UNIVERSIDAD DE VALENCIA

"Biomarcadores precoces de fracaso renal agudo tras angiografía coronaria o cirugía cardiaca en pacientes con síndrome coronario o fallo cardiaco agudo"

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MANIFIESTAN:

Que el licenciado en Medicina y Cirugía ISIDRO A. TORREGROSA MAICAS ha realizado, bajo su supervisión, el trabajo **"Biomarcadores precoces de fracaso renal agudo tras angiografía coronaria o cirugía cardiaca en pacientes con síndrome coronario o fallo cardiaco agudo"** y reúne todos los requisitos necesarios para presentación y defensa pública.

Dr Guillermo T. Sáez   Dr Alfonso Miguel Carrasco   Dra Carmina Montoliu Félix

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A mi familia

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AKI: acute kidney injury

AKIN: Acute Kidney Injury Network

AUC: area under the ROC curve (área bajo la curva ROC)

CEC: circulación extracorpórea

CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration

ELISA: Enzyme-Linked ImmunoSorbent Assay

FR: función renal

FRA: fracaso renal agudo

eFG: filtrado glomerular estimado

ERC: enfermedad renal crónica

ERCT: enfermedad renal crónica terminal

IL-18: interleucina 18

kD: kiloDalton

KDIGO: Kidney Disease Improving Global Outcomes

KIM-1: kidney injury molecule-1

L-FABP: liver fatty acid-binding protein

MDRD: Modification of Diet in Renal Disease

MMP: metaloproteinasa de matriz

NGAL: neutrophil gelatinase-associated lipocalin

RIFLE: R (risk), I (injury), F (failure), L (loss), E (end stage renal disease)

ROC: receiver operating characteristic

S Cr: Creatinina sérica

TMB: tetrametil-bencidina

TRS: tratamiento renal sustitutivo

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INTRODUCCIÓN

1. Fracaso renal agudo: definición, fisiopatología, evolución clínica, importancia y detección

1.1. Definición de fracaso renal agudo

Se ha definido el fracaso renal agudo (FRA) de múltiples formas. Una definición clásica podría ser "síndrome clínico, secundario a múltiples etiologías, que se caracteriza por un deterioro brusco de las funciones renales, que altera la homeostasis del organismo, se asocia con frecuencia con un descenso de la diuresis y tiene como expresión común un aumento de la concentración de los productos nitrogenados en sangre"[1]. El problema surge a la hora de establecer una definición instrumental u operativa. Así, se ha definido FRA como un incremento absoluto o relativo de la creatinina sérica (estableciendo el dintel en valores tan dispares como 1.5 y 6 mg/dl) o una disminución del filtrado glomerular estimado (eFG) o de la diuresis.

En la literatura anglosajona el término *acute renal failure* (fallo renal agudo) ha sido sustituido por el de *acute kidney injury* o *AKI* (lesión aguda del riñón), remarcando la importancia de que incluso pequeños cambios en la creatinina sérica influyen de manera importante en el pronóstico. Ante el gran número de definiciones existentes y los problemas que esta falta de uniformidad conlleva, especialmente a la hora de comparar series o resultados de intervenciones, dos grupos propusieron criterios para definir más específicamente la entidad. Representantes de la nefrología y de la medicina intensiva

formaron un grupo de trabajo llamado the Acute Dialysis Quality Initiative[2] y propusieron una clasificación, la clasificación RIFLE, basada en parámetros bioquímicos y clínicos como la creatinina sérica o la diuresis[3]. Esta clasificación propone que el incremento en el nivel de creatinina durante los siete días que siguen al insulto original (como puede ser la cirugía cardiaca, la angiografía coronaria o el shock séptico) se correlaciona con la gravedad de la enfermedad. Se establecen tres estadios en función del grado de deterioro de la función renal: R (risk), I (injury) y F (failure) y dos evolutivos L (loss) y E (end stage renal disease) (Tabla 1).

La segunda clasificación, propuesta por el grupo Acute Kidney Injury Network (AKIN) [4] eliminó las dos últimas categorías y cambió los tres primero estadios por 1, 2 y 3 (Tabla 1). La diferencia fundamental con la clasificación RIFLE estriba en el hecho de que se define AKI como un incremento en la creatinina sérica en las primeras 48 horas en lugar de 7 días. Estas clasificaciones han mostrado en diversos estudios epidemiológicos[5-8] ser útiles para predecir el riesgo de mortalidad aunque no permiten distinguir entre el fracaso renal agudo funcional, parenquimatoso u obstructivo.

En 2012 la guía internacional Kidney Disease Improving Global Outcomes (KDIGO 2012)[9] propuso una nueva clasificación, resultado de la fusión de RIFLE y AKIN. Está por determinar si esta nueva clasificación mejora la capacidad diagnóstica o pronóstica de las dos anteriores. De todas formas, cuando se ha comparado la capacidad de las definiciones AKIN, RIFLE y KDIGO de FRA para diagnosticar y estadiar el FRA y también para predecir un pronóstico adverso en adultos se ha encontrado una buena concordancia entre ellas, comportándose cada definición de manera similar a las otras en distintos contextos clínicos[10].

En la literatura en castellano no existe aún una traducción unánimemente aceptada para el término AKI por lo que en el texto seguiremos usando el de FRA.

Tabla 1. Clasificación RIFLE y AKIN del fracaso renal agudo

	RIFLE		AKIN		
	Cr S	Diuresis		Cr S	Diuresis
Risk	↑x 1.5	<0.5 ml/kg/h x 6 h	Estadio 1	↑x 1.5 ó ↑Cr≥ 0.3 mg/dl	<0.5 ml/kg/h x 6 h
Injury	↑x 2	<0.5 ml/kg/h x 12 h	Estadio 2	↑x 2	<0.5 ml/kg/h x 12 h
Failure	↑x 3 ó ↑Cr≥ 0.5 mg/dl si Cr Basal > 4 mg/dl	<0.3 ml/kg/h x 24 h ó anuria x 12 h	Estadio 3	↑x 3 ó ↑Cr≥ 0.5 mg/dl si Cr Basal > 4 mg/dl	<0.3 ml/kg/h x 24 h ó anuria x 12 h
Loss	Pérdida completa de FR > 4 semanas		Pacientes que reciben TRS se incluyen en el estadio 3		
End-Stage	ERCT				

S Cr: Creatinina sérica. FR: función renal. TRS: tratamiento renal sustitutivo. ERCT: enfermedad renal crónica terminal.

1.2. Fisiopatología del fracaso renal agudo

La nefrona es la unidad funcional del riñón. Cada nefrona está compuesta por diversas poblaciones celulares que funcionan de manera coordinada para llevar a cabo un gran número de procesos complejos y estrechamente controlados. El FRA es una complicación común de múltiples patologías que altera esta homeostasis y conlleva un marcado aumento de la morbimortalidad. Probablemente, la causa más importante de FRA es la isquemia, que a menudo se acompaña de otras como la acción de diversos

nefrotóxicos y que puede ser consecuencia de diversos procesos como la sepsis, la pérdida de sangre tras una cirugía o el uso de drogas vasoactivas o radiocontrastes. El organismo es capaz de adaptarse a la reducción del flujo sanguíneo hasta un cierto nivel, pero cuando el suministro de oxígeno y substratos metabólicos disminuye de manera crítica, la lesión celular lleva a la disfunción renal[11,12].

En respuesta a la hipoperfusión se activan baroreceptores que ponen en marcha una cascada de respuestas neurohumorales, que incluyen la activación del sistema nervioso simpático y la producción de catecolaminas, la liberación de vasopresina y la activación del sistema renina-aniotensina-aldosterona[11,12]. La puesta en marcha de todos estos mecanismos induce vasoconstricción y retención de sodio y agua con el objetivo de mantener la perfusión de los distintos órganos y tejidos. La activación del sistema renina-angiotensina-aldosterona induce un incremento de la producción de angiotensina II, un potente vasoconstrictor a nivel sistémico que tiene la peculiaridad a nivel renal de aumentar preferentemente la resistencia de la arteriola eferente[13]. De esta manera aumenta la presión hidrostática glomerular y se preserva, al menos inicialmente, el filtrado glomerular. Sin embargo, si la hipoperfusión es suficientemente intensa o mantenida la constricción de la arteriola aferente reduce el flujo plasmático renal y el filtrado glomerular[14], lo que lleva al fracaso renal agudo funcional. A la vez que se ponen en marcha los mecanismos presores, lo hacen también mecanismos compensatorios de autoregulación renal para preservar la perfusión glomerular[15] (Figura 1). En condiciones fisiológicas la autoregulación renal mantiene el filtrado glomerular cuando la presión arterial sistémica media es superior a 75-80 mmHg. Por debajo de esta presión el filtrado glomerular cae rápidamente. En repuesta, aumenta la

producción renal de prostaglandinas, calicreina y óxido nítrico que ejercen un efecto vasodilatador[16] (Figura 1).

