuel Diaz, José Ballarin, Ricard Marcos, Susana Pastor

3.4. Resumen del artículo 4

Genetic Damage Follow-up in Kidney Transplant Patients with Chronic Kidney Disease

El trasplante renal se ha asociado con una menor mortalidad y una mejor calidad de vida en pacientes con enfermedad renal crónica (ERC), en comparación con el tratamiento de diálisis. Sin embargo, la evaluación del daño sobre el DNA y la inestabilidad genómica después trasplante renal (TR) ha sido poco documentada. En dicho contexto, el objetivo de este estudio fue analizar el efecto del trasplante renal sobre el estado metabólico y el daño genómico, en pacientes trasplantados. El estudio se realizó en 53 pacientes con enfermedad renal crónica. El seguimiento se realizó en 41 pacientes después de 6 meses del trasplante, en 42 pacientes después de un año trasplantados, y en 11 casos específicos, 24 meses después del trasplante. El daño genómico de los pacientes se analizó mediante el ensayo del cometa alcalino; para el estudio del daño oxidativo se utilizó en ensayo del cometa con la enzima FPG; mientras que para el estudio de la inestabilidad cromosómica se utilizó el ensayo de micronúcleos. Los resultados obtenidos mostraron diferencias significativas entre el periodo pre-trasplante y a los seis meses después de la cirugía para algunos marcadores bioquímicos, hasta lograr niveles normales, como son: glucosa (P=0,032), albúmina (P=0,000), calcio (P=0,000), presión arterial (sistólica P=0,011, diastólica P=0,013), y urea (P=0,004). Además, algunos pacientes lograron también normalizar los niveles de fósforo (P=0,000) y HDL (P=0,000). Estas diferencias se mantuvieron después de un año del trasplante. El daño en el DNA analizado antes del trasplante y después de un año, mostró un aumento estadísticamente significativo tanto del daño oxidativo (P=0,031), como de la inestabilidad cromosómica (BNMN) (P=0,041), mientras que disminuyó la proliferación celular (CBPI) (P=0,003). Por último, los pacientes que recibieron un trasplante de un donante fallecido mostraron mayor daño oxidativo (21,10 ± 3,19; N=17) que los que lo recibieron de un donante vivo (12,29 ± 1,75; N=19) (P=0,023), después de 6 meses del trasplante de riñón. Para la inestabilidad genómica, el origen del riñón recibido también mostró diferencias estadísticamente significativas, ya que después de un año de trasplante, los pacientes que recibieron

el injerto de un donante fallecido, tenían niveles más altos de micronúcleos (BNMN *P*=0,032). En conclusión, este estudio muestra una mejora sustancial de los valores bioquímicos a los 6 meses y un año después de la intervención, en comparación con los niveles antes del trasplante. Sin embargo, después de un año los pacientes sometidos a trasplante de riñón, mostraron un aumento de los niveles de daño oxidativo y la inestabilidad genómica. Esto sugiere que el daño genómico sigue presente posiblemente por predisposición genética a la ERC, a pesar de que la terapia de reemplazo renal mejora el estado metabólico.

ANEXO 3

ARTÍCULO 5

Genetic Variants Associated with Chronic Kidney Disease in a Spanish Population

Zuray Corredor, Miguel Inácio da Silva Filho, Lara Rodríguez-Ribera, Andrea Woltmann, Kari Hemminki, Elisabeth Coll, Irene Silva, Juan Manuel Díaz, José Ballarin, Martí Valles Prat, Jordi Calabia Martínez, Asta Försti, Ricard Marcos, Susana Pastor

3.5. Resumen del artículo 5

Genetic Variants Associated with Chronic Kidney Disease in a Spanish Population

Los pacientes con ERC tienen afectadas muchas vías fisiológicas y, en consecuencia, variantes en los genes que regulan tales vías pueden afectar la incidencia, predisposición y su progresión. En este contexto, los estudios de asociación del genoma completo (GWAS) de grandes poblaciones europeas, han identificado polimorfismos que confieren riesgo para diferentes patologías relacionadas con la ERC como: diabetes, hipertensión, enfermedad de la arteria coronaria, enfermedad vascular y también para los rasgos renales funcionales en pacientes con ERC.

Para entender más la predisposición a la ERC, en este estudio se analizaron 38 SNPs en genes candidatos relacionados con: la respuesta inflamatoria (citoquinas: *IL-1A, IL-4, IL- 6, IL-10, TNF-α, ICAM-1*), el sistema renina-angiotensina-aldosterona *(CYP11B2, AGT*), la fibrogénesis (*TGFB1*), la síntesis de homocisteína (*MTHFR*), la reparación del DNA (BER: *OGG1, MUTYH, XRCC1*; NER: *ERCC2/XPD, ERCC4*); y metabolismo fase II (*GSTP1, GSTO1, GSTO2)*. Además de algunos genes previamente descritos como involucrados en la ERC (*GLO1, SLC7A9, SHROOM3, UMOD, VEGFA, MG, KL)*.

Mediante el análisis de casos y controles, ajustado por edad y sexo, los resultados mostraron asociación con la ERC de los siguientes SNPs: rs17080528 (*GPX1*), rs2164624 (*GSTO1*), rs156697 (*GSTO2*), rs12917707 (*UMOD*) y rs4236 (*MGP*). Por lo que se refiere a la asociación de los SNPs con patologías relacionadas con la ERC, hemos encontrado asociación con la hipertensión (rs713041, *GPX4*; rs17999988, *CYP11B2*; rs3136166, *ERCC4*), con las enfermedades cardiovasculares (rs5050, *AGT;* rs1799793, *ERCC2/XPD*, con la *diabetes mellitus* (rs5050, *AGT*; rs1801133, *MTHFR*; rs1799793, *ERCC2/XPD*), y con la predisposición al cáncer (rs13181, *ERCC2/XPD)*. El análisis lineal y logístico también mostró asociación entre los parámetros bioquímicos representativos de la ERC, con algunos de los SNPs estudiados. De estos resultados se desprende que nuestro estudio ha sido capaz de determinar la asociación de diferentes variantes

alélicas con la susceptibilidad a la enfermedad renal crónica en una población española.

ANEXO 4

ARTÍCULO 6

Loci Associated With Genomic Damage Levels in Spanish Chronic Kidney Disease Patients

Zuray Corredor, Miguel Inácio da Silva Filho, Lara Rodríguez-Ribera, Andrea Woltmann, Kari Hemminki, Elisabeth Coll, Irene Silva, Juan Manuel Díaz, José Ballarin, Asta Försti, Ricard Marcos, Susana Pastor

3.6. Resumen del artículo 6

Loci Associated With Genomic Damage Levels in Spanish Chronic Kidney Disease Patients

Aunque se han identificado variantes genéticas que causan diferentes formas de enfermedad renal monogenética, identificar variantes que confieren susceptibilidad a la ERC ha sido más difícil, ya sea por la falta de reproducibilidad o por el componente étnico. Aunque existen estudios de asociación entre parámetros fisiológicos y variantes genéticas, no hay estudios de este tipo que hayan tenido en cuenta la fragilidad genómica de los pacientes con ERC. Este enfoque es de vital interés ya que estudios previos han demostrado que los pacientes con ERC se caracterizan por poseer elevados niveles de daño genético e inestabilidad cromosómica.

Por lo tanto, el objetivo de este estudio fue analizar la posible asociación entre el daño genómico observado previamente en pacientes con ERC y polimorfismos en genes candidatos. Entre estos se encuentra genes implicados en la reparación por escisión de bases (BER), la reparación por escisión de nucleótidos (NER), y en la producción de enzimas antioxidantes, como protectores de daño oxidativo del DNA. Para ello se seleccionaron diversos SNPs de genes relacionados con la vía BER: (*OGG1,* rs1052133; *MUTYH,* rs3219489; *XRCC1,* rs25487), vía NER: (*ERCC2/XPD,* rs1799793, rs171140, rs13181; *ERCC4,* rs3136166); codificadores de enzimas antioxidantes: (*SOD1,* rs17880135, rs1041740, rs202446; *SOD2,* rs4880; *CAT,* rs1001179; *GPX1,* rs17080528; *GPX3,* rs870406; *GPX4,* rs713041), y genes relacionados con el metabolismo de fase II: (*GSTP*1, rs749174; *GSTO1,* rs2164624; *GSTO2,* rs156697). Además, se seleccionaron algunos genes previamente implicados en la ERC: (*AGT,* rs5050; *GLO1,* rs386572987; y *SHROOM3,* rs17319721).

Los resultados obtenidos han demostrado que algunos SNPs de los genes implicados en la reparación por escisión de bases (*XRCC1*, rs25487; P =0,018) y de los implicados en la reparación por escisión de nucleótidos (*ERCC2/XPD,* rs13181; P=0,022) mostraron altos niveles de asociación con el daño en el DNA (incluido el daño oxidativo) y con la inestabilidad cromosómica.

IV. DISCUSIÓN

4. DISCUSIÓN

La enfermedad renal crónica (ERC) es un importante problema de salud pública en todo el mundo. Hace décadas la glomerulonefritis era considerada como una de las principales causas de la enfermedad renal (Rivera et al., 2000). Sin embargo, hoy en día se contemplan muchas más causas entre las que destacan la hipertensión y la diabetes (Haroun et al., 2003). Dada la negativa evolución de la enfermedad renal, en la fase final los pacientes requieren diálisis o trasplante de riñón para mantener la supervivencia a largo plazo. La ERC tiene, además, una interrelación compleja con otras enfermedades como la enfermedad cardiovascular (ECV), por lo que la disfunción renal debe ser un objetivo adicional para la intervención y prevención de las enfermedades cardiovasculares (De Zeeuw et al., 2005).

Según la fundación nacional del riñón de los Estados Unidos (*National Kidney Foundation - Kidney Disease Outcomes Quality Initiative* (NKF KDOQI)), la prevalencia global de la ERC en estadios del 3 al 5, es del 6,8%, con un intervalo de confianza del 95% (IC) de 5,4 a 8,2. Si se tiene en cuenta la edad, se obtiene un prevalencia del 3,3% entre los 40-64 años, y del 21,4% para edades mayores de 64 años. Además, se han estimado como factores de riesgo la edad, la obesidad y la hipertensión previamente diagnosticada (Otero et al., 2010).

Una de las características de los pacientes con ERC es que presentan elevados niveles de daño genómico, posiblemente debido a la acumulación de toxinas urémicas, e independientemente de si se encuentran o no sometidos a diálisis (Rangel-López et al., 2013). Así, con el fin de investigar más a fondo las causas y consecuencias del daño genómico observado en la ERC, en esta Tesis hemos determinado los niveles de daño genómico y su relación con diversos marcadores de la patología. Cabe resaltar que a la fecha de publicado el artículo 1, contamos con la población de mayor tamaño de las encontradas en la literatura. Por otro lado, hemos analizado el efecto modulador de diferentes técnicas y/o suplementos sobre este daño, así como el papel que juegan diferentes polimorfismos genéticos en la enfermedad.

En esta Tesis, que se presenta en formato de artículos, en el primero de ellos (Artículo 1) hemos analizado los niveles de daño genómico mediante la técnica del cometa. Este ensayo permite detectar fácilmente roturas en el DNA, así como sitios

álcali-lábiles, o productos intermedios de la reparación por escisión de bases o nucleótidos (Kan et al., 2002). Esta misma técnica nos permitió evaluar la eficacia de la protección antioxidante en pacientes con ERC (Artículo 2), así como el efecto de diferentes terapias de remplazo renal como la hemodiafiltración on-line y el trasplante (Artículos 3 y 4). Un aspecto relevante de la Tesis lo constituyen los estudios de asociación entre distintas variables de la enfermedad con variantes genéticas (Artículos 5 y 6). Cabe señalar que también hemos utilizado el ensayo de micronúcleos (MN) (Artículos 4 y 6) para medir el daño cromosómico (clastogénico/aneugénico). La sensibilidad y fiabilidad del ensayo de MN en linfocitos humanos, mediante el bloqueo de la citocinesis con citocalasina B (cyt-B) ha demostrado ser una herramienta eficaz para medir el daño cromosómico (Pastor et al., 2003), siendo reconocido como un buen biomarcador de riesgo de cáncer (Bonassi et al., 2007).

Todo lo anterior muestra la complejidad del trabajo desarrollado en la presente Tesis.

Daño genómico asociado con biomarcadores de la enfermedad renal crónica

La presencia del daño genómico en pacientes con ERC se ha asociado con diversos factores, como la inflamación (Stenvinkel y Alvestrand, 2002), las infecciones (Sarnak y Jaber, 2000), mecanismos de reparación alterados (Trabulus et al., 2012), incrementos en los niveles de especies reactivas de oxígeno (ROS), defensas antioxidante reducidas (Morena et al., 2002; Sung et al., 2013), así como a la acumulación de toxinas urémicas (Schupp et al., 2010).

Estas variables no actúan del mismo modo a lo largo del tiempo. Hay que recordar que los pacientes presentan un avance de la enfermedad desde sus estadios iniciales donde se ve el deterioro paulatino de la función renal (1-5 TFG < 60 mL/min/1,73 m^2) hasta el estado 6, en el que ya la tasa de filtrado glomerular está tan disminuida que se requieren terapias de remplazo. Esto hace necesario analizar la evolución del daño genómico en dos poblaciones diferentes: en aquellos pacientes en estados iniciales (pacientes prediálisis, PD), y en aquellos que ya requieren el tratamiento de hemodiálisis (HD). Esta terapia se aplica para lograr la eliminación de solutos tóxicos y, dado que no puede reemplazar completamente las

funciones de los riñones, los pacientes en HD tienen que llevar una dieta especial y necesitan medicación suplementaria (Schupp et al., 2010).

Teniendo en cuenta este escenario, consideramos necesario explorar los niveles de daño genómico en un número elevado de pacientes. Nuestra investigación abarcó 187 individuos sanos, 206 pacientes en PD y 209 en HD. La evaluación del daño genómico se llevó a cabo mediante el ensayo del cometa (electroforesis en gel de una sola célula) en su versión alcalina, complementándose con la inclusión de la enzima FPG (formamidopirimidina-DNA glicosilasa) para la detección de bases oxidadas en el DNA. Este ensayo ha ganado creciente aceptación debido a que es un método muy útil para cuantificar el daño en el DNA, donde se detecta la presencia de roturas de simple o de doble hebra, sitios lábiles alcalinos, y la cromatina relajada por la fragmentación del DNA (Stopper et al., 2001; Burlinson et al., 2007). Así, en el Artículo 1 se buscó establecer asociaciones entre los niveles de daño en el DNA y diferentes parámetros relacionados con la patología. De ese primer trabajo se pudo concluir que los niveles de creatinina, albúmina, hormona paratiroidea (HPT), y ácido úrico se asocian con los niveles de daño en el DNA, en los pacientes en PD. Respecto a los pacientes en HD, los niveles de ferritina, vitamina B12 y el índice de saturación de transferrina (IST), se correlacionaron positivamente con el daño en el DNA, observándose correlaciones negativas con las lipoproteínas de alta densidad (HDL) y la capacidad total de fijación del hierro (CTFI).

En individuos sanos la creatinina, que proviene de la creatina del músculo esquelético, se elimina por el riñón (Taguchi et al., 2016). En los pacientes con la función excretora del riñón comprometida, como es el caso de los pacientes PD, es de esperar una acumulación de creatinina en el suero y por lo tanto, se esperarían mayores niveles de daño genómico. La relación proteína/creatinina se sabe que predice con exactitud el ritmo de descenso de la tasa de filtración glomerular y el riesgo de progresión de la insuficiencia renal en pacientes no diabéticos con proteinuria y enfermedad renal crónica (Ruggenenti et al., 1998). Al igual que la creatinina, la albumina entra dentro de los parámetros bioquímicos referentes de la ERC, por lo que se sabe que los individuos hipertensos con microalbuminuria manifiestan una mayor incidencia de eventos cardiovasculares y una mayor disminución de la función renal que los pacientes con excreción urinaria normal de

albúmina (Bigazzi et al., 1998). Esto se relacionaría con lo observado en nuestro trabajo, donde los pacientes con microalbuminuria presentan elevados niveles de daño genómico. Dada la importancia de la albúmina se recomienda medirla, ya sea en orina o en suero, para evaluar el daño renal y para estimar la TFG y el riesgo de ERC (Stevens et al., 2006). Dentro de los principales parámetros clínicos que mostraron correlación con el daño genómico en nuestro estudio se encuentra el ácido úrico, el cual es un producto final del metabolismo endógeno de las purinas. Debido a la falta de genes que codifican la uricasa, los humanos somos incapaces de convertir el ácido úrico en alantoína soluble, lo que conduce a niveles elevados de ácido úrico en suero (Oda et al., 2002). El ácido úrico puede entrar en las células del músculo liso vascular, a través de transportadores específicos de aniones orgánicos, y activar quinasas intracelulares y factores de transcripción nuclear, dando como resultado un fenotipo proliferativo y proinflamatorio, en donde las células del músculo liso vascular producen factores de crecimiento, sustancias vasoconstrictoras, moléculas proinflamatorias (como la Proteína C-reactiva, PCR), y el receptor de tipo I de la angiotensina II (Kanellis et al., 2003). Valores elevados de ácido úrico también tienen efectos negativos en las células endoteliales, lo que resulta en una inhibición de la proliferación y la migración, la estimulación de la PCR, y la inhibición de la liberación de óxido nítrico (Kang et al., 2005). Este escenario puede llegar a producir hiperuricemia, la cual está estrechamente relacionada con la disminución de la función renal, aumentando el riesgo de la ERC (Obermayr et al., 2008), tanto en sujetos sanos (Oh et al., 2014), como en pacientes con otras enfermedades relacionadas, como son la hipertensión (Sundström et al., 2005), y la diabetes tipo 2 (Lin et al., 2016).

Dentro de las anormalidades endocrinas presentes en pacientes con ERC está la deficiencia en 1.25-dihidroxivitamina D (1.25-OH_2D_3). Dicha deficiencia se produce durante la progresión de la enfermedad renal, debido a que el paso final de hidroxilación de 25-hidroxivitamina D (25(OH)D_3) a 1.25-OH_2D_3 (calcitriol) se lleva a cabo por la 1 α-hydroxalasa del riñón (Levin et al., 2007). Es así como bajos niveles de calcitriol pueden conducir a un hiperparatiroidismo secundario, en donde la deficiencia de vitamina D promueve el crecimiento de la glándula paratiroidea (hiperplasia) y el aumento de la síntesis de la hormona paratiroidea (HPT) a través de la pérdida de la capacidad para regular el aumento de la expresión del receptor

de vitamina D en las células paratiroideas. Esto da como resultado que los niveles de HTP en suero sean elevados y un desequilibrio entre los niveles de calcio (Ca) y de fósforo (P) (Llach y Forero, 2001). Todo lo anterior podría explicar la correlación positiva de elevados niveles de HPT con el incremento del daño genómico en los pacientes PD.

Por otro lado hemos observado que en pacientes PD el daño genómico era menor cuanto mayor eran los niveles de vitamina B12, mientras que en los pacientes en HD el daño genómico se incrementaba con los niveles de vitamina B12. Esta vitamina está implicada en reacciones de transmetilación y se necesita para el transporte celular y el almacenamiento del folato teniendo una función clave en el metabolismo del ácido fólico. Su función esencial es la desmetilación del metiltetrahidrofolato y la metilación de la homocisteína (Sánchez et al., 2007). La deficiencia de vitamina B12 está entre las causas más comunes de la hiperhomocisteinemia, la cual a su vez juega un papel patogénico en el desarrollo de la enfermedad cardiovascular (Brattström y Wilcken, 2000) Todo esto podría explicar la relación directa observada entre el daño genómico y los niveles de vitamina B12 donde los paciente PD, al estar en las etapas más tempranas de la ERC, presentan bajos niveles de daño y aún conservan óptimos niveles de vitamina B12, mientras que los altos niveles de daño en los pacientes en HD se debería su avanzado estado de la insuficiencia renal.

En los pacientes en HD también se observó una relación directa entre los niveles de daño genómico y los niveles de ferritina y del índice de saturación de la tranferrina (IST). La ferritina sérica y el IST son las principales herramientas para la evaluación del metabolismo del hierro en pacientes con anemia y ERC (National Kidney Foundation, 2001). La ferritina es una proteína que almacena el hierro dentro de la célula y el intercambio permanente de éste es mediado por la transferrina, proteína transportadora de hierro. Después de una ingesta de hierro, la transferrina transporta una cantidad significativa de éste a los órganos de depósito, mientras que cuando hay una demanda de dicho metal por algún tejido, la transferrina captará el hierro de los depósitos para transferirlo a dicho tejido (Boccio et al., 2003). Los niveles de ferritina sérica pueden variar debido a la oferta y la demanda metabólica, y no a la deficiencia de hierro corporal total. Aún con niveles normales de hierro, pero en el contexto de la estimulación de la médula

ósea por el agente estimulante de la eritropoyesis, a la velocidad a la que el hierro se libera y con la que está siendo entregado por la transferrina de la médula eritroide, los niveles de hierro pueden llegar a ser insuficientes para mantener la producción de glóbulos rojos (Wish, 2006). Estudios previos han demostrado una relación entre los niveles de ferritina con la evolución de la ERC (Branten et al., 2004), encontrándose que en pacientes sometidos a HD los elevados niveles de ferritina sérica estarían causados por la inflamación (Kirschbaum, 2002). Otro estudio con pacientes en HD observó que los pacientes con ferritina sérica elevada (>800 ng/mL) presentaban mayores niveles de PCR y mayor status de malnutrición e inflamación que los pacientes con menores niveles de ferritina sérica (<800 ng/mL) (Kalantar-Zadeh et al., 2004). Lo anterior estaría de acuerdo con la relación directa en el incremento de estos parámetros indirectos del metabolismo del hierro y los niveles del daño genómico.

Por otro lado, en nuestro trabajo también ha sido posible observar correlaciones inversamente proporcionales en pacientes en HD, como fue el caso de los niveles de HDL. Muntner et al. (2000) mostraron que las personas con niveles bajos de colesterol, HDL e hipertrigliceridemia tienen un mayor riesgo de tener pérdida de la función renal. Además, un seguimiento durante 10 años de los factores de riesgo para la ERC, mostró que la hipercolesterolemia a largo plazo, hipertrigliceridemia y niveles bajos de HDL fueron factores de riesgo para el desarrollo de proteinuria, tanto en hombres como en mujeres (Yamagata et al., 2007).

Además de la correlación entre el daño genómico y distintos factores bioquímicos, característicos de la ERC, hay que recordar que existen distintas patologías asociadas a la ERC, de las que se desconocen si son causa o efecto de la patología, y que podrían relacionase con los niveles de daño genómico. En este contexto cabe recordar que en nuestros pacientes la incidencia de diabetes, hipertensión y enfermedades cardiovasculares fue particularmente elevada. Los incrementos en los niveles de presión arterial se pueden explicar porque en los sujetos con enfermedad renal existe una elevada retención de sodio, junto con la activación del sistema renina angiotensina (Schiffrin et al., 2007). La enfermedad cardiovascular es otra característica asociada con la ERC y estudios previos han establecido que la tasa de mortalidad por enfermedad cardiovascular es de 10 a 20 veces mayor en los pacientes urémicos en comparación con la población general

(Noce et al., 2013). Los pacientes con enfermedad renal crónica experimentan una forma secundaria de dislipidemia que se caracteriza por un aumento de los triglicéridos en suero con niveles elevados de LDL, y niveles bajos de colesterol HDL. Es de destacar que la dislipidemia no es sólo secundaria a la enfermedad renal, sino que también los es a la diabetes (Trevisan et al., 2006), lo que ligaría a ambas patologías. Además, la concentración de colesterol total y triglicéridos, junto con el aumento de la tasa de excreción de albúmina también se observa en los pacientes con diabetes tipo 1 (Jenkins et al., 2003).

Los pacientes con ERC presentan una alta incidencia de diferentes tipos de cáncer, dentro de los cuales los seis más frecuentes son los de mama, próstata, colorectal, melanoma, pulmón y tracto urinario (Wong et al., 2009). Paralelo a ello, se ha evidenciado que estos pacientes presentan altos niveles de daño en el DNA, así como de inestabilidad genómica (Sandoval et al., 2012; Rodríguez-Ribera et al., 2014). Además, en un estudio sobre la insuficiencia renal y el uso de medicamentos antitumorales, se observó que de entre los 4.684 participantes con cáncer, con un promedio de edad de 58 años, el 12% tenían una TFG <60 mL/min por 1,73 m^2 (Launay-Vacher et al., 2007), lo que conectaría ambas patologías.

En cuanto a las asociaciones entre los niveles de daño genómico y la ERC, es de destacar que no encontramos correlación entre la tasa de filtrado glomerular y el porcentaje de DNA en cola, dado que ya en las etapas más tempranas de la enfermedad (etapas 2-3) se encontraron elevados niveles de daño. Nuestro estudio confirmaría que el daño en el DNA puede ser considerado como un biomarcador intrínsecamente asociado con la ERC sin estar asociado con su desarrollo. Nuestros resultados, mostraron que los individuos con ERC tiene mayores niveles de daño genómico que los individuos sanos, coinciden con casi todos los estudios previos con pacientes similares (Stopper et al., 2001; Sandoval et al., 2010; Rangel-Lopez et al., 2013). Un aspecto que genera controversia es el papel que puede jugar el estrés oxidativo en la ERC. Nuestros resultados muestran que los pacientes sometidos a HD tienen incrementados los niveles de daño oxidativo en el DNA, respecto a los pacientes PD. El estrés oxidativo generado por la oxidación de hidratos de carbono y lípidos puede dar lugar a la formación de compuestos carbonilo reactivos, lo que resulta en la formación de productos de glicosidación avanzada y productos finales de lipoxidación (Schwedler et al., 2001),

observándose que los niveles de estos productos están elevados en el plasma de pacientes urémicos (Bayés et al., 2003). Además, el estrés oxidativo (peróxidos de lípidos) se asocia con la calcificación de la arteria coronaria en pacientes en HD (Taki et al., 2006) y se ha demostrado que los pacientes en HD están expuestos a estrés oxidativo e inflamación (Danielski et al., 2003). Cuando se utilizan los niveles de 8-OHdG, como biomarcador de daño en el DNA, se encuentran niveles significativamente mayores en leucocitos de pacientes en HD respecto a pacientes no dializados (Tarng et al., 2000b).

Es obvio que la tasa de supervivencia es un buen marcador del riesgo de la ERC. Cuando se comparó la supervivencia de los pacientes con ERC sometidos a HD y los niveles de daño genómico de estos pacientes, se pudo evidenciar que los pacientes fallecidos durante el estudio presentaban niveles de daño genómico significativamente mayores que los pacientes que continuaron con vida hasta la finalización de mismo. La incidencia de mortalidad entre los pacientes con ERC ha sido ampliamente estudiada. En una revisión sistemática se observó una relación directa entre la severidad de la disfunción renal y la mortalidad, sugiriendo que el riesgo absoluto de muerte aumenta con la disminución de la función renal (Tonelli et al., 2006). En un estudio previo, y utilizando un análisis multivariante, se observó que sólo el daño genómico, la edad y la PCR se asociaron de forma independiente con la mortalidad. Esto evidenciaría que la presencia de altos niveles de daño genómico son un fuerte predictor de la mortalidad (Coll et al., 2013). Otro estudio ha demostrado que la edad, la presencia de diabetes y bajos niveles de albúmina en suero, fueron predictores independientes de mortalidad en pacientes en HD (Lin et al., 2009); asimismo, en otro análisis se observó que los niveles de 8-OHdG en suero fueron menores en los pacientes en PD que en los pacientes en HD, lo que mostraría un efecto predictivo de la mortalidad mediante los niveles de 8-OHdG (como indicador de daño genómico) en pacientes en HD, pero no en pacientes con PD (Xu et al., 2015). Todos estos estudios estarían de acuerdo con nuestros resultados, encontrando una correlación positiva entre los niveles de daño en el DNA y el riesgo de mortalidad en pacientes con ERC.

Efecto antioxidante del mosto

Como hemos demostrado los pacientes con ERC presentan elevados niveles de daño genómico y oxidativo. Dados los altos niveles de daño oxidativo en el DNA de los pacientes sometidos a HD, respecto a los no dializados, nos propusimos un nuevo objetivo de estudio como es el determinar el papel de los compuestos antioxidantes como potenciales agentes reductores de estos efectos. Se ha indicado que los pacientes con ERC presentan un desequilibrio entre los niveles de especies reactivas de oxígeno (ROS) y los mecanismos de defensa ante el daño causado por estos radicales (Massy et al., 2009). Por lo tanto, se hace necesario estudiar el papel protector de agentes exógenos con potencial antioxidante. Existe una amplia gama de compuestos con probada capacidad antioxidantes (polifenoles, tocoferoles, ácido ascórbico, glutatión y otros grupos de proteínas tiol) diseñados para proteger la integridad funcional y estructural de las diferentes moléculas biológicas tales como proteínas, lípidos y ácidos nucleicos (Hassan, 2013). En los pacientes con ERC la HD es el tratamiento de preferencia, en aquellos que no se someten a trasplante renal. Factores de la propia diálisis como pueden ser el contacto de la sangre con la membrana de diálisis, la pureza del agua de diálisis y las limitaciones dietéticas a que se ven sometidos los pacientes, hacen que éstos sean susceptibles al estrés oxidativo y a la inflamación (Nagane et al., 2013). Para reducir los efectos indeseados de este estrés se ha propuesto la búsqueda de posibles blancos terapéuticos para disminuir este estrés oxidativo, en miras de la mejora de la calidad de vida de los pacientes. En este sentido, el Artículo 2 se diseñó para evaluar si el efecto antioxidante del mosto (jugo de uva sin fermentar) era capaz de reducir los niveles de daño genético, ya sea general u oxidativo, en nuestros pacientes.