Figura 1. Autoregulación del filtrado glomerular en respuesta a la hipoperfusión renal
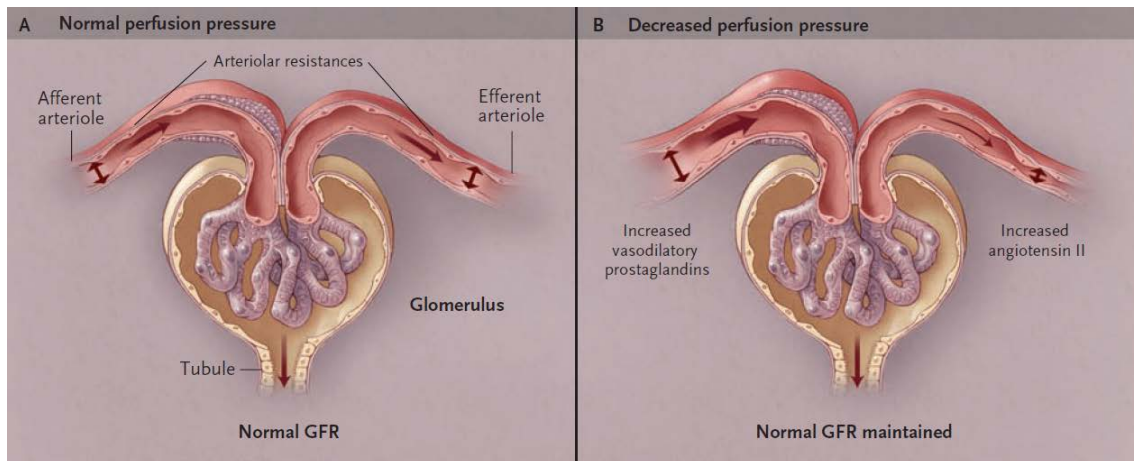
Abuelo JG. Normotensive Ischemic Acute Renal Failure. N Engl J Med 2007; 357:797-805

La caída del filtrado glomerular se acompaña de una disminución del flujo sanguíneo a nivel del lecho capilar postglomerular, que perfunde los túbulos, pero estos permanecen intactos. Sin embargo, si la isquemia es suficientemente intensa o mantenida puede acabar causando daño estructural. Los restos de las células epiteliales tubulares forman cilindros que obstruyen los túbulos y el filtrado glomerular vuelve a la circulación desde la luz tubular a través de las paredes tubulares desnudas[11,17]. Además, el sodio no puede ser reabsorbido en el túbulo lesionado y su concentración aumenta en la luz, lo que favorece la polimerización de la proteína de Tamm-Hosfall secretada en el asa de Henle, que forma un gel y contribuye a la formación de cilindros[18].

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El mecanismo por el que la isquemia y la falta de oxígeno lesionan las células tubulares comienza con la pérdida de ATP, que conduce a la lesión celular y, si es suficientemente grave, a la muerte celular por necrosis o apoptosis. Todos los segmentos de la nefrona pueden verse afectados pero el más susceptible al daño isquémico es el túbulo proximal[19]. El agotamiento del ATP intracelular produce una serie de alteraciones en el metabolismo celular[20,21]. La desestructuración del citoesqueleto conlleva la pérdida de las microvellosidades del borde en cepillo y de las uniones intercelulares en el epitelio tubular así como una translocación de integrinas y de las ATPasas sodio-potasio desde la superficie basal a la apical. Como resultado las membranas del borde en cepillo y las células dañadas se desprenden y obstruyen los túbulos. La pérdida de ATP también activa proteasas y fosfolipasas que causan daño oxidativo no sólo a las a las células tubulares sino también a las células endoteliales de los capilares peritubulares. El daño oxidativo secundario al desequilibrio entre las sustancias vasoconstrictoras y las vasodilatadoras, con una preponderancia de las primeras como la endotelina, da lugar a vasoconstricción, congestión, hipoperfusión y expresión de moléculas de adhesión, que a su vez inicia la infiltración leucocitaria que se amplifica por citoquinas proinflamatorias y quimiotácticas generadas por las células tubulares isquémicas. Estos leucocitos obstruyen la microcirculación y liberan citoquinas citotóxicas, especies reactivas de oxígeno y enzimas proteolíticas, que aumentan el daño tubular[21] (Figura 2).

Figura 2. Fisiopatología del fracaso renal agudo isquémico
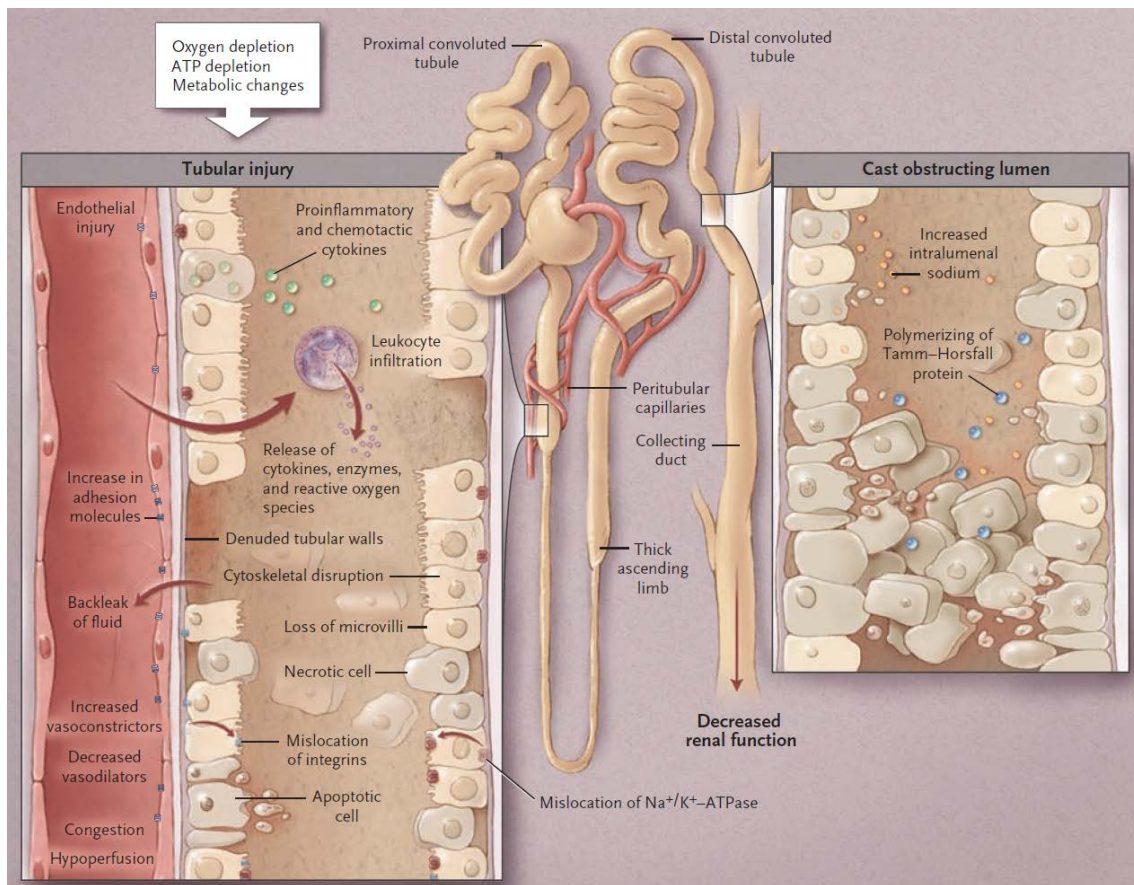
Abuelo JG. Normotensive Ischemic Acute Renal Failure. N Engl J Med 2007; 357:797-805

1.3. Relación entre la fisiopatología y la evolución clínica en el fracaso renal agudo

Se ha propuesto un esquema evolutivo del curso clínico del FRA isquémico[22,23] en el que se relacionan los sucesivos eventos fisiopatológicos con 4 fases clínicas (Figura 3). En este esquema el FRA comienza con una primera fase temprana de nefropatía vasomotora en la que las alteraciones en la hemodinámica y la perfusión renal llevan al fracaso renal prerenal. Si la hipoperfusión renal es suficientemente intensa o mantenida en el tiempo se agota el ATP celular y aparece daño oxidativo (fase de iniciación). Este

proceso lleva a la activación de células derivadas de la médula ósea, células endoteliales y células epiteliales renales induciendo un estado proinflamatorio. Las células inflamatorias se adhieren al endotelio activado en los capilares peritubulares de la médula externa lo que produce congestión medular y mayor daño hipóxico en el segmento S3 del túbulo proximal (fase de extensión). Adicionalmente, la elaboración de mediadores inflamatorios induce mayor daño celular. Las células tubulares comienzan entonces el proceso de proliferación (fase de mantenimiento) y rediferenciación. Finalmente se reconstituyen la polaridad y la función (fase de reparación).

Figura 3. Relación entre las fases clínicas y celulares del fracaso renal agudo isquémico y el impacto temporal en la función renal representada por el filtrado glomerular
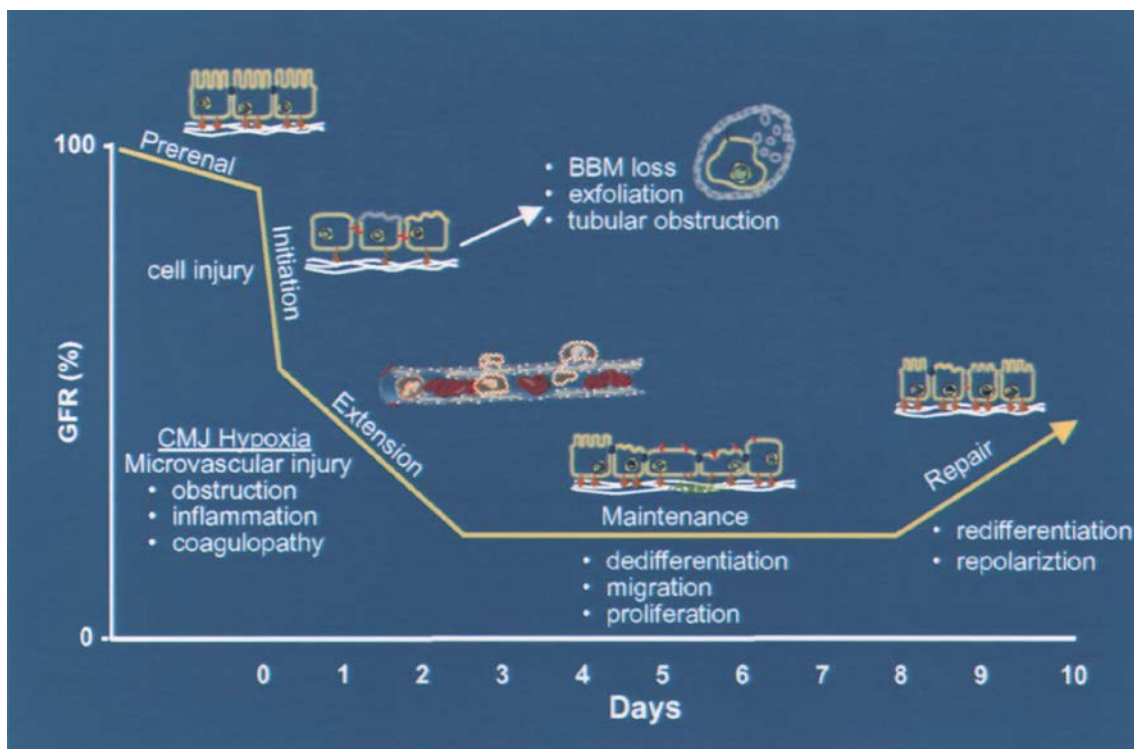
Sutton et al. Microvascular endothelial injury and dysfunction during ischemic acute renal failure. Kidney Int 2002; 62:1539-1549

1.4. Importancia del fracaso renal agudo

Numerosos trabajos demuestran que la aparición del FRA conlleva una mayor utilización de recursos y un incremento del riesgo de muerte en distintos contextos clínicos. Uchino el al.[24] estudiaron de manera prospectiva 29.269 pacientes ingresados por distintas patologías en unidades de cuidados intensivos de 54 hospitales en 23 países con el objetivo de determinar la incidencia del FRA y su influencia sobre la evolución. Casi un 6 % de los pacientes incluidos presentó FRA grave, asociándose la aparición del mismo a una altísima mortalidad hospitalaria, de manera que el 60% de los pacientes que lo sufrieron falleció durante su estancia en el hospital. En un segundo estudio publicado por Chertow et al. en el mismo año[25] y que incluyó a 9210 pacientes hospitalizados en distintos servicios médicos y quirúrgicos de un único centro hospitalario, se mostró que la presencia de FRA se asociaba de forma significativa con la mortalidad, la duración de la estancia hospitalaria y los costes, incluso después de ajustar por edad, sexo, diagnóstico, gravedad de la enfermedad o presencia de enfermedad renal crónica (ERC). Un incremento de la cifra de creatinina sérica mayor o igual a 0.5 mg/dl aumentaba el riesgo de mortalidad 6.5 veces y un incremento mayor o igual a 2 mg/dl, 16.4 veces. Otros estudios han obtenido resultados similares[26-28]. Más aún, este incremento del riesgo de muerte se relaciona con la gravedad del FRA[6,25,26,29] e incluso pequeños decrementos agudos de la función renal se asocian a un peor pronóstico. En el estudio de Chertow et al. un incremento de la cifra de creatinina sérica de tan sólo 0.3 mg/dl multiplicaba el riesgo de muerte 4.1 veces[25]. En un metaanálisis que incluía estudios observacionales de cohortes y ensayos randomizados y controlados publicados entre 1990 y 2007 en los que se ofrecía información sobre pequeños cambios en la creatinina plasmática (<0.5 mg/dl o <25%) los autores concluyeron que existía una relación entre

la mortalidad a corto plazo y los cambio agudos en la creatinina sérica de manera que incluso pequeños cambios en ésta implicaban un peor pronóstico en una variedad de contextos clínicos y tipos de pacientes[30]. Se ha demostrado también que el FRA rápidamente reversible, considerado habitualmente como un problema clínico menor y que no tiene una influencia apreciable sobre el pronóstico, se asocia de manera independiente al riesgo de muerte[31]. Una proporción considerable de los individuos que sobreviven a un FRA grave sufre una drástica disminución de la calidad de vida[32]. Otro aspecto que ha adquirido gran importancia en los últimos años es la relación existente entre fracaso renal agudo y enfermedad renal crónica. Ishani el al.[33] analizaron datos de 233.803 pacientes mayores de 66 años, beneficiarios de *Medicare* y hospitalizados por distintos motivos en el año 2000. Un 3.1% recibieron un diagnóstico de FRA al alta. Tras ajustar por distintos factores, el riesgo de llegar a la enfermedad renal terminal en los siguientes dos años era 41.2 veces superior para los pacientes que desarrollaban FRA y ya tenían ERC previa respecto a los pacientes sin FRA, 13 veces superior para los que desarrollaban FRA sin ERC previa y 8.4 para los pacientes con ERC previa sin FRA. El impacto es aún mayor si se evalúa el FRA que requiere diálisis. En un estudio en el que se incluyeron 556.090 pacientes adultos miembros del Kaiser Permanente de California Norte hospitalizados durante un periodo de 8 años, con un eFG basal mayor de 45 ml/min/1.73 m^2 y que sobrevivieron a la hospitalización, la presencia de FRA que requirió diálisis se asoció de modo independiente a un incremento de 28 veces del riesgo de desarrollar ERC estadio 4 o 5[34]. Así la ERC es un factor de riesgo para el FRA y el FRA lo es para el desarrollo de ERC. En la figura 4 se muestra la interconexión existente entre el FRA y la ERC. Ambos (y probablemente su combinación) se asocian con un aumento del riesgo de muerte y pueden conllevar complicaciones

como desarrollo de enfermedad cardiovascular, disminución progresiva de la función renal y de la calidad de vida y la autonomía personal[35].

Figura 4. Fracaso renal agudo y enfermedad renal crónica como síndromes interconectados
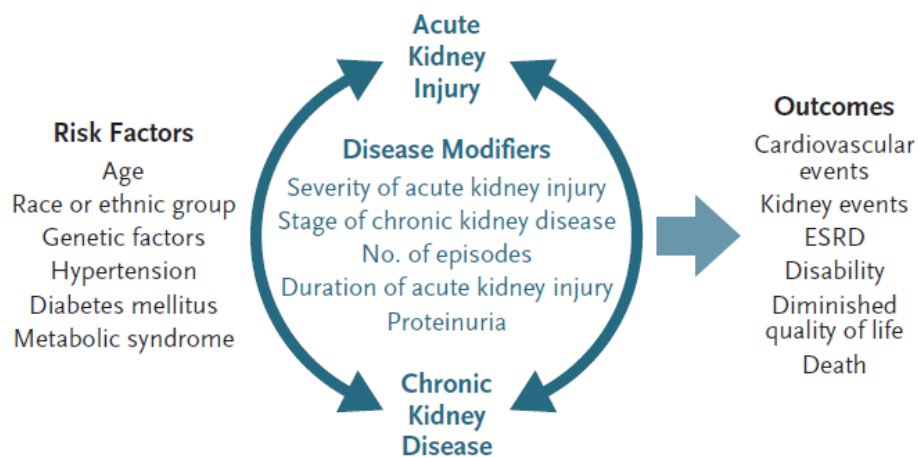
Chawla et al. Acute Kidney Injury and Chronic Kidney Disease as Interconnected Syndromes. N Engl J Med 2014; 371:58-66

1.5. Fracaso renal agudo tras cirugía cardiaca

El FRA es una complicación frecuente tras la cirugía cardiaca, con una incidencia comunicada que oscila, dependiendo tanto de la definición de FRA utilizada como de la población estudiada, entre el 8.9 y el 39%[36]. Los factores de riesgo preoperativos para el desarrollo de FRA tras cirugía cardiaca son edad avanzada, sexo femenino, función ventricular izquierda reducida o fallo cardiaco congestivo, diabetes, enfermedad vascular periférica, enfermedad pulmonar obstructiva crónica, cirugía no programada y función renal alterada[23,36]. Otros factores, asociados a la propia cirugía y cuya

relevancia es más controvertida son aquellos específicamente relacionados con la circulación extracorpórea (CEC), tales como el uso de técnicas con o sin CEC, el flujo no pulsátil, la CEC normotérmica versus hipotérmica, la hemólisis y la hemodilución durante la CEC y la duración de la misma[23,36]. Los mecanismos que contribuyen al fracaso renal incluyen toxinas exógenas y endógenas, factores metabólicos, isquemia y reperfusión, activación neuro-humoral, inflamación y estrés oxidativo[37]. El FRA prolonga la estancia hospitalaria y es un predictor independiente de mortalidad tras la cirugía cardiaca[38]. La cirugía coronaria aislada presenta la menor incidencia de FRA, seguida de la cirugía valvular y de la cirugía combinada valvular y coronaria[39]. Tanto los criterios RIFLE como AKIN son predictores precoces de mortalidad en este contexto[40,41]. Aplicando los criterios RIFLE y AKIN, la mortalidad comunicada en pacientes que desarrollan FRA tras cirugía cardiaca se sitúa entre el 3.8 y el 54.4% y se incrementa progresivamente con la gravedad del fracaso renal[36]. Se ha demostrado también en esta población que incluso pequeñas elevaciones de la creatinina sérica conllevan un peor pronóstico[42,43]. Además, la supervivencia a largo plazo también es diferente en función de la duración del FRA[44] y la recuperación temprana del mismo se asocia a una mejoría de la misma[45].