En condiciones normales, las especies reactivas de oxigeno (ROS) se generan en las mitocondrias de las células en el proceso de producción de energía, al reducir el oxígeno durante la respiración aeróbica. Sin embargo, aunque son componentes esenciales de la célula, niveles excesivos de ROS pueden producir daño celular, mediante la interacción con las distintas biomoléculas. Esta interacción tiene efectos negativos sobre la función del tejido y su estructura, jugando un papel importante en el desarrollo de una amplia variedad de enfermedades renales, como pueden ser la glomerulonefritis, la insuficiencia renal aguda o progresiva (Nath y

Norby, 2000), o la nefritis tubulointersticial, que contribuye a la proteinuria (Lahera et al., 2006). Por otra parte, debido a su impacto en la regulación del ciclo celular (Shackelford et al., 2000), las ROS pueden contribuir a la hipertrofia de las células tubulares (Hannken et al., 2000). En el sistema vascular, la interacción de superóxido (O_2^-) con monóxido de nitrógeno (NO) parece ser de gran importancia, en particular en el ajuste de la hipercolesterolemia, la aterosclerosis (Harrison et al., 2003) y la hipertensión (Landmesser et al., 2003). El NO es un importante vasodilatador endotelial, inactivado por O_2^- mediante una reacción que produce peroxinitrito ($ONOO^-$) que a su vez favorece la formación de más ROS (Griendling y FitzGerald, 2003). La interacción entre el NO y O_2^- se produce a una velocidad extremadamente rápida, tres veces más rápido que la velocidad de reacción de $O2^-$ con la superóxido dismutasa (SOD). Teniendo en cuenta este tipo de reacción es posible que haya siempre algún O_2^- libre que pueda reaccionar con el NO dentro de las células y en el espacio extracelular. En condiciones fisiológicas, las defensas antioxidantes endógenas minimizan esta interacción y mantienen lo que parece ser un tenue equilibrio entre O_2 y NO, pero la pérdida de ese equilibrio genera la disfunción endotelial y alteraciones de la renovación celular en el sistema vascular (Cai y Harrison, 2000; Taniyama y Griendling, 2003). Al mismo tiempo dentro de las biomoléculas que juegan un papel importante están las lipoproteínas aterogénicas como el LDL, que no sólo se someten a modificación oxidativa en la pared vascular, sino que también son capaces de generar potenciadores de ROS por medio de la estimulación de oxidasas NADPH-dependientes en las células endoteliales del músculo liso y en las células mesangiales. Con este escenario, el estrés oxidativo juega un papel importante en diferentes situaciones patológicas como son las enfermedades cardiovasculares (Jeremy et al., 2004), el envejecimiento (Dugan y Quick, 2005) o las enfermedades renales (Forbes et al., 2008). Otro de los efectos asociados al estrés oxidativo es la inflamación, también típica de los pacientes con ERC sometidos a HD. Dado que la HD es un proceso no selectivo, ciertas modalidades de HD inducen pérdidas de solutos, tanto productos de desecho como sustancias esenciales, incluyendo antioxidantes. Estas pérdidas durante la HD contribuyen a anormalidades en las vías enzimáticas antioxidantes, por lo que el deterioro en antioxidantes en HD, lejos de mejorar el estado oxidativo, podría ser responsable de las pérdidas antioxidantes no enzimáticas. Debido a la

sobreproducción de ROS, no es sorprendente que los pacientes con ERC presenten valores elevados para muchos de los marcadores de estrés oxidativo normalmente utilizados (Morena et al., 2005). Así, productos de la peroxidación lipídica como el malonildialdehido, el 4-hidroxinonenal, y los F2-isoprostanos, se encuentran elevados en pacientes en HD (Paul et al., 1993; Neiva et al., 2002).

De lo anterior se puede deducir que en pacientes con ERC los efectos negativos del estrés oxidativo se asocian con la progresión de la enfermedad, al encontrarse una correlación con el nivel de la función renal (Dounousi et al., 2006). En el Artículo 1 hemos encontrado que el daño oxidativo en el DNA medido por el ensayo del cometa, complementado con la enzima FPG, es mayor en los pacientes en HD con respecto a los pacientes PD. Estudios recientes, que comparan los niveles de estrés oxidativo bajo distintos tipos de modalidades de diálisis, muestran elevados niveles de subproductos de la oxidación de proteínas en los pacientes en HD convencionales, en comparación con aquellos sometidos a diálisis peritoneal (Zhou et al., 2012; Marques de Mattos et al., 2012). Sin embargo, otros estudios han encontrado niveles similares o inferiores de distintos marcadores de estrés oxidativo (como subproducos de la oxidación de proteínas, mieloperoxidasa, y niveles de 8-OHdG) en los pacientes en HD, en comparación con los pacientes sometidos a diálisis peritoneal (Castoldi et al., 2010; Samouilidou et al., 2012). Por lo tanto, la contribución del daño oxidativo a la enfermedad y viceversa es todavía controvertida.

El tratamiento de diálisis, por sí mismo, parece contribuir al estrés oxidativo mediante la creación de alteraciones en el equilibrio entre la generación de radicales libres y los sistemas de protección antioxidantes (Sung et al., 2013). De hecho, estudios previos han mostrado aumentos en los niveles de malondialdehído (producto de la peroxidación lipídica) y disminución de los niveles del antioxidante α-tocoferol primario soluble en lípidos, lo que sugiere que el procedimiento HD está asociado con el estrés oxidativo (Westhuyzen et al., 1997). Teniendo en cuenta que el procedimiento de HD no elimina de manera eficiente ni los productos finales de la glicación avanzada (AGES) ni los lipoperóxidos (Gugliucci et al., 2007), es de suponer que en los pacientes en HD se producen alteraciones en los niveles de lipoproteínas del plasma así como productos resultados de la peroxidación de lípidos (Kaysen, 2011).

Frente a esta situación se han propuesto diferentes terapias de suplementación con antioxidantes, para ser utilizadas en pacientes con ERC. Los suplementos a base de jugo de uva se han indicado como una propuesta adecuada para reducir los niveles de estrés oxidativo en este tipo de pacientes (Castilla et al., 2006, 2008), y también para disminuir los niveles de daño genómico (Weisel et al., 2006). Las uvas contienen un gran número de metabolitos secundarios, tales como flavonoides, que constituyen un gran grupo de compuestos polifenólicos que también se encuentran en las frutas y otros alimentos (Rice-Evans et al., 2000; Georgiev et al., 2014). El consumo de alimentos ricos en flavonoides se ha asociado con un menor riesgo de varias enfermedades crónicas (Hertog, 1995). Los beneficios protectores de los flavonoides, a través de la dieta, pueden ser debidos en parte a sus propiedades antioxidantes y a su capacidad para reducir el estrés oxidativo (Rice-Evans et al., 1996).

En este contexto, el objetivo de nuestro estudio fue evaluar el efecto de la suplementación con mosto, en un grupo de pacientes de HD, durante un período de seis meses. Los resultados obtenidos indican que algunos de los parámetros clínicos analizados, tales como LDL y colesterol, se redujeron significativamente en los pacientes estudiados después de la suplementación. Sin embargo, ya que los niveles de colesterol también se redujeron en los controles no podemos atribuir esta disminución de forma única a la ingestión de mosto. Como se indica en el Artículo 2, observamos un ligero descenso (no significativo), tanto en el daño genómico medido por el ensayo del cometa como en el cromosómico medido por el ensayo de micronúcleos. Sin embargo, sí que se observó una disminución estadísticamente significativa en los niveles del daño oxidativo (ensayo del cometa con FPG) en nuestros pacientes después de 6 meses de la suplementación con mosto.

Varios estudios han demostrado que la ingesta de extractos ricos en polifenoles reducen las concentraciones de colesterol-LDL en plasma (O'Byrne et al., 2002; Castilla et al., 2006); y un efecto similar se encontró para los polifenoles del vino tinto en hámsteres (Vinson et al., 2001). Esto podría explicarse dado que los polifenoles pueden actuar a través de la disminución de la actividad de la HMG-CoA reductasa y la disminución en la expresión de su mARN. La HMG-CoA reductasa cataliza la reducción del HMG-CoA a mevalonato CoA que modula la velocidad de la síntesis de novo del colesterol. (Cho et al., 2008). Además se ha

observado que los hámsteres hiperlipidémicos alimentados con vino tinto desalcoholizado tenían una reducción del 45% en las concentraciones de LDL en plasma respecto al control (hámsteres suplementados con agua) (Vinson et al., 2001). Otro estudio *in vivo* sugiere que la disminución observada en las lipoproteínas circulantes en hámsteres alimentados con vino tinto desalcoholizado puede ser el resultado de una mayor actividad de unión del receptor de LDL y una supresión de la producción de lipoproteína hepática (Pal et al., 2003). Estos resultados están de acuerdo con los nuestros y con un estudio enfocado en antocianidinas, flavanonas, y alimentos ricos en flavonoides que se asociaron con una menor mortalidad por enfermedad cardiovascular (Mink et al., 2007). Aunque en nuestro estudio observamos una mayor disminución de los valores de LDL en los pacientes suplementados con mosto que en los controles, no observamos aumentos en los niveles de colesterol-HDL en plasma, como se ha descrito por otros autores. Se ha visto que el jugo de uva roja mejora el perfil de lipoproteínas, reduce las concentraciones plasmáticas de biomarcadores inflamatorios y la LDL oxidada, tanto en pacientes en HD como en controles (Castilla et al., 2006). La discrepancia observada entre nuestros resultados y los anteriormente citados se podría deber a las diferentes dosis de mosto suministradas: 1.766 mg/semana en nuestro caso, respecto a los 4.508 mg/semana administrados por Castilla et al. (2006). Son distintos los estudios que han demostrado los efectos protectores de los suplementos con zumos de fruta en pacientes en HD. Pacientes en HD suplementados durante un año con jugo de granada mostraron una reducción significativa de los subproductos de la oxidación de proteínas, de la oxidación de lípidos y de biomarcadores de inflamación (Shema-Didi et al., 2012). A pesar de estos estudios, nuestros resultados no apoyan la idea de que los cambios en el perfil lipídico se atribuyen únicamente a la ingestión de mosto. Este punto de vista estaría de acuerdo con los resultados del meta-análisis realizado por Liu et al., (2013) en el que no se encontró un efecto directo de los zumos de frutas sobre colesterol total, lipoproteínas de alta y de baja densidad.

Es de destacar que en nuestro estudio los pacientes en HD redujeron significativamente los niveles de daño oxidativo en el DNA, tras la ingesta de mosto, mientras que estos niveles se mantuvieron sin cambios en el grupo de referencia. Aunque el efecto antioxidante del mosto en pacientes en HD se ha demostrado

anteriormente (Castilla et al., 2008), no hay otros estudios que hayan evaluado el efecto del mosto sobre los niveles de daño genómico en los pacientes en HD. Sin embargo, hay estudios que han observado cambios en los niveles de daño genómico en los pacientes en HD después de la suplementación con vitamina E (Kan et al., 2002), vitamina C (Tarng et al., 2004), o selenio (Zachara et al., 2011). También se ha observado que en las personas sanas el jugo de uva ejerce una actividad antioxidante persistente hasta dos horas después de la suplementación (Ko et al., 2005). Estos estudios sugieren que el consumo de zumo de fruta rica en polifenoles puede reducir el estrés oxidativo, lo que disminuye el daño en el DNA, y que este efecto puede ser el resultado de su actividad antioxidante sobre las especies reactivas de oxígeno generadas. En general, las publicaciones en esta área, muestran que la suplementación con antioxidantes reduce los niveles de daño genómico.

Efectos del cambio a hemodiafiltración on-line sobre el daño genómico

Después de tres décadas de rápido desarrollo técnico en la terapia de reemplazo renal, los pacientes en diálisis todavía se enfrentan con una alta morbilidad y riesgo de mortalidad. La morbilidad se refleja en las hospitalizaciones, eventos cardiovasculares y otras patologías relacionadas con la diálisis. La mortalidad se ha registrado en promedio alrededor de 17% por año en todo el mundo, aunque con grandes variaciones internacionales (Japón 7%, Europa 15% y EE.UU 25%) (Canaud, 2011). Dentro de los diferentes factores de riesgo a solucionar está la reducción de los efectos generados por el estrés oxidativo, incluyendo el daño genómico. Hoy en día, se sabe que cuando estos pacientes reciben HD están siendo sometidos a un estrés oxidativo adicional, no presente en los estadios tempranos de la ERC, como observamos en el Artículo 1. Actualmente se dispone de diferentes técnicas de eliminación de elementos tóxicos para el paciente con ERC. Entre ellas destacan las técnicas convectivas, donde la infusión directa en sangre del baño de diálisis, han supuesto un gran avance. En particular, la técnica de infusión on-line permite utilizar grandes volúmenes de reposición sin incremento del costo, superando así una de las principales limitaciones de la convección

clásica, y consiguiendo un alto rendimiento en la eliminación de sustancias por convección (Lucas et al., 2010).

Para visualizar el efecto de esta técnica sobre los diferentes factores de riesgo y la progresión en el avance de la ERC se utilizan distintas biomoléculas, como productos avanzados de la oxidación de proteínas (PAPO), que son el resultado del entrecruzamiento, agregación y fragmentación de las proteínas como consecuencia de su oxidación, lo que genera cambios en la hidrofobicidad, conformación, alteración en la susceptibilidad a la acción de enzimas proteolíticas o formando nuevos grupos reactivos como son los hidroperóxidos (Delgado Roche et al., 2009). Otros compuestos son los productos finales de la glicación avanzada (AGES), que constituyen un gran y heterogéneo grupo de sustancias producidas por las reacciones no enzimáticas de los azúcares con grupos amino libre en las proteínas, péptido o en aminoácidos (la denominada reacción de Maillard). Los AGES se pueden formar a través de varias otras vías, incluyendo la oxidación de azúcares, lípidos, y aminoácidos para crear aldehídos reactivos que se unen covalentemente a las proteínas (Mallipattu et al., 2012). Se considera que los AGES están involucrados en muchas complicaciones de la enfermedad renal, fundamentalmente en su etapa terminal. En un estudio llevado a cabo durante seis meses, se observó que los niveles de AGES en suero antes de la diálisis fueron significativamente menores en los pacientes tratados con HDF on-line en comparación con los tratados con HD convencional y de alto flujo (Lin et al., 2003). Por otro lado, el glutatión que se encuentra principalmente en su estado reducido (GSH) y, en mucha menor proporción en su estado oxidado (GSSG) gracias a la glutatión reductasa, en situaciones de estrés oxidativo sirve como indicador del estado oxidativo celular. Esta relación GSSG/GSH se ha estudiado antes y después de la HDF on-line tres veces/semana, observándose que la diálisis aumenta la concentración de GSSG de manera significativa, disminuyendo favorablemente el estrés oxidativo (H_2O_2) celular (Torregrosa et al., 2007).

Así, en el Artículo 3 se analizó por medio del ensayo del cometa, con FPG, los efectos de la HDF on-line sobre los niveles de daño genómico. En el estudio, llevado a cabo en 34 pacientes, se pudo observar una leve disminución del daño genómico total, seis meses después de empezar la terapia renal sustitutiva con HDF on-line.

Al contrario de lo observado en el grupo control, el cual se mantuvo tratado con HD de bajo flujo, y donde el daño genómico no mostró cambios.

Un aspecto interesante de nuestro estudio es que los efectos beneficiosos de la HDF on-line fueron positivamente evidentes en aquellos pacientes que partían de niveles de daño genómico más altos. Esto se observó tanto para los niveles de daño genómico global como para el daño genómico oxidativo. Lo cual sugiere que el tratamiento de HDF on-line tiene un efecto visiblemente beneficioso sobre el daño genómico en aquellos pacientes con altos niveles de daño. Debe tenerse en cuenta que estos resultados todavía deben ser demostrados con un número mayor de pacientes para corroborar las tendencias observadas.

Tan sólo dos estudios previos han evaluado el papel de la HDF on-line sobre el daño genómico. El primero es un estudio preliminar llevado a cabo en tan sólo siete pacientes donde los autores encontraron una ligera reducción, aunque significativa, en los niveles de daño genómico evaluado mediante el ensayo del cometa, sin encontrar variaciones en los niveles de daño cromosómico evaluado con el ensayo de micronúcleos (Kobras et al., 2006). El segundo estudio es de nuestro grupo, donde en 33 pacientes se observó que la frecuencia de micronúcleos disminuyó significativamente tras seis meses en HDF on-line (Rodríguez-Ribera et al., 2016). El poco impacto de la HDF on-line en la disminución de los niveles de daño se podría asociar con los ligeros cambios en la expresión de distintas proteínas del plasma, tales como la hemo-oxigenasa-1 que juegan un papel relevante en la defensa antioxidante (Calò et al., 2007).

Dentro de los parámetros clínicos comúnmente estudiados en las terapias de remplazo, está el índice de la dosis de diálisis basado en el aclaramiento fraccional de urea, que se expresa comúnmente como la relación de reducción de urea intradiálisis (Kt/V), donde K representa la tasa de aclaramiento de urea por el dializador en mililitros por minuto (t), la duración en minutos de la sesión de tratamiento, y V el volumen de distribución de urea en el paciente en mililitros. Según las recomendaciones de las principales guías clínicas los valores adecuados deben ser Kt/V>1,3 (Maduell et al., 2008). Lo que hemos visto en nuestro estudio longitudinal, es que los niveles de Kt/V mejoran con el tiempo que el paciente lleve en HDF on-line, lo que nos indicaría que las membranas utilizadas para el

transporte convectivo son más biocompatibles, eliminan mayor cantidad de β2-microglobulina (y se genera menor cantidad de la misma por su mayor biocompatibilidad), a la vez que eliminan mayor cantidad de mediadores inflamatorios (IL-6, IL- 8, y TNF), así como mayor cantidad de productos finales de la glicación avanzada (AGES), mejoran el estado anémico y de nutrición por la depuración de leptina, se consigue mejoría de la dislipemia, y por tanto del riesgo cardiovascular y mejora la tolerancia hemodinámica (Barroso, 2007).

En referencia a los parámetros bioquímicos analizados en sangre, se observó una disminución estadísticamente significativa de los triglicéridos, seis meses después del tratamiento en HDF on-line, lo que no se observó en el grupo control. Los triglicéridos son moléculas grasas de triple cadena que circulan en la sangre y también se almacenan en el tejido graso. Un nivel alto de triglicéridos en la sangre, se conoce como hipertrigliceridemia y se asocia con un mayor riesgo a desarrollar enfermedades cardiovasculares (Weiner et al., 2008b). Un par de trabajos sugieren que niveles elevados de triglicéridos y colesterol-HDL bajo, predicen un mayor riesgo de disfunción renal (Muntner et al., 2000; Fried et al., 2001).

Estudios en los que se han analizado los niveles de productos avanzados de oxidación de proteínas (AOPP) y AGES, han encontrado que durante la HDF on-line, estos niveles se redujeron de manera significativa (Lin et al., 2003). Uno de los aspectos fundamentales en la obtención de mejores resultados con el tratamiento de HDF on-line se podría asociar con el uso del agua ultrapura empleada, lo que conlleva una disminución en estímulos de procesos inflamatorios con la subsecuente disminución de la formación de AGES y menor oxidación (Vaslaki et al., 2006).

Algunos estudios indican que la utilización de HDF on-line no aporta ningún beneficio significativo sobre la anemia ni sobre el metabolismo mineral, comparado con la HD de alto flujo (Vilar et al., 2009). Tampoco se han encontrado disminuciones en los niveles de fosfato sérico ni variaciones en la estabilidad hemodinámica, cuando se comparó la HDF on-line con la HD convencional (Jean et al., 2015). En cambio, un estudio reciente sugiere que la HDF on-line reduce la mortalidad en comparación con la hemodiálisis convencional (Maduell et al., 2013).

Lo cual demuestra que los beneficios o no de una terapia de remplazo renal pueden variar dependiendo de los marcadores utilizados.

En el caso de la HDF on-line, como utiliza grandes cantidades de solución de sustitución, se pueden esperar los siguientes efectos: (a) la supresión de la amplificación de los radicales hidroperóxidos y la supresión de la amplificación de radicales hidroxilo, y (b) la supresión de la oxidación de las grasas por los propios AGES. Estos efectos anti-radicales se presume que se deben a la eliminación eficaz de proteínas portadoras de radicales, y proteínas desnaturalizadas, por la el uso de grandes cantidades del líquido de solución de sustitución (Tomo et al., 2004).

Finalmente, debido a que no se observaron diferencias entre los parámetros clínicos de los pacientes tratados con HDF on-line y los que continuaron con la HD convencional se podría inferir que las sutiles diferencias observadas pueden deberse más al tratamiento que a otra posible variante, hecho que podría estar relacionado con la pequeña disminución observada en los niveles de daño oxidativo en pacientes tratados con HDF on-line.

Daño genómico en pacientes sometidos a trasplante renal

Los pacientes en el último estadio de la ERC deben ser sometidos a diferentes terapias de remplazo renal, dentro de las cuales están los diferentes tipos de diálisis y el trasplante. En el Artículo 1 se demostró un aumento del daño en el DNA en los pacientes en HD. Además se ha citado un descenso en la capacidad de reparación del DNA en los linfocitos irradiados de los pacientes con ERC, ya sea en PD o HD (Rodríguez-Ribera et al., 2015), e igualmente se ha observado un aumento significativo en las frecuencias de intercambios entre cromátidas hermanas junto con una reducción significativa en los índices mitóticos en pacientes con ERC, en comparación con individuos sanos (Lialiaris et al., 2010). A nivel mundial, la tasa de mortalidad en diálisis es de 10-15% por año, en comparación con el 2-4% en pacientes con trasplante renal (TR) (Webster et al., 2007). Esta intervención se ha reconocido como el mayor avance en la lucha contra la enfermedad, al proporcionar años de supervivencia con una elevada calidad de vida en todo el mundo. El registro de la UNOS (*United Network for Organ Sharing*) muestra que la supervivencia de paciente e injerto a 5 años es del 80,7% y 65,7% para el trasplante renal de donante

cadáver, y de 90,1% y 78,6% para el trasplante renal de donante vivo (Cecka, 2005). Los beneficios en la supervivencia de pacientes con trasplante renal (TR) sobre la terapia de diálisis están bien establecidos para los pacientes con enfermedad renal terminal (Goldfarb-Rumyantzev et al., 2005). El trasplante renal (TR) es el tratamiento comúnmente usado en la etapa 5 de la ERC. Además, se ha indicado que el riesgo de muerte por TR es menos de la mitad que el de los pacientes de diálisis (Kidney Disease: Improving Global Outcomes (KDIGO) Transplant Work Group, 2009). Es importante tener en cuenta que, además de la reducción en el riesgo de muerte, el trasplante conduce a un aumento significativo en la esperanza de vida estimada del paciente. Los estudios sugieren que los receptores de trasplante de edad avanzada aunque tienen una ventaja de supervivencia menor que la de trasplantados más jóvenes tienen asegurada su supervivencia libre de diálisis (Oniscu et al., 2004).

El Registro de Enfermos Renales de Cataluña, adscrito a la Organización Catalana de Trasplantes indica, en el registro de enfermos renales de Cataluña, reportó 28.719 pacientes trasplantados hasta diciembre de 2013. Entre los enfermos hay un predominio de hombres (62,7%) y los grupos de edad con mayor número de casos son los de 55 a 79 años en ambos sexos, lo que representa un 55,4% del total de pacientes (Generalitat de Catalunya, Departament de Salut, 2015). A pesar del éxito de esta terapia, el trasplante de riñón no está exento de riesgos, ya que los receptores tienen un mayor riesgo de enfermedades cardiovasculares, cáncer e infecciones. La tasa de mortalidad relativa para los receptores de riñón después del primer año postrasplante sigue siendo 4-6 veces mayor que la de la población general, con un 33 % de muertes atribuibles a la enfermedad cardiovascular y un 32% al cáncer (Webster et al., 2007). Aunque hay diversos estudios encaminados a determinar los niveles de daño genómico en los pacientes con ERC, en sus diferentes estadios, tanto PD como HD (Stopper et al., 2001; Stopper et al., 2005; Kobras et al., 2006; Schupp et al., 2008; Buemi et al., 2010; Sandoval et al., 2010; Stoyanova et al., 2010; Coll et al., 2013), éstos no contemplan al colectivo de los pacientes con ERC trasplantados. Ante esta falta de información, se hace evidente la necesidad de dichos estudios.

En este sentido en el Artículo 4 se planteó analizar mediante el ensayo del cometa y el ensayo de micronúcleos, la evolución de los niveles de daño genómico de estos

pacientes. Para ello se siguieron a 53 pacientes sometidos a trasplante renal durante un año. Todos los pacientes tenían menos de 75 años de edad, no presentaban comorbilidades importantes a nivel cardíaco, pulmonar, hepático y neurológico, y poseían una TFG inferior a 12 mL/min/1,73 m^2. Los pacientes después de seis meses del trasplante de riñón vieron normalizados sus niveles de fósforo, HDL y presión arterial. Además, los niveles de urea se redujeron casi a la mitad aunque sin alcanzar los niveles normales. Un año después del trasplante, el fósforo, HDL, y la presión arterial (sistólica y diastólica) mostraron una mejora en comparación con los niveles antes de la intervención. La mejora en el perfil lipídico puede deberse al tratamiento con estatinas, tal y como se ha visto en estudios previos. El uso de estatinas podría atenuar la vasoconstricción asociada con la disfunción endotelial (Douglas et al., 2006) y reducir significativamente las concentraciones de lípidos en pacientes con ERC (Strippoli et al. 2008). En contraste con lo anterior, no se observaron cambios significativos en los parámetros bioquímicos en los últimos 6 meses del seguimiento. Sin embargo, si se observaron mejoras sustanciales a los seis meses y un año después de la intervención, en comparación con los niveles antes del trasplante.

En cuanto a los niveles de daño genómico, se encontró que éstos aumentan con el tiempo. Sin embargo, sólo fueron estadísticamente significativos al comparar los niveles de daño antes y después de un año del trasplante, tanto para el daño oxidativo en el DNA como para la frecuencia de células binucleadas con micronúcleos. En los pocos casos que se obtuvo información a los 24 meses después del trasplante, pudimos observar un aumento de los niveles de daño genómico total. A partir de nuestros resultados, sugerimos que, a pesar de la mejora metabólica de los pacientes con trasplante de riñón, el daño genómico continuó aumentando, llegando a ser significativamente mayor que el obtenido antes del trasplante un año después de la intervención.

Lo anterior estaría de acuerdo con lo observado en otro estudio con pacientes con rechazo intersticial agudo, donde las células tubulares proximales mostraron fragmentación del DNA y tinción positiva para 8-OHdG, lo que sugiere estrés oxidativo (Ott et al., 2007). Además, se ha demostrado que el número de roturas de doble cadena en células endoteliales glomerulares se correlaciona positivamente con la duración del período postraplante, lo que sugieren que a largo plazo el TR

induce roturas de doble cadena en el DNA y acumulación de colágeno tipo VI en los capilares glomerulares, pudiendo progresar a fibrosis glomerular (Matsui et al., 2015). Lo que podría verse reflejado en los niveles de daño oxidativo incrementado en los pacientes estudiados.

Cabe recordar que el trasplante supone la inmunosupresión de los pacientes. En nuestro grupo de estudio, la inmunosupresión se basó en la administración de prednisona, que inhibe la proliferación de linfocitos activados por la presencia de aloantígenos del injerto (Ramírez López, 2015) y corticosteroides, que inhiben la interleucina 1 por los monocitos, y que afectan selectivamente a los primeros eventos inmunorreguladores (Cupps y Fauci, 1982). Algunos estudios muestran un mayor riesgo de rechazo agudo en aquellos pacientes a los que se retiraron los corticoesteroides (Marcén, 2009). Los inmunosupresores tacrolimus y micofenolato mofetilo, han demostraron reducir la tasa de mortalidad en los receptores de trasplante renal (Gonzalez-Molina et al., 2012), mientras que el micofenolato sódico se ha mostrado útil para reducir complicaciones gastrointestinales en los pacientes tratados con micofenolato mofetilo (Burg et al., 2009). Éste ha sido el tratamiento que se administró en el 98% de los casos durante los primeros días del trasplante, y continuó sin cambios en el 71,7% de los pacientes a los 6 meses después del trasplante, y en el 67% de los casos después de un año. Además, en los casos que fue necesaria una mayor inmunosupresión, en el momento del trasplante se administró suero anti-linfocitos, timoglobulina en el 45,3% de los pacientes, la globulina antitimocitos (ATG) en el 11,3% y el basiliximab en el 26,4%, mientras que en el 17% de los pacientes no fue necesario este tratamiento.

La tasa de proliferación celular (CBPI) es un indicador de toxicidad. En nuestro estudio ésta disminuyó tanto a los 6 meses como al año del trasplante Esto podría asociarse con la inmunosupresión dado que el micofenolato mofetilo (MMF) actúa como un inhibidor selectivo de la inosina monofosfato deshidrogenasa, conocida por inhibir la síntesis de DNA en linfocitos T y B mediante el bloqueo de la síntesis de guanosina, lo que podría producir inhibición de la proliferación celular y un aumento de la tasa de apoptosis (Cohn et al., 1999). En experimentos *in vitro* se ha visto que el MMF causa retraso en el ciclo celular en cultivos de linfocitos. Esto estaría de acuerdo con lo observado en un estudio previo con pacientes después de un trasplante renal, donde solamente entraron en división mitótica el 53% de los

cultivos de linfocitos (Rath y Oliveira-Frick, 2009). Esto reflejaría el efecto antiproliferativo de la terapia inmunosupresora aplicada; sin embargo, también puede aludir a la mutagenicidad de los fármacos aplicados (Rath y Oliveira-Frick, 2009). Un estudio reciente ha demostrado que tanto el tacrolimus como el MMF inhiben la proliferación celular, incrementan los radicales libres, la producción de la peroxidación lipídica, inducen lesiones en el DNA y reducen el potencial de membrana mitocondrial (Ferjani et al. 2015), lo cual explicaría el daño genómico presente en pacientes con trasplante de riñón.

Un último aspecto de este artículo ha consistido en evaluar la importancia del origen del órgano trasplantado, dado que el tipo de donante es una variable importante de la supervivencia del injerto. En un estudio realizado en los Estados Unidos, donde se evaluó la supervivencia superior a cinco años después de trasplante de riñón, se obtuvo que el 59,9% de los pacientes habían recibido un injerto de donante vivo, respecto el 40,3% que lo habían recibido de un donante fallecido (Macrae et al., 2005). Un estudio español de hace un par de años obtuvo un 75,9% de supervivencia en pacientes trasplantados con injerto proveniente de donante fallecido, frente al 93,6% de supervivencia con injertos provenientes de donantes vivos (Gil et al., 2014). En nuestro estudio hemos observado que seis meses después de la cirugía, el daño oxidativo fue significativamente mayor en los pacientes que recibieron el trasplante de donante fallecido, respecto a los que recibieron injerto de donante vivo. Igualmente, un año después del trasplante, los niveles de BNMN resultaron ser significativamente mayores en los pacientes que recibieron injertos de donante fallecido respecto al donante vivo. Por lo tanto, se sugiere que los niveles más bajos de daño en el DNA están en los pacientes que recibieron trasplantes de riñón de donante vivo en comparación con el trasplante renal de donante fallecido.

Según distintos estudios existen diversos factores que podrían contribuir a explicar la mejor supervivencia y filtrado glomerular de los trasplantados a partir de donante vivo. Entre éstos está la mejor compatibilidad HLA y la edad del receptor del donante (Guirado et al., 2008). Debido a que la mayoría de donantes se seleccionan dentro del ámbito familiar, se podría obtener un mayor número de compatibilidades HLA. Por otra parte está la menor edad del receptor dado que la media de edad de los receptores de donante vivo fue de 44,4 ± 2,50 años, unos 10 años, inferior a la

edad media de los receptores de donante fallecido años (54,8 ± 2,53). Asimismo, la edad promedio de los donantes vivos fue inferior a la edad promedio de los donantes fallecidos (53,03 ± 1,75 *vs* 60,76 ± 3,16). En este sentido, se observó una correlación positiva entre la edad del receptor y los niveles de BNMN antes del trasplante así como a los 6 y 12 meses después. Igualmente la edad del donante se correlacionó positivamente con los niveles de BNMN, a los 6 y 12 meses postraplante. Por lo que se refiere a los índices de proliferación celular después de un año del trasplante, éste mostró ser mayor en los receptores más jóvenes, lo que sugeriría que receptores más jóvenes presentan menor inestabilidad genómica postrasplante.