1.6. Fracaso renal agudo tras angiografía coronaria

Al igual que ocurre tras la cirugía cardiaca, se ha comunicado una incidencia variable de FRA tras angiografía coronaria dependiendo de la definición utilizada y de la población estudiada[46-52]. El FRA que ocurre tras la exposición a contrastes iodados es, en la mayoría de los casos, transitorio. A pesar de ello, su aparición se ha relacionado con un peor pronóstico y un aumento del riesgo de muerte[47-49]. La aparición de FRA tras angiografía coronaria se asocia también a riesgo aumentado de desarrollo de ERC[51]. Los

predictores más potentes para el desarrollo de FRA por contraste iodado son el filtrado glomerular basal alterado, la presencia de diabetes y el volumen de contraste utilizado[53]. Otros factores implicados son la edad avanzada, el sexo femenino, la presencia de proteinuria y la insuficiencia cardiaca[54]. En su patogenia se ha implicado a la hipoxia medular inducida por la acción vasoconstrictora directa del contraste. Además el iodo es directamente citotóxico tanto para las células tubulares como para las endoteliales. Por último, las propiedades viscosas del contraste exacerban la vasoconstricción y la citotoxicidad[54]. Es muy probable que tras angiografía coronaria la incidencia del FRA sea superior y el pronóstico peor que tras exploraciones con contraste por vía venosa por la comorbilidad asociada de estos pacientes, el mayor volumen de contraste habitualmente utilizado, la administración intraarterial o la posibilidad de otras complicaciones asociadas como el ateroembolismo. Como ocurre en el FRA que se produce tras la cirugía cardiaca el aumento de la creatinina sérica es un marcador tardío del daño renal.

1.7. Detección del fracaso renal agudo

El diagnostico de FRA se basa fundamentalmente en la detección del incremento de la creatinina sérica, que es tardío y no refleja adecuadamente el filtrado glomerular en el paciente agudo[10,55]. Al inicio del FRA, asumiendo un insulto simple a los riñones como podría ser un periodo recortado de isquemia en el contexto de una cirugía cardiaca, el filtrado glomerular disminuye rápidamente hasta llegar a un nadir. La creatinina sérica sin embargo ira ascendiendo durante un periodo más o menos largo de horas o días hasta alcanzar un nuevo equilibrio[10]. Existe por tanto un decalaje entre los cambios que se producen en el filtrado glomerular y en el valor de la creatinina sérica. En la figura 5

se muestra un ejemplo hipotético de evolución de filtrado glomerular y creatinina sérica antes y durante un episodio de FRA. Además, los pacientes con FRA, especialmente en el caso de pacientes ingresados en unidades de críticos, sufren a menudo múltiples insultos renales sucesivos, complicando aún más la valoración de la función renal.

Figura 5. Ejemplo hipotético de evolución del filtrado glomerular y creatinina sérica antes y durante un episodio de fracaso renal agudo
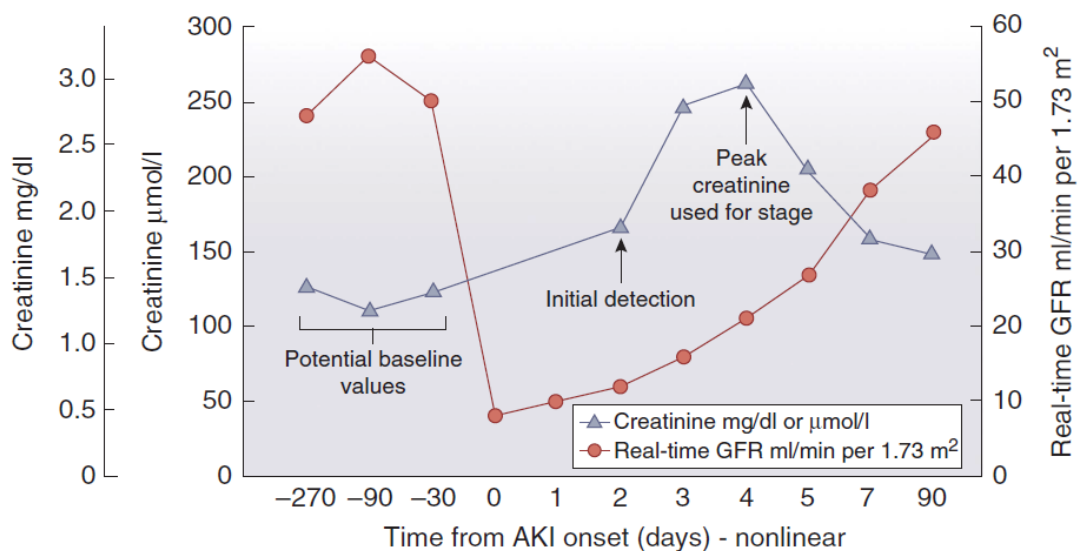
Thomas ME et al. The definition of acute kidney injury and its use in practice. Kidney Int 2015; 87:62-73

Numerosos factores, incluyendo la sepsis y la sobrecarga de volumen pueden influir sobre los niveles de creatinina sérica independientemente de la función renal[10,56]. Estos factores pueden tener un efecto agudo sobre la cifra de creatinina o un efecto crónico, afectando a la estimación del filtrado glomerular basal o a la capacidad de generar creatinina durante el FRA (Figura 6).

Figura 6. Factores que afectan a la interpretación de la creatinina sérica en el fracaso renal agudo

Factors having an acute effect on creatinine

Acute rise in creatinine:
• Dietary creatine intake
  – a meat meal[50]
• Increased creatinine generation
  – rhabdomyolysis[51]
• Decreased glomerular filtration
  – AKI
• Reduced tubular secretion
  – trimethoprim and cimetidine

False elevation of creatinine:
• Jaffe assay interference
  – hyperglycemia and DKA[52]
  – delayed centrifugation
  – other: hemolysis; high total protein
• Enzymatic assay interference
  – high total protein, lidocaine

Acute fall / blunted rise in creatinine:
• Reduced creatinine generation
  – sepsis[53]
• Increased volume of distribution
  – edematous states*
  – acute fluid overload[54–57]

Blood creatinine
Renal elimination
Creatinine
Muscle creatine

Factors having a chronic effect on creatinine – affecting baseline eGFR and ability to generate creatinine rise during AKI

Chronic 'elevation' of creatinine:
• Increased creatinine generation
  – muscular body habitus
  – Afro-Caribbean ethnicity
• Decreased glomerular filtration
  – chronic kidney disease

False reduction of creatinine:
• Jaffe assay interference
  – hyperbilirubinemia
• Enzymatic interference
  – hyperbilirubinaemia, hemolysis

Chronic 'reduction' in creatinine:
• Low dietary protein (cooked meat) intake
• Reduced creatinine generation with lower muscle mass
  – old age and female sex
  – muscle-wasting conditions
  – amputation
  – malnutrition and critical illness[58]

Thomas ME et al. The definition of acute kidney injury and its use in practice. Kidney Int 2015; 87:62-73

Sin embargo, estudios experimentales han demostrado que aunque el FRA se puede prevenir o tratar con diferentes intervenciones, éstas deben iniciarse tempranamente tras la lesión renal[21,57], mientras que en estudios clínicos también se ha demostrado que el retraso en el diagnóstico del FRA conlleva un aumento de complicaciones y un peor pronóstico[56]. El mejor tratamiento para los pacientes con FRA incipiente en distintos contextos clínicos como la sepsis, la cirugía cardiaca o la angiografía coronaria es, en el momento actual, la prevención de la progresión del daño renal evitando nefrotóxicos y asegurando un adecuado aporte de volumen pero evitando a la vez la

sobrecarga de fluidos y la consiguiente congestión tisular y orgánica. El poder disponer de biomarcadores precoces permitiría la implementación temprana de estas medidas y además podría facilitar la exploración de nuevas terapias y abrir nuevos caminos para favorecer la recuperación de la integridad renal y prevenir las complicaciones a corto y largo plazo[57,58].

Las distintas fórmulas propuestas para la estimación del filtrado glomerular, como las ecuaciones MDRD (*Modification of Diet in Renal Disease*) y CKD-EPI (*Chronic Kidney Disease Epidemiology Collaboration*) fueron desarrolladas en pacientes con ERC estable[59,60] y no son apropiadas para el diagnóstico de FRA.

Otras definiciones de FRA se basan en la disminución de la diuresis. Se ha definido clásicamente la oliguria como una diuresis inferior a 400 ml en 24 horas, que equivale en una persona de 70 kg a 0.24 ml/kg/h. En las clasificaciones RIFLE y AKIN se propone una definición más restrictiva, siendo uno de los criterios para definir el FRA una diuresis <0.5 ml/kg/h durante 6 horas[3,4]. Sin embargo la disminución de la diuresis como criterio diagnóstico de FRA no está exenta de problemas porque es bien sabido que una parte muy importante de los casos no cursan con oliguria[61] y además en el postoperatorio inmediato puede haber una disminución de la diuresis compatible con el diagnóstico de FRA (<0.5 ml/kg/h en 6 h) independientemente de la función renal.

La oliguria también es una complicación frecuente en las unidades de críticos pero en muchos casos esta disminución de la diuresis no se sigue de FRA definido como elevación de la creatinina[62]. De todos modos, la oliguria es un predictor independiente de mortalidad en este contexto[63].

1.7.1. La búsqueda de nuevos biomarcadores

A pesar de los avances realizados en los últimos años en el conocimiento de la patogenia del FRA y de la importancia de su aparición sobre el pronóstico del paciente en distintos contextos clínicos, persiste un problema fundamental a la hora de abordarlo, que es la disponibilidad de un biomarcador precoz, sensible y específico. Como ya se ha dicho, la creatinina sérica asciende tardíamente en el FRA y está sujeta a otras variables como la masa muscular o el estado de hidratación. Además, da información únicamente acerca del filtrado glomerular, pero no de la existencia de daño tubular. Para intentar resolver este problema, en los últimos años diferentes grupos han intentado identificar nuevos marcadores para el diagnóstico temprano y estratificación del riesgo del FRA. En numerosos estudios se ha comunicado la utilidad de distintos biomarcadores precoces de FRA tras cirugía cardiaca, pero existe mucha menos información acerca del FRA relacionado con otros procesos, como la angiografía coronaria. Los marcadores más estudiados son cistatina-C, que en realidad no es un marcador de daño renal, sino de filtrado glomerular, *neutrophil gelatinase-associated lipocalin* (NGAL), interleucina-18 (IL-18), *kidney injury molecule-1* (KIM-1) y *liver fatty acid-binding protein* (L-FABP), en suero o en orina. Sin embargo, y pese al esfuerzo invertido y los progresos realizados, en la actualidad aún no está bien establecida la utilidad real de los distintos marcadores en diferentes contextos clínicos de FRA[64].

1.7.1.1. NGAL *(Neutrophil Gelatinase-Associated Lipocalin)*

De entre todos los biomarcadores que están siendo estudiados **NGAL** es probablemente el que más interés ha generado. NGAL es una proteína de 25 kD de la familia de las lipocalinas que se une covalentemente a la gelatinasa de los neutrófilos[65]. Se expresa

habitualmente en muy bajas concentraciones en diversos tejidos como riñón, pulmón, estómago o colon pero se encuentra en niveles muy superiores en riñones con daño isquémico o tóxico. Tras un insulto renal tóxico o isquémico, NGAL se sobreregula de manera muy marcada a nivel tanto transcripcional como de síntesis proteica[66-68]. La proteína NGAL se detecta en orina precozmente, a las pocas horas tras la lesión renal. NGAL se produce a nivel intrarenal en el túbulo, específicamente en la porción gruesa ascendente del asa de Henle y en el tubo colector, aunque se ha demostrado también in vitro su secreción por parte de las células tubulares proximales en respuesta a la pérdida de ATP[67,69,70]. Aunque el nivel máximo de NGAL en orina se obtiene aproximadamente 6 horas tras la lesión renal, se pueden encontrar niveles elevados durante varios días[65,71-73]. NGAL también se eleva en el FRA en plasma como consecuencia en parte de un aumento de su producción hepática. Además, NGAL se filtra a nivel glomerular y se reabsorbe en el túbulo proximal por un mecanismo dependiente de megalina[69,70,74]. Así pues, NGAL tanto en plasma como en orina puede ejercer un efecto a nivel de los procesos moleculares y celulares que ocurren durante el FRA y la determinación de sus niveles se ha utilizado para intentar detectar de manera precoz la aparición del FRA.

NGAL es un agente bacteriostático que participa en la respuesta inmune innata. Actúa secuestrando complejos hierro-sideróforo evitando así su captación por patógenos bacterianos[75,76]. Por otro lado, NGAL también se ha implicado en el tráfico de hierro durante el desarrollo del epitelio renal[77,78]. Estudios realizados en modelos animales de FRA han mostrado que la administración parenteral de NGAL cargado de hierro-sideróforo protege el riñón del daño inducido por isquemia-reperfusión[68,69]. Aunque el mecanismo por el que NGAL ejerce esta acción protectora no es totalmente conocido,

se ha demostrado que disminuye la apoptosis y aumenta la proliferación de células del epitelio tubular[68,69]. NGAL también aumenta la expresión de la enzima renoprotectora hemeoxigenasa 1[69]. Por otro lado, la proliferación de células epiteliales observada tras la administración de NGAL sugiere que facilita la recuperación del tejido renal dañado. Este papel en la regeneración del tejido renal podría explicar la elevación mantenida de NGAL urinario que se observa durante días tras la lesión renal.

1.7.1.2. IL-18 (Interleucina 18)

La **interleucina 18** es una citoquina proinflamatoria de 22 kD. Aumenta en riñón tras daño inducido por isquemia-reperfusión o nefrotóxicos por un mecanismo dependiente de caspasa-1[79,80]. IL-18 se produce como una procitoquina que debe ser activada proteolíticamente por la caspasa-1 antes de su secreción[81]. La IL-18 promueve daño tisular agudo a través de NF-kB, induciendo la sobreexpresión de otros mediadores inflamatorios como TNF-α, que conducen a la infiltración intrarenal por macrófagos y neutrófilos[80].

IL-18 podría ser es una diana adecuada en el FRA porque parece jugar un papel prominente en el proceso inflamatorio que exacerba el daño renal durante la fase de extensión del mismo[65]. Distintos trabajos en modelos animales han demostrado que actuando sobre IL-18 se puede prevenir o atenuar el daño renal inducido[79,82]. IL-18 se eleva en las primeras 6 horas tras la lesión renal y alcanza su pico a las 12-18 horas[65,72,73].

1.7.1.3. Cistatina-C

Como la creatinina y a diferencia de NGAL, IL-18, KIM-1 y L-FABP, **cistatina-C** no es un marcador de daño tubular sino de filtrado glomerular. La cistatina-C es una proteína no glicosilada de 122 aminoácidos y 13.3 kD que se produce a un ritmo constante por todas las células nucleadas. Es un miembro de la familia de los inhibidores de la cisteína proteasa[83]. Su pequeño peso molecular y su carga positiva a pH fisiológico permiten que se filtra libremente en el glomérulo. Posteriormente se reabsorbe y cataboliza casi completamente en el túbulo proximal, de manera que sólo una pequeña cantidad se excreta en orina[84]. Por su ritmo constante de producción, la filtración glomerular libre y la ausencia de secreción tubular[85,86], cistatina-C tiene el potencial de ser un marcador de filtrado glomerular superior a creatinina[87,88]. También su vida media es más corta que la de la creatinina por lo que cuando disminuye el filtrado glomerular asciende y alcanza un nuevo estado de equilibrio más rápidamente[89].

En los últimos años se ha explorado su utilidad como posible sustituto de la creatinina para la determinación del filtrado glomerular, tanto en ERC como en FRA, por su menor dependencia de la masa muscular[90,91]. Sin embargo, aunque se ha propuesto que su concentración es independiente de otras variables como el sexo o la edad, se ha comunicado que en su valor sí pueden influir otras variables como la edad, el sexo, la raza y la presencia de hiper o hipotiroidismo, diabetes o inflamación[92,93].

1.7.1.4. KIM-1 (*Kidney Injury Molecule-1*)

KIM-1 es una glicoproteína transmembrana de 38.7 kD que contiene mucina y dominios de inmunoglobulina[94]. Su expresión basal es muy baja en el riñón normal pero aumenta de forma marcada en células tubulares tras daño isquémico o tóxico. KIM-1 puede

encontrase en células epiteliales proliferativas desdiferenciadas del túbulo proximal 48 horas tras la lesión renal[94]. El componente extracelular de KIM-1 se libera de la membrana por un mecanismo dependiente de metaloproteinasa de matriz (MMP)[95]. Este es el mecanismo por el cual se produce el aumento de KIM-1 que aparece tras el daño renal isquémico o tóxico[96,97]. KIM-1 no es sólo un marcador de daño renal, sino que parece ejercer un papel funcional en la biología molecular y celular del FRA. La expresión de KIM-1 confiere un fenotipo fagocítico en células cultivadas de riñón, promoviendo la fagocitosis de cuerpos apoptóticos y restos necróticos[98]. Se ha propuesto por ello que KIM-1 puede jugar un papel en la regeneración tubular y la recuperación renal tras el FRA[98]. Este papel es congruente con el pico tardío de concentración de KIM-1 en orina que se produce en el FRA, dos o tres días tras la lesión[65,99]. Las intervenciones farmacológicas destinadas a promover el efecto de KIM-1 podrían ser beneficiosas facilitando la limpieza de detritos en los túbulos lesionados. La inhibición de la liberación de KIM-1 parece un mecanismo lógico porque aumentaría la cantidad de KIM-1 anclado a la membrana en las células epiteliales supervivientes[65]. Esto se podría conseguir inhibiendo MMP-3 (metaloproteinasa de matriz 3) que acelera la liberación de KIM-1 de las células epiteliales renales[100]. Por otro lado existen indicios de que la libración acelerada de KIM-1 se produce en respuesta a la producción de factores de crecimiento implicados en la proliferación y recuperación celular. Así, se ha propuesto que la concentración urinaria de KIM-1 podría ser útil para diferenciar entre la fase de extensión y la fase de mantenimiento y recuperación del FRA[65].

1.7.1.5. L-FABP (*Liver-type fatty acid binding protein*)

L-FABP es una proteína de 14 kD. Su gen se expresa en condiciones normales en el córtex renal y la proteína se produce fundamentalmente en el túbulo proximal[101,102]. La función principal de L-FABP se relaciona con el metabolismo de los ácidos grasos pero actúa también como un agente renoprotector. L-FABP tiene propiedades antioxidantes[103,104] y su expresión se induce por la hipoxia[105]. En modelos animales se ha demostrado que L-FABP reduce la gravedad del daño renal isquémico[105]. Se ha propuesto que L-FABP es capaz de ligar peróxidos y promover su paso desde el interior celular a la luz tubular, protegiendo así al epitelio tubular del daño producido por las especies reactivas de oxígeno[105].

L-FABP se eleva precozmente en orina tras la lesión renal y alcanza su pico aproximadamente en 6 horas[65,99,106].

1.7.1.6. Klotho

La proteína Klotho apenas se ha estudiado como posible biomarcador precoz de FRA. El gen Klotho fue identificado en 1997 como un gen antienvejecimiento[107]. Se expresa en múltiples tejidos, siendo el riñón el lugar donde lo hace de manera más marcada[108], especialmente en el túbulo distal pero también en el proximal[109] y en el colector[110,111]. Klotho es una proteína transmembrana que funciona como un correceptor para el *fibroblast growth factor-23* (FGF-23)[112,113]. El ectodominio pude ser liberado al espacio extracelular por proteasas ancladas a la membrana, dando lugar al Klotho soluble[114]. El Klotho soluble es una proteína pleiotrópica que ejerce funciones paracrinas y endocrinas, tanto a nivel renal como extrarrenal[115]. Cada vez se conoce mejor el papel de Klotho en envejecimiento y metabolismo fosfocálcico[109,116,117]. También ha sido

objeto de intenso estudio en los últimos años su papel en la evolución de ERC y en las complicaciones extrarrenales de la misma[115,118-120] así como la relación entre los niveles circulantes y la progresión de la ERC[121]. Respecto al FRA, se ha demostrado déficit renal de Klotho en diversos modelos animales de FRA inducido por isquemia-reperfusión, obstrucción ureteral o nefrotóxicos[122-129]. En 2010 Hu et al.[125] midieron Klotho urinario en 17 pacientes con FRA y encontraron unos valores marcadamente reducidos frente a los obtenidos de 14 voluntarios sanos. Hasta el momento no se ha publicado ningún otro estudio con humanos en el que se haya testado Klotho en orina como biomarcador de FRA.