De este análisis se puede concluir que, efectivamente, hay un incremento en los niveles de daño en el DNA y en la inestabilidad genómica, así como una disminución en la proliferación linfocitaria, en pacientes con trasplante de riñón. Estos valores llegan a ser significativos un año después de la intervención, aunque esto no afectaría a la mejora metabólica que presentan estos pacientes.

Variantes genéticas asociadas con la enfermedad renal crónica en una población española

Además de los conocidos factores de riesgo, demográficos, bioquímicos y de estilo de vida, las características genéticas de los pacientes también contribuyen al riesgo de padecer ERC y a su pronóstico. En los últimos años los estudios de asociación del genoma completo (GWAS) han proporcionado un nuevo e importante enfoque para identificar variantes genéticas comunes que podrían explicar el componente de riesgo genético de enfermedades como la enfermedad renal crónica. La identificación de estas variantes podría revelar los procesos biológicos de la insuficiencia renal subyacente y podría ayudar en la mejora de las estimaciones de riesgo de la ERC. El componente genético de la enfermedad renal crónica se ha demostrado en estudios de agregación familiar. En familias con diabetes o hipertensión se estiman heredabilidades para la ERC de 0,30-0,44 y 0,49, respectivamente. Para la tasa de filtración glomerular (TFG) los valores de heredabilidad son de 0,45-0,75 en familias con miembros diabéticos (Satko et al., 2007). Sin embargo, la marcada variabilidad en el desarrollo de la enfermedad renal crónica en individuos con hipertensión y diabetes demuestra que otros factores

subyacentes adicionales contribuyen a su etiología. Recientemente se han identificado variantes de susceptibilidad para la función renal e insuficiencia renal crónica en *UMOD* (*Uromodulin*), un inhibidor de la cristalización constitutiva del calcio en los fluidos renales, en *SHROOM3* (*shroom family member 3*), que puede estar implicado en la regulación de la forma celular en ciertos tejidos y en *STC1* (*stanniocalcin 1*), que puede desempeñar un papel en la regulación de calcio renal e intestinal y en el transporte de fosfato, así como en el metabolismo celular, o homeostasis celular de calcio/fosfato. En conjunto, polimorfismos de un sólo nucleótido (SNPs) en estos *loci* explican sólo el 0,43% de la varianza en la tasa de filtrado glomerular, lo que sugiere que aún faltan por identificar muchos loci (Köttgen et al., 2010c).

En el Artículo 5 hemos estudiado 38 SNPs de 31 genes diferentes, en un total de 722 adultos españoles, siendo 548 pacientes con ERC (TFG<60 mL/min/1,73 m^2) y 174 controles sanos. Los genes seleccionados están relacionados con procesos patológicos característicos de la ERC como citoquinas (*IL-1A, IL-4, IL-6, IL10, TNF-α* y *ICAM-1*), o se habían encontrado asociados con la ERC en estudios GWAS (*GLO1*, *SHROOM3, UMOD, VEGFA,* y *SLC7A9)* (Köttgen et al., 2009; Köttgenb, 2010; Köttgen et al., 2010c; Böger y Heid, 2011). Otros genes seleccionados estaban implicados en el sistema renina-angiotensina-aldosterona (*AGT* y *CYP11B2*), en proteínas implicadas en la fibrogénesis (*TGFB1*), o en la síntesis de homocisteína (*MTHFR*). También se incluyeron algunos genes que codifican para enzimas antioxidantes (*SOD1, SOD2, CAT, GPX1, GPX3* y *GPX4*), o genes implicados en las vías de reparación del DNA (*ERCC2, ERCC4, OGG1, MUTYH y XRCC1),* o en genes del metabolismo de fase II *(GSTP1, GSTO1 y GSTO2*). Por último también se incluyeron genes relacionados con la mortalidad en pacientes en hemodiálisis, con la calcificación vascular y con el envejecimiento *(KL y MGP*) (Friedman et al., 2009; Wang et al., 2013). En los casos en que no fue posible hacer el genotipado con el sistema TaqMan SNP genotyping assays de Life Technologies, se remplazó el SNP original por uno con desequilibrio de ligamiento mayor de 0,90 (Artículo 5, Tabla 2).

De los 38 SNPs candidatos seleccionados, los genes que mostraron asociación con la ERC fueron: *GPX1, GSTO1, GSTO2, UMOD* y *MGP.* GPX1 es la principal isoforma de GPX que se expresa en el riñón normal, representando el 96% de la

actividad GPX, y tiene un papel protector contra el estrés oxidativo (De Haan et al., 1998). El polimorfismo estudiado genera una variante asociada con una reducción de 40% de la actividad de GPX1 y confiere una elevada susceptibilidad a tumores (Takata et al., 2012). Este SNP se ha asociado significativamente con la densificación mineral de la cadera (Mullin et al., 2009) y se ha sugerido que *GPX1* es un posible gen candidato para el riesgo de ECV (Zhang et al., 2014) que, como se ha indicado anteriormente, es una patología fuertemente ligada a la ERC.

Las glutatión transferasas (GST) son enzimas de desintoxicación que juegan un papel importante en la conjugación de toxinas xenobióticas endógenas o exógenas al glutatión (GSH). La familia de las GST citosólicas incluye diferentes clases, como la clase Omega (GSTO) (Hayes et al., 2005). La proteína GSTO1, está altamente expresada en células del hígado, en macrófagos, células gliales, y endocrinas, y está codificada por un único gen (*GSTO1*). El gen que codifica para la glutatión S-transferasa omega 2 (*GSTO2*) puede presentar la transición A>G en la posición 424 en el exón 4. Este SNP provoca la sustitución Asn142Asp que altera la función de la enzima GSTO2 (Rezazadeh et al., 2015). Por lo tanto, polimorfismos en estos genes podrían influir en el nivel de estrés oxidativo (Whitbread et al., 2005). Los polimorfismos estudiados en *GSTO1* y *GSTO2* se asocian con un peor pronóstico y menor supervivencia en pacientes con cáncer de vejiga (Djukic et al., 2013), lo que coincidiría con nuestros resultados. Aunque también se ha indicado que el genotipo mutante para *GSTO1* tiene función protectora en cáncer de mama (Chariyalertsak et al., 2009).

El gen *UMOD* codifica para la proteína uromodulina, la cual actúa como inhibidor de la cristalización constitutiva de calcio en los fluidos renales (Rampoldi et al., 2011). El SNP rs12917707 de *UMOD* se ha asociado con la tasa de filtración glomerular y una mejor función renal (Köttgen et al., 2009; Gorski et al., 2014). Siete SNPs en el extremo 5´- del gen *UMOD*, fuertemente ligados a nuestro SNP también se han asociado con la ERC (Köttgen, 2010). Muchos estudios corroboran la asociación entre *UMOD* y la ERC (Reznichenko et al., 2012). Estudios previos han demostrado que mutaciones en *UMOD* contribuyen a la nefropatía juvenil familiar, a la hiperuricemia, y a la enfermedad renal quística medular 2 (Hart et al., 2002). Además, variantes en el promotor del gen *UMOD* se han asociado con los niveles de presión sanguínea, y de ácido úrico en plasma (Padmanabhan et al., 2010),

observándose que los pacientes con el genotipo GG, presentan dos veces más uromodulina en orina que los portadores de los otros genotipos (Olden et al., 2014).

El gen *MGP* codifica la proteína gla de la matriz extracelular que actúa como un inhibidor de la formación del hueso. El SNP rs4236 se encuentra en el exón 4 del gen y la transición del alelo T a C conduce a un cambio de aminoácido de Thr a Ala, que altera la modificación postraduccional de la proteína, y afecta a su función (Lu et al., 2012). Esto da como resultado una mutación sin sentido que influye en el proceso de calcificación y que afecta a las placas ateroscleróticas (Herrmann et al., 2000). La variante se ha asociado con una disminución de la calcificación de las arterias coronarias indicándose que los portadores del alelo C tenían 1,39 veces menor riesgo de cálculos renales en comparación con los no portadores (Crosier et al., 2009). En este contexto, nuestros resultados son consistentes con los citados en la literatura, mostrando un ligero aumento del riesgo en pacientes con ERC.

Dado que en los pacientes con ERC se observa una alta incidencia de hipertensión (HT), enfermedades cardiovasculares (ECV), diabetes, y cáncer, se buscaron asociaciones entre los polimorfismos seleccionados y estos parámetros. Los resultados indican asociación entre HT y los genes *GPX4* (rs713041), *CYP11B2* (rs1799998) y *ERCC4* (rs3136166).

El gen *GPX4* está implicado en la protección de las células contra el daño oxidativo. La glutatión peroxidasa (GPx), de hidroperóxidos en fosfolípidos (GPX4) es una selenoproteína intracelular expresada en la mayoría de los tejidos, que reduce los hidroperóxidos de lípidos y regula la biosíntesis de leucotrienos y la señalización de citoquinas vía GPX4 (Brigelius-Flohé, 1999). El polimorfismo rs713041 causa una sustitución de C-T en la región 3'-UTR del gen (Bermano et al., 2007). En nuestra población, el alelo recesivo T mostró asociación con la HT en pacientes con ERC. Sin embargo, datos obtenidos en una población japonesa no revelaron asociación entre polimorfismos en *GPX4* y la ERC, lo que sugeriría una componente étnica importante (Hishida et al., 2013).

El gen *CYP11B2* codifica la aldosterona sintasa, una enzima del citocromo P450 que cataliza los pasos terminales de la síntesis de aldosterona en las células de la zona glomerular de la glándula suprarrenal (Kawamoto et al., 1992). Se han descrito diversos polimorfismos en el gen *CYP11B2,* entre ellos, el polimorfismo

rs179998, que se encuentra en un sitio de unión putativo para el factor de transcripción esteroidogénico 1 (SF-1), relacionado con las enfermedades cardiovasculares (Alvarez-Madrazo et al., 2013). Algunos estudios han sugerido que el alelo G de este polimorfismo está asociado con la predisposición genética a la enfermedad cardiovascular (Delles et al., 2001), aunque existen resultados contradictorios (Munshi et al., 2010). En nuestro estudio el polimorfismo se encuentra asociado con un mayor riesgo de hipertensión en los pacientes con enfermedad renal crónica.

Aunque el SNP rs3136166 del gen *ERCC4* (involucrado en la vía de reparación por escisión de nucleótidos, NER), ha mostrado asociación con la HT, no existen datos en la literatura que liguen ambos parámetros. Sin embargo, este polimorfismo muestra asociación significativa con el cáncer, asociándose con el carcinoma de células escamosas de cabeza y cuello (Vaezi et al., 2011; Wyss et al., 2013), el cáncer de mama (Milne et al., 2006) y el cáncer gástrico (Chu et al., 2013). En nuestro caso se ha asociado con pacientes con antecedentes de cáncer, lo que demuestra la importancia de la ruta NER en la patología de la enfermedad renal.

Respecto a la enfermedad cardiovascular: se observó asociación con los genes *AGT* (rs5050) y *ERCC2* (rs1799793). El producto del gen *AGT*, el precursor angiotensinógeno AGT, se expresa en el hígado y se escinde por la enzima renina en respuesta a una presión sanguínea baja. El sistema renina-angiotensina-aldosterona desempeña un papel crucial en la regulación del tono vascular y la presión arterial. Nuestros resultados demuestran que la presencia del alelo mutante aumenta el riesgo de enfermedades cardiovasculares en los pacientes con ERC. Este resultado es consistente con lo obtenido en otro estudio donde este polimorfismo se asoció con un mayor riesgo de deterioro de la función renal cuando dos alelos de riesgo estaban presentes (Worobey et al., 2009).

El gen *ERCC2*, que es un componente esencial de la vía NER de reparación del DNA, mostró asociación con el aumento del riesgo de enfermedad vascular. Aunque este polimorfismo no se ha relacionado directamente con la insuficiencia renal, sí que se ha relacionado con distintos cánceres como el cáncer oral (Zhang et al., 2013), como es habitual con genes de la ruta NER.

La incidencia de cáncer en pacientes con ERC se ha asociado con el gen *MTHFR*, y además con los genes *AGT* y *ERCC2*, anteriormente citados. El gen *MTHFR* codifica para la metilentetrahidrofolato reductasa, que es una enzima folato-dependiente que juega un papel importante en la conversión de homocisteína a metionina (Frosst et al., 1995). Según el genotipo recesivo se observó un aumento del riesgo de DM en pacientes con enfermedad renal crónica homocigotos para la variante del polimorfismo rs1801133. Variantes del gen *MTHFR* ya se habían encontrado asociadas con la susceptibilidad a la DM2 (Wang et al., 2014) y con un aumento del riesgo de la enfermedad arterial (Chen et al. 2014) indicándose que en sujetos con diabetes, el alelo mutante incrementa el riesgo de nefropatía diabética (El-Baz et al., 2012). Hay que indicar que los polimorfismos previamente indicados de los genes *AGT* y *ERRC2* también mostraron asociación con la DM2.

Por lo que respecta a la asociación con la incidencia de cáncer nuestro estudio ha encontrado asociación con el polimorfismo rs13181 (T>G) del gen *ERCC2*. Este SNP se ha encontrado asociado con un mayor riesgo de leucoplasia oral, cáncer de esófago (Zhu et al., 2014) o cáncer de cabeza y cuello (Wyss et al., 2013).

Cuando se han buscado asociaciones con distintos parámetros clínicos relacionados con la ERC, hemos encontramos asociaciones significativas para diferentes polimorfismos de genes involucrados en múltiples rutas. Con la finalidad de visualizar de manera más simple los resultados obtenidos, estos se indican de forma simplificada en la Tabla 5.

Así, para los niveles de creatina se han encontrado asociaciones para cuatro genes, *GPX1* (rs17080528), *GSTO1* (rs2164624), *GSTO2* (rs156697) y *KL* (rs577912).

El gen *GPX1* está implicado en diferentes procesos cardiovasculares (Polonikov et al., 2012, Méplan et al., 2013, Zhang et al., 2014) y, en nuestro caso, el polimorfismo estudiado aumentó el riesgo de presentar altos niveles de creatinina en pacientes con ERC. Los polimorfismos de los genes *GSTO1* y *GSTO2* también mostraron asociación con los niveles de creatinina encontrándose las formas variantes ligeramente más representadas en pacientes con niveles de creatinina inferior a 100 µmol/L. Estos polimorfismos se han encontrado asociados con mayor riesgo al rechazo del injerto renal (Nekooie-Marnany et al., 2013).

Tabla 5. SNPs asociados con diferentes parámetros clínicos de enfermedad renal crónica (ERC)

Gen	SNP	Parámetros Clínicos (Valor normal) (valor de corte) (N) *P*								
		Creatinina (45-80 µmol/L) (100 µmol/L)*	TFG (>60mL/min/1,75m²) (60mL/min/1,75m²)*	Hemoglobina (120-160g/L) (130g/L)*	IRE (<10) (10)*	Albúmina (37-47 g/L) (41,70 g/L)*	Fósforo (0,8-1,3mol/L) (1,19mol/L)*	HPT (7-53ng/L) (123,5ng/L)*	PCR (<10mg/L) (10 mg/L)*	Ferritina (25-250ug/L) (154,5ug/L)*
AGT	rs5050	(84) **0,021**								
GPX1	rs17080528	(103) **0,009**	(136) **0,004**							
GPX4	rs713041									(95) **0,036**
GSTO1	rs2164624	(102) **0,001**	(135) **0,007**							
GSTO2	rs156697	(115) **0,017**				(115) **0,045**				
GSTP1	rs749174								(14) **0,046**	(92) **0,017**
ERCC2	rs171140			(125) **0,006**	(118) **0,034**	(118) **0,017**		(125) **0,026**		
ERCC2	rs1799793								(86) **0,036**	
ERCC4	rs3136166						(27) **0,023**			
ICAM-1	rs5498						(123) **0,040**			
IL-4	rs2070874						(40) **0,040**			
IL6	rs1800797							(71) **0,029**		
TGFB1	rs1800468								(3) **0,033**	
OGG1	rs1052133				(62) **0,036**					
SOD1	rs17880135	(17) **0,046**				(20) **0,008**				
SOD1	rs202446					(54) **0,001**				
SOD1	rs1041740					(97) **0,011**				
SOD2	rs4880				(116) **0,027**				(109) **0,018**	(107) **0,016**
KL	rs577912	(8) **0,024**	(6) **0,050**							
KL	rs1207568				(7) **0,028**					
MPG	rs4236									(82) **0,041**
SHROOM3	rs17319721							(130) **0,013**		
SLC7A9	rs12460876									(110) **0,028**
UMOD	rs12917707							(13) **0,033**		
VEGFA	rs881858				(99) **0,033**					

N= grupo de riesgo. * Valor de Corte normal o mediana. TFG: Tasa de filtrado glomerular, IRE: Índice de resistencia a la eritropoyetina, HPT: hormona paratiroidea, PCR: Proteína C-Reactiva.

El gen *Klotho* (*KL*) se ha relacionado con la supresión del proceso de envejecimiento, y se expresa predominantemente en el túbulo distal del riñón y el plexo coroideo del cerebro, y en menor grado en los órganos reproductivos y endocrinos (Kurosu et al., 2005), la glándula paratiroidea y el músculo esquelético (Izquierdo et al., 2012). La expresión del genotipo dominante del gen *KL* en el riñón, puede tener un papel importante en la patogénesis de las enfermedades renales (Koh et al., 2001). Nuestros estudios revelaron asociación entre este SNP y los altos niveles de creatinina, lo que contradice lo observado por Friedman et al. (2009) quienes asociaron el genotipo GG con una mayor mortalidad en pacientes en hemodiálisis crónica.

Respecto a las asociaciones con la tasa de filtrado glomerular (TFG), hemos encontrado asociaciones con cinco genes: *AGT* (rs5050), *SOD1* (rs17880135), *GPX1* (rs17080528), *GSTO1* (rs2164624) y *KL* (rs57791). Estos marcadores son muy dispares en su función, lo que demuestra la elevada complejidad de la enfermedad. Hay que tener en cuenta el bajo número de individuos con el que cuentan algunos análisis y, por lo tanto, es natural encontrar discrepancias con otras publicaciones (Izquierdo et al., 2012).

La principal causa de la anemia asociada con la ERC es la disminución de la eritropoyetina (EPO), mostrando estos pacientes una mala respuesta al tratamiento con Darbepoetina, consecuencia de su resistencia a la EPO. Cuando hemos tenido en cuenta parámetros relacionados con el estado anémico de los pacientes con ERC (niveles de hemoglobina e índice de resistencia a la eritropoyetina, IRE), hemos observaron asociaciones de cinco genes para un mayor riesgo de resistencia a la eritropoyetina: *SOD2* (rs4880), *VEGFA* (rs881858), *OGG1* (rs1052133), *KL* (rs1207568) y *ERCC2* (rs171140); este último también asociado con los niveles de hemoglobina. Por lo que respecta al gen *ERCC2,* aunque diferentes SNPs se han asociado con varias patologías crónicas como carcinoma de células renales (Hirata et al., 2006), glioma de adultos (Wrensch et al. 2005), y cáncer de pulmón (Chang et al. 2008), no se ha encontrado ningún estudio que lo relacione con la ERC. El polimorfismo rs4880 del gen *SOD2* causa un cambio conformacional en la secuencia diana, que supone una disminución del 30 al 40% en la actividad SOD2 (Shimoda-Matsubayashi et al., 1996; Sutton et al., 2003). Algunos estudios han demostrado la importancia de este SNP en patologías

renales, ya sea por un mayor riesgo o efecto protector, lo que puede variar en función de la población de estudio. Algunos autores atribuyen un efecto protector, asociado con el alelo mutante, con un menor riesgo de complicaciones diabéticas microvasculares (Tian et al., 2011); otros también han visto que el alelo mutante, que codifica para el aminoácido alanina, confiriere un efecto protector contra la microalbuminuria (Ascencio-Montiel et al., 2013). Por lo que hace referencia al gen *VEGFA*, éste se ha relacionado con la nefrogénesis (Köttgen et al., 2010ac). Este gen codifica para un factor de crecimiento endotelial vascular, que tiene un papel en la angiogénesis y la permeabilidad vascular y podocitos renales, que producen grandes cantidades de VEGFA, que es esencial para glomerulogénesis y la formación de la barrera de filtración glomerular en modelos animales (Eremina et al. 2007). Además, el gen *VEGFA* regula el crecimiento de la yema uretral durante la embriogénesis y, por lo tanto, puede afectar el número de nefronas (Karihaloo et al., 2005). En base a esta función, no es de extrañar la asociación encontrada con la ERC (Köttgen et al. 2010b). El gen *OGG1* también ha mostrado asociación con la resistencia a la EPO. Se cree que alteraciones en *OGG1* pueden influir en el desarrollo del estrés oxidativo y, de este modo, contribuir a la fisiopatología de muchas enfermedades crónicas y el cáncer. Si bien son muchas las variantes del gen *OGG1* identificadas, la variante Ser326Cys ha sido objeto de muchos estudios asociándose con muchos tipos de cáncer, incluyendo riñón, colon y pulmón (Weiss et al., 2005). Se ha encontrado que los individuos homocigotos del alelo menos frecuente tienen menor capacidad para reparar 8-OHdG que los indiciduos homocigotos para el alelo más frecuente, lo que contribuye al riesgo de cáncer (Lee et al., 2005). Recientemente, se ha observado que la variante Ser326Cys se asocia con una disminución de la sensibilidad a la insulina en sujetos con tolerancia normal a la glucosa, lo que sugiere que las alteraciones genéticas en *OGG1* pueden contribuir a la resistencia a la insulina y, potencialmente a la DM2 (Wang et al., 2006), lo que explicaría nuestros resultados. Finalmente hay que señalar el interés de la asociación encontrada con el gen *KL*. Éste juega un papel importante en el proceso de envejecimiento (Kuro-o et al., 1997; Christensen et al., 2006). Es interesante indicar que en los pacientes con insuficiencia renal crónica la expresión de *KL* en los riñones se encuentra reducida (Koh et al., 2001). Estos hallazgos

implicarían que la reducción de la proteína KL es relevante para la fisiopatología de la enfermedad renal.

En relación con la albuminuria, tres genes han mostrado asociaciones *SOD1* (rs17880135, rs202446, rs1041740), *GSTO2* (rs156697) y *ERCC2* (rs171140). Los polimorfismos de *SOD1* ya se han observado asociados con microalbuminuria persistente (Mohammedi et al., 2011, Al-Kateb et al., 2008, Neves et al., 2012), lo que estaría de acuerdo con nuestros resultados. No se han encontrado estudios que relacionen las asociaciones encontradas para los otros genes.

Por otro lado, anomalías en las concentraciones de fósforo sérico, son comunes en pacientes con ERC y se han asociado con una mayor morbilidad y mortalidad (Kestenbaum et al., 2005; Levin et al., 2007; Tentori et al., 2008). En nuestro estudio hemos observado asociación de los niveles de fósforo con tres genes *ICAM-1* (rs5498), *IL4* (rs2070874) y *ERCC4* (rs3136166). El gen *ICAM-1* codifica para una molécula de adhesión intercelular 1 (ICAM-1) que se expresa en niveles altos en las células endoteliales del riñón normal e interactúa con las integrinas ß2 y mejora la activación de células T (McLaren et al., 1999). Nuestros resultados estarían de acuerdo con un estudio previo donde se ha observado la asociación del genotipo menos frecuente en pacientes con enfermedad renal en etapa terminal respecto a los controles (Ranganath et al., 2009). Otro polimorfismo asociado fue el de la interleuquina *IL4*, que es un estimulador de crecimiento para las células B y T, entre otras (Mittal y Manchanda, 2007). Este polimorfismo se ha asociado con la función renal y la prevalencia de la ERC en una gran población japonesa (Okada et al., 2012b).

Dentro de las anormalidades endocrinas en los pacientes con ERC, se sabe que la deficiencia de calcitriol juega un papel importante en el desarrollo del hiperparatiroidismo. En nuestro trabajo hemos observado cinco genes asociados con las variaciones en los niveles de HPT: *IL1A* (rs1800587), *IL6* (rs1800797), *SHROOM3* (rs17319721), *UMOD* (rs12917707) y *ERCC2* (rs171140). IL1A es una citoquina proinflamatoria producida por monocitos, macrófagos y células epiteliales, en respuesta a la invasión microbiana, la inflamación y el daño tisular (Sáenz López et al., 2009). Polimorfismos en *IL-1* se han asociado con distintos tipos de cáncer (El-Omar et al., 2000; Zienolddiny et al., 2004; Grimm et al., 2004), aunque no se

ha asociado con el riesgo y la progresión de cáncer renal y de próstata (Sáenz López et al., 2009). Funcionalmente, la IL-6 regula la producción de moléculas de adhesión celular e interviene en la liberación de otras citoquinas implicadas en la respuesta inflamatoria (Moriyama et al. 1995). Estas citoquinas son factores clave en la malnutrición, la aterogénesis acelerada, y la morbilidad y la mortalidad en pacientes con ERC en hemodiálisis (Bologa et al., 1998). Este gen se ha asociado con el riesgo de enfermedad renal terminal (Mittal y Manchanda, 2007). El gen *SHROOM3* se ha asociado con la función renal e insuficiencia renal crónica (Köttgen et al., 2009), lo que corroboraría nuestros resultados de asociación con altos niveles de HPT. Finalmente, el gen *UMOD* también mostró asociación con los niveles de HPT. Este gen ya se ha encontrado asociado con la ERC (Lever y Sheer, 2010). Éste es un hallazgo interesante, ya que *UMOD* codifica la uromodulina (proteína de Tamm-Horsfall) que es la proteína más abundante en la orina (Devuyst et al., 2005) y se ha asociado de forma significativa con la tasa de filtración glomerular (Deshmukh et al., 2013).

La proteína C-reactiva (PCR) junto con otras proteínas, se origina en el hepatocito, al estímulo de citoquinas como IL-6, IL-1β, TNF-α, y se encuentra elevada en los pacientes con insuficiencia renal, considerándose como un predictor de la mortalidad por eventos cardiovasculares en pacientes con ERC (Menon et al., 2005). En nuestro estudio hemos obtenido asociación de la PCR con cuatro genes: *TGFβ1* (rs1800468), *GSTP1* (rs749174), *ERCC2* (rs1799793) y *SOD2* (rs4880). El factor de crecimiento transformante beta (TGFβ1), es una citoquina multifuncional implicada en la patogénesis de muchas formas de enfermedad renal progresiva, incluyendo la nefropatía diabética (Syukri et al., 2014) y los niveles de TGFβ1 están incrementados significativamente en el glomérulo renal y túbulo-intersticial en pacientes con diabetes (Reeves y Andreoli, 2000). Este papel relevante justificaría la asociación observada en nuestro estudio.

Uno de los principales biomarcadores para la evaluación del metabolismo del hierro en pacientes con anemia y ERC es la ferritina sérica (Hemodialysis Adequacy 2006 Work Group). Ésta refleja el hierro almacenado y, de acuerdo con las directrices KDOQI, indica que la deficiencia de hierro se correlaciona con los niveles de ferritina sérica (Wish, 2006). Cinco genes mostraron asociación con este parámetro: *SOD*2 (rs4880), *GSTP*1 (rs749174), *SLC7A9* (rs12460876), *MPG* (rs4236) y *GPX4*

(rs713041). Un estudio reciente ha reconocido que la ferritina y SOD juegan un papel en la carcinogénesis pulmonar y en la supervivencia de los pacientes, encontrándose mayores niveles de ferritina y SOD en las células tumorales (Carpagnano et al., 2012). El gen *SLC7A9* se ha asociado con la función renal y la insuficiencia renal crónica (Köttgen, 2010a), al igual que sucede con otros genes también relacionados con el trasporte de solutos (Köttgen et al., 2010b). Esta importante función apoyaría el interés de la asociación encontrada en nuestro trabajo. La proteína GLA de la matriz (MGP) es un regulador clave de la calcificación vascular por lo que variaciones en este gen podrían modular el desarrollo de la calcificación de las arterias coronarias. Un estudio previo ha revelado una asociación entre los portadores del alelo menos frecuente, y una disminución de la calcificación de las arterias coronarias (Crosier et al., 2009; Wang et al., 2013). Finalmente el gen *GPX4* ha mostrado una asociación estadísticamente significativa con un mayor riesgo de tener niveles altos de ferritina en pacientes con ERC. Nuestro SNP se ha asociado con un mayor riesgo de cáncer colorectal (Méplan et al., 2010) y de infarto cerebral en pacientes hipertensos (Polonikov et al., 2012). Es de destacar el estudio que ha mostrado un efecto protector del alelo menos frecuente para la disfunción renal en sujetos con DM1 (Monteiro et al., 2013), aunque nuestros resultados indican que este polimorfismo aumenta la probabilidad de tener altos niveles de ferritina en pacientes con ERC.

Variantes genéticas asociadas con la enfermedad renal crónica en la población española

Nuestros estudios de biomonitorización de pacientes con ERC han demostrado que éstos presentan mayores niveles de daño genómico que los individuos sin la patología (Sandoval et al., 2012; Corredor et al., 2015; Rodríguez-Ribera et al., 2015). En este contexto, es interesante identificar polimorfismos genéticos asociados con este hecho.

En este trabajo hemos observado correlaciones significativas entre los valores de daño genómico y diferentes parámetros bioquímicos característicos de la ERC, como la ferritina y la presión arterial. Lo cual era de esperar debido al estrés celular producido por el metabolismo del hierro y las patologías cardiovasculares presentes

en los pacientes (Cakmak Demircigil et al., 2011). Además, también hemos encontrado asociaciones con los niveles de hormona paratiroidea (HPT). Por otra parte, los marcadores de daño cromosómico (BNMN) han mostrado correlación directa con los niveles de urea, y correlación inversa con los niveles de filtrado glomerular. Estos resultados corroboran lo publicado anteriormente, relacionando la frecuencia de MN en los pacientes con ERC con la progresión de la enfermedad, de acuerdo con la tasa de filtrado glomerular (Sandoval et al., 2010). Es conocido que se necesita un sistema complejo de enzimas de reparación para proteger el genoma de los diferentes agentes genotóxicos, tanto de origen exógeno como endógenos (Hoeijmakers, 2001). Además, se sabe que la deficiencia en enzimas de reparación aumenta el riesgo de desarrollar neoplasias (Kraemer et al., 1994). En base a ello se ha postulado que los polimorfismos en genes de reparación del DNA reducen su capacidad para reparar el daño y, de este modo, incrementan la probabilidad de cáncer u otras enfermedades (Ladiges y Wiley, 2003; Stern et al., 2006, Trabulus et al. 2012), estableciendo a los genes de reparación del DNA como piezas clave en el desarrollo de enfermedades crónicas (Hakem, 2008).

Nuestros resultados muestran la existencia de asociaciones significativas entre algunos de los genes seleccionados, con el daño genómico en pacientes con ERC (Tabla 6).