HIPÓTESIS DE TRABAJO.

OBJETIVOS DEL ESTUDIO

HIPÓTESIS DE TRABAJO. OBJETIVOS DEL ESTUDIO

El FRA es una complicación que aparece con una elevada frecuencia en los pacientes sometidos a cirugía cardiaca o angiografía coronaria, tiene un impacto muy negativo sobre la morbimortalidad de los mismos y prolonga la estancia hospitalaria. Incluso el FRA considerado tradicionalmente como leve o pasajero implica un peor pronóstico. En el momento actual la detección del FRA se basa en la determinación de la creatinina sérica, que asciende tardíamente y no refleja bien el filtrado glomerular real en el paciente agudo. Por tanto la creatinina sérica no es un marcador adecuado en este contexto. La carencia de biomarcadores precoces de FRA tiene importantes repercusiones en la clínica porque impide implementar medidas tempranas y eficaces para resolverlo. La imposibilidad de detectar precozmente el FRA también supone un problema de primera magnitud a la hora de desarrollar y evaluar estrategias o tratamientos para el mismo. Por todo ello en los últimos años numerosos grupos han intentado encontrar un biomarcador que permita el diagnóstico precoz del FRA. Sin embargo, a pesar de todo el esfuerzo realizado en la búsqueda de biomarcadores, quedan importantes cuestiones por resolver:

1. El mejor biomarcador (o combinación de biomarcadores) en distintos contextos clínicos y el punto de corte que ofrece mejor sensibilidad y especificidad.

2. La capacidad de los biomarcadores para distinguir entre FRA funcional o prerenal y la necrosis tubular aguda.

3. La conveniencia de determinarlos en sangre o en orina y el mejor momento para la obtención de la muestra.

4. Los factores extrínsecos que pueden influir en los resultados y el rendimiento en distintos contextos clínicos.

5. La capacidad pronóstica además de la diagnóstica.

Hipótesis

Planteamos la hipótesis de que NGAL, IL-18, KIM-1 y Klotho en orina y cistatina-C en suero, o alguno de ellos, son capaces de detectar de manera precoz el FRA en pacientes con síndrome coronario o fallo cardiaco agudo sometidos a cirugía cardiaca o angiografía coronaria.

Objetivos

1. Evaluar la utilidad de distintos biomarcadores - NGAL, IL-18, KIM-1 en orina y cistatina-C en suero - que están siendo estudiados en distintas poblaciones y situaciones clínicas para la detección precoz del FRA, en una población de pacientes con síndrome coronario o fallo cardíaco agudo y sometidos a cirugía cardiaca.

2. Evaluar la utilidad de estos biomarcadores para la detección precoz del FRA en una población de pacientes con síndrome coronario o fallo cardiaco agudo y sometidos a angiografía coronaria.

3. Comparar los resultados obtenidos para los distintos biomarcadores en ambos grupos de pacientes.

4. Comparar los resultados obtenidos con los publicados en la literatura y analizar las diferencias y similitudes encontradas.

5. Explorar la posible aplicación de la proteína Klotho en orina como biomarcador precoz del FRA en una población de pacientes con síndrome coronario o fallo cardiaco agudo y sometidos a cirugía cardiaca.

MATERIAL Y MÉTODOS

MATERIAL Y MÉTODOS

El trabajo presentado es un estudio observacional prospectivo de pacientes ingresados en la Unidad de Cuidados Intensivos por síndrome coronario o fallo cardíaco agudo por patología coronaria o valvular y a quienes se les realizó una angiografía coronaria con o sin angioplastia o implantación de endoprótesis o una cirugía cardíaca de revascularización o recambio valvular. Se ha desarrollado en tres fases sucesivas.

En la primera se ha investigado la utilidad de **interleucina-18 (IL-18)** en orina**, *neutrophil gelatinase-associated lipocalin* (NGAL)** en orina y **cistatina-C** en suero para la detección precoz del FRA en una población de pacientes con síndrome coronario o fallo cardíaco agudo y sometidos a cirugía cardíaca o angiografía coronaria.

En la segunda fase se ha evaluado la utilidad de otros dos marcadores que han generado mucho interés, *kidney injury molecule-1* **(KIM-1) y** *liver-type fatty acid binding protein* **(L-FABP)** en orina, comparándolos con el marcador con el que mejores resultados se había obtenido en la fase anterior, NGAL.

En la última parte de trabajo se ha investigado el posible uso como biomarcador de la proteína **Klotho** en orina.

Este estudio fue aprobado por el comité ético del Hospital Clínico Universitario de Valencia y se siguieron los principios de la declaración de Helsinki[130].

1. Pacientes

En la primera fase del estudio se incluyeron 135 pacientes ingresados entre mayo de 2008 y diciembre de 2009 en la Unidad de Cuidados Intensivos del Hospital Clínico

Universitario de Valencia por síndrome coronario o fallo cardíaco agudo por patología coronaria o valvular y a quienes se les realizó una angiografía coronaria con o sin angioplastia y/o implantación de endoprótesis (grupo 1, n = 89) o una cirugía cardiaca de revascularización o recambio valvular (grupo 2, n = 46).

Los criterios de exclusión fueron: edad menor de 18 años, ERC preexistente en tratamiento sustitutivo renal con diálisis o trasplante y FRA por shock cardiogénico en el momento del ingreso. También se excluyeron los pacientes a los que se había realizado angiografía y cirugía consecutivas para evitar confusión.

Se utilizó suero salino isotónico antes de la angiografía para la prevención de la aparición del FRA. La elección del contraste fue realizada por el médico que realizó la angiografía. El volumen de contraste utilizado fue muy variable, en función de la necesidad.

Todos los pacientes fueron seguidos de manera prospectiva desde la inclusión. Se monitorizó la creatinina desde el día previo de la intervención hasta seis días después y se siguió la evolución clínica del paciente hasta el alta. Se recogieron muestras de suero y orina a las 12 horas de finalizada la intervención para la determinación de los biomarcadores. Se recogieron también muestras de orina de 20 voluntarios sanos para obtener los valores de normalidad en orina.

La creatinina basal se definió como el valor de la creatinina el día anterior a la intervención. Se recogieron en todos los casos: datos demográficos y de comorbilidad, parámetros de la cirugía y complicaciones durante o tras la angiografía o la cirugía cardiaca.

El criterio de valoración primario del estudio fue la aparición de FRA definido como un aumento de la creatinina igual o mayor del 50%, siguiendo los criterios de la clasificación RIFLE (Risk, Injury, Failure, Loss, End-stage renal disease)[3] (Tabla 1).

Para la segunda parte del estudio se incluyó entre enero y junio de 2011 a 58 pacientes más hasta llegar a un total de 193 (144 en el grupo de angiografía coronaria y 49 en el de cirugía cardiaca). Los datos recogidos, los criterios de exclusión y el criterio de valoración primario fueron los mismos que en el primer estudio. Se recogieron también muestras de orina antes de la intervención a algunos pacientes de manera aleatoria en el grupo de angiografía coronaria.

Para la última fase se incluyó 60 pacientes de la cohorte previa. El grupo de angiografía coronaria estaba compuesto por 30 pacientes: 18 que presentaron FRA tras la intervención y 12 que no lo presentaron. Otros 30 pacientes fueron también seleccionados en el grupo de cirugía cardiaca: 15 con y 15 sin FRA.

2. Obtención y procesamiento de las muestras

Las muestras de sangre se obtuvieron por punción venosa y se recogieron en tubos BD Vacutainer® de 8.5 ml conteniendo gel separador (BD, Referencia 366468). Tras centrifugación durante 10 minutos a 1.500 g se recogió el sobrenadante (suero) y se guardaron varias alícuotas de 0,5 ml en tubos de polipropileno para almacenarlas a –80 ºC para su posterior ensayo.

Las muestras de orina se recogieron en tubos estériles de 9 ml (URIDRAW, ref. VF-109SURI) y se centrifugaron durante 10 minutos a 1.500 g. Se recogió el sobrenadante y se guardaron varias alícuotas de 0,5 ml en tubos de polipropileno para almacenarlas a –80 ºC para su posterior ensayo.

3. Determinación de Biomarcadores

Todas las determinaciones de los biomarcadores a excepción de la Cistatina-C se realizaron en el laboratorio de Deterioro Neurológico del INCLIVA. La Cistatina-C en suero se midió en el Laboratorio de Bioquímica del Hospital Clínico Universitario de Valencia, donde se realizaron también las determinaciones de creatinina, tanto en suero como en orina.

3.1. Determinación de Biomarcadores mediante ensayos ELISA

La técnica de enzimo-inmunoanálisis de adsorción o ELISA (acrónimo del inglés *Enzyme-Linked ImmunoSorbent Assay*) se basa en el uso de antígenos o anticuerpos marcados con una enzima, de forma que los conjugados resultantes tengan actividad tanto inmunológica como enzimática. Al estar uno de los componentes (antígeno o anticuerpo) marcado con una enzima e insolubilizado sobre un soporte (inmunoadsorbente) la reacción antígeno-anticuerpo quedará inmovilizada y, por tanto, será fácilmente revelada mediante la adición de un substrato específico que al actuar la enzima producirá un color observable a simple vista o cuantificable mediante el uso de un espectrofotómetro o un colorímetro.

Pasos generales de un ELISA (Figura 7)

1. Tapizado del pocillo con el antígeno o anticuerpo. Se utilizan placas de 96 pocillos.

2. Adición de la muestra problema con la mezcla de antígenos o anticuerpos.

3. Unión del antígeno o anticuerpo específico al anticuerpo o antígeno tapizado en el pocillo.

4. Lavado del pocillo para eliminar el exceso de antígeno o anticuerpo no unido.

5. Adición del anticuerpo secundario marcado con la enzima.

6. Unión del anticuerpo secundario al antígeno o anticuerpo.

7. Lavado del pocillo para eliminar el exceso de enzima no unida.

8. Adición del substrato.

9. Unión del substrato a la enzima.

10. Desarrollo del color. El sustrato utilizado es el TMB (tetrametil-bencidina), que desarrolla color azul. Tras la adición de una solución ácida se detiene la reacción obteniéndose un cambio de color a amarillo. La densidad óptica se mide normalmente a 450 nm.

Figura 7. Pasos generales de un ensayo ELISA
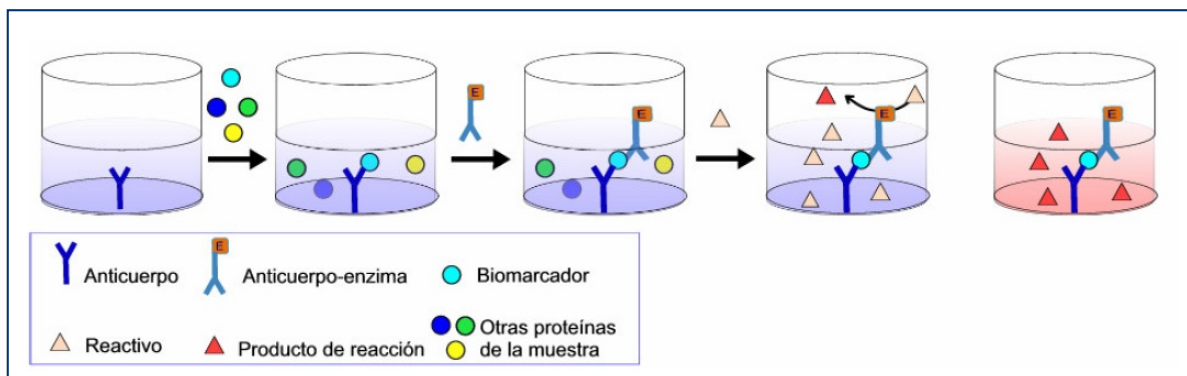
En la Figura 8 se muestran dos ejemplos de placas en los que se ha desarrollado un ensayo ELISA. La cantidad del biomarcador en la muestra se mide evaluando la cantidad del producto final coloreado, analizando la densidad óptica de la placa e interpolando en una curva de calibración realizada en la misma placa.

La lectura de la absorbancia final se realizó mediante un espectrofotómetro *Multiskan*

Ascent (Thermo) (Figura 9).

Figura 8. A. Resultado de ensayo ELISA utilizando como sustrato TMB (tetrametil-bencidina). **B.** Resultado final de un ensayo ELISA tras parar la reacción por adición de solución ácida
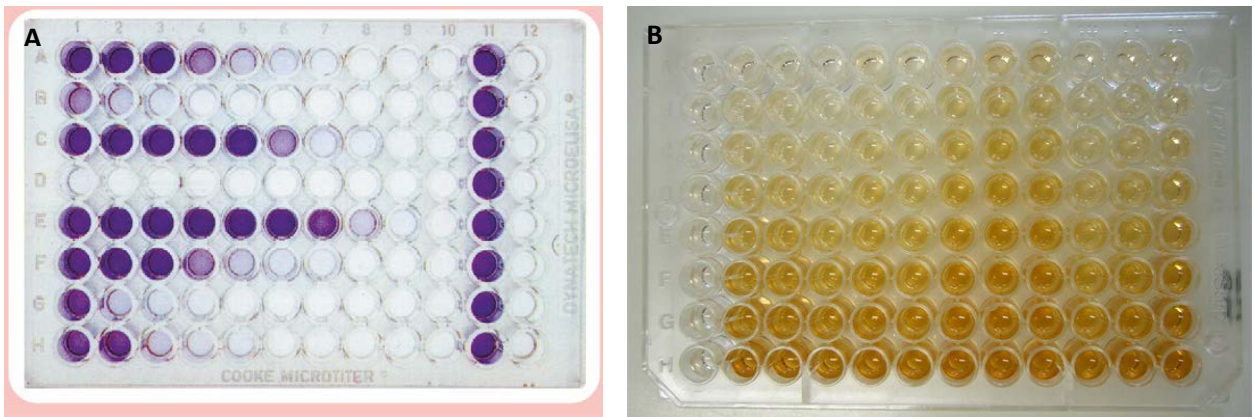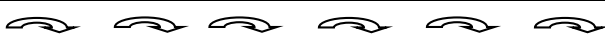
Figura 9. Espectrofotómetro Multiskan Ascent (Thermo)

3.1.1. Determinación de NGAL (*Neutrophil Gelatinase-Associated Lipocalin*) en orina

La determinación de NGAL en muestras de orina se realizó con un ensayo ELISA (Human NGAL ELISA, Hycult biotechnology b.v.). Las muestras de orina se diluyeron 1:20 en tampón de dilución suministrado por el proveedor, y de esta dilución se tomaron 100 µl para el ensayo.

El ensayo contiene NGAL recombinante purificado que se utiliza para realizar la curva patrón. Para preparar la curva patrón se preparan diluciones seriadas en tubos de polipropileno según el siguiente esquema:

Nº tubo	1	2	3	4	5	6	7	8 Blanco
Concentración (ng/ml)	100	40	16	6.4	2.6	1.0	0.4	0
Tampón de dilución	*	225 µl	225 µl	225 µl	225 µl	225 µl	225 µl	500 µl
Volumen de Calibrador a añadir	0	150 µl de tubo 1	150 µl de tubo 2	150 µl de tubo 3	150 µl de tubo 4	150 µl de tubo 5	150 µl de tubo 6	

* En las instrucciones de cada kit se indica la cantidad de tampón de dilución a añadir en el tubo 1.

En la figura 10 se muestra un resumen del protocolo utilizado. La determinación se realiza en un tiempo aproximado de 3 horas y media. Las muestras y diferentes concentraciones de la curva patrón se incuban en placa de 96 pocillos tapizados con anticuerpos que reconocen al NGAL humano. Tras lavar se incuba con un anticuerpo marcado con biotina que se une al NGAL humano. Se elimina tras la incubación el anticuerpo no unido y se adiciona un conjugado de Estreptavidina-peroxidasa, que se

une al anticuerpo marcado con biotina. Este conjugado reacciona con el sustrato, tetrametil-bencidina (TMB), desarrollándose color azul de mayor o menor intensidad según la concentración de NGAL del pocillo. La reacción se detiene con la adición de ácido oxálico y se mide la absorbancia a 450 nm en un espectrofotómetro (*Multiskan Ascent,* Thermo). Para obtener la curva patrón se representa la absorbancia a 450 nm frente a las correspondientes concentraciones de NGAL humano de la curva patrón. Para calcular la concentración de NGAL (ng/ml) en las muestras de orina se interpola la absorbancia obtenida en la curva patrón y se multiplica por el factor de dilución. Los resultados se expresan en ng/ml. El nivel mínimo de detección del ensayo es de 0.4 ng/ml.

Figura 10. Esquema del protocolo utilizado para la determinación de NGAL en orina
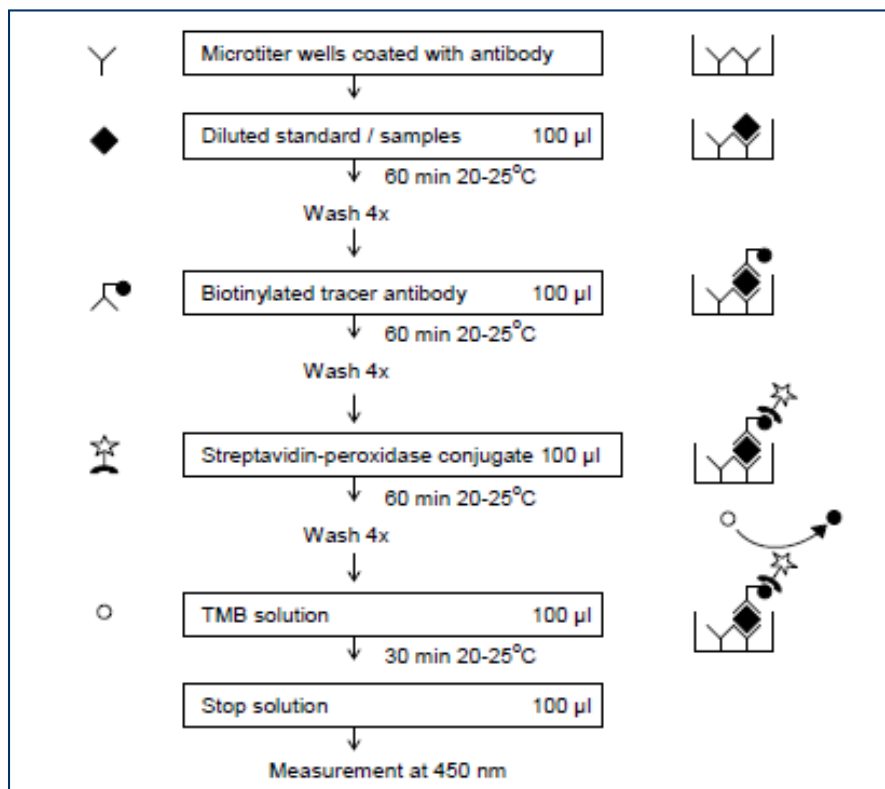
3.1.2. Determinación de L-FABP (*Liver-type fatty acid binding protein*) en orina

La determinación de L-FABP en orina se realizó con un ensayo ELISA (*Human L-FABP HK404 ELISA, Hycult biotechnology b.v.*). Las muestras de orina se diluyeron 1:20 en tampón de dilución suministrado por el proveedor, y de esta dilución se tomaron 100 µl para el ensayo. La curva patrón abarca un rango de concentraciones de 0.102-25 ng/ml. El nivel mínimo de detección del ensayo es de 102 pg/ml. El esquema del protocolo utilizado para la determinación de L-FABP en orina es el mismo que el descrito en la Figura 10 para la determinación de NGAL. Para calcular la concentración de L-FABP en las muestras de orina se interpola la absorbancia obtenida en la curva patrón y se multiplica por el factor de dilución. Los resultados se expresan en ng/ml.