Tabla 6. SNPs asociados con daño genómico en pacientes con enfermedad renal crónica (ERC)

Gen	SNP	Parámetros de daño genómico (valor de corte) (N) *P*			
		Daño genómico (% DNA en cola) (13,46)*	Daño oxidativo (Daño genómico–FPG) (8,59)*	BNMN (6)*	BNMN neto (BNMN 0,5Gy–BNMN) (21)*
AGT	rs5050			(9) **$4,01E^{-08}$**	(7) **0,014**
GLO1	rs4746		(49) **0,011**		
SHROOM3	rs17319721		(134) **0,038**		(143) **0,042**
XRCC1	rs25487	(111) **0,018**	(39) **0,023**		(158) **0,018**
ERCC2	rs171140				(65) **0,045**
ERCC2	rs13181				(39) **0,013**

N= grupo con altos niveles de daño. * Valor de Corte: mediana.

En estas asociaciones intervienen genes tanto de reparación: *ERCC2* (rs13181, rs171140) y *XRCC1* (rs25487), como otros no implicados directamente en la misma: *AGT* (rs5050), *GLO1* (rs4746) y *SHROOM3* (rs17319721). Dentro de los

SNPs de genes de la vía BER, que mostraron asociación con el daño genómico aparece el gen *XRCC1*. La proteína codificada por este gen está implicada en la reparación de roturas de cadena simple, que son las lesiones más frecuentes en el DNA (Brem y Hall, 2005). Se conoce que el polimorfismo rs25487 del gen *XRCC1* está relacionado con la susceptibilidad a diferentes tipos de cáncer (Przybylowska et al., 2013, Tengström et al., 2014). Nuestros resultados indican que la presencia del alelo menos frecuente está significativamente relacionada con niveles más altos de daño genómico y de daño oxidativo, evaluado con el ensayo del cometa. También se ha encontrado asociación con el daño inducido por la irradiación, evaluado mediante la técnica de MN, en los pacientes con ERC. De todo ello podemos sugerir que el SNP rs25487 además de estar estrechamente relacionado con la susceptibilidad a diferentes tipos de canceres, podría también tener relación con la susceptibilidad a la ERC.

El gen *ERCC2* es uno de los genes más comunes en el proceso de reparación por escisión de nucleótidos, y forma parte (XPD) de los genes involucrados en la patología conocida como *Xeroderma pigmentosum*. La proteína de este gen elimina y corrige fragmentos de oligonucleótidos en multitud de lesiones, tales como lesiones inducidas por UV, aductos en el DNA y enlaces cruzados (Duell et al., 2000), siendo a su vez un miembro integral del complejo factor de transcripción BTF2/TFIID (Hu et al., 2012). En nuestro estudio los SNPs seleccionados han mostrado asociación con el daño genético en pacientes con ERC. Diversos estudios han mostrado que el polimorfismo rs13181 se asocia significativamente con mayor riesgo de melanoma (Li et al., 2006, Tomescu et al., 2001), lo que explicaría nuestros resultados previos, donde observamos que el daño genético inducido por la radiación fue significativamente mayor en los pacientes con ERC que en los controles (Rodríguez-Ribera et al., 2015). En su momento sugerimos la existencia de una base genética subyacente que modularía los niveles de daño en el DNA causado por irradiación en pacientes con ERC. Así, la predicha base genética podría deberse al polimorfismo rs13181 del gen *ERCC2*. Por otro lado, a pesar que la asociación con daño genómico no fue significativa, el polimorfismo rs171140 de *ERCC2* se ha asociado con una menor supervivencia en pacientes con cáncer de vejiga tratados con quimioterapia (Sacerdote et al., 2013).

A parte de los SNPs asociados con genes de reparación, también hemos encontrado asociación entre los niveles de daño genómico y otros genes, entre los que hay el responsable del angiotensinógeno AGT. AGT es una proteína del hígado que interactúa con la renina para producir angiotensina I, la prohormona de la angiotensina II, que es la molécula principal de sistema renina angiotensina (RAS). Variantes en el gen *AGT* pueden modificar su concentración en plasma, que está directamente relacionada con la presión arterial. Además, desempeña un papel importante en la mediación de diversas funciones fisiológicas, incluyendo la vasoconstricción, la homeostasis, la secreción de aldosterona, la inflamación, la fibrosis y el estrés oxidativo (Paul et al., 2006). Existen pocos estudios de asociación que mencionen este gen, sin embargo, éste se ha relacionado con el riesgo de hipertensión (Gu et al., 2011). En nuestro estudio, el alelo menos frecuente del polimorfismo rs5050 mostró asociación con los niveles de daño genético en los pacientes con ERC, evaluado con el ensayo de micronúcleos.

Otro de los genes relacionados con la enfermedad renal y el envejecimiento es el codificador de la glioxalasa I (*GLO1*), que mostró asociación con el daño oxidativo en los pacientes con ERC. Esta enzima es responsable de convertir los intermediarios dicarbonilos reactivos, en intermediarios no tóxicos y, por lo tanto, protege las células de la producción y acumulación de productos finales de la glicación avanzada (AGES) (Thornalley, 2003). Se sabe que la actividad disminuida de la GLO1 promueve la formación de los AGES y, por lo tanto, el daño tisular (Thornalley, 2003), El SNP rs4746 cambia el aminoácido alanina por el ácido glutámico en la posición 111 de la secuencia de aminoácidos, lo que reduce la actividad de la enzima en líneas celulares linfoblastoides inmortalizadas (Peculis et al., 2013). Esto podría explicar su asociación al daño oxidativo en pacientes con ERC. Pese a que este SNP no se ha asociado con patologías renales, existe un estudio que muestra que confiere vulnerabilidad al autismo (Gabriele et al. 2014). Además, otro SNP del mismo gen, el rs2736654, ha mostrado conferir un modesto riesgo de cáncer de próstata, un riesgo notable de la progresión del cáncer y bajo tiempo de supervivencia (Baunacke et al., 2014). Por otro lado, la asociación observada con el daño oxidativo, estaría de acuerdo con un estudio previo que relaciona polimorfismos en *GLO1* con estrés oxidativo (Antognelli et al.2013).

El último polimorfismo que mostró asociación con el daño genómico, pertenece a la familia Shroom 3 (*SHROOM3*). Esta es una proteína de unión a la actina, que juega un papel clave en la constricción del epitelio apical, aunque aún no se sabe bien como ésta actúa (Nishimura y Takeichi, 2008). En nuestro estudio hemos encontrado una asociación entre el SNP rs17319721 y los niveles de daño oxidativo y de daño cromosómico (BNMN). Hay que recordar que este polimorfismo lo encontramos asociado en la sección anterior con la ERC. Es de destacar que los estudios de asociación del genoma completo (GWAS) del Consorcio CKDGen lo han identificado entre los 16 genes encontrados para ERC en poblaciones europeas (Köttgen et al., 2010c; Köttgen et al., 2009). Además, este gen también se ha encontrado asociado con la relación albúmina-creatinina (Böger et al., 2011a) y con los niveles de albuminuria más bajos (Köttgen et al., 2010d; Köttgen et al. 2009).

Por lo tanto, podemos concluir que entre los genes seleccionados hemos encontrado que los individuos portadores de variantes de algunos de los genes estudiados muestran mayores niveles de daño en su DNA, ya sea utilizando el ensayo del cometa o el ensayo de micronúcleos.

V. CONCLUSIONES

5. CONCLUSIONES

De acuerdo con los objetivos inicialmente propuestos, y en base al análisis realizado con los resultados obtenidos, se pueden extraer las siguientes conclusiones:

Respecto al estudio del daño genético en pacientes con enfermedad renal crónica (ERC):

1- Los niveles de daño en el DNA pueden ser usados como un biomarcador de la enfermedad renal, y como un indicador de mal pronóstico en los pacientes con ERC.

En cuanto al efecto antioxidante del suplemento con jugo de uva sin fermentar:

2- La suplementación con mosto, durante 6 meses, en pacientes sometidos a hemodiálisis, reduce los niveles de daño oxidativo.

Respecto a la terapia renal sustitutiva HDF on-line:

3- El cambio de hemodiálisis de bajo flujo a hemodiafiltración on-line durante 6 meses, produce una ligera reducción en los niveles de daño genómico basal y una mejora en el perfil lipídico de los pacientes. El beneficio de la HDF on-line sobre el daño oxidativo sólo se observó en pacientes con niveles iniciales de daño significativamente altos.

Acerca de la evolución del daño genómico en pacientes con trasplante renal:

4- Los pacientes sometidos a trasplante de riñón, después de un año, muestran un aumento de los niveles de daño oxidativo.

5- Los pacientes que recibieron el riñón de donante vivo, presentan niveles más bajos de daño en el DNA, en comparación con aquellos pacientes que recibieron el injerto de donante fallecido.

En cuanto a las variantes alélicas asociadas con la ERC y con el daño genómico:

6- Polimorfismos en los genes *GPX1, GSTO1, GSTO2, UMOD y MPG* muestran asociación con la ERC.

7- Dentro de los genes seleccionados, se han encontraron SNPs asociados con diferentes patologías: *GPX4, CYP11B2* y *ERCC4* estarían asociados con hipertensión; *ERCC2/XPD* con cáncer; *AGT* y *ERCC2/XPD* con enfermedad cardiovascular; *AGT, MTHFR* y *ERCC2/XPD* con diabetes tipo 2.

8- Polimorfismos en los genes *GPX1, AGT, GSTO1, ERCC2/XPD, SOD1, GSTP1 y GPX4* se han asociado con distintos parámetros bioquímicos alterados en la *ERC*.

9- SNPs de genes implicados en la reparación del DNA por BER (*XRCC1*) y NER (*ERCC2/XPD*) se han asociado con el daño genómico y oxidativo evaluado mediante el ensayo del cometa y el de micronúcleos, en pacientes con ERC.

CONCLUSIONS

According with the planed objectives, and based on the analysis carried out, we can summarize the following conclusions:

Regarding the study on the genetic damage in patients with chronic kidney disease (CKD):

1- The levels of DNA damage can be used as a biomarker of CKD, as well as an indicator of bad prognosis in patients with CKD.

Regarding the antioxidant effect of the unfermented grape juice supplement:

2- Oxidative damage was decreased in patients with CKD on HD therapy, supplemented during six months with unfermented grape juice.

Regarding renal replacement therapy (HDF on-line):

3- Changing from low-flow HD to on-line HDF on-line, produces a slight reduction in the basal levels of genomic damage and an improved lipid profile. The benefit of the on-line HDF on oxidative damage was observed only in those patients with initial significantly high levels of DNA damage.

About genomic damage changes in kidney transplant patients:

4- Patients undergoing kidney transplantation, after one year of follow-up, show an increase in the oxidative DNA damage levels.

5- Patients which received kidney from living donors, showed lower DNA damage levels compared with those that received allograft from deceased donor.

Concerning the allelic variants associated with CKD and genomic damage:

6- Polymorphisms in *GPX1, GSTO1, GSTO2, UMOD* and *MPG* showed associations with CKD.

7- We have identified several genes associated with different pathologies characteristics of CKD: *GPX4, CYP11B2* and *ERCC4* associated with hypertension; *ERCC2/XPD* with cancer; *AGT* and *ERCC2/XPD,* with cardiovascular disease; *AGT, MTHFR* and *ERCC2XPD* with diabetes type 2.

8- Polymorphisms in *GPX1, AGT, GSTO1, SOD1, ERCC2/XPD, GSTP1* and *GPX4* genes have been related with altered biochemical parameters in CKD.

9- SNPs involved in BER (*XRCC1*) and NER (*ERCC2/XPD*) DNA repair pathways have been associated with genomic and oxidative DNA damage, as measured using the comet and micronucleus assays.

VI. BIBLIOGRAFÍA

6. BIBLIOGRAFÍA

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VII. ANEXOS

7. ANEXOS

7.1. Anexo 1. Artículo 3

Changing To On-Line Hemodiafiltration Affect the Levels of Genomic Damage in Patients Undergoing Hemodialysis

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ABSTRACT

Chronic kidney disease (CKD) patients exhibit high levels of genetic damage. Part of this genetic damage is supposed to be caused by the hemodialysis (HD) therapy. Different and more efficient HD procedures could reduce the genetic damage and improve health status of CKD patients. In the present study, we analyzed if changing to online hemodiafiltration (OL-HDF) has a beneficial effect on the levels of genetic damage. The levels of genetic damage (DNA breaks and oxidatively damaged DNA) were analysed in peripheral blood lymphocytes by using the comet assay. Forty-nine patients submitted to HD, 34 of them changing to OL-HDF and 15 patients continuing in low-flux HD, were included in the study. Plasma antioxidant capacity was also determined. Second sampling period was established after 6 months on the new or traditional HD protocol. A slight decrease in the levels of DNA damage was observed in patients who switched to OL-HDF (P=0.048) in relation to the reference group. This reduction is indicative that OL-HDF shows greater efficiency than low-flux HD in the reduction of basal levels of genetic damage.

INTRODUCTION

Millions of end-stage renal patients over the world survive thanks to hemodialysis therapies (1). In chronic kidney disease (CKD) patients with renal failure, conventional hemodialysis (HD) is the main renal replacement modality used to remove solutes (2,3). Despite the different HD alternatives that exist, patients still suffer several and serious health problems such as malnutrition, diabetes mellitus and cardiovascular diseases, among other, and exhibit high rates of mortality (4), some of these alterations are due to the accumulation of solutes that cannot be removed by the HD techniques.

One of the proposed extracorporeal HD alternatives is the on-line hemodiafiltration (OL-HDF), which combines the advantages of classical hemodialysis (small solute removal, by diffusion) with those of hemofiltration (large solute removal, by convection) (5). OL-HDF uses high-flux synthetic membranes and ultrapure dialysis fluids that allow the clearance of medium to large molecules that are impossible to remove using exclusively diffusive techniques. OL-HDF showed other advantages such as an improvement of the hemodynamics stability, better removal of phosphates, decrease of amyloidosis and markers of chronic inflammation, and improvements in survival and nutritional status (6). In fact, four recent studies have found a reduction in mortality with OL-HDF that correlates with the convection volumes achieved during therapy (7-10).

High levels of genomic damage are present in CKD patients and recently this value has been proposed as biomarker of chronic renal disease status (11). Such high levels of DNA damage can be due to the presence of toxins not removed from blood, such as advanced glycation end-products (AGEs) (12), or to deficiencies in DNA repair ability (13). In addition, the high levels of DNA damage present in CKD patients have been directly associated with all-type mortality (14). Although an important part of the increased level of genomic damage can be associated to the renal pathology itself (2), differences in the HD protocol can also influence the final levels of DNA damage (15). In this context it seems interesting to evaluate whether moving from conventional HD to OL-HDF acts as a factor modulating the levels of genomic damage.

In a previous study we observed that patients with chronic kidney disease (CKD), which were changed to OL-HDF therapy, showed a significant reduction of the basal levels of genetic damage using the micronucleus assay, that detects both structural chromosomal breakage as well as aneuploidy (16). In addition, a greater increase in plasma antioxidant capacity was detected with the Trolox equivalent antioxidant capacity (TEAC) assay (16). In the present study we extend the previous work using the comet assay, in peripheral blood lymphocytes from patients, as a tool to determine the levels of genomic damage. This approach permits detection of DNA strand breaks as well as oxidized DNA bases, when the comet assay is complemented with the use of formamidopyrimidine DNA-glycosylase (FPG) (17).

MATERIALS AND METHODS

Study population

The study was carried out in 49 patients with chronic kidney disease underwent thrice weekly low-flux HD treatment. The inclusion criteria were as follows: aged over 18 years, undergoing HD for

more than 3 months who were stable on 3 weekly sessions of 3.5-4 hours and a stable regimen of anticoagulation and erythropoietin with a vascular access allowing flows greater than 250 mL/min. Patients with a survival rate lower than 18 months or with an expected kidney transplantation in the next 6 months or with significant residual renal function (diuresis greater than 400 mL/day or creatinine clearance >2 mL/min) were excluded from the study. From the overall group, 34 patients with a previous average period of 26.06 ± 5.28 months submitted to low-flux HD therapy, changed to OL-HDF. Another group of 15 patients, with a previous average period of 17.87 ± 6.98 months submitted to low-flux HD therapy, continued in low-flux HD, and it was used as a reference group. Patients were randomized by a computer program, and those who started OL-HDF had not previously been submitted to OL-HDF. Two blood samples were obtained for each patient: the first one was obtained before the first OL-HDF session and the second one after 6 months on OL-HDF therapy. For the group that remain in conventional HD, the period of time between both samples was of 7.9 months. Usually CKD patients attended dialysis sessions on Monday, Wednesday and Friday. It should be mentioned that generally blood samples were obtained before the Wednesday dialysis session. Characteristics of the dialyser for low-flux HD: Vitapes®BF 200 was a polyetersulphone with an area of 2 m^2 and ultrafiltration ratio of 24 mL/h/mm Hg. On-line HDF was performed with Vitapes®HF190 that was a polyetersulphone with an area of 1.9 m^2 and ultrafiltration rate of 80 mL/h/mm Hg. The length of dialysis session was between 3.5 and 4 h and was not modified during the study period. When patients switched to post-dilution OL-HDF a minimum of 18 L/session of replacement volume was requested. Both OL-HDF and HD were performed with ultrapure dialysis fluids defined by colony forming unit levels less than 0.1 and endotoxins less than 0.03 UE/mL. All participants (aged over 18) were recruited at the hospital Fundació Puigvert and provided written informed consent and blood samples were collected under protocols approved by the Ethics Committee of the Puigvert Foundation. Standard blood analysis was also carried out at the Puigvert Foundation, including the determination of relevant parameters such as calcium, phosphorus, glucose, cholesterol, triglycerides, albumin and haemoglobin, ferritin, iron, transferrin saturation, parathyroid hormone and C-reactive protein. Blood samples were collected simultaneously for comet and micronucleus tests, previously published (16), and sent to the Universitat Autònoma de Barcelona for its processing.

Comet assay

DNA breaks present in peripheral blood lymphocytes were measured using the comet assay. The assay was performed following the standard protocol previously described (17,18) with minor modifications. Briefly, isolated lymphocytes from 2 mL of blood from each patient were cryopreserved in 500 µL with 90% serum and 10% DMSO, until use. Gelbond® films (with 48 agarose drops, 6 drops per individual) were used instead of microscopic slides as a support for the agarose gel. The use of hydrophilic films facilitates the rapid processing of numerous samples, increasing the efficiency of the alkaline comet technique, without sacrificing the reliability or sensitivity of the assay (19,20). Samples were immediately processed after their arrival to the laboratory. A total of one hundred randomly selected cells from 8 replicas (16-17 nuclei per replica), were analysed per patient. The % tail DNA was used as a measure of DNA damage and computed using the Komet version 5.5 Software.

Detection of oxidatively damaged DNA

The comet assay was performed following the standard protocol previously described (13) with minor modifications. Formamidopyrimidine DNA-glycosylase (FPG) was used as lesion-specific repair enzyme to detect oxidatively damaged DNA, as recommended by the European Standards Committee on Oxidative DNA Damage (ESCODD) (21). FPG-modified comet converts oxidized purines, ring-opened purines and also some alkylation damage in single strand breaks. The FPG cellular extract was produced in our laboratory, from transformed *E. coli* and was used at a final concentration of 0.0089 µg/µL for the treatment. Net oxidatively damaged DNA values were calculated by subtracting the damage scored in the samples incubated with buffer from those incubated with FPG. Positive and negative controls were included on each FPG experiment to assure the procedure. For oxidatively damaged DNA values, positive controls had an average of 47.37±6.01. As previously mentioned, 100 randomly selected cells were scored for each patient.

Trolox equivalent antioxidant capacity (TEAC) assay

The antioxidant capacity of the plasma was measured by using the TEAC assay as we have already described (16). Venous blood samples collected in EDTA tubes were centrifuged at 170 g during 5 min to obtain plasma that was stored at -80 ºC. Ten µL of plasma or Trolox standard reacted with 6.20 µM myoglobin solution (20 µL), 183 µM ABTS solution (150 µL) and 10 mM H_2O_2 (25 µL) on a microplate. Reaction was followed at 405 nm with the plate reader Sunrise (Tecan Trading AG, Switzerland). Lag time from kinetic curves and Trolox calibration curve was calculated and plasma TEAC was expressed as Trolox equivalent.

Statistical analysis

Data were assessed for normality using the Kolmogorov-Smirnov test. Two-tailed Fisher test and t-Student test were used for analysis of categorical and continuous data, respectively. Comet assay data were analysed using the parametric t-Student test for paired samples. Correlations were evaluated using the Pearson correlation parameter. Data are presented as mean ± standard error, if not otherwise indicated. All data were analysed using the Statistic Package of Social Science (SPSS) software for windows version 19.0. Statistical significant was defined as *P*-value lower than 0.05.

RESULTS

The study was carried out with 49 patients on HD treatment. All patients underwent conventional low-flux HD using synthetic membranes with a bicarbonate dialysate. Thirty-four patients changed to a post-dilution OL-HDF for 6 months, with a mean infusion of 20.2 ± 0.9 L/session. The etiology on patients who switched to OL-HDF, as well as of those maintained as reference group, was characterized by ischemic nephropathy (13.3%, 13.3%), diabetes mellitus (14.3%, 13.3%) and glomerulonephritis (31.4%, 46.7%), among others. The general characteristics of both populations are described in Table 1. As observed, there was a high incidence of hypertension (88.6%, 93.3%), cardiovascular pathologies (62.9%, 66.7%) and dyslipidemia (71.4%, 60%) among the studied groups. The only statistical significant difference was observed in the levels of BMI, which were higher in the group that moved to OL-HDF, showing that those individuals had light overweigh. Furthermore 22.9% and 33.3% of patients had suffered cancer, which is a characteristic of this type of patients, and may be related to genomic instability. It should be clarified that no patient was receiving cancer medication during the study, and they were currently considered as cancer-free patients. Medications and supplements received by the HD patients before and during the study are listed in Table 2. As observed, few differences were found between the two sampling periods, with respect to the percentages of individuals receiving such treatments. In the group of patients that moved to OL-HDF the percentage of patients supplemented with L-carnitine (31.4% *vs* 48.6% after 6 months, $P<0.05$), and vitamin D (51.4% *vs* 74.3% after 6 months, $P<0.01$) were increased. Regarding the reference group, the only supplement that changed during the 6 month study was folic acid that showed statistical differences between the percentages of patients who took folic acid. None of the other administered drugs showed statistical differences.

Blood chemistry data are shown in Table 3. As indicated, the levels of some values such as Kt/V, urea reduction ratio (URR) and ferritin levels showed a significant increase in the second sampling time, as well as triglyceride levels that significantly improved after treatment (1.62 ± 0.12 *vs* 1.29 ± 0.06, after 6 months, $P=0.038$). Despite no significant differences in blood chemistry were observed in the reference group, a slight increase in the levels of ferritin, and a slight decrease in the levels of triglycerides are shown, following the same tendency in both studied groups.
The levels of genomic damage observed in the selected groups of patients for both sampling times are shown in Table 4. As indicated, a significant borderline decrease in the levels of genomic damage ($P=0.048$), was observed after 6 month in OL-HDF treatment, for the group of 34 patients. No variation was obtained when patients remained using the same HD membranes. It is remarkable to point out that no significant variations were observed when the levels of oxidized DNA bases were evaluated between sampling times. Due to technical reasons for the oxidatively damaged DNA we only had data of 27 patients. When the basal damage of those 27 individuals was analyzed, no variation was detected after 6 months on OL-HDF The TEAC assay showed a slight tendency to increase the antioxidant capacity (0.11 ± 0.01 *vs* 0.18 ± 0.03, $P=0.058$).
When we selected those patients with the greatest differences in their levels of genomic damage between both samples we found that 35.7% of the studied patients showed very high levels of basal

damage (32.37 ± 3.48). After 6 months on OL-HDF, basal DNA damage of these patients decreased to 9.61 ± 1.56 (P<0.001). The other 64.3% of patients, increased their basal DNA damage (7.76 ± 1.23 *vs* 11.98 ± 1.06, P=0.006) (see Figure 1). On the other hand, 29.6% of patients changed the levels of oxidatively damaged DNA from 39.91 ± 3.45 to 9.87 ± 3.18 (P=0.019), after 6 months HDF-OL. The other 70.4% of patients increased from 15.73 ± 3.00 to 24.61 ± 3.32 (P=0.008).Potential associations between the changes in the levels of genomic damage and biochemical parameters were determined. In OL-HDF patients when the total iron-binding capacity (TIBC) increases, basal DNA damage decreases (r= -0.381, P=0.025), while no correlation exists (r= -0.050, P=0.889) in the control group. None of the other biochemical parameters showed any correlation with the levels of genomic damage.

DISCUSSION

On HD patients it is known that the main causes of endothelial dysfunction, among other adverse effects, are due to the increased levels of pro-inflammatory and pro-oxidant uremic toxins. The HD-based diffusion technique is not fully efficient in removing uremic toxins (22), therefore, new forms of dialysis have been developed, looking to improve the state of uremic patients with CKD. The decrease of the inflammatory state is associated with the use of high-flux dialysis, rather than low-flux membranes dialysis (23), and with the reduction of cardiovascular mortality (24). In this context, haemodiafiltration (HDF), which combines diffusive and convective transports, has been proposed as a useful approach to further reduce uremic toxins, in particular those of middle molecular weight, and protein-bound solutes (25).

OL-HDF performed using high-flux biocompatible membrane and high-quality ultrapure dialysate, is suggested to provide superior reduction in inflammation and oxidative stress than high-flux HD (26). Recent studies have demonstrated a reduction of the mortality in patients treated by OL-HDF with regard to conventional HD (7-10), and a reduced inflammatory activity over time (27). The question is if this amelioration of the health status of CKD patients submitted to OL-HDF can be translated to reductions in the levels of genetic damage. At this point it must be indicated that the presence of high levels of genetic damage in CKD patients seems to be a general characteristic of such kind of patients (16), and it has been related with cancer development and cardiovascular problems and mortality (14).

The first approach to determine the potential benefits of OL-HDF to reduce the levels of genomic damage was carried out by Kobras et al. (28) in a small group of 7 HD patients who moved from HD to HDF. Authors observed a slight but significant reduction of the levels of genomic damage detected by the comet assay, without effects on the frequency of chromosome damage detected in the micronucleus assay. These results slightly differ from our previous study (16) where in a larger population including 33 HD patients who moved to OL-HDF, a significant reduction on the levels of chromosome damage measured by the MN assay was observed.

The results of the present study by using the comet assay would agree with the above reported studies. Thus, our slight borderline significant reduction would coincide with the study of Kobras et al. (28) and, at the time, would reinforce our previous study (16) supporting the view that OL-HDF reduce the levels of genomic damage. When we analysed only the 27 patients who matched with the patients that have data for the levels of oxidatively damaged DNA, we observed that the borderline significance was lost, showing that the size of the population is critical for detecting slight changes. Nevertheless, although a non-significant effect was detected, better plasma antioxidant capacity was observed when moving to OL-HDF, although this does not translate to reduce the levels of oxidized DNA bases.

Results seem indicate that patient benefits from such a therapy (at least at the molecular level) are those patients that had more DNA damage. It should be noted that these results must be still demonstrated with a larger population to corroborate the observed trends.

The benefits of OL-HDF respect to other forms of HD are quite contradictory (29). We could mention the little impact of OL-HDF on the expression levels of plasma proteins such as haeme-oxygenase-1 (30). More recently, no differences were observed in haemodynamic stability, or in serum phosphate levels, in patients which changed to OL-HDF compared with conventional HD (31); while another study suggests that OL-HDF reduces all-cause mortality compared with conventional HD (32).

It is important to determine if this ameliorating effect on the levels of genetic damage translates to the clinical condition of patients. In the present study we have observed a statistically significant improvement in the Kt/v index of patients after moving to OL-HDF (P<0.001). This would support that the efficacy of this technique could also have a clinical translation. This fact has also been observed

in other studies comparing both techniques where an improvement of Kt/V values were found (33, 34).
The dyslipidemia present in 71.4% of the patients studied is characterized by hypertriglyceridemia (35). It must be indicated that at the end of the OL-HDF period the triglycerides levels were significantly lowered (1.62 ± 0.12 *vs* 1.29 ± 0.06, *P*=0.038), while in the control group such improvement was not observed (1.67 ± 0.24 *vs* 1.59 ± 1.20, *P*=0.678). A decrease in triglyceride levels as a result of OL-HDF treatment was observed previously by Pedrini et al. in a prospective study comparing OL-HDF vs low-flux HD (36). The improvement in the lipid profile could be due to removal by convection of circulating inhibitors of lipoprotein lipase and also to decrease of inflammation and oxidative stress related to the technique (37, 38).
Another biochemical parameter suffering a significant change is ferritin levels. These values significantly increase after the 6 months in OL-HDF (217.68 ± 28.24 vs 297.07 ± 33.67, *P*=0.011) without changes in the administered dose of intravenous iron and not increase in the level of C-reactive protein. Nevertheless, the improvement in the ferritin levels was not accompanied by an increase in the hemoglobin levels or a decrease in ESA dose. In our study we found significant correlations between the total iron binding capacity (TIBC) levels and the levels of DNA damage in the OL-HDF patients (r = -0381, *P*=0.026) but not in HD patients (r = -0.050, *P*=0.889). This means that when the TIBC levels decrease, the DNA damage levels detected by the comet assay increase.
The level of C-reactive protein (CRP) is another important parameter to have into account in HD patients. Such levels increase in response to inflammation and are subjected to enhanced oxidative stress, as a result of both insufficient antioxidant defense mechanisms and excessive generation of oxidant compounds (39). In our study CRP did change neither in OL-HDF nor in HD patients. Nevertheless previous studies have suggested a beneficial effect of OL-HDF on inflammatory parameters such as CRP and interleukin-6 (40-42), which could be related to the observed little decrease in the levels of oxidatively damaged DNA in patients submitted to OL-HDF (21.80 ± 4.20 *vs* 18.25 ± 2.81, *P*=0.761). On the contrary this damage was borderline significantly in the control group (9.70 ± 2.22 *vs* 15.84 ± 2.88, *P*=0.050).

CONCLUSIONS

As a summary we can conclude that in patients switching from low-flux HD to OL-HDF a slight but significant reduction on the levels of basal genomic damage was observed. Nevertheless, due to the borderline *P* value significance, more patients should be studied to obtain a better conclusion. It is remarkable the finding showing that moving to OL-HDF supposes a greater benefit only to those patients with high levels of basal genomic damage. In addition, an amelioration of the lipidic profile was also detected in patients moving to OL-HDF confirming the usefulness of this technique.

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CONFLICT OF INTEREST STATEMENT

None declared.

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TABLE 1. General characteristics of the studied populations

	HD - OL-HDF N=34	**HD – HD N=15**
Sex (men/women)	25 (71.4%) / 10 (28.6%)	9 (60%) / 5 (40%)
Age (years)	61.85 ± 2.36	59.26 ± 4.31
BMI	26.35 ± 0.64	23.57 ± 1.05*
Time in HD (months)	26.06 ± 5.28	17.86 ± 6.97
RT previous (% yes/no)	20 / 80	26.7 / 73.3
Hypertension (% yes/no)	88.6 / 11.4	93.3 / 6.7
CV pathology (% yes/no)	62.9 / 37.1	66.7 / 33.3
Diabetes (% yes/no)	20 / 80	33.3 / 66.7
Dyslipidemia (% yes/no)	71.4 / 28.6	60 / 40

Mean ± S.E. *t*-student test for independent samples: $^{*}P \leq 0.05$. No differences were observed between the two groups (Two-tailed Fisher test). RT, renal transplantation; CV, cardiovascular.

TABLE 2. Medication and supplementation received during the study.

	HD - OL-HDFa		**HD - HD**	
	First sample	**Second sample**	**First sample**	**Second sample**
Folic acid	17.1 / 82.9	22.9 / 77.1	33.3 / 66.7	41.7 / 58.6*
Vitamin B and C	20 / 80	22.9 / 77.1	26.7 / 73.3	8.3 / 91.7
L-carnitine	31.4 / 68.6	48.6 / 51.4*	20 / 80	41.7 / 58.3
ACE inhibitor	48.6 / 51.4	37.1 / 62.9	46.7 / 53.3	50 / 50
Statins	80 / 20	82.9 / 17.1	73.3 / 26.7	83.3 / 16.7
Vitamin D	51.4 / 48.6	74.3 / 25.7**	53.3 / 46.7	66.7 / 33.3
Sevelamer hydrochloride	71.4 / 28.6	71.4 / 28.6	46.7 / 53.3	58.3 / 41.7
Calcium	48.6 / 51.4	48.5 / 51.5	53.3 / 46.7	40 / 60
Venofer	91.4 / 8.6	88.6 / 11.4	93.9 / 6.7	80 / 20
ESA	94.3 / 5.7	97.1 / 2.9	93.3 / 6.7	73.3 / 26.7

Values are expressed as % yes/no. Two-tailed Fisher test: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$. ACE inhibitor, angiotensin-converting-enzyme inhibitor; Venofer, iron sucrose injection; ESA, Erythropoiesis stimulating agents.

TABLE 3. Blood chemistry data of studied population

	HD - OL-HDF		Paired t-test	HD - HD		Paired t-test
Kt/V (>1.3)	1.48±0.04	1.76±0.05	**0.001**	1.50±0.08	1.63±0.07	0.061
URR (%>70)	70.03±0.99	76.22±0.86	**<0.000**	70.60±2.01	73.00±1.97	0.121
BMI (Kg/m^2)	26.35±0.64	28.38±0.64	0.081	23.57±1.05	ND	
Albumin (37-47 g/L)	40.68±0.53	40.25±0.49	0.350	36.64±0.90	39.08±1.33	0.121
Phosphorus (0.8-1.3 mmol/L)	1.65±0.07	1.58±0.07	0.456	2.21±0.13	1.63±0.13	0.569
Calcium (2.1-2.55 mmol/L)	2.25 ± 0.03	2.28 ±0.03	0.328	1.81 ±0.05	1.63±0.16	0.390
PTH (7-53 ng/L)	240.52±35.39	211.6±25.53	0.315	149.49±29.43	201.46±46.47	0.382
CRP (<10 mg/L)	9.33±3.50	8.74±2.33	0.884	7.93±3.71	10.28±4.65	0.666
Homocysteine (<15 µmol/L) N=23	30.65±3.65	33.60±5.25	0.795	24.96±1.25	19.57±2.27	0.121
Hemoglobin (120-160 g/L)	121.91±2.55	121.20±0.18	0.812	117.60±4.34	118.66±3.38	0.121
Iron (9-27 µmol/L)	12.37±0.98	11.83±0.83	0.648	9.80±1.39	11.51±1.10	0.569
TIBC (47-79 µmol/L)	43.28±1.72	43.65±1.52	0.638	45.38±3.73	41.72±3.24	0.390
Transferrin saturation (12-44%)	29±2.53	27.71±2.38	0.657	23.92±0.03	28.17±0.03	0.569
Ferritin (25-250 µg/L)	217.68±28.24	297.07±33.67	**0.011**	175.14±57.71	276.42±58.00	0.077
Cholesterol (3.20-5.20 mmol/L)	3.97±0.14	3.81±0.18	0.324	4.51±0.36	3.58±0.26	0.390
Triglycerides (0.30-1.40 mmol/L)	1.62±0.12	1.29±0.06	**0.038**	1.67±0.24	1.59±1.20	0.678
HDL (>1.40 mmol/L)	1.21±0.06	1.91±0.14	0.199	1.14±0.08	1.08±0.08	0.212
LDL(<4.13 mmol/L)	1.94±0.12	1.90±0.15	0.820	2.47±0.32	1.85±0.22	0.057

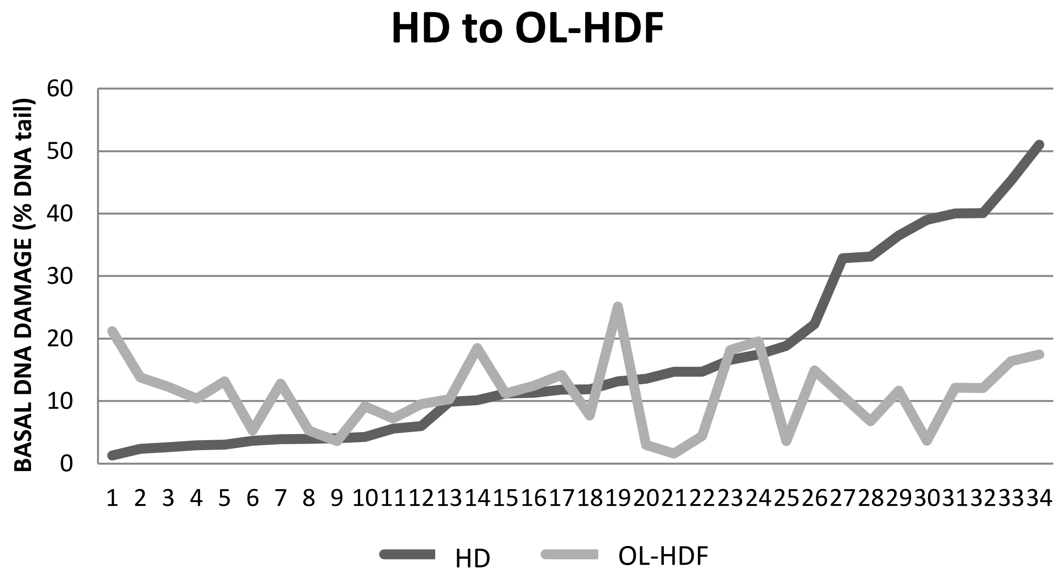
Mean ± S.E, paired samples *t*-student
PTH, Parathyroid hormone; CRP, C-reactive protein; TIBC, Total iron-binding capacity; HDL, high-density lipoprotein; LDL, low-density lipoprotein; URR, Urea reduction ratio, Kt/V treatment adequacy. Values reaching statistical significance are in bold.

TABLE 4. Comet assay DNA damage levels. Before and after 6 months on OL-HDF.

	HD	OL-HDF	*t*-paired test	HD	HD	*t*-paired test
Basal damage (% DNA tail)	(34) 16.44±2.46	11.14±0.98	**0.048**	(15) 13.29±1.71	13.48 ±2.01	0.949
Basal damage (% DNA tail)	(27) 15.20±2.80	11.75±1.05	0.271			
Oxidative damage (% DNA tail)	(27) 21.80±4.20	18.25±2.81	0.761	(15) 9.70±2.22	15.84 ± 2.88	0.050

Mean ± Standard Error.

Figure 1. Pair comparison within individuals and between therapies of hemodiafiltration.



7.2. Anexo 2. Artículo 4

Genetic Damage Follow-Up in Kidney Transplant Patients with Chronic Kidney Disease

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Keywords: Kidney transplantation (KT); Genomic damage; Oxidative damage; Comet assay; Micronucleus Assay

ABSTRACT

Kidney transplantation (KT) is associated with lower mortality and improved quality of life in chronic kidney disease (CKD) patients, compared with those submitted to chronic dialysis treatment. Nevertheless the evaluation of DNA damage and genomic instability in kidney transplant patients has been poorly documented. To cover this gap, 53 KT patients were follow-up over time to determine variations in biomarkers of genomic damage. The alkaline comet assay was used to determine DNA breaks and oxidatively damaged DNA. The Micronucleus (MN) assay was used to determine chromosomal breakage and/or aneuploidy. The results obtained in this study showed ameliorating effects on different biomarkers like glucose, albumin, calcium, systolic and diastolic blood pressure and urea (P=0.004). The determination of DNA damage levels before and after kidney transplantation showed statistically significant increases in the levels of oxidative damage and in the levels of micronuclei (MN). In addition, we have also determined higher levels of genomic damage in those patients who received a transplant from a deceased donor with regard to those who received it form a living donor. Our conclusion is that the genomic instability of CKD patients is maintained in spite of KT. This instability, and perhaps the role of immunosuppression drugs, would account for the increasing levels of genomic damage present in kidney transplant patients over time.

INTRODUCTION

The number of people affected by chronic kidney disease (CKD) is steadily increasing. Several factors as cardiovascular diseases, dyslipidemia, hypertension, and diabetes, have been implicated in the increased CKD risk (Strippoli et al., 2008). As renal disease worsens over time, kidney functions deteriorate and at the end-stage of renal failure process, kidney replacement or dialysis therapies are required (Levey et al., 2007). Individual studies indicate that kidney transplantation (KT) is associated with lower mortality and improved patients quality of life compared with chronic dialysis treatment (Merion et al., 2005). In recent decades, KT treatment has gradually spread over a larger number of patients. In Spain, about half of the CKD patients are carriers of a successful KT (Moreso and Hernández, 2013). At present, Spain is the European country with more kidney donors (Ramírez López, 2015). The Spanish Registry of Renal Patients shows that the incidence of KT has evolved significantly in Catalunya by a prevalence rate per million population (pmp) from 446.6 in 2001 to 676.5 in 2013 (http://www.registrorenal.es/informes/). However, the factors associated with higher or lower benefits of KT have been poorly described. Among the different factors that can influence the improvement of the quality of life for kidney transplant patients and their survival, the type of donor is an important one. It has been reported that the survival rate (five years after KT) is higher in those KT from a living donor (59.9%) compared with recipients of kidneys from deceased donors (40.3%) (Macrae et al., 2005). On the other hand, long-term complications of KT have assumed increasing importance as short-term patient and graft survival have improved. Although the immunosuppressive regimens have steadily reduced the incidence of acute rejection and have extended the life expectancy of allograft recipients, post-transplant malignancy has become an important cause of mortality. The etiology of post-transplant malignancy is believed to be multifactorial, like neoplastic cells, as well as depressed antiviral immune activity with a number of common post-transplant malignancies being related (Buell et al., 2005). There are reasons to believe that immunosuppressive agents may cause DNA damage and interfere with normal DNA repair mechanism (Kasiske et al., 2004). Until now, only few studies analyzed the genomic damage in CKD patients with KT. The study of Ott et al., (2007) revealed a high degree of tubular DNA fragmentation associated with oxidative stress in acute interstitial rejection. More recently Matsui et al., (2015) suggested that the long-term kidney pots-transplant induces double-strand breaks and human glomerular endothelial cells secreted collagen type VI accumulation in the glomerular capillaries, which might progress to intractable glomerular fibrosis. In this context, the aim of our study was analyze, in a follow-up study, the effect of KT on the metabolic status of patients, as well as variations in the levels of genomic damage. The levels of genomic damage of patients were analyzed by the alkaline comet and micronucleus assays; for oxidative damage, the formamidopyrimidine-DNA glycosylase (FPG) enzyme was included; and for chromosomal instability, the micronucleus assay was carried out. Samples were taken before the transplantation,and 6 and 12 months after surgery.

MATERIALS AND METHODS

Study population

The study was carried out in 53 patients with chronic kidney disease. Blood samples were collected just before the KT, and the follow-up was done 6, 12 and 24 months after the KT. From the 53 initial patients, 41 were monitored after 6 months; 42 patients after one year, and 11 patients after 24 months of the KT.
The criterion for selection of patients to be candidates for kidney transplantation is a very complex and individualized study. Patients were less than 75 years old, and had not significant comorbidities on the heart, lung, liver and neurology and on the status of their iliac arteries. All patients of this study had a GFR ≤ 12 mL/min/1.73 m^2. Some patients required hemodialysis after KT, 2% need 10 sessions, and 13% less than the 7 sessions.
The immunosuppression was based on prednisone as corticosteroids, and tacrolimus, mycophenolate mofetil, and mycophenolate sodium as active compounds (European Medicines Agency, http://www.ema.europa.eu). This treatment was provided to 98% of the cases during the first's days after the KT, and continued unchanged in 71.7% of patients after 6 months, and in 67% of cases after one year of the KT.
Those patients needing greater immunosuppression at the time of KT required the administration of antilymphocyte serum: thymoglobulin (in 45.3% of the patients), antilymphocyte globulin (AGT) (in 11.3%) and basiliximab (in 26.4%). Antilymphocyte serum was not administered in a 17% of the patients. Only one patient was treated with cyclosporine.
General characteristics of the selected group are indicated in Table 1. Supplements and medications received during the follow-up are indicated in Table 2. All participants (aged over 18) were recruited at the hospital Fundació Puigvert and provided written informed consent. Blood samples were collected under protocols approved by the Ethics Committee of the Puigvert Foundation. Standard blood analysis included the determination of calcium, phosphorus, glucose, cholesterol, triglycerides, albumin and hemoglobin, ferritin, iron, transferrin saturation, parathyroid hormone and C-reactive protein. For the analysis of genetic damage, blood samples were immediately sent to the Universitat Autònoma de Barcelona where were appropriately processed.

Comet assay

DNA present in peripheral blood lymphocytes were measured using the comet assay performed following the standard protocol, as previously described (Stoyanova et al., 2010) with minor modifications. Isolated lymphocytes from 2 mL of blood from each patient were cryopreserved in 500 µL of medium containing 90% serum and 10% DMSO, until use. Comet assay was carried out using Gelbond® films (GF) instead of microscopic slides as a support for the agarose gel. The use of hydrophilic films facilitates the rapid processing of numerous samples, increasing the efficiency of the alkaline comet technique, without sacrificing the reliability or sensitivity of the assay (McNamee et al., 2000; Azqueta et al., 2013). Lymphocytes were isolated using Ficoll–Paque density gradient from 500 µL of whole blood; cells were adjusted to a concentration of 17,800 cells in 25 µL PBS and carefully re-suspended in 225 µL of 0.75% low melting agarose (LMA) at 37 ºC and dropped onto a GF (10.5 x 7.5 cm). Forty-eight drops (7 µL each) were placed on each GF and samples of eight donors were run simultaneously, each donor being represented by six drops. Lymphocytes were lysed for a minimum of 1 h at 4 ºC in a dark chamber containing a cold fresh lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl and 1% Triton X-100, adjusted to pH 10). To allow DNA denaturation, unwinding and exposure of alkali-labile sites, GF were placed in a horizontal gel electrophoresis tank filled with freshly cold (4 ºC) electrophoresis solution (1 mM Na_2EDTA and 300 mM NaOH, adjusted to pH 13) for 35 min. Electrophoresis was performed in the same buffer for 20 min at 20 V and 300 mA. After electrophoresis, GF were neutralized with two 5-min washes with PBS 1X, followed by 1 min wash with water and then incubated overnight in 100% ethanol for fixation. Sheets were then dried and stored in the dark at room temperature until scoring. Just before the microscopic analysis, GF were stained with 20 µL of SybrGold. The images were examined at 20x magnification with a Komet 5.5 Image Analysis System (Kinetic Imaging Ltd, Liverpool, UK) fitted with an Olympus BX50 fluorescence microscope equipped with a 480–550 nm wide band excitation filter and a 590 nm barrier filter. A total of one hundred randomly selected cells were analysed per patient and the % tail DNA was used as a measure of DNA damage.

Detection of oxidative damage

To determine the levels of oxidized bases present in the lymphocytes, GF were washed two times (10 and 50 min, 4 ºC) after cell lysis in an enzyme buffer solution (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8.0) containing the enzyme FPG (formamidopyrimidine DNA glycosylase) (Azqueta et al., 2013). FPG enzyme was produced in our laboratory and the final concentration of enzyme extract used for each treatment was 0.0089 µg/µL.

Each sample was analysed using two GF. One GF remained in the cell lysis solution in order to assess basal DNA damage. The second one was treated with the enzyme buffer without FPG, to control for any effects of the buffer alone. GF were incubated with enzyme buffer (with and without FPG) for 30 min at 37 ºC. After that, samples were processed as in the standard alkaline comet assay procedure. Net oxidative DNA damage was calculated by subtracting the damage scored in the samples incubated with buffer from those incubated with FPG.

Lymphocyte culture and micronucleus assay

Blood samples from heparinized vacutainers were processed as described previously using cytochalasin-B to arrest cytokinesis (Rodríguez-Ribera et al., 2014). Two of the four cultures set up were irradiated with 0.5 Gy ^{137}Cs gamma rays in an irradiator IBL 437C, type H, No. 701 (SCHERING CIS Bio International) at the Unitat Tècnica de Protecció Radiològica (UTPR-UAB). To determine the frequency of binucleated cells with micronuclei (BNMN), 1000 binucleated lymphocytes per sample (irradiated and non-irradiated) were blind scored on coded slides, according to standard criteria (Fenech, 2007). In addition, 500 cells with one, two or more nuclei were scored to determine the cytokinesis-block proliferation index (CBPI) (Surrallés et al. 1995). The effect induced by irradiation was calculated by subtracting the background BNMN values in the non-irradiated samples from the values obtained in the irradiated samples (Rodríguez-Ribera et al., 2014).

Of some patients, and mainly due to the inmunosupression received treatment, lymphocytes were not properly stimulated, thus the final number of studied patients was reduced, and variations are observed between the different period times.

Statistical analyses

For the statistical analysis of discrete variables the chi-square test was performed (Tables 1 and 2), and for continuous variables t-test for paired samples was used (Tables 3 to 5). Data are presented as mean ± standard error. Pearson correlation was used to determine relationship between variables. All the analyses were done using the Statistic Package of Social Sciences (SPSS) software for Windows version 19.0.

RESULTS

Fifty-three chronic kidney patients were selected for a KT. Blood samples were obtained before and 6, 12 and in very few cases, 24 months after transplant. Some of the patients died and other was removed from the study due to graft malfunction. Thus, after 6 months of the transplant, only 41 patients were recruited, and after one year, 42 patients. The origin of their renal failure was mainly unknown (30.2%), interstitial nephropathies (7.5%), glomerular (24.5%), polycystic (15.1%), related to diabetes (13.2%), nephroangiosclerosis (7.5%), and congenital (2%).

Sixty-two percent of the CKD transplanted patients were previously submitted to hemodialysis therapy, 11.3 % to peritoneal dialysis, and 26.4% did not need any renal replacement therapy before the transplant. Most of the half of the kidney graft comes from alive donors (53%) in front of the 47% of kidney graft that comes from deceased donors.

Fifty-eight and a half percent of the KT patients were men (N=31) and the 41.5% (N=22) were women. It should be mention that a 15% of the CKD patients have had a previous transplant. The group of patients presented an average age of 49.34±1.90 years old, and a body mass index of 26.15±0.76. The donor group had an average of 56.64±1.90 years old (P=0.008).

The mean creatinine levels (µmol/L) of the KT patients, six months after transplantation were of 157.48±8.02, while it was 149.71±7.13 twelve months after. No statistically significant differences were observed between these two sampling points (P=0.521 N=33), and mean creatinine levels in the donors were 72.68±3.24, while creatinine levels depending on the type of donor if showed significant differences (live 65.84±2.68 vs. deceased 81.38±6.14 P=0.028), while levels of glomerular filtration rate did not differ (live 95.06±2.36 vs. deceased 78.75±8.10 P=0.075). In the 11 patients

analyzed after 24 months the observed creatinine values were 151.09±16.21. In terms of glomerular filtration rate (GFR), the average six and twelve months after transplantation were 40.78±2.57 and 41.26±2.05 (*P*=0.866 N=33). Additionally, in the eleven patients analyzed after 24 months GFR was 41.00±5.11.

The presence of CKD related pathologies in the patients submitted to KT are described in Table 1. The patients studied showed no new cancer after transplantation. Acute rejection after transplantation was presented in 11.3% of the patients while in 88.7% did not occur, which shows the success of this procedure.

No important differences in supplementation and medication received by the KT patients were observed at the time intervals studied (Table 2).

When several clinical parameters commonly studied in CKD patients were evaluated in KT patients significant differences 6 months after surgery were observed for most of them (Table 3). Nevertheless, no differences were observed between 6 and 12 month after KT.

With regard to the parameters associated with genomic damage, the rate of cell proliferation (CBPI) was reduced significantly 6 months after transplantation (*P*=0.003), and this reduction remains when blood samples were obtained after 12 month of KT.

As observed in Table 4 all parameters measuring genetic damage showed increased values over time after KT. Basal DNA breaks detected by the comet assay increases in the follow-up study but without attain statistical significance. Nevertheless, when the levels of oxidized DNA bases were determined by using FPG enzyme, statistical increases over time were observed. Besides a significant correlation was observed between the levels of total and oxidative DNA damage before and after 6 months of KT (r= 0.381, *P*=0.020, N=37) and (r= 0.563, *P*=0.002, N=31) respectively. Likewise, patients with high levels of binucleated lymphocyte before transplantation, had high levels 6 months after KT (r=0.510, *P*=0.009, N=25) and this remained even one year later (r=0.914, *P*=0.000, N=14).

These increases in DNA damage were confirmed when the MN assay was used. In this case the frequency of binucleated cells with micronucleus (BNMN) attained statistical significance in the sample obtained 12 months after KT. It is interesting to indicate that this increase in chromosome damage is not due to an increase in the levels of genomic instability since the effects induced by radiation (0.5 Gy) did not show significant increases over time.

The levels of DNA damage were strongly dependent on the initial characteristics of the donors. We tried to determine the potential role of kidney donor type on the levels of genomic damage of the recipient patient (Table 5). Interestingly, statistically significant differences (*P*=0.023) were observed in the levels of oxidative damage after 6 months of kidney transplant between those who received a transplant from a deceased donor (21.10±3.19, N=17) than those who received it from an alive donor (12.29±1.75, N=19). Similar effect was observed when genomic instability was evaluated (P=0.032) with higher values among those patients receiving transplant from a deceased donor (13.63±3.66, N=10) than in those who received it from a living donor (4.17±0.71, N=14), 12 months after KT.

DISCUSSION

Kidney transplantation (KT) is the replacement therapy that has shown greater benefits to patients with chronic kidney disease (CKD) (Blancas et al. 2015). However, CKD transplanted patients have not fully resolved their health problems and whose quality of life is associated with different factors. It is important to emphasize that kidney transplanted patients comprise a complex subgroup of CKD patients selected as suitable candidates for transplantation according to different parameters. Among CKD patients, it is well-known the existence of high levels of genomic damage, in comparison with the overall population; nevertheless, this parameter is not considered among the selection criteria for KT. At this point it should be pointed out the evidences that exist linking genetic damage levels with adverse health outcomes (Fenech, 2008). In fact, the accumulation of changes at the genome level, due to both endogenous and exogenous factors, it is recognized as a fundamental underlying cause of developmental defects and accelerated aging as well as of an increased risk of degenerative conditions such as infertility, immune dysfunction, cancer, and cardiovascular and neurodegenerative disease (Fenech, 2010). In spite of that, few data exist on follow-up studies carried out on kidney transplanted patients with regard to variations in genomic damage values, accordingly to their KT status.

Our results show that the patients included in the present study moved to the acquisition of normal levels for different parameters just after six months of KT. This occurs for phosphorus, HDL and blood pressure levels. Other parameters such as urea and proteinuria although suffer significant reductions they do not reach standard levels. This improvement may be due to statin treatment as

previously seen in two meta-analysis, where the use of statins among participants with albuminuria and proteinuria, may moderately reduce pathologic excretion within a median of 6 months after initiation of therapy (Douglas, et al. 2006), reducing significantly lipid concentrations in CKD patients (Strippoli et al. 2008). With regard to other biochemical parameters, substantial improvements were also observed at 6 months and one year after the intervention, compared to the levels present before transplantation.

For CKD patients an important amount of literature has been generated relating different types of dialysis, as a renal replacement therapy, with changes on the levels of genomic damage (Miyazaki et al. 2000; González-Diez et al. 2008; Yeates et al. 2012; Rodríguez-Ribera et al. 2016). However, at present, there are no studies evaluating this genomic damage after transplantation and its follow-up after the intervention. Taking into account the relevance of genomic damage levels on the health status, our main interest was to assess the evolution of genomic damage in kidney transplanted patients as indicative of potential amelioration of their health status.

According to the complexity of the genomic damage we have used the comet and the micronucleus assays to cover a wide range of effects.

The standard comet assay measures DNA strand breaks and alkali-labile sites, but a simple modification of the assay allows for the detection of oxidized DNA bases resulting from pro-oxidant factors or different types of exposures (Collins, 2005). For example, the addition of FPG detects modified purines (mainly 8-oxodG). On the other hand, the micronucleus (MN) assay measures chromosomal breakage due to misrepair of DNA lesions or loss of chromosomal segregation due to mitotic errors (Bonassi et al., 2007). The possible causative factors in MN formation are induction of oxidative stress, exposure to clastogens or aneugens, genetic defects in cell cycle checkpoints or DNA repair genes, deficiency of essential cofactors in DNA metabolism and chromosomal segregation resulting from exposure to genotoxic agents or in genetically susceptible subjects (Fenech et al., 1999). According to the relevance of these biomarkers, our findings showing that the levels of DNA damage in kidney transplanted patients increasing over time are of paramount importance, as indicative of the future health status of kidney transplanted patients. Our results would suggest that in spite of the metabolic improvement of transplanted patients, genomic damage continues to evolve showing a tendency to increase over time. This would agree with other studies showing high degree of DNA fragmentation associated with oxidative stress in acute interstitial rejection (Ott et al. 2007) what connect with the increased levels of oxidatively damaged DNA observed in our transplanted patients. Likewise, it has also been reported that long-term kidney transplant induces double-strand breaks, and collagen type VI accumulation in glomerular capillaries, which might progress to glomerular fibrosis (Matsui et al. 2015). This could explain the significant increases observed in the total genomic damage values of the few individuals who were analyzed for 24 months after KT.

Immunosuppression in transplanted patients has been proposed as a potential source of genomic damage. In this way, cyclosporine has been proposed as a responsible for the inhibition of DNA repair and induction of apoptosis of activated T cells, thereby promoting the genesis and spread of cancer (Rath and Oliveira-Frick 2009). This would support the finding showing that patients who have received cyclosporine presented higher levels of sister-chromatid exchange compared to that in the control group, suggested that cyclosporine was responsible of the increased genotoxic effects in kidney transplanted patients (Ozturk et al. 2008). Likewise tacrolimus and mycophenolate mofetil treated individuals exhibit enhanced free radicals contributing to the oxidative damage, lipid peroxidation production, induced DNA lesions and reduced mitochondrial membrane potential (Ferjani et al. 2015). These studies would support our findings showing over time increases in the levels of genetic damage in kidney transplanted patients. It should be remembered our previous findings showing that CKD patients present genomic instability (Rodriguez-Ribera et al., 2015) and deficiencies in base excision repair (Stoyanova et al., 2014), together with a recent unpublished data in a large group of CKD patients indicating association between the levels of genetic damage and polymorphisms at the *XRCC1* and *ERCC2* genes. This genetic susceptibility, together with potential exposure to genotoxic agents (including immunosuppressors) would explain the tendency to accumulate genetic damage in kidney transplanted patients over time.

Another important finding of our study is the relevance of the kidney donor. It is known that the type of donor can be an important variable for graft survival. A study in USA showed better survival after five years post KT in a geriatric living donor cohort compared with recipients of kidneys from deceased donors (Macrae et al. 2005). This has been recently confirmed in Spain in 2014 showing a lower survival in KT from deceased donor grafts versus those from living donor grafts (Barrera et al. 2014). From our results we detect that both the oxidative damage levels and the BNMN values

showed by patients who received the deceased donor transplant are higher than in patients who received kidney graft from living donors.

CONCLUSION

The conclusion of the present study is that, in spite of the substantial improvements observed in kidney transplanted patients with regard to different markers associated with CKD, increased levels of oxidative damage and genomic instability are observed. This suggests that their genomic instability is still present, although renal replacement therapy improves metabolism. Moreover, lower levels of DNA damage are observed in patients who received kidney transplants from living donors compared with those who received it from deceased donors.

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CONFLICT OF INTEREST STATEMENT

None declared.

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TABLE 1. General characteristics of the patients.

	Before KT (N=53)	**6 months after KT (N=41)**
CV pathology	18.9 / 81.1	7.3 / 92.7
Cancer	17 / 83	0 / 100
Diabetes	22.6 / 77.4	(N=30) 10 / 90 ***
Dyslipidemia	54.7 / 45.3	ND
Hypertension	90.6 / 9.4	ND

Values are expressed as percentage yes/no. CV pathology: Cardio Vascular pathology; ND, no data. Chi-square test, ****P*<0.001. Not information available at 12 and 24 months after transplant.

TABLE 2. Medication and supplementation received during the study

	Before transplant (N=53)	**6 months after transplant (N=41)**	**12 months after transplant (N=42)**
Folic acid	7.5 / 92.5	ND / 77.4	1.9 / 77.4
Calcium	32 / 68	52.8 / 24.5	45.3 / 34.0$^{\wedge\wedge}$
L-carnitine	2 / 98	ND / 77.4	ND / 79.2
Vitamin B and C	5.7 / 94.3	ND / 77.4	ND / 79.2
Vitamin D	22.6 / 77.4	56.6 / 20.8	56.6 / 22.6$^{\wedge\wedge}$
ESA	41.5 / 58.5	19/ 58	18.9 / 60.4$^{+\wedge}$
Venofer	4 / 96	9.4 / 67.9	9.4 / 69.8^{+}
Statins	40 / 60	30 / 47**	41/ 37$^{++\wedge\wedge\wedge}$
Sevelamer hydrochloride	15 / 85	ND /77.4	ND / 79.2

Values expressed as percentages of patients treated / untreated at each time of the study. Venofer: iron sucrose injection; ESA, Erythropoiesis stimulating agents. Chi-square test, ***P*<0.01 before *vs* 6 months after transplant; $^{+}$*P*<0.05, $^{++}$*P*<0.01, before *vs* 12 months after transplant. ^^^*P*<0.01, 6 months after transplant *vs* 12 months after transplant.

TABLE 3. Biochemical blood data of the patients studied

	Before transplant *vs* 6 months after transplant		Before transplant *vs* 12 months after transplant		6 months after transplant *vs* 12 months after transplant	
Hemoglobin(120-160 g/L)	121.56 ± 2.55	(N=41) 127.34 ± 3.76	121.47±2.36	(N=42) 131.23±1.17**	130.03±2.74	(N=33) 130.09±2.42
Glucose (4-5.8 µmol/L)	5.13 ± 0.24	(N=38) 5.78 ± 0.41*	5.18±0.23	(N=38) 6.29±0.54**	6.04±0.45	(N=33) 6.52±0.61
Albumin (37-47 g/L)	41.08 ± 0.89	(N=32) 45.79 ± 0.56***	41.94±0.87	(N=36) 44.62±0.45**	45.43±0.52	(N=29) 44.86±0.51
Calcium (2.1-2.55 mmol/L)	2.18 ± 0.05	(N=39) 2.46 ± 0.02***	2.22±0.05	(N=38) 2.42±0.01**	2.47±0.02	(N=33) 2.42±0.02
Phosphorus (0.8-1.3mmol/L)	1.71 ± 0.06	(N=30) 1.06 ± 0.04***	1.83±0.15	(N=30) 1.07±0.03***	1.02±0.03	(N=33) 1.07±0.03
Ca x P ($mmol^2/L^2$)	3.78 ± 0.13	(N=30) 2.61 ± 0.08***	3.95±0.26	(N=30) 2.59±0.08***	2.53±0.07	(N=33) 2.58±0.08
Parathyroid hormone (7-53 ng/L)	262.98 ± 35.73	(N=12) 183.35 ± 41.58	223.58±27.21	(N=16) 134.16±22.64**	158.87±41.94	(N=13) 132.55±30.19
HDL (>1.40 mmol/L)	1.21±0.08	(N=21) 1.54±0.09***	1.26±0.08	(N=21) 1.62±0.10***	1.57±0.08	(N=31) 1.65±0.07
LDL (<4.13 mmol/L)	2.48±0.16	(N=21) 2.54±0.15	2.63±0.17	(N=21) 2.29±0.18	2.57±0.13	(N=31) 2.40±0.12
Cholesterol (3.20-5.20 mmol/L)	4.40±0.26	(N=24) 4.77±0.16	4.58±0.24	(N=26) 4.56±0.14	4.85±0.14	(N=31) 4.70±0.12
Triglycerides (0.30-1.40 mmol/L)	1.50±0.17	(N=23) 1.59±0.16	1.60±0.16	(N=25) 1.63±0.14	1.54±0.15	(N=31) 1.48±0.12
Systolic blood pressure (<140mmHg)	139.40±4.61	(N=30) 126.83±2.19*	139.40±4.44	(N=32) 127.06±2.42*	127.09±1.96	(N=33) 129.42±2.39
Diastolic blood pressure (<90 mmHg)	82.91±2.68	(N=30) 74.80±1.72*	83.29±2.58	(N=32) 75.21±1.75**	75.48±1.66	(N=33) 75.36±1.66
Proteinuria (<0.15 g/L)	1.99±1.15	(N=13) 0.27±0.08	1.86±1.15	(N=13) 0.21±0.06	0.26±0.04	(N=33) 0.23±0.03
Urea (2.5-7 mmol/L)	25.28±3.79	(N=9) 10.21±1.11*	ND	ND	ND	ND

Mean± standard error. Paried-samples *t*-test **P*<0.05, ***P*<0.001; CaxP, calcium phosporous product; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ND, no data.

TABLE 4. DNA damage levels, evaluated by comet and micronucleus assays, on the different stages of the study

		Before transplant	6 months after transplant	Paired *t*-test	Before transplant	12 months after transplant	Paired *t*-test	6 months after transplant	12 months after transplant	Paired *t*-test
Comet assay (% DNA tail)	Basal damage	(N=37) 9.01±0.77	9.56±0.80	0.557	(N=39) 9.32±1.50	11.16±0.88	0.299	(N=32) 9.31±0.86	11.11±1.04	0.125
	Oxidative damage	(N=31) 12.82±1.82	16.52±1.97	0.053	(N=34) 14.68±2.12	21.40±1.91	**0.031**	(N=30) 18.31±2.03	23.31±2.51	0.089
Micronucleus assay	BNMN	(N=25) 5.02±1.01	7.10±1.43	0.115	(N=22) 5.40±1.25	8.49±1.96	**0.041**	(N=14) 7.64±1.89	9.56±2.94	0.204
	Induced BNMN	(N=25) 22.14±5.66	25.17±4.77	0.702	(N=19) 24.41±6.90	26.56±4.32	0.808	(N=13) 23.84±4.58	20.75±5.36	0.607
	CBPI	(N=25) 1.47±0.03	1.32±0.02	**0.003**	(N=22) 1.52±0.04	1.31±0.03	**0.003**	(N=14) 1.33±0.04	1.32±0.05	0.797

Values are expressed as mean ± standard error. BNMN, binucleated cells with micronuclei; Induced BNMN, is the difference between BNMN after 0.5 Gy of irradiation and the basal BNMN values.

Table 5. Genomic damage according to the origin of the graft.

		Living donor	Deceased donor	*t*-student
6 month post-transplant	Basal damage (% DNA tail)	(N=22) 9.68±1.19	(N=17) 9.04±1.17	0.703
	Oxidative damage (% DNA tail)	(N=19) 12.29±1.75	(N=17) 21.10±3.19	**0.023**
	BNMN	(N=19) 5.52±1.25	(N=2) 10.37±3.20	0.097
	Induced BNMN	(N=19)	(N=8) 26.37±6.79	0.519
	CBPI	(N=19) 1.36±0.02	(N=8) 1.26±0.04	0.062
12 month post-transplant	Basal damage (% DNA tail)	(N=22) 10.10±1.23	(N=19) 12.32±1.11	0.190
	Oxidative damage (% DNA tail)	(N=22) 22.29±2.79	(N=19 24.06±2.99	0.668
	BNMN	(N=14) 4.17±0.71	(N=10) 13.63±3.66	**0.032**
	Induced BNMN	(N=12)	(N=10) 30.63±6.58	0.283
	CBPI	(N=14) 1.35±0.05	(N=10) 1.23±0.05	0.113

Values are expressed as mean ± standard error, binucleated cells with micronuclei; Induced BNMN, is the difference between BNMN after 0.5 Gy of irradiation and the basal BNMN values.

7.3. Anexo 3. Artículo 5

Genetic Variants Associated With Chronic Kidney Disease in a Spanish Population

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Key words: CKD patients; single nucleotide polymorphisms.

ABSTRACT

Chronic kidney disease (CKD) patients have many physiological pathways affected. Variations in the genes regulating these pathways might affect the incidence and predisposition. To better understand the genetic predisposition to this pathology, a total of 722 Spanish adults, including 548 patients and 174 controls were genotyped. We analysed 38 SNPs in candidate genes associated with the inflammatory response (*IL-1A, IL-4, IL-6, IL-10, TNF-α, ICAM-1),* fibrogenesis (*TGFB1*), homocysteine synthesis (*MTHFR*), DNA repair (*OGG1, MUTYH, XRCC1, ERCC2, ERCC4*), renin-angiotensin-aldosterone system (*CYP11B2, AGT*), phase-II metabolism *(GSTP1, GSTO1, GSTO2),* antioxidant capacity (*SOD1, SOD2, CAT, GPX1, GPX3, GPX4*), and some other genes previously reported involved in the CKD (*GLO1, SLC7A9, SHROOM3, UMOD, VEGFA, MG, KL*).

The results showed associations of *GPX1, GSTO1, GSTO2, UMOD*, and *MGP* with CKD. Additionally, associations with CKD related pathologies, such as hypertension (*GPX4, CYP11B2, ERCC4*), cardiovascular disease (*AGT, ERCC2*), diabetes (*AGT, MTHFR, ERCC2*) and cancer predisposition (*ERCC2*) were also observed. Other genes showed association with biochemical parameters characteristic for CKD, such as creatinine (*GPX1, GSTO1, GSTO2*), glomerular filtration rate (*AGT, SOD1, GPX1, GSTO1*), haemoglobin (*ERCC2*), resistance index erythropoietin (*SOD2*), albumin (*SOD1*), parathyroid hormone (*IL-1A, SHROOM3, UMOD, ERCC2*), C-reactive protein (*GSTP1*),and ferritin (*SOD2, GSTP1, SLC7A9, GPX4*).

INTRODUCTION

Chronic kidney disease (CKD) is becoming a major public health problem worldwide. CKD is defined as a progressive loss of renal function, measured by a decline in glomerular filtration rate (GFR < 60 mL/min/1.73 m^2) (Smyth et al. 2014), which is typically associated with irreversible pathological changes within the kidney. This pathology has a complicated interrelationship with other diseases (Tonelli et al. 2005; Snively and Gutierrez, 2014). Diabetes and hypertension are the primary risk factors for CKD (Adler et al. 2003), and CKD is also associated with cardiovascular morbidity and mortality (Mequid El Nahas and Bello, 2005; Levey et al., 2007), even in early stages and in young patients (London, 2003).

CKD patients are also characterized by a high genomic instability (Sandoval et al. 2010; Sandoval et al. 2012; Lialiaris et al. 2010; Moffitt et al. 2014). This instability could be translated to high levels of genetic damage measured by the incidence of micronuclei (MN) when their cells are challenged with ionizing radiation (Rodríguez-Ribera et al. 2015), and could be the cause or the consequence of renal pathologies. In addition, it has been observed that CKD patients repair less efficiently DNA damage (Stoyanova et al. 2014).

CKD patients present increased levels of C-reactive protein (CRP), that is indicative of an inflammatory status (Tonelli et al. 2005; Vidt, 2006; Dungey et al., 2013). Oxidative stress is also a characteristic usually shown by CKD patients (Spittle et al. 2001; Morena et al. 2002; Sung et al. 2013; Corredor et al. 2015). All this information suggests that many physiological pathways are affected in CKD patients and, consequently, variants in genes regulating such pathways can affect CKD incidence and/or its progression. In this context recent genome-wide association studies (GWASs) on large European populations have identified novel genetic risk single-nucleotide polymorphisms (SNPs) associated with different CKD related pathologies like hypertension (Ehret et al. 2011), coronary artery disease (Schunkert et al. 2011), subclinical vascular disease (Bis et al. 2011), and kidney functional traits in CKD patients (Köttgen et al. 2010b; Ellis et al. 2012; Olden et al. 2013). Other studies have shown an overlap between genetic variants underpinning kidney traits and cardiovascular pathologies (Olden et al. 2013).

Aiming to determine possible association between allelic variants and susceptibility to CKD, we selected 38 SNPs from 31 candidate genes in a Spanish population. From the selected genes, 6 were related with inflammatory response (*IL-1A, IL-4, IL-6, IL-10, TNF-α,* and *ICAM)*, 2 related with the renin-angiotensin-aldosterone system (*CYP11B2* and *AGT)*, 1 with fibrogenesis (*TGFB1)*; 1 with homocysteine synthesis (*MTHFR)*, 5 with DNA repair (*OGG1, MUTYH, XRCC1, ERCC2* and *ERCC4),* 3 with phase-II metabolism *(GSTP1, GSTO1,* and *GSTO2)* and 6 with the antioxidant capacity directly related with CKD (*SOD1, SOD2, CAT, GPX1, GPX3* and *GPX4*). In addition, seven genes previously reported as being involved in CKD (*GLO1, SLC7A9, SHROOM3, UMOD, VEGFA, KL* and *MGP)* were also selected.

MATERIALS AND METHODS

Ethics statement
All individuals participating in the study provided written informed consent, and blood samples were collected under protocols approved by the Ethics Committee of the Puigvert Foundation from Barcelona, and Josep Trueta Hospital from Girona. In addition to the genotyping studies, peripheral blood samples were also used to determine standard biochemical parameters relevant for CKD.
Study populations
The study involved a total of 722 European-Spanish adults, including 548 patients suffering kidney pathologies at different stages and 174 controls. All patients had a reduced glomerular filtration rate (GFR<60 mL/min/1.73 m^2). General characteristics of all patients are indicated in Table 1. In addition to the 133 patients recruited from the hospital J. Trueta (Girona), the rest of the patients and the controls were randomly recruited at the Puigvert Foundation, Barcelona, over a period of 7 years. Controls were selected from urology clinical outpatients suffering from either prostatic pathology, urinary tract infections or kidney stones, and all had normal GFR according to their ages. All controls and 415 patients (over the included 548) belong to our previous work (Corredor et al., 2015).

Gene and SNP selection and genotyping

Gene and SNP selection
A total of 38 SNPs from 31 candidate genes were selected. Some of them were previously reported in a GWAS to be associated with CKD (*SHROOM3, UMOD, VEGFA,* and *SLC7A9*) (Köttgen 2010a; Köttgen et al. 2009, 2010b, Böger and Heid 2011); other were related to pathological processes characteristic of CKD, such as cytokines (*IL-1A, IL-4, IL-6, IL10, TNF-a* and *ICAM-1*), renin-angiotensin-aldosterone system (*AGT* and *CYP11B2*), proteins involved in fibrogenesis (*TGFB1*), and in homocystein synthesis (*MTHFR*). Some genes that coded for antioxidant enzymes were also included (*SOD1, SOD2, CAT, GPX1, GPX3* and *GPX4*). Moreover, genes involved in DNA repair pathways such as *nucleotide excision repair (ERCC2, and ERCC4) and base excision repair (OGG1, MUTYH, XRCC1), and phase-II metabolism (GSTP1, GSTO1 and GSTO2) were included. Finally, other genes related with mortality in haemodialysis patients, vascular calcification and aging (KL and MGP) (Friedman et al. 2009; Wang et al. 2013) were incorporated.* The SNP selection was mainly based on the minor allele frequency (MAF>10%) of the SNPs, as well as on published studies reporting associations of SNPs with CKD or related phenotypes. Table 2 shows details the SNPs studied in our population. When no genotyping assay was available for the selected SNP another SNP in a high linkage disequilibrium (r^2>0.8) was genotyped instead (alternative SNPs in Table 2).
Genotyping was carried out using the TaqMan SNP genotyping assays (Life Technologies) according to the manufacturer's guidelines. To assure the genotyping reliability, repeated analysis was performed in a randomly selected 10% of samples (quality controls), and samples whose genotypes were inconsistent, were excluded from the study. KASP allelic discrimination method (LGCgenomics, Middelsex, UK) was used to genotype the SNPs rs1800896, rs1800470, rs1799793, and rs1207568. DNA amplification was performed according to the LGC genomics' PCR conditions. Genotype detection for all SNPs was performed using a ViiA™ 7 v1.2.1 (Applied Biosystems) and allelic discrimination was performed with 95% confidence.

Data analysis (Statistical analysis)
For the comparison of means of the different clinical parameters, between cases and controls, the Mann Whitney test was used. For the analysis of the pathologies associated with CKD, Fisher test was performed.
In the association study, observed genotype frequencies in controls were tested for Hardy-Weinberg equilibrium using the Chi-square test. Odds ratios (ORs) and 95% confidence intervals (95% CIs) for associations between genotypes and CKD, associated phenotypes and clinical parameters converted to binary variables were estimated by logistic regression. The calculations were done for unadjusted model as well as with adjustment for age and gender. Linear regression model was used for continuous variables. Statistical significance was determined by a *P* value lower than 0.05. The analyses were performed using the following statistical software's: the Statistic Package of Social Sciences (SPSS) software for Windows version 19.0, PLINK 1.90, https://www.cog-genomics.org/plink2, (Chang et al. 2015) and Rx64 3.1.3 for Windows, http://www.r-project.org/.

RESULTS

Population

Table 1 shows some general characteristics of the individuals under study. Among CKD patients there were more men than women, reflecting the well-known higher incidence of CKD in males. As expected, statistically significant differences were observed between cases and controls for different parameters related with the pathology. The differences were statistically significant (*P*<0.001) for the levels of creatinine, glomerular filtration rate, parathyroid hormone, uric acid, proteinuria and urea. Other biomarkers such as C-reactive protein showed smaller differences between cases and controls (*P*<0.05).

SNPs associated with CKD

General information of the 38 SNPs included in the study, with their allelic frequencies and their location in the genome is described in Table 2. As indicated, we used alternative SNPs in strong linkage disequilibrium with the selected one, when no assay corresponding to the originally selected SNP was available.

Table 3 shows the observed associations (*P*<0.05) between the candidate SNPs and CKD susceptibility in the entire study population. When the analysis was adjusted for age and gender, three SNPs showed an association under the dominant model. These SNPs were rs17080528 in the *GPX1* gene, that encodes one of the most important antioxidant enzymes in humans (OR=1.87, *P*=0.001) and rs2164624 and rs156697 in the *GSTO1* and *GSTO2* genes, that are involved in the metabolism of xenobiotics and carcinogens (OR=0.50, *P*=0.001 and OR=0.60, *P*=0.021, respectively). For the SNPs rs12917707 in *UMOD* that acts as a constitutive inhibitor of calcium crystallization in renal fluids and rs4236 in *MGP* encoding a protein acting as an inhibitor of bone formation, the associations were according to the allelic model (OR=0.72, *P*=0.041 and OR=0.75, *P*=0.025, respectively).

SNPs associated with related pathologies

It is known that patients with CKD have at the same time other diseases, which are related with the presence of renal failure, either as a cause or as a consequence. Among them we can indicate hypertension (HT), cardiovascular disease (CVD), and diabetes mellitus (DM) and, in some cases, a medical history of cancer. In our study we observed a high incidence of HT (91.5%), CVD (45.2%), DM (32%), and previous cancer (30%) in patients with CKD.

When the associations between candidate SNPs and pathologies related with CKD were considered, some significant associations were observed (Table 4). For HT two genes, *GPX4,* implicated in the protection of cells against oxidative damage, *CYP11B2,* with the encoded enzyme catalysing many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids, showed an association in the dominant model and *ERCC4,* involved in nucleotide excision repair pathway, only among minor allele homozygotes. With regard to previous cancer history, the *ERCC2* gene, also involved in nucleotide excision repair, showed an association in an allelic model. For CVD, associations with the *AGT* gene coding for an angiotensinogen precursor, expressed in the liver, and cleaved by the enzyme renin in response to lowered blood pressure, and with the *ERCC2* gene were observed. Also DM was associated with the *ERCC2* and *AGT* SNPs. In addition we observed association with the *MTHFR* gene, which has previously been reported to be associated with DM in other populations (Zhou et al. 2015), although in our study only minor allele homozygotes seemed to be affected.

SNPs associated with clinical/biochemical parameters

CKD patients are characterized by a defined biochemical profile acting as a clinical indicator. To detect associations between the selected SNPs and clinical parameters both linear and logistic regression models with the median or normal value as a cut-off were used. The obtained results are shown in Table 5. As indicated, nine biochemical parameters showed any kind of statistical association with defined genes: creatinine, glomerular filtration rate, haemoglobin, resistance erythropoietin index, albumin, phosphorus, parathyroid hormone, C-reactive protein and ferritin.

When creatinine values were considered three genes showed associations according to dominant model using both linear and logistic regression: *GPX1* (linear *P*=0.003; logistic OR=1.77, *P*=0.009), *GSTO1* (*P*=0.001; OR=0.47, *P*=0.001), *GST02* (*P*=0.045; OR=0.56, *P*=0.017). Glomerular filtration rate values showed some associations, but only in the logistic regression analysis for *AGT* and *SOD1*

genes, while *GTO1* showed association according to the dominant model in both linear and logistic regression models, respectively. In addition, the *KL* gene showed association with these two biochemical parameters characteristic for CKD as well as with the resistance erythropoietin index, however only according to the allelic model in the linear regression analysis.
Haemoglobin values were only associated with the *ERCC2* gene. The same *ERCC2* SNP showed association with the resistance erythropoietin index. This parameter showed also associations with the *SOD2, VEGFA* and *OGG1* genes either in the linear or the logistic regression analysis.
The levels of albumin were associated with *SOD1* (rs17880135 and rs202446) and *ERCC2* genes in the dominant model in both the linear and logistic regression analysis; while *GSTO2* (rs15669) gene only showed association under the linear regression model and the *SOD1* (rs1041740) in the logistic regression analysis.
Phosphorus levels showed association with *ICAM-1* and *IL-4* genes according to the dominant model in the logistic regression analysis. On the other hand, the *ERCC4* gene displayed an association only in the linear regression analysis.
The levels of parathyroid hormone showed associations with *IL-1A, IL-6*, and *SHROOM3* genes in the linear regression analysis, while *ERCC2* did that when the logistic regression was used. Finally, an association with the *UMOD* gene was observed in the logistic regression, although only among 19 homozygous individuals.
C-reactive protein was associated with *SOD2 and ERCC2* according to the dominant model and with GSTP1 according to the allelic model when logistic regression was used. An association with *TGFB1* was observed only among minor allele homozygotes in the lineal regression model.
Finally, ferritin values were associated with *SOD2*, *GSTP1*, *SLC7A9* and *MPG* genes in the linear regression analysis following the dominant model. On the other hand, a *GPX4* association was observed in the logistic regression analysis.

DISCUSSION

This study succeeded to demonstrate associations between five SNPs and CKD in the list of 38 SNPs selected in the 31 candidate genes. Genes showing associations were *GPX1*, *GSTO1*, *GSTO2*, *UMOD* and *MGP*.
GPX1 is the major isoform of *GPX* expressed in normal kidney, accounting for 96% of kidney GPX activity, and showing a protective role against oxidative stress (De Haan et al. 1998). Pro198Leu and Pro197Leu variants (strongly associated to our variant, LD r^2=0.98) have been reported to be associated with reduction of GPX1 activity (Takata et al. 2012), and it has been suggest that *GPX1* is a possible candidate gene for CVD risk (Zhang et al. 2014) that, as previously indicated, is a pathology strongly linked to CKD.
Glutathione S-transferase (GST) are detoxification enzymes playing an important role in the conjugation of endogenous or exogenous xenobiotic toxins to glutathione (GSH). The family of cytosolic GSTs has different classes, including the Omega (GSTO) class (Hayes et al. 2005), and polymorphisms in genes encoding Omega class members *GSTO1* and *GSTO2* might influence the level of oxidative stress (Whitbread et al. 2005). *GSTO1* (rs4925) and *GSTO2* (rs156697) genotypes have been associated with worse prognosis and shorter survival in bladder cancer patients (Djukic et al. 2013).
The *UMOD* gene encodes for uromodulin protein acting as a constitutive inhibitor of calcium crystallization in renal fluids (Rampoldi et al. 2011). rs12917707 was associated in some GWASs with both glomerular filtration rate and better kidney function (Köttgen et al. 2009; Gorski et al. 2014). Seven SNPs in or upstream of the *UMOD* gene in high LD with rs12917707 were also associated with CKD at a genome-wide significant level (Köttgen, 2010a) and, in general, many studies corroborate earlier evidence and independently confirm the association between *UMOD* and CKD (Reznichenko et al. 2012).
The *MGP* gene encodes a protein acting as an inhibitor of bone formation. rs4236 results in a missense mutation influencing the calcification process and affecting atherosclerotic plaques (Herrmann et al. 2000). It is also known that the variant form is associated with a decreased quantity of coronary artery calcification (Crosier et al. 2009). In this context, our results are consistent with those reported in the literature showing protective effect with respect to CKD (Arcidiacono et al. 2014).
Different pathologies like hypertension, cardiovascular disease, diabetes mellitus, and cancer are strongly linked with CKD. Although in our study none of the above genes was associated with these pathologies, positive associations with some candidate SNPs and genes were observed. *GPX4,*

CYP11B2 and *ERCC4* genes were associated with hypertension. The phospholipid hydroperoxide GPX (GPX4) is an intracellular selenoprotein expressed in most tissues where it reduces lipid hydroperoxides and regulates leukotriene biosynthesis and cytokine signalling pathways. The SNP rs713041 causes a C to T substitution in a region of the *GPX4* gene corresponding to the 3´-untranslated region of the messenger RNA altering protein binding (Bermano et al. 2007). Although no association was observed in a Japanese CKD population (Hishida et al. 2013), a direct relationship between the rate of change of plasma GPX activity and the rate of change of glomerular filtration rate has been observed (Crawford et al. 2011). *The CYP11B2* gene, which encodes the human aldosterone synthase, is a cytochrome P450 enzyme which catalyzes the terminal steps of aldosterone synthesis in the zona glomerulosa cells of the adrenal cortex (Kawamoto et al. 1992). The rs1799998 polymorphism has been suggested to be associated with genetic predisposition to cardiovascular diseases, such as hypertension and myocardial infarction (Delles et al. 2001). Our study agrees with those researches revealing an association with increased risk of hypertension among CKD patients (Tsukada et al. 2002). Finally, *ERCC4* is involved in nucleotide excision repair pathway, with a reported association with cancer (Shi et al., 2012). No previous reports have found associations between this SNP and kidney diseases or hypertension.

With regard to cardiovascular disease, associations with *AGT* and *ERCC2* were observed. AGT angiotensinogen precursor is expressed in the liver and is cleaved by the enzyme renin in response to lowered blood pressure. The renin-angiotensin-aldosterone system plays a crucial role in the regulation of vascular tone and blood pressure (Li et al. 2014) and it has been associated with cardiovascular diseases (Visvikis-Siest and Marteau, 2006). It has been suggested that genetic variants in this gene may contribute to loss of renal function in the general female Caucasian population (Cooper Worobey et al. 2009). On the other hand, *ERCC2* is an important DNA repair gene in the NER pathway that has been associated with cancer incidence (Zhang et al. 2013), but no previous reports linked this gene with kidney failure or cardiovascular disease in humans. Nevertheless in a mouse model *ERCC* has been shown to be associated with age-related vascular dysfunction (Durik et al. 2012). Interestingly in our study this gene was associated with both CVD and DM as well as with a previous cancer history among CKD patients.

MTHFR, a folate-dependent enzyme, plays an important role in the conversion of homocysteine to methionine. Studies have found that specific genetic polymorphisms in the *MTHFR* gene lead to change of MTHFR enzyme activity (Shahzad et al. 2013). *MTHFR* variants are associated with susceptibility of type 2 diabetes mellitus in diabetic nephropathy patients (Zhou et al., 2015) which would support our findings. These finding also agree with those linking *MTHFR* polymorphisms with cardiovascular disease in patients with end-stage renal disease (Gao et al., 2014).

Since CKD is characterized by changes in clinical parameters both a linear and a logistic regression model, with either the median or the normal value as a cut-off, were carried out. Several associations between clinical parameters and selected SNPs were observed as indicated in Table 5. All genes showing association with CKD showed also at least one association with the evaluated clinical parameters. *GPX1* showed associations with the creatinine levels and with the glomerular filtration rate, which are strongly linked to CKD. *GSTO* genes were also associated with creatinine levels, glomerular filtration rate and albumin levels. Finally, the UMOD and MGP genes were also associated with the levels of parathyroid hormone and ferritin, respectively.

According to the importance of cytokines in inflammatory diseases we have found that *IL1A* and *IL-6* were associated with parathyroid hormone (PTH) levels. Interestingly, both cytokines have been implicated as key factors linking malnutrition, accelerated atherogenesis, and excessive morbidity and mortality in ESRD patients on hemodialysis (Bologa et al. 1998). In addition, *ICAM-1* and *IL-4* were also associated with phosphorus levels. *IL-4* has been found associated with glomerulonephritis (Mittal and Manchanda 2007) and phosphorus levels increases parathyroid cell proliferation (Roussanne et al. 2001), what would support our findings.

With regard to the selected antioxidant genes, in addition of the role of *GPX* gene, *SOD* genes were also associated with biomarkers such as ferritin levels, glomerular filtration rate, erythropoietin resistance index, C-reactive protein and albumin levels. These associations support the role of oxidative stress in the progression of several diseases, including CKD (Himmelfarb, 2004) and agree with the results showing that *SOD* was associated with advanced nephropathy (Mohammedi et al. 2011).

Some genes involved in DNA repair were associated with the same parameters. *OGG1* was associated with erythropoietin resistance index, while *ERCC2* were associated with erythropoietin resistance index, C-reactive protein, parathyroid hormone, albumin and haemoglobin levels and *ERCC4* with phosphorus levels. Alterations in *OGG1* are thought to influence the development of

oxidative stress and thus contribute to the pathophysiology of many diseases including kidney cancer (Weiss et al. 2005). In this way, this association can be linked to the other associations with genes involved in regulation of oxidative stress. Moreover, *OGG1* has also been associated with decreased insulin sensitivity suggesting that genetic alterations in *OGG1* may contribute to insulin resistance and potentially type 2 DM (Wang et al. 2006). On the other hand, *ERCC* genes are involved in DNA repair, in particular in base excision repair, and different SNPs have been associated with diverse pathologies, such as renal cell carcinoma. *ERCC1* and *ERCC4* may play important roles in the development of nephropathies, as demonstrated in mammalian models (Wang et al. 2012).

In our study the *GSTP1* gene was associated with ferritin and C-reactive protein levels. This gene is involved in a wide range of detoxification reactions which protect cells from carcinogens (Peng et al. 2013). GSTs provide protection against the electrophilic metabolites of carcinogens and reactive oxygen species. Interestingly the role of *GSTP1* (together with *GSTA1*, *GSTM1*, and *GSTT)* genotypes were determined in a group of end-stage renal disease patients showing that those carrying the null alleles showed increased susceptibility towards oxidative and carbonyl stress (Suvakov et al. 2013)

Klotho (*KL*) gene was associated with high creatinine levels, glomerular filtration rate and erythropoietin resistance index. *KL* expression in kidneys was reduced in patients with chronic renal failure (Koh et al. 2001).These findings would imply that the reduction of KL protein may be relevant to the pathophysiology of kidney disease. Our results would agree with those indicating an increased risk in different pathologies associated with CKD (Ko et al. 2012).

Additionally, *AGT* was associated with glomerular filtration rate and *TGFB1* with C-reactive protein levels. Urinary levels of angiotensinogen (AGT) were suggested as a biomarker reflecting CKD (Park et al., 2015) which would agree with the observed role on the glomerular filtration rate. On the other hand, since renal fibrosis is a common pathological consequence of CKD with tissue fibrosis closely associated with chronic inflammation in numerous pathologies, overexpression of *TGFB1* accelerates the progression of renal fibrosis and, consequently, CKD (Morinaga et al. 2016) what would support our findings.

SLC7A9, *VEGFA* and *SHROOM3* and *UMOD* were associated with ferritin levels, with erythropoietin resistance index, and with parathyroid hormone levels, respectively. Interestingly these are four genes associated to CKD in GWAS studies (Chambers et al. 2010; Köttgen et al. 2010c; Köttgen et al. 2010d; Böger et al. 2011).

SLC7A9 gene encodes the neutral and basic amino acid transport protein (rBAT) involved in the transport of the urinary dibasic amino across the renal tubular membrane (Wong et al. 2015). *VEGFA* encodes vascular endothelial growth factor A, and some variants have been identified related with nephrogenesis (Köttgen et al. 2010b). *SHROOM3* encodes an actin-binding protein expressed in the kidney, where it may have an important role in the morphogenesis of epithelial tissues during development (Lee et al. 2009). *UMOD* encodes uromodulin which is the most abundant protein in normal urine (Devuyst et al, 2005) having antimicrobial properties providing defence against uropathogens responsible for urinary tract infection. In addition it may also play a role in preventing crystallization of calcium and uric acid in kidneys and urine (Schlieper et al. 2007). In spite of the important role on kidney physiology, in our study these genes were not associated with CKD; nevertheless we have been able to detect their modulatory role on some of the biochemical parameters of CKD patients.

A detailed discussion of the pros and cons of SNPs association studies in the clinical context of CKD is outside the scope of this article and the large number of comparisons tested may hinder the clarity of the study. These studies usually rely on smaller samples, often being limited by the logistics of clinical study designs. Therefore, the ratio of the number of variables to the number of individuals/observations grows even higher, placing an additional strain on the analysis methods. The additional difficulty here lies in incorporating different data types (e.g., SNPs from different kind of genes and metabolite measurements from metabolomics studies) into the same analysis framework, which is something that the traditional parametric statistical analysis methods are not particularly efficient at either. Thus, effects of small numbers of patients in some analysis, multiple variables analysed and interconnected, are perhaps the most difficult challenge, requiring in this study, to associate complex variants to the CKD.

The overall conclusion of this study is that variants in *GPX1, GSTO1, GSTO2, UMOD,* and *MGP* genes are associated with CKD. In addition, other genes have been found to be associated with CKD related pathologies, such as hypertension (*GPX4, CYP11B2, ERCC4*), cardiovascular disease (*AGT, ERCC2*), diabetes (*AGT, MTHFR, ERCC2*) and cancer predisposition (*ERCC2*). Finally, associations with classical CKD biochemical parameters have been found for creatinine (*GPX1, GSTO1, GSTO2*),

glomerular filtration rate (*AGT, SOD1, GPX1, GSTO1*), haemoglobin (*ERCC2*), resistance index erythropoietin (*SOD2*), albumin (*SOD1*), parathyroid hormone (*IL-1A, SHROOM3, UMOD, ERCC2*), C-reactive protein (*GSTP1*), and ferritin (*SOD2, GSTP1, SLC7A9, GPX4*).

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CONFLICT OF INTEREST STATEMENT

None declared.

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Table 1. Description of the study population. Differences in biochemical and clinical parameter of the studied groups are indicated.

Characteristic	Controls N= 174 (N) mean±SD	Cases N=548 (N) mean±SD
Age (years)	(169) 56.059±15.31	(547) 66.34±13.40***
Gender (# men / women)	105 / 67	338 / 210
Creatinine (45-80 µmol/L)[a]	(169) 69.86±14.36	(184) 200.95±92.92***
Glomerular Filtration Rate (>60 mL/min/1.75 m^2)	(169) 86.82±6.98	(184) 31.22±13.69***
Erythropoietin/month (µg Darbepoetin/month)	ND	(400) 207.61±689.91
Erythropoietin Resistance Index (<10)	ND	(379) 13.50±37.45
Hemoglobin (120-160 g/L)	(96) 144.18±11.96	(539) 128.41±18.23***
Glucose (4-5.8 µmol/L)	(113) 5.86±1.93	(412) 5.60±1.85*
Cholesterol (3.20-5.20 mmol/L)	(86) 5.22±1.04	(542) 4.48±1.16**
Triglycerides (0.30-1.40 mmol/L)	(84) 1.34±1.19	(542) 1.50±0.79**
Albumin (37-47 g/L)	(66) 44.06±4.28	(405) 40.70±4.52***
Calcium (2.1-2.55 mmol/L)	(70) 2.33±0.11	(542) 2.29±0.25
Phosphorus (0.8-1.3 mmol/L)	(68) 1.07±0.14	(542) 1.30±0.40**
Parathyroid hormone (7-53 ng/L)	(25) 61.23±23.58	(401) 190.19±179.09***
Ferritin (25-250 µg/L)	(3) 88.56±49.32	(325) 245.87±257.66
C-Reactive Protein (<10 mg/L)	(13) 2.80±3.21	(362) 10.16±19.40*
Homocysteine (<15 µmol/L)	(2) 6.14±1.77	(239) 25.69±12.82*
B12 (175-750 pmol/L)	(2) 181.50±4.94	(220) 384.05±195.49
Folic acid (600-2350 nmol/L)	(2) 263.00±65.05	(218) 1075.61±736.33
Uric acid (210-420 µmol/L)	(98) 302.54±85.50	(286) 385.80±109.60***
Iron (9-27 µmol/L)	(2) 15.60±1.83	(302) 11.78±5.45
Total Iron-Binding Capacity (47-79 µmol/L)	(2) 54.70±0.28	(302) 43.54±10.71
Transferrin Saturation Index (0.12-0.14)	(2) 0.28±0.03	(301) 0.28±0.15
Proteinuria/24 h (<0.15 g/L)	(116) 0.14±0.73	(165) 0.81±1.43***
Urea (2.5-7 Mmol/L)	(56) 5.67±1.63	(179) 15.32±6.88***
Hba1c glycosylated hemoglobin (<5.7%)	(10) 5.18±2.43	(158) 4.80±1.62
Fibrinogen (2-4 µmol/L)	(2) 4.35±1.11	(91) 4.47±0.98

Mann-Whitney test; cases *vs* controls; ****P*<0.001, ***P*<0.01, **P*<0.05. ND: no data. [a] normal values are shown in parentheses

Table 2. Description of the SNPs selected for this study

Gene	SNP original	SNP alternative	LD (r^2)	Chr.	Position (NCBI dbSNP GRCh38)	Consequence of the original SNP*	Minor allele	Major allele	Minor allele frequency (NCBI dbSNP)
IL-1A	rs1800587	rs17561	0.99	2	112779646	5'-UTR	A	C	0.2175
IL-4	rs2243250	rs2070874	0.99	5	132674018	2KB 5'IL-4 [a]	T	C	0.4012
IL-6	rs1800795	rs1800797	0.97	7	22726602	intron variant	A	G	0.1382
IL-10	rs1800896			1	206773552	1.1kb 5' of IL10	C	T	0.2722
TNF-α	rs1800629			6	31575254	312bp 5' of TNF	A	G	0.0903
ICAM-1	rs5498			19	10285007	missense Glu469lys	G	A	0.3588
CYP11B2	rs1799998			8	142918184	340bp 5' of CYP11B2	G	A	0.3472
AGT	rs5050			1	230714140	5'UTR	G	T	0.1759
	rs1800470			19	41353016	Missense Pro10Leu	G	A	0.4547
TGFB1	rs1800468			19	41354682	3'UTR	T	C	0.0413
	rs1800469			19	41354391	intron variant	A	G	0.368
MTHFR	rs1801133			1	11796321	missense	T	C	0.2454
	rs17880135			21	31669690	758bp 3' of SOD1	G	T	0.0276
SOD1	rs1041740			21	31667849	intron variant	T	C	0.2428
	rs202446			21	31656328	intron variant [a]	T	G	0.0755
SOD2	rs4880			6	159692840	missense Val16Ala	G	A	0.4107
CAT	rs1001179			11	34438684	240bp 5' of CAT	T	C	0.1256
GPX1	rs1050450	rs17080528	0.98	3	49352409	Downstream gene variant [b]	T	C	0.2175
GPX3	rs870406			5	151021040	intron variant	A	G	0.0974
GPX4	rs713041			19	1106616	synonymous	T	C	0.401
OGG1	rs1052133			3	9757089	missense Ser326Cys	G	C	0.3021
MUTYH	rs3219489			1	45331833	missense Gln338His	G	C	0.3135
XRCC1	rs25487			19	43551574	missense Gln399Arg	T	C	0.2604
GSTP1	rs1695	rs749174	0.92	11	67585782	missense Ile105Val	A	G	0.2438
GSTO1	rs4925	rs2164624	0.93	10	104253687	intron variant [b]	A	G	0.1879
GSTO2	rs156697			10	104279427	missense Asn142Asp	G	A	0.4407
	rs1799793			19	45364001	Missense Asp312Asn	T	C	0.1945
ERCC2 (XPD)	rs171140			19	45361744	intron variant	C	A	0.367
	rs13181			19	45351661	missense 500pb 3'of ERCC2	G	T	0.2366
ERCC4	rs3136166			16	13938236	intron variant	G	T	0.4249
GLO1	rs386572987	rs4746	1.00	6	38682852	missense Glu111Ala [a]	G	T	0.2873
SLC7A9	rs12460876			19	32865985	intron variant	C	T	0.4235
SHROOM3	rs17319721			4	76447694	intron variant	A	G	0.2238
UMOD	rs12917707			16	20356368	66bp 5' of UMOD	T	G	0.0982
VEGFA	rs881858			6	43838872	13kb 3' of RP11-344J7.2	G	A	0.3626
MGP	rs4236			12	14882147	missense Thr83Ala	C	T	0.3854
KL	rs1207568			13	33016046	22bp 5' of KL	A	G	0.1601
	rs577912			13	33036014	intron variant	T	G	0.1953

Chr. chromosome; *according to Haploreg; LD linkage disequilibrium; [a]http://www.ncbi.nlm.nih.gov/pubmed. [b] http://www.ensembl.org/index.html

Table 3. Positive associations found between candidate SNPs and chronic kidney disease (CKD) susceptibility

	Without co-variables							Adjusted for age and sex		
Gene	**SNP**	**Geno-type**	**Affected**	**Un-affected**	**OR**	**95%CI**	***P***	**OR**	**95%CI**	***P***
GPX1	rs17080528	CC	212	91	1.00					
		CT	247	62	1.71	1.18-2.48	**0.005**	1.91	1.28-2.84	**0.001**
		TT	58	16	1.56	0.85-2.85	0.152	1.70	0.89-3.27	0.109
		T			1.41	1.08-1.86	**0.013**	1.52	1.13-2.04	**0.005**
		TT+CT	305	78	1.68	0.65-1.04	**0.004**	1.87	1.28-2.72	**0.001**
GSTO1	rs2164624	GG	215	48	1.00					
		GA	241	99	0.54	0.37-0.80	**0.002**	0.48	0.31-0.73	**0.001**
		AA	71	26	0.61	0.35-1.05	0.076	0.58	0.32-1.05	0.071
		A			0.73	0.57-0.94	**0.014**	0.70	0.53-0.91	**0.008**
		AA+GA	312	125	0.56	0.38-0.81	**0.002**	0.50	0.33-0.75	**0.001**
GSTO2	rs156697	AA	173	39	1.00					
		AG	254	90	0.64	0.42-0.97	**0.036**	0.62	0.39-0.98	**0.041**
		GG	84	34	0.56	0.33-0.94	**0.030**	0.54	0.30-0.95	**0.031**
		G			0.74	0.57-0.95	**0.020**	0.72	0.55-0.96	**0.023**
		GG+AG	338	124	0.61	0.41-0.92	**0.018**	0.60	0.39-0.92	**0.021**
UMOD	rs12917707	GG	337	97	1.00					
		GT	156	50	0.90	0.61-1.33	0.589	0.82	0.54-1.23	0.33
		TT	15	12	0.36	0.16-0.79	**0.011**	0.39	0.17-0.90	**0.026**
		T			0.74	0.55-1.01	0.054	0.72	0.52-0.99	**0.041**
		TT+GT	171	62	0.79	0.55-1.15	0.219	0.74	0.50-1.09	0.12
MGP	rs4236	TT	230	67	1.00					
		TC	186	55	0.99	0.66-1.48	0.942	0.84	0.55-1.30	0.440
		CC	84	38	0.64	0.40-1.03	0.066	0.55	0.33-0.91	**0.019**
		C			0.82	0.65-1.0	0.105	0.75	0.59-0.97	**0.025**
		CC+TC	270	93	0.85	0.59-1.21	0.362	0.72	0.49-1.07	0.101

Case-control analysis, OR, odds ratio; CI, confidence interval.

Table 4. Positive associations observed between candidate SNPs and pathologies related with chronic kidney disease (CKD), case-only analysis.

Pathology	Gene	SNP	Genotype	Group 1	Group 2	OR	95%CI	*P*
Hypertension	***GPX4***	rs713041	CC	8	191	1.00		
			CT	14	128	2.61	1.06-6.40	**0.036**
			TT	15	85	4.21	1.72-10.31	**0.001**
			T			2.01	1.31-3.07	**0.001**
			TT+CT	29	213	3.25	1.45-7.28	**0.004**
	CYP11B2	rs1799998	AA	22	138	1.00		
			AG	18	229	0.49	0.25-0.95	**0.035**
			GG	5	105	0.30	0.11-0.81	**0.018**
			G			0.53	0.33-0.83	**0.006**
			GG+AG	23	334	0.43	0.23-0.80	**0.007**
	ERCC4	rs3136166	TT	201	13	1.00		
			TG	221	21	1.46	0.36-0.71	0.293
			GG	46	10	3.36	1.38-8.14	**0.007**
			G			1.79	1.13-2.83	**0.012**
			GG+TG	267	31	1.80	0.91-3.51	0.088
Previous cancer	***ERCC2***	rs13181	TT	23	204	1.00		
			TG	17	212	1.20	0.77-1.88	0.408
			GG	3	54	3.47	1.39-8.67	**0.007**
			G			1.52	1.09-2.12	**0.013**
			GG+TG	20	266	1.43	0.93-2.19	0.101
Cardiovascular disease	***AGT***	rs5050	TT	31	302	1.00		
			TG	11	144	0.67	0.45-0.98	**0.040**
			GG	0	18	0.60	0.23-1.56	0.297
			G			0.70	0.51-0.97	**0.033**
			GG+TG	11	162	0.66	0.45-0.95	**0.028**
	ERCC2	rs1799793	CC	28	232	1.00		
			CT	12	188	0.58	0.40-0.84	**0.004**
			TT	4	45	0.80	0.43-1.48	0.483
			T			0.76	0.58-0.99	**0.045**
			TT+CT	16	233	0.62	0.43-0.87	**0.007**
Diabetes mellitus	***AGT***	rs5050	TT	31	302	1.00		
			GT	11	144	1.32	0.87-2.02	0.188
			GG	0	18	1.72	0.55-5.36	0.345
			G			1.32	0.92-1.88	0.122
			GG+GT	11	162	1.36	0.90-2.07	**0.050**
	MTHFR	rs1801133	CC	18	160	1.00		
			TC	19	204	0.90	0.58-1.39	0.649
			TT	6	86	0.56	0.33-0.96	**0.034**
			T			0.76	0.58-0-99	**0.049**
			TT+TC	25	290	0.78	0.52-1.17	0.233
	ERCC2	rs1799793	CC	28	232	1.00		
			TC	12	188	1.54	1.03-2.30	**0.033**
			TT	4	45	1.10	0.58-2.08	0.766
			T			1.21	0.91-1.61	0.181
			TT+TC	16	233	1.44	0.99-2.08	**0.050**

Case-case analysis, OR, odds ratio; CI, confidence interval. Group 1: With the pathology; Group 2: Without the pathology.

Table 5. Positive associations observed between candidates SNPs and different clinical parameters related with chronic kidney disease (CKD). Available data from both cases and controls were used for the analysis.

Clinical Parameters (normal values) (Cut-off value)	Gene	SNP	Genotype	Group1	Group 2	OR	95%CI	$P_{logistic}$	P_{linear}
Creatinine (45-80 µmol/L) (100 µmol/L*)	***GPX1***	rs17080528	CC	93	69	1.00			
			CT	62	88	1.91	1.22-2.99	**0.005**	**0.003**
			TT	15	15	1.21	0.55-2.64	0.634	0.434
			T			1.38	0.99-1.93	0.060	**0.028**
			TT+CT	77	103	1.77	1.15-2.71	**0.009**	**0.003**
	GSTO1	rs2164624	GG	50	75	1.00			
			GA	99	75	0.44	0.28-0.7	**0.001**	**0.001**
			AA	25	27	0.58	0.3-1.11	0.099	0.098
			A			0.67	0.49-0.92	**0.014**	**0.015**
			AA+GA	124	102	0.47	0.3-0.73	**0.001**	**0.001**
	GST02	rs156697	AA	40	59	1.00			
			AG	90	85	0.57	0.35-0.95	**0.029**	0.059
			GG	33	30	0.52	0.27-0.99	**0.045**	0.130
			G			0.70	0.51-0.96	**0.029**	0.088
			GG+AG	123	115	0.56	0.35-0.9	**0.017**	**0.045**
	KL	rs577912	GG	119	113	1.00			
			GT	47	48	1.18	0.73-1.91	0.494	0.144
			TT	1	8	3.88	0.79-19.1	0.095	**0.024**
			T			1.36	0.9-2.06	0.138	**0.019**
			TT+GT	48	56	1.29	0.81-2.06	0.276	0.053
Glomerular filtration rate (>60mL/min/ 1.75m²) (60mL/min/ 1.75m²)**	***AGT***	rs5050	TT	86	141	1.00			
			TG	48	72	1.77	1.10-2.85	**0.019**	**0.047**
			GG	2	12	1.34	0.50-3.60	0.563	0.955
			G			1.45	1.00-2.11	**0.050**	0.165
			GG+TG	50	84	1.70	1.08-2.67	**0.021**	0.070
	SOD1	rs17880135	TT	125	207	1.00			
			TG	14	15	ND	ND	ND	0.345
			GG	0	2	ND	ND	ND	0.107
			G			2.31	1.06-5.00	0.034	0.114
			GG+TG	14	17	2.29	1.02-5.16	**0.046**	0.191
	GPX1	rs17080528	CC	60	92	1.00			
			CT	61	114	2.02	1.29-3.18	**0.002**	**0.002**
			TT	19	22	1.35	0.62-2.94	0.454	0.661
			T			1.46	1.04-2.04	**0.028**	**0.049**
			TT+CT	80	136	1.89	1.23-2.90	**0.004**	**0.006**
	GSTO1	rs2164624	GG	53	99	1.00			
			GA	70	101	0.50	0.31-0.80	**0.004**	**0.001**
			AA	19	34	0.70	0.36-1.34	0.276	0.090
			A			0.75	0.55-1.02	0.065	**0.012**
			AA+GA	89	135	0.54	0.35-0.84	**0.007**	**0.001**
	KL	rs577912	GG	94	157	1.00			
			GT	43	62	1.04	0.64-1.68	0.875	0.239
			TT	3	6	8.14	1.00-66.12	**0.050**	**0.013**
			T			1.33	0.88-2.00	0.180	**0.025**
			TT+GT	46	68	1.19	0.75-1.89	0.469	0.086
Hemoglobin (120-160 g/L) (130 g/L)*	***ERCC2***	rs171140	AA	54	41	1.00			
			AC	74	80	1.71	0.4-0.86	**0.007**	**0.038**
			CC	44	45	1.58	0.4-1	**0.048**	**0.035**
			C			1.27	0.63-0.99	**0.038**	**0.030**
			CC+AC	118	125	1.67	0.42-0.86	**0.006**	**0.019**
Resistance index erythro-poietin (<10) (10)**	***SOD2***	rs4880	AA	58	59	1.00			
			AG	70	78	0.86	0.54-1.37	0.527	**0.034**
			GG	41	38	0.92	0.53-1.60	0.761	0.120
			G			0.95	0.72-1.25	0.699	0.076
			GG+AG	111	116	0.88	0.57-1.35	0.556	**0.027**
	ERCC2	rs171140	AA	41	54	1.00			
			AC	80	74	1.80	1.09-2.97	**0.022**	0.120
			CC	45	44	1.41	0.77-2.58	0.264	0.214
			C			1.21	0.89-1.63	0.219	0.193
			CC+AC	125	118	1.68	1.04-2.70	**0.034**	0.105

Clinical Parameters (normal values) (Cut-off value)	Gene	SNP	Genotype	Group1	Group 2	OR	95%CI	$P_{logistic}$	P_{linear}
Resistance index erythro-poietin (<10) (10)**	***VEGFA***	rs881858	AA	70	71	1.00			
			AG	85	85	0.91	0.59-1.40	0.669	**0.027**
			GG	15	14	1.43	0.67-3.02	0.351	0.476
			G			1.07	0.78-1.48	0.665	0.095
			GG+AG	100	99	0.98	0.65-1.48	0.926	**0.033**
	OGG1	rs1052133	CC	108	111	1.00			
			CG	60	53	1.62	1.05-2.49	**0.030**	0.167
			GG	7	9	1.18	0.40-3.46	0.770	0.929
			G			1.40	0.97-2.01	0.072	0.274
			GG+CG	67	62	1.57	1.03-2.38	**0.036**	0.189
	KL	rs1207568	GG	116	129	1.00			
			GA	49	36	1.12	0.68-1.83	0.514	0.195
			AA	2	7	0.83	0.22-3.16	0.824	**0.028**
			A			1.04	0.69-1.57	0.695	**0.026**
			AA+GA	51	43	1.09	0.67-1.74	0.581	0.070
Albumin (37-47 g/L) (41.70 g/L)*	***SOD1***	rs17880135	TT	150	151	1.00			
			TG	10	18	ND	ND	ND	**0.047**
			GG	0	2	ND	ND	ND	0.449
			G			0.40	0.21-0.76	**0.006**	**0.035**
			GG+TG	10	20	0.40	0.20-0.78	**0.008**	**0.037**
	SOD1	rs202446	GG	113	111	1.00			
			GT	50	49	0.50	0.33-0.77	**0.001**	**0.003**
			TT	0	5	0.61	0.21-1.81	0.378	0.975
			T			0.59	0.41-0.84	**0.004**	**0.022**
			TT+GT	50	54	0.51	0.34-0.77	**0.001**	**0.006**
	SOD1	rs1041740	CC	71	71	1.00			
			CT	81	73	1.48	1.00-2.21	0.052	0.151
			TT	18	24	2.42	1.27-4.59	**0.007**	0.659
			T			1.53	1.15-2.03	**0.004**	0.324
			TT+CT	99	97	1.63	1.12-2.38	**0.011**	0.176
	GSTO2	rs156697	AA	40	59	1.00			
			AG	90	85	0.84	0.55-1.27	0.404	0.177
			GG	33	30	0.62	0.36-1.07	0.087	**0.008**
			G			0.79	0.61-1.04	0.092	**0.009**
			GG+AG	123	115	0.77	0.52-1.15	0.204	**0.045**
	ERCC2	rs171140	AA	41	54	1.00			
			AC	80	74	1.75	1.10-2.76	**0.017**	**0.004**
			CC	45	44	1.61	0.94-2.74	0.081	**0.012**
			C			1.27	0.97-1.66	0.080	**0.012**
			CC+AC	125	118	1.70	1.10-2.63	**0.017**	**0.002**
Phosphorus (0.8-1.3 mol/L) (1.19 mil/L)*	***ICAM-1***	rs5498	AA	48	50	1.00			
			AG	89	77	1.52	1.02-2.26	**0.040**	0.100
			GG	35	46	1.40	0.90-2.18	0.140	0.204
			G			1.18	0.95-1.47	0.143	0.267
			GG+AG	124	123	1.47	1.02-2.13	**0.040**	0.092
	IL-4	rs2070874	CC	127	132	1.00			
			CT	42	39	1.52	1.05-2.22	**0.029**	0.239
			TT	4	1	0.96	0.32-2.90	0.938	0.207
			T			1.34	0.96-1.85	0.081	0.754
			TT+CT	46	40	1.47	1.02-2.12	**0.040**	0.420
	ERCC4	rs3136166	TT	68	64	1.00			
			TG	76	74	0.83	0.58-1.17	0.285	0.462
			GG	26	27	0.70	0.40-1.20	0.191	**0.023**
			G			0.83	0.65-1.07	0.144	**0.048**
			GG+TG	102	101	0.80	0.57-1.12	0.187	0.175
Parathyroid hormone (7-53 ng/L) (123.5 ng/L)*	***IL1A***	rs17561	CC	96	82	1.00			
			CA	61	73	0.98	0.68-1.41	0.908	0.257
			AA	10	14	1.78	0.88-3.59	0.107	0.052
			A			1.15	0.88-1.52	0.308	**0.044**
			AA+CA	71	87	1.07	0.76-1.52	0.693	0.092
	IL6	rs1800797	GG	84	86	1.00			
			GA	60	58	0.69	0.48-1.01	0.057	0.086
			AA	17	13	0.82	0.47-1.43	0.493	**0.026**
			A			0.84	0.65-1.08	0.172	**0.016**
			AA+GA	77	71	0.72	0.51-1.03	0.070	**0.029**

Clinical Parameters (normal values) (Cut-off value)	Gene	SNP	Genotype	Group1	Group 2	OR	95%CI	$P_{logistic}$	P_{linear}
Parathyroid hormone (7-53 ng/L) (123.5 ng/L)*	***SHROOM3***	rs17319721	GG	79	76	1.00			
			GA	98	93	0.91	0.62-1.33	0.624	**0.016**
			AA	27	37	0.71	0.42-1.20	0.204	0.401
			A			0.86	0.67-1.10	0.228	**0.016**
			AA+GA	125	130	0.86	0.60-1.22	0.393	**0.013**
	UMOD	rs12917707	GG	106	97	1.00			
			GT	55	50	0.70	0.48-1.02	0.063	0.269
			TT	6	13	9.50	1.20-74.99	**0.033**	0.378
			T			0.94	0.68-1.32	0.739	0.372
			TT+GT	61	63	0.79	0.55-1.15	0.220	0.253
	ERCC2	rs171140	AA	54	41	1.00			
			AC	74	80	1.63	1.07-2.48	**0.022**	**0.023**
			CC	44	45	1.44	0.88-2.37	0.150	**0.048**
			C			1.21	0.94-1.55	0.132	0.175
			CC+AC	118	125	1.57	1.06-2.33	**0.026**	0.253
C-reactive protein (<10mg/L) (10 mg/L)**	***SOD2***	rs4880	AA	59	58	1.00			
			AG	79	69	0.57	0.36-0.91	**0.020**	0.336
			GG	39	40	0.64	0.37-1.12	0.116	0.345
			G			0.77	0.59-1.02	0.067	0.305
			GG+AG	118	109	0.59	0.39-0.91	**0.018**	0.266
	TGFB1	rs1800468	CC	150	138	1.00			
			CT	19	30	0.87	0.49-1.55	0.643	0.826
			TT	3	3	0.73	0.16-3.32	0.684	**0.033**
			T			0.87	0.54-1.39	0.550	0.159
			TT+CT	22	33	0.86	0.49-1.48	0.577	0.388
	GSTP1	rs749174	GG	80	76	1.00			
			GA	70	82	0.80	0.48-1.33	0.377	0.721
			AA	22	14	0.37	0.15-0.94	**0.046**	0.861
			A			0.68	0.47-0.98	**0.046**	0.100
			AA+GA	92	96	0.68	0.42-1.11	0.143	0.706
	ERCC2	rs1799793	CC	77	85	1.00			
			TC	75	67	1.54	0.99-2.38	0.054	0.816
			TT	15	19	1.65	0.81-3.40	0.170	0.484
			T			1.37	1.00-1.89	**0.050**	0.531
			TT+TC	90	86	1.56	1.03-2.36	**0.036**	0.671
Ferritin (25-250 (ug/L) (154.5 ug/L)*	***SOD2***	rs4880	AA	59	50	1.00			
			AG	65	72	0.77	0.46-1.27	0.298	**0.026**
			GG	35	35	0.85	0.46-1.55	0.590	0.067
			G			0.90	0.67-1.22	0.507	**0.042**
			GG+AG	100	107	0.79	0.5-1.26	0.326	**0.016**
	GSTP1	rs749174	GG	78	65	1.00			
			GA	64	70	0.76	0.48-1.22	0.259	**0.029**
			AA	20	22	0.76	0.38-1.51	0.430	0.104
			A			0.84	0.61-1.15	0.279	**0.030**
			AA+GA	84	92	0.76	0.49-1.18	0.226	**0.017**
	SLC7A9	rs12460876	TT	64	47	1.00			
			TC	71	89	0.59	0.36-0.96	**0.032**	**0.009**
			CC	26	21	0.91	0.46-1.81	0.786	0.843
			C			0.86	0.62-1.19	0.353	0.307
			CC+TC	97	110	0.65	0.41-1.03	0.067	**0.028**
	MPG	rs4236	TT	64	69	1.00			
			TC	60	57	1.14	0.69-1.87	0.618	**0.023**
			CC	25	25	1.08	0.56-2.07	0.821	0.485
			C			1.06	0.78-1.44	0.725	0.187
			CC+TC	85	82	1.12	0.71-1.76	0.633	**0.041**
	GPX4	rs713041	CC	84	67	1.00			
			CT	51	57	1.77	1.04-3.02	**0.037**	0.096
			TT	32	38	1.52	0.81-2.84	0.194	0.659
			T			1.29	0.95-1.76	0.109	0.453
			TT+CT	83	95	1.67	1.03-2.71	**0.036**	0.166

*Median as a cut-off, **normal value as a cut-off in the logistic regression analysis; Group 1: individuals with low low risk biochemical parameter; Group 2: individuals with high-risk biochemical parameter indicative of poor prognosis. ND, No data.

7.4. Anexo 4. Artículo 6

Loci Associated With Genomic Damage Levels in Spanish Chronic Kidney Disease Patients

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Key words: CKD patients; genomic damage, single nucleotide polymorphisms;

ABSTRACT

Chronic kidney disease (CKD) is a multifactorial disorder with an important genetic component, and several studies have demonstrated potential associations with allelic variants. Nevertheless, no studies have stablished relationships between DNA damage or genomic instability present in CKD patients and gene SNPs. To fill in this gap the potential role of polymorphisms in genes involved in base excision repair (*OGG1*, rs1052133; *MUTYH*, rs3219489; *XRCC1*, rs25487), nucleotide excision repair (*ERCC2/XPD*, rs1799793, rs171140, rs13181; *ERCC4*, rs3136166); phase II metabolism (*GSTP1*, rs749174; *GSTO1*, rs2164624; *GSTO2*, rs156697), and antioxidant enzymes (*SOD1*, rs17880135, rs1041740, rs202446; *SOD2*, rs4880; *CAT*, rs1001179; *GPX1*, rs17080528; *GPX3*, rs870406: *GPX4*, rs713041) were genotyped. In addition, some genes involved in CKD (*AGT*, rs5050; *GLO1*, rs386572987; *SHROOM3*, rs17319721) were also evaluated. Our results showed significant associations with *XRCC1* and *ERCC2/XP* (rs13181) as genes directly involved in DNA repair pathways. Interestingly the three genes associated to CKD (*AGT*, *GLO1*, and *SHROOM3*) showed associations with high levels of DNA damage, oxidatively damaged DNA and genomic instability. These results support our view that genomic instability can be considered a biomarker of CKD status.

INTRODUCTION

In Europe and North America, chronic kidney disease (CKD) affects about 11% of adults. CKD is associated with high morbidity and in the advanced stages requires life-support treatment by renal dialysis or transplantation (Levey et al., 2007). CKD is a multifactorial disorder with an important genetic component (Köttgen et al., 2009). Multiple studies, such as familial aggregation studies, have provided evidence for a genetic component to kidney disease. Heritability estimates of serum creatinine (eGFRcrea), the most commonly used measure of kidney function, are reported between 0.41 and 0.75 in individuals with major CKD risk factors such as hypertension or diabetes (Bochud et al., 2005), and 0.33 in a general population-based sample (Fox et al., 2004). Although rare genetic variants causing different forms of monogenetic kidney disease have been identify, common CKD susceptibility variants have been difficult to detect by linkage or candidate gene studies (Friedhelm, 2010). Nevertheless, recent studies have identified common variants at the *SHROOM3* and *AGT* genes, among others loci, associated with kidney function in European and African-American populations (Köttgen et al., 2009; Böger et al., 2011; Shaikh, et al. 2014). Additionally *GLO1* gene has also found to be associated with renal cancer (Chocholatý et al., 2014).

Techniques such as the comet and micronucleus (MN) assays in peripheral blood lymphocytes have demonstrated that CKD patients present elevated levels of genomic damage (Corredor et al., 2015). In addition, these patients also showed genomic instability and the genomic damage induced by *in vitro* irradiation of patients cells was significantly higher than observed in controls (Rodríguez-Ribera et al., 2015). Furthermore, CKD patients show deficiencies in repair oxidatively damages DNA (Stoyanova et al., 2014). In this scenario, the looking for genetic variants explaining the genomic instability of CKD patients seems urgent.

The role of different polymorphism (SNPs), as modulation factors in the development of end-stage renal disease (ESRD), has been demonstrated. Nevertheless, data on the role of genetic variants modulating the levels of genomic damage in CKD patients are practically inexistent. Until now there is only one study showing the role of the Ser326Cy polymorphism in the *OGG1* gene, modulating the levels of 8-oxodG in leukocytes of CKD patients (Tarng et al., 2001). Therefore, it is necessary to determine further genetic variants involved in the modulation of the genomic damage observed in CKD patients.

With this aim we have evaluated the role of 21 single nucleotide polymorphisms (SNPs from 17 genes related with BER (*OGG1, MUTYH, XRCC1*), NER (*ERCC2/XPD), ERCC4*), antioxidant enzymes (*SOD1, SOD2, CAT, GPX1, GPX3, GPX4*), and phase II metabolism (*GSTP1, GSTO1, GSTO2*). Moreover, some genes previously reported as being involved in CKD were selected: *AGT, GLO1* and *SHROOM3*. The *AGT* (renin-angiotensin-aldosterone system) gene was reported as inducing susceptibility to essential hypertension (Li et al., 2015) and accelerated risk of renal function decline (Cooper Worobey et al., 2009). The *GLO1* (Glyoxalase I) allelic variables of this gene have been associated with renal cell carcinoma (Chocholatý et al., 2014). Finally, the *SHROOM3* gene (Shroom family member 3) has been identifying as susceptibility loci for glomerular filtration rate in CKD patients in a genome-wide association study (GWAS) (Böger et al., 2011).

MATERIALS AND METHODS

Study populations

The study involved a total of 589 Caucasian Spanish adults, including 415 patients suffering kidney pathologies at different stages and 174 controls. All patients had a reduced glomerular filtration rate (GFR < 60 mL/min/1.73 m^2). Controls were selected from urology clinical outpatients suffering from either prostatic pathology, urinary tract infections or kidney stones, and all had normal GFR according to their age.

In total we had 255 men and 160 women (61.4% and 38.5%, respectively) CKD patients with an average age of 66.79±14.29 years. Healthy controls had 106 men and 68 women (61% and 39%, respectively), with an average age of 56.059±15.31 years. The general characteristics of all patients are indicated in Table 1. Patients and controls were randomly recruited at the Puigvert Foundation, Barcelona, over a period of 6 years. All individuals participating in the study provided written informed consent, and blood samples were collected under protocols approved by the Ethics Committee of the Puigvert Foundation. To determine the levels of genetic damage, DNA was extracted from peripheral blood lymphocytes. Standard biochemical blood analyses were also carried out for the determination of routine parameters relevant to the illness. Patients from our previous work were included in the present study (Corredor et al., 2015).

DNA damage determination

Comet assay

DNA breaks present in peripheral blood lymphocytes were measured using the comet assay performed following the standard protocol, as previously described (Stoyanova et al., 2010). Briefly, isolated lymphocytes from 2 mL of blood from each patient were cryopreserved until use, in 500 µL of medium containing 90% serum and 10% DMSO. Comet assay was carried using Gelbond® films (GF) instead of microscopic glass slides as a support for the agarose gel. The use of hydrophilic films facilitates the rapid processing of numerous samples, increasing the efficiency of the alkaline comet technique, without sacrificing the reliability or sensitivity of the assay (McNamee, McLean et al., 2000; Azqueta et al., 2013). Lymphocytes were isolated using Ficoll–Paque density gradient from 500 µL of whole blood; cells were adjusted to a concentration of 17,800 cells in 25 µL PBS and carefully re-suspended in 225 µL of 0.75% low melting agarose (LMA) at 37 ºC and dropped onto a GF (10.5 x 7.5 cm). Forty-eight drops (7 µL each) were placed on each GF and samples of eight donors were run simultaneously. Each donor was represented by six drops. Lymphocytes were lysed for a minimum of 1 h at 4 ºC in a dark chamber containing a cold fresh lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl and 1% Triton X-100, adjusted to pH 10). To allow DNA denaturation, unwinding and exposure of alkali-labile sites, GF were placed in an horizontal gel electrophoresis tank filled with freshly cold (4 ºC) electrophoresis solution (1 mM Na_2EDTA and 300 mM NaOH, adjusted to pH 13) for 35 min. Electrophoresis was performed in the same buffer for 20 min at 20 V and 300 mA. After electrophoresis, GF were neutralized with two 5-min washes with PBS 1X, followed by 1 min wash with water, and then incubated overnight in 100% ethanol for fixation. Sheets were then dried and stored in the dark at room temperature until scoring. Just before the microscopic analysis, GF were stained with 20 µL of SybrGold. The images were examined at 20x magnification with a Komet 5.5 Image Analysis System (Kinetic Imaging Ltd, Liverpool, UK) fitted with an Olympus BX50 fluorescence microscope equipped with a 480–550 nm wide band excitation filter and a 590 nm barrier filter. A total of one hundred randomly selected cells were analyzed per patient and the % tail DNA was used as a measure of DNA damage.

Detection of oxidative damage

To determine the levels of oxidized DNA bases present in lymphocytes, GF were washed two times (10 and 50 min, 4 ºC) after cell lysis in an enzyme buffer solution (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8.0) containing the enzyme FPG (formamidopyrimidine DNA glycosylase) (Azqueta et al., 2013). FPG enzyme was produced in our laboratory, and a concentration of 0.0089 µg/µL of enzyme extract was used in each treatment.

Each sample was analyzed using two GF. One GF remained in the cell lysis solution in order to assess basal DNA damage. The second was treated with the enzyme buffer without FPG, to control

for any effects of the buffer alone. GF were incubated with enzyme buffer (with and without FPG) for 30 min at 37 ºC, after that, samples were processed as in the standard alkaline comet assay procedure. Net oxidative DNA damage was calculated by subtracting the damage scored in the samples incubated with buffer from those incubated with FPG.

Lymphocyte culture and micronucleus assay

Blood samples from heparinized vacutainers were processed as described previously using cytochalasin-B to arrest cytokinesis (Rodríguez-Ribera et al., 2015). Two of the four cultures set up were irradiated with 0.5 Gy ^{137}Cs gamma rays in an irradiator IBL 437C, type H, No. 701 (SCHERING CIS Bio International) at the Unitat Tècnica de Protecció Radiològica (UTPR-UAB). To determine the frequency of binucleated cells with micronuclei (BNMN), 1000 binucleated lymphocytes per sample (irradiated and non-irradiated) were blind scored on coded slides, according to standard criteria (Fenech, 2007). In addition, 500 cells with one, two or more nuclei were scored to determine the cytokinesis-block proliferation index (CBPI) (Surrallés et al., 1995). The net effect of irradiation (IR) was calculated by subtracting the background BNMN values in the non-irradiated samples from the values obtained in the irradiated samples (induced BNMN) (Rodríguez-Ribera et al., 2015). The resulting induced BNMN value represents the frequency of BNMN produced by IR.

SNP selection and genotyping

SNP selection

Selection of SNPs with the candidate gene is essential, because only some of they may be related to the response. The selection was focus mainly in the frequency of the SNP with a high probability to be detected and the number of previous genotyping studies. Thus, most of the selected SNPs have been previously reported to have some association directly with the disease or reported. In Table 2, there are indicated the selected SNPs. In cases where primers were not available we selected SNPs with the highest linkage disequilibrium (called SNPs alternative).
A total of 21 SNPs from 17 genes were selected. Some of them have been previously reported to be associated with CKD such as *SHROOM3* (shroom family member 3) (Köttgen, 2010), *AGT* (renin-angiotensin-aldosterone system) (Li et al., 2015), and *GLO1* (glyoxalase I) (Chocholatý et al., 2014). Other were related to DNA repair process like, *nucleotide excision repair (ERCC2/XPD and ERCC4) and base excision repair (OGG1, MUTYH and XRCC1); to antioxidant enzymes (SOD1, SOD2, CAT, GPX1, GPX3 and GPX4), and phase II metabolism enzymes (GSTP1, GSTO1 and GSTO2)* (Table 2).
Genotyping was carried out using the TaqMan SNP genotyping assays (Life Technologies) according to the manufacturer's guidelines. To assure the genotyping reliability, repeated analysis was performed in a randomly selected 10% of samples (quality controls), and samples whose genotypes were inconsistent, were excluded. KASP allelic discrimination method (LGCgenomics, Middelsex,UK) was used to genotype the SNPs rs1800896, rs1800470, rs1799793, and rs1207568. DNA amplification was performed according to the LGCgenomics'PCR conditions. Genotype detection for all SNPs was done using a ViiA™ 7 v1.2.1 (Applied Biosystems) and with an allelic discrimination was performed with 95% confidence interval.

Data analysis (Statistical analysis)

For the comparison of means between the different clinical and genomic parameters between cases and controls, the Mann Whitney test was done for most cases, due to, lack of normality, (Kolmogorov–Smirnov-Test. Continuous phenotypes were analyzed using linear regression and logistic regression for each SNP. Statistical significance was determined by a P value lower than 0.05. Odds ratios (ORs) and 95% confidence intervals (95% CIs) for associations between genotypes and genomic damage phenotypes.
The analyses were performed using the Statistic Package of Social Sciences (SPSS) software for Windows version 19.0. Statistical significance was defined as a P value lower than 0.05., PLINK 1.90 Beta Windows 64-bits (Chang et al., 2015) (https://www.cog-genomics.org/plink2) and Rx64 3.1.3 for Windows (http://www.r-project.org/).

RESULTS

In the present study, we analyzed a group of 415 CKD patients that was compared with a group of 174 healthy donors. A slight difference between CKD patients and controls was observed for age (66.79 *vs* 56.06 years, respectively), but no differences were observed in the gender percentage between the studied groups. When those blood biochemical parameters commonly used in CKD patients were compared, significant differences were observed between groups, as expected. Creatinine, glomerular filtrated rate (FGR), HDL, PTH, proteinuria/24 h, urea, C-reactive protein (CRP), and homocysteine were significantly outside the normal values in the CKD patients group (Table 1). This fact confirms the status of patients with chronic kidney disease. The 89.9% of the patients showed hypertension, a 62.9% presented dyslipidemia, a 55.2% suffered cardiovascular disease, a 29.9% had cancer before their inclusion in the study and 28% showed *diabetes mellitus*.
When the levels of genetic damage were measured in CKD patients, the results showed a significant increase of the DNA damage (Table 2). Although no effects were detected in the levels of oxidatively damaged DNA, we were able to detect genomic instability among CKD patients since when their lymphocytes were challenged with ionising radiation, higher levels of chromosome damage were obtained with regard to controls. Significant correlations were observed between comet and BNMN for both CKD patients and controls.
To demonstrate potential associations between genetic variants and the levels of genomic damage we selected a group of genes/variants, as indicated in Table 3. Due to the previously demonstrated radiosensitivity of CKD patients, we included genes involved in base-excision repair (BER): (*OGG1, MUTYH, XRCC1*) and nucleotide-excision repair (NER): (ERCC2/XPD, ERCC4). In addition, genes related to antioxidant enzymes (*SOD1, SOD2, CAT, GPX1 GPX3, CPX4,*) and to phase II metabolism enzymes (*GSTP1, GSTO1, GSTO2*) were also selected. According to the demonstrated role of *AGT, GLO1* and *SROOM3* genes in CKD they were also included in the study.
To increase the strength of the assay, data from CKD patients and controls were included in the association study. The logistic analysis divided the distribution into two groups: group 1 (patients with low level of DNA damage) and group 2 (patients with high levels of DNA damage). The cut off was the median. The SNPs that showed association with the genomic damage are indicated in Table 4. Those SNPs that were not significant are included in the supplementary table (Table S1). From our results it is shown that some genes related to DNA repair showed significant associations. This occurred with the rs25487 variant from the base-excision repair *XRCC1* gene and with two variants (rs171140 and rs13181) from the nucleotide-excision repair *ERCC2* gene. Interestingly the genes initially associated with CKD (*AGT, GLO1, SHROOM3*) also showed association with the levels of DNA damage. *XRCC1* showed association with the basal levels of DNA breaks detected by the comet assay. In spite that no differences were observed between CKD patients and controls with regard to the levels of oxidatively damaged DNA, *XRCC1, GLO1* and *SHROOM3* showed associations with the levels of such kind of DNA damage. With regard to the levels of chromosome damage, only *AGT* showed association with the basal levels of BNMN. Nevertheless, *AGT, SHROOM3, XRCC1* and *ERCC2/XPD* were associated with radiosensitivity measured through the micronucleus assay.

DISCUSSION

From our results we confirmed that CKD patients have higher levels of genomic damage than controls, as previously reported (Sandoval et al., 2012; Corredor et al., 2015; Rodríguez-Ribera et al., 2015). In addition, we reported for first time the modulating role of different genes (*XRCC1, ERCC2/XPD, AGT, GLO1* and *SHROOM3*) on the levels of DNA damage as detected using both comet and micronucleus assays. We have also shown the presence of common underlying mechanisms since positive associations were observed between the levels of genomic/oxidative DNA damage (comet) and chromosome damage (MN). This association was also observed with regard to the radiosensitivity values what would confirm our previous results suggesting the importance of genomic instability in the status of CKD patients (Rodríguez-Ribera et al., 2015). According to this general association between CKD and genomic damage, it is expected to find associations also between the most characteristics CKD biomarkers, such as creatinine and urea

(Cakmak Demircigil et al., 2011) and glomerular filtration rate (Sandoval et al., 2010), and high values of genetic damage.
We have already reported that DNA damage levels in CKD patients are in part associated with the repair capacity of the patients, as determined by their ability to repair 8OHdG lesions (Stoyanova et al., 2014). In this way, our association study showing positive association for *XRCC1* and *ERCC2/XPG* genes would support our previous finding. It is well-known that a complex system of DNA repair enzymes is needed to protect the genome from the consequences of exogenous and endogenous mutagenic influences (Hoeijmakers, 2001). Polymorphisms in DNA repair genes that reduce their capacity to repair DNA damage lead to increased cancer or other disease susceptibility (Hakem, 2008; Riceri et al., 2012). Germ-line alterations in DNA repair-related genes would be responsible of the so called 'mutator phenotype' (Loeb et al., 2008).
Exposure to both endogenous and exogenous agents leads to single-strand DNA breaks followed by base excision repair (NER), in which the XRCC1 enzyme is involved (Brem and Hall, 2005). The SNP rs25487 in exon 10, where A substitutes G, lead to the amino acid substitutions Arg399Gln and has been repeatedly associated with increased risk of numerous types of cancer (Hung, 2005), including colorectal cancer (Przybylowska et al., 2013), and a worse overall survival in breast cancer patients receiving postoperative radiotherapy (Tengström et al., 2014). In addition high levels of DNA damage have been reported in Arg399Gln carriers (Hanssen-Bauer et al., 2012). This would agree with our results showing higher levels of DNA damage, oxidative DNA damage, and genomic instability in those individuals carrying the variant allele.
The protein encoded by the *ERCC2/XPD* gene removes and corrects oligonucleotide fragments containing a variety of lesions such as, UV-induced lesions, chemical adducts and crosslinks (Duell et al., 2000), and it is an integral member of the basal transcription factor BTF2/TFIIH complex (Hu et al., 2012). The variant rs13181 has been associated with increased risk of cancer (Riceri et al., 2012) and it has shown to modulate the basal frequency of chromosomal aberrations in healthy individuals (Vodicka et al., 2015), as well as in lymphocytes from healthy individuals when they were treated with bleomycin (Angelini et al., 2008). In addition, its role as genomic instability factor has also been demonstrated in lymphocytes from ovarian cancer patients when exposed to beomycin (Monteriro et al., 2014). This particular sensitivity would agree with our results, showing association between high levels of induced BNMN and *ERCC2* variants (rs13181 and rs171140).
The role of *XRCC1* and *ERCC2/XPD* variants, would explain the genomic instability in CKD patients. It is interesting to point out the finding that the three selected genes involved in CKD predisposition (*AGT*, rs5050; *GLO1*, rs4746; and *SHROOM3*, rs17319721) have also an important role in both genomic damage and genomic instability. These three genes were identified in a GWAS carried out with European populations by the CKDGen Consortium (Köttgen et al., 2009, 2010).
The protein encoded by the *AGT* gene is the angiotensinogen precursor, expressed in liver and cleaved by the enzyme renin in response to lowered blood pressure. The resulting product is the angiotensin I, whicht is cleaved by the angiotensin converting enzyme (ACE), to generate the physiologically active enzyme angiotensin II in the renin–angiotensin–aldosterone system (RAAS). RASS plays an important role in mediating diverse physiological functions, including vasoconstriction, sodium homeostasis, fluid balance, aldosterone secretion, inflammation, fibrosis, and oxidative stress (Paul et al., 2006).
Many variants in the *AGT* gene can modify the plasma AGT concentrations that are directly linked with arterial blood pressure. The rs5050 SNPs in the promoter region, influence *AGT* transcriptional activity and, consequently, AGT levels in plasma (Jeunemaitre et al., 1992). No evidences exist on associations of this SNP with genetic damage values. Nevertheless, studies with *Agt*$^{-/-}$ mice indicated lower levels of angiotensin in plasma (Uchida et al., 2009). It is known that angiotensin is a potent inducer of transforming growth factor-β (TGF-β) and recently it has been demosntrated that inhibition of TGF-β inhibits also NER repair (Quinag et al., 2015). This would be consistent with that observed in our study where the mutant allele showed association with genomic instability.
Glyoxalase I (GLO1) is the main enzyme of glyoxalase system that is an efficient enzymatic detoxification system, suppressing the formation of methylglyoxal and glyoxal-derived (advanced glycation end-products, AGEs). GLO1 is responsible for converting reactive dicarbonyls into non-toxic intermediates protecting living cells from the production and accumulation of toxic AGEs (Thornalley, 2003). Gene deletion of *GLO1* is embryonically lethal and *GLO1* silencing increases methylglyoxal concentration. When *GLO1* was knockdown in primary human aortic endothelial cells, ROS production was increased. This effect is mediated by ROS-induced methylglyoxal (MG), the major substrate of glyoxalase 1 (Yao and Brownlee, 2009). MG reacts with DNA producing the MGdG adduct that increases the frequency of DNA strand breaks (Rabbani and Thornalley 2014). GLO1

metabolizes >99% methylglyoxal and thereby protects the proteome and genome. From this point of view variants on *GLO1* can act as genomic instability factors as we have determined in this study. Despite the importance of this gene, there is an important gap on the study of its polymorphisms. A recent study showed that *GLO1* expression levels in prostate cancer were associated with the pathological grade, and assigned *GLO1* as a risk factor for prostate cancer development and progression (Baunacke et al., 2014). According to our results we would confirm that *GLO1* is not only a risk factor for CKD but also for genomic damage levels and genomic instability.
The Shroom family member 3, *SHROOM3* gene, encodes a PDZ-domain-containing protein that belongs to the family of Shroom-related proteins. This protein may be involved in regulating cell shape in certain tissues and was previously associated with CKD (Böger et al., 2011, Yeo et al., 2015). The importance of *SHROOM3* has been determined in *Shroom3* null (*Shroom3*$^{Gt/Gt}$) mice who showed marked glomerular abnormalities, including cystic and collapsing/degenerating glomeruli. Additionally, heterozygous (*Shroom3*$^{Gt/+}$) mice showed developmental irregularities manifested as adult-onset glomerulosclerosis and proteinuria (Uchida et al., 2009; Khalili et al., 2016). Our findings strongly support the GWAS results suggesting a role for *SHROOM3* in human kidney disease. Due to its role as actin-binding protein, *SHROOM3* may play an important role in renal aging by inducing fibrosis. At this point, it should be indicated that other acting-binding proteins have been shown to be involved in DNA repair processes as occurs with the actin depolymerizing factor (ADF) (Chang et al., 2015), the junction-mediating and regulatory protein (JMY), that is an actin nucleation promoting factor (Lin et al., 2014), and the actin-dependent regulator of chromatin, subfamily A-like1 (SMARCAL1), a recently identified DNA damage response protein involved in remodeling stalled replication forks (Feldkamp et al., 2014). All this information would reinforce the importance of the role of *SHROOM3* not only in the genesis of kidney pathologies but also in controling DNA integrity

CONFLICT OF INTEREST

There are no conflicts of interest, and the results presented in this paper have not been published previously in whole or part.

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Table 1. Description of the study population. Clinical and biochemical data for CKD cases and controls

Characteristics	Controls (N) (mean±SD)	CKD (N) (mean±SD)
Age (years)	(169) 56.059±15.31	(414) 65.79±14.29***
Sex (# men / women)	106 / 68	255 / 160
BMI (kg/m^2)[b]	(49) 27.29±4.79	(352) 25.61±4.85*
Creatinine (45-80 µmol/L)[a]	(169) 69.86±14.36	(184) 200.95±92.92***
Filtered Glomerular Ratio (>60 mL/min/1.75 m^2)[a]	(169) 86.82±6.98	(184) 31.22±13.69***
Erythropoietin/month (µg Darbepoetin/month)[a]	ND	(400) 207.61±689.91
Erythropoietin Resistance Index (<10)[a]	ND	(379) 13.50±37.45
Hemoglobin (120-160 g/L) [a]	(96) 144.18±11.96	(411) 125.04±17.76***
Glucose (4-5.8 µmol/L)[a]	(113) 5.86±1.93	(412) 5.60±1.85*
Cholesterol (3.20-5.20 mmol/L)[b]	(86) 5.22±1.04	(409) 4.26±1.08***
Triglycerides (0.30-1.40 mmol/L)[a]	(84) 1.34±1.19	(409) 1.47±0.76
HDL (>1.40 mmol/L)[a]	(38) 1.51±0.41	(397) 1.27±0.46**
LDL (<4.13 mmol/L)[a]	(37) 2.88±0.86	(394) 2.32±0.88***
Albumin (37-47 g/L)[a]	(66) 44.06±4.28	(405) 40.70±4.52***
Calcium (2.1-2.55 mmol/L)[b]	(70) 2.33±0.11	(409) 2.29±0.15***
Phosphorus (0.8-1.3 mmol/L)[a]	(68) 1.07±0.14	(409) 1.38±0.42***
Parathyroid hormone (PTH) (7-53 ng/L)[a]	(25) 61.23±23.58	(401) 190.19±179.09***
Ferritin (25-250 µg/L)[a]	(3) 88.56±49.32	(325) 245.87±257.66*
C-Reactive Protein (CRP) (<10 mg/L)[a]	(13) 2.80±3.21	(362) 10.16±19.40*
Homocysteine (<15 µmol/L)[a]	(2) 6.14±1.77	(239) 25.69±12.82*
B12 (175-750 pmol/L)[a]	(2) 181.50±4.94	(220) 384.05±195.49
Folic acid (600-2350 nmol/L)[a]	(2) 263.00±65.05	(218) 1075.61±736.33
Uric acid (210-420 µmol/L)[a]	(98) 302.54±85.50	(286) 385.80±109.60***
Iron (9-27 µmol/L)[a]	(2) 15.60±1.83	(302) 11.78±5.45
Total Iron-Binding capacity (47-79 µmol/L)[b]	(2) 54.70±0.28	(302) 43.5410.71
Transferrin Saturation Index (0.12-0.14)[a]	(2) 0.28±0.03	(301) 0.28±0.15
Proteinuria/24h (<0.15 g/L)[a]	(116) 0.14±0.73	(165) 0.81±1.43***
Urea (2.5-7 Mmol/L)[a]	(56) 5.67±1.63	(179) 15.32±6.88***
Systolic blood pressure (≤140 mmHg)[a]	(10) 122.90±13.90	(340) 135.54±18.57*
Diastolic blood pressure (≤90 mmHg)[a]	(10) 73.60±5.71	(340) 74.98±11.69
Hba1c glycosylated hemoglobin (<5.7%)[b]	(10) 5.18±2.43	(158) 4.80±1.62
Fibrinogen (2-4 µmol/L)[b]	(2) 4.35±1.11	(91) 4.47±0.98

[a]Mann-Whitney test; cases *vs* controls; ***P<0.001, *P<0.05. ND no data. [b]Student´s *t*-test; cases *vs* controls; +P<0.05.

Table 2. Genomic damage levels observed in CKD patients and controls for both the Comet and the MN assays

Genomic damage	Controls (N) (mean±SD)	Cases (N) (mean±SD)
DNA Damage (Mean % DNA Tail)	(138) 7.89±4.61	(352) 21.05±13.05***
Net Oxidative DNA Damage	(124) 11.20±9.13	(304) 12.00±11.65
BNMN	(164) 5.63±5.47	(375) 9.51± 8.48***
Induced BNMN	(169) 18.67± 14.99	(330) 26.10± 18.37+++

Z test K-S p<0.05; Mann-Whitney test case vs controls ***P<0.001.

Z test K-S p>0.05; Student´s t-test case vs controls +++P<0.001.

Table 3. Description of the SNPs analyzed selected for this study

Gene	SNP original	SNP alternative	LD (r^2)	Chr.	Position (NCBI dbSNP GRCh38)[a]	Consequence of the original SNP*	Minor allele	Major allele	Minor allele frequency (NCBI dbSNP)[a]
AGT	rs5050			1	230714140	5´UTR	G	T	0.175
SOD1	rs17880135			21	31669690	758bp 3' of SOD1	G	T	0.027
SOD1	rs1041740			21	31667849	intron variant	T	C	0.242
SOD1	rs202446			21	31656328	intron variant[a]	T	G	0.075
SOD2	rs4880			6	159692840	Missense Val16Ala	G	A	0.410
CAT	rs1001179			11	34438684	240bp 5' of CAT	T	C	0.125
GPX1	rs1050450	rs17080528	0.98	3	49352409	Downstream gene variant [b]	T	C	0.217
GPX3	rs870406			5	151021040	intron variant	A	G	0.097
GPX4	rs713041			19	1106616	synonymous	T	C	0.401
OGG1	rs1052133			3	9757089	missense Ser326Cys	G	C	0.302
MUTYH	rs3219489			1	45331833	missense Gln338His	G	C	0.313
XRCC1	rs25487			19	43551574	missense Gln399Arg	T	C	0.260
GSTP1	rs1695	rs749174	0.92	11	67585782	missense Ile105Val	A	G	0.243
GSTO1	rs4925	rs2164624	0.93	10	104253687	intron variant [b]	A	G	0.187
GSTO2	rs156697			10	104279427	missense Asn142Asp	G	A	0.440
ERCC2 (XPD)	rs1799793			19	45364001	missense Asp312Asn	T	C	0.194
ERCC2	rs171140			19	45361744	intron variant	C	A	0.367
ERCC2	rs13181			19	45351661	missense 500pb 3´of ERCC2	G	T	0.236
ERCC4	rs3136166			16	13938236	intron variant	G	T	0.424
GLO1	rs386572987	rs4746	1.00	6	38682852	Missense [a] Glu111Ala	G	T	0.287
SHROOM3	rs17319721			4	76447694	intron variant	A	G	0.223

*According to Haploreg; LD linkage disequilibrium; [a]http://www.ncbi.nlm.nih.gov/pubmed. [b] http://www.ensembl.org/index.html

Table 4. Positive associations found between candidate SNPs and genomic damage susceptibility

Genomic damage Parameters	Gene	SNP	Genotype	Group1	Group2	OR	95%CI	*P linear*	*P logistic*
DNA damage (Mean %DNA tail, 13.46)*	XRCC1	rs25487	CC	79	62				
			CT	61	89	0.59	0.40-0.88	**0.041**	0.010
			TT	30	22	0.78	0.45-1.33	0.746	0.366
			T			0.81	0.62-1.05	0.695	0.116
			TT+CT	91	111	0.63	0.44-0.92	0.137	**0.018**
Oxidative DNA damage (mean %DNA tail basal damage–FPG damage, 8.59)*	XRCC1	rs25487	CC	86	70				
			CT	95	99	0.78	0.51-1.19	0.845	0.252
			TT	25	39	0.52	0.29-0.94	**0.023**	**0.031**
			T			0.74	0.56-0.97	0.054	0.031
			TT+CT	120	138	0.71	0.47-1.06	0.328	0.089
	SHROOM3	rs17319721	GG	82	70				
			GA	89	101	0.75	0.49-1.15	**0.048**	0.192
			AA	35	33	0.91	0.51-1.61	0.169	0.733
			A			0.91	0.69-1.2	0.081	0.500
			AA+GA	124	134	0.79	0.53-1.18	**0.038**	0.250
	GLO1	rs4746	TT	66	72				
			TC	115	90	1.39	0.9-2.15	0.311	0.132
			CC	26	49	0.58	0.32-1.04	**0.011**	0.065
			C			0.84	0.64-1.11	**0.014**	0.217
			CC+TC	141	139	1.11	0.74-1.66	0.086	0.626
BNMN (6)*	AGT	rs5050	TT	162	168				
			TG	79	82	1.00	0.69-1.46	0.998	0.996
			GG	14	9	1.61	0.68-3.83	**4.01E-06**	0.278
			G			1.11	0.82-1.5	**0.005**	0.508
			GG+TG	93	91	1.06	0.74-1.52	0.180	0.752
Induced BNMN (21)*	AGT	rs5050	TT	147	163				
			TG	69	75	1.02	0.69-1.52	0.412	0.921
			GG	15	7	2.38	0.94-5.99	**0.014**	0.066
			G			1.22	0.89-1.67	**0.037**	0.216
			GG+TG	84	82	1.14	0.78-1.66	0.138	0.507
	XRCC1	rs25487	CC	99	88				
			CT	104	115	0.80	0.54-1.19	**0.027**	0.273
			TT	34	43	0.70	0.41-1.2	0.100	0.195
			T			0.83	0.64-1.07	**0.038**	0.154
			TT+CT	138	158	0.78	0.54-1.12	**0.018**	0.176
	ERCC2	rs171140	CC	67	58				
			CA	115	119	0.84	0.54-1.29	0.111	0.421
			AA	54	65	0.72	0.43-1.19	**0.045**	0.199
			A			0.85	0.66-1.09	0.044	0.199
			AA+CA	169	184	0.80	0.53-1.2	0.050	0.271
	ERCC2	rs13181	TT	105	97				
			TG	109	107	0.94	0.64-1.38	0.654	0.756
			GG	21	39	0.50	0.27-0.9	**0.013**	**0.022**
			G			0.77	0.59-1.01	**0.035**	0.056
			GG+TG	130	146	0.82	0.57-1.18	0.221	0.292
	SHROOM3	rs17319721	GG	78	103				
			GA	121	101	1.58	1.07-2.35	0.053	0.023
			AA	34	42	1.07	0.62-1.83	0.173	0.808
			A			1.13	0.87-1.46	0.082	0.355
			AA+GA	155	143	1.43	0.99-2.08	**0.042**	0.058

*Median as a cut-off, **normal value as a cut-off in the logistic regression analysis; Group 1: Selected to present the lowest levels of genomic damage; Group 2: Selected to present highest levels of genetic damage. CDK cases and controls were used in the analysis.