3.1.3. Determinación de IL-18 (Interleucina 18) en orina

La determinación de IL-18 en muestras de orina se realizó mediante un ensayo ELISA (*Human IL-18 ELISA, Bender MedSystems*), y se utilizó 50 µl de cada muestra de orina para el ensayo. El límite de detección de este ensayo era de 9 pg/ml.

El ensayo se realizó según el protocolo proporcionado por el fabricante. El kit consta de una placa tapizada con el anticuerpo frente a la IL-18 humana a la que se añaden 50 µl de muestra (orina) y 50 µl de diluyente de muestra. La curva patrón abarca un rango de concentraciones de 0-5000 pg/ml. Posteriormente se añade el anticuerpo secundario conjugado con biotina a todos los pocillos y se incuba en agitación, 2 h a temperatura ambiente. Una vez finalizada la incubación, se lava y se adiciona estreptavidina marcada con peroxidasa de rábano (HRP) y se vuelve a incubar en agitación, 1 h a temperatura ambiente. Finalmente, se añade el reactivo revelador TMB (tetrametil-bencidina). La

reacción se detiene por adición de reactivo 'Stop solution' (ácido) y se lee la absorbancia a 450 nm en un lector de placas.

La concentración de IL-18 en orina se calcula interpolando la absorbancia a 450 nm en la curva estándar. Los resultados se expresan en pg/ml.

3.1.4. Determinación de KIM-1 (*Kidney Injury Molecule-1*) en orina

La medición de KIM-1 en orina se realizó con un ensayo ELISA diseñado para medir KIM-1 humano en orina (Human TIM-1/KIM-1/HAVCR, R&D Systems Europe Ltd. UK). El ensayo se realizó según el protocolo proporcionado por el fabricante. El kit consta de una placa tapizada con el anticuerpo frente a KIM-1 humano a la que se añaden 50 µl de muestra de orina o de soluciones patrón y 100 µl de tampón de dilución suministrado con el kit. Se preparó la curva patrón como indica la Figura 11 mediante diluciones seriadas en tampón de dilución a partir una solución de 100 ng/ml de KIM-1, para tener un rango de concentraciones de 0-10 ng/ml.

Figura 11. Preparación de diluciones seriadas a partir de solución estándar de KIM-1 a 100 ng/ml para obtener la curva patrón
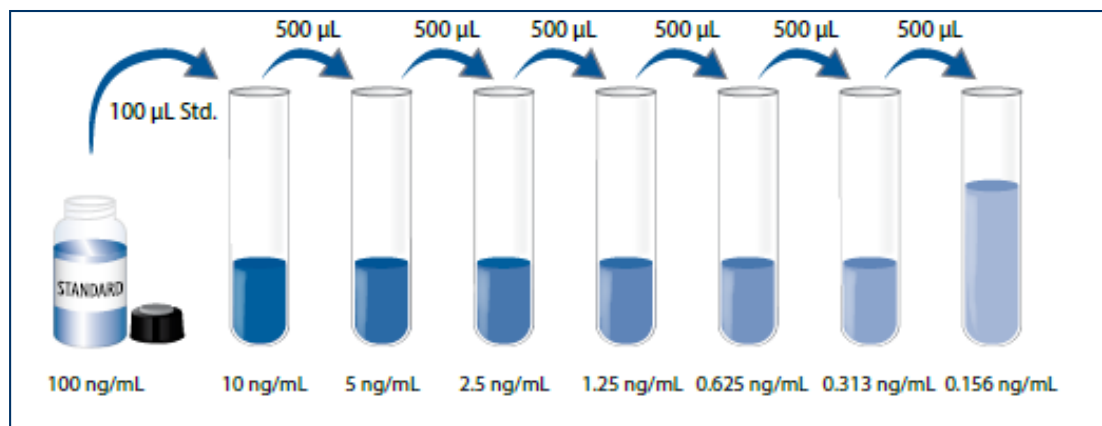
Tras incubación de 2 horas a temperatura ambiente se realizaron 3 lavados con el tampón de lavado suministrado con el kit y se añadieron 200 µl del conjugado de KIM-1 humano a cada pocillo. Tras otras 2 horas de incubación y 3 lavados para eliminar el conjugado no unido se adicionaron 200 µl de la solución del sustrato a cada pocillo, incubándose a temperatura ambiente durante 30 min. Mediante la adición de 50 µl de *'Stop solution'* se detuvo la reacción, virando el color de la reacción de azul a amarillo. Finalmente se realizó la lectura de la absorbancia a 450 nm en lector de placas.

Para el cálculo de los resultados se representó la curva patrón y se interpolaron los valores de absorbancia de las muestras en la curva, obteniéndose la concentración de KIM-1 en ng/ml. El nivel mínimo de detección del ensayo es de 0.009 ng/ml.

3.1.5. Determinación de Klotho en orina

Los niveles de Klotho humano se determinaron en orina usando dos ensayos ELISA comerciales, uno de *Shanghai Sunred Biological Technology Co., Ltd*, con sensibilidad de 0.05 ng/ml y un rango del ensayo de 0.1-20 ng/ml, y otro de *IBL International* (Human soluble α-Klotho Assay Kit-IBL, *Immuno-Biological Laboratories Co., Ltd*), con sensibilidad de 0.006 ng/ml y un rango del ensayo de 0.093-6 ng/ml.

Para la determinación de los niveles de Klotho en muestras de orina mediante el kit ELISA de *Shanghai Sunred Biological Technology* se utilizaron 40 µl de muestra, a los que se adicionaron 10 µl del anticuerpo marcado con biotina y 50 µl de Estreptavidina-HRP. Tras incubación de 60 minutos a 37ºC se realizaron varios lavados y se añadieron a cada pocillo 50 µl de solución cromógena A y 50 µl de solución cromógena B. Se incubaron durante 10 minutos a 37ºC, protegiendo de la luz. La reacción se detuvo mediante la adición de 50 µl de *'Solución Stop'* (el color de la reacción cambia de azul a amarillo) y

se midió la absorbancia a 450 nm. Los valores de los niveles de Klotho en las muestras se calcularon interpolando en la curva patrón.

La curva patrón y el procedimiento y para la determinación de Klotho en orina mediante el kit ELISA de *IBL International* se resumen en las Figuras 12 y 13, respectivamente. En este kit ELISA se utilizaron 100 µl de muestra, se incubaron 60 minutos a temperatura ambiente y tras 7 lavados se volvieron a incubar durante 30 minutos con 100 µl de anticuerpo marcado. Se realizaron 9 lavados y se añadieron 100 µl del cromógeno por pocillo, incubándose durante 30 minutos más. Una vez desarrollado el color, se paró la reacción con 100 µl de *'Stop Solution'* y se midió la absorbancia a 450 nm.

Figura 12. Curva patrón utilizada para la determinación de Klotho en orina mediante el kit ELISA de IBL International
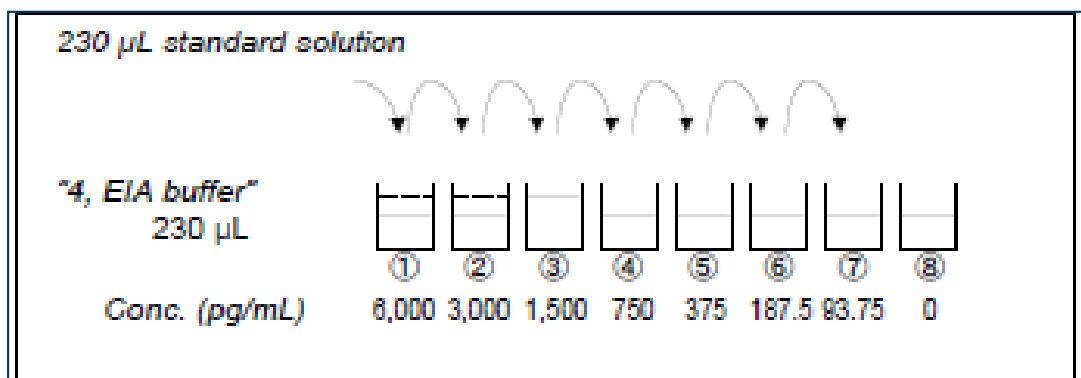
Figura 13. Resumen del procedimiento utilizado para la determinación de Klotho en orina mediante el kit ELISA de IBL International

Reagents	Test Sample	Standard	Test Sample Blank	Reagent Blank
	Test sample 100 µL	Diluted standard (Tube 1-7) 100 µL	EIA buffer (Tube-8) 100 µL	EIA buffer 100 µL
Incubation for 60 minutes at room temperature with plate lid				
Washing 7 times				
Labeled Antibody	100 µL	100 µL	100 µL	-
Incubation for 30 minutes at room temperature with plate lid				
Washing 9 times				
Chromogen	100 µL	100 µL	100 µL	100 µL
Incubation for 30 minutes at room temperature (shielded)				
Stop solution	100 µL	100 µL	100 µL	100 µL
Read the plate at 450nm against a Reagent Blank within 30 minutes after addition of Stop solution.				

3.2. Determinación de Cistatina-C en suero

La determinación de cistatina-C en suero se realizó en el Laboratorio de Bioquímica del Hospital Clínico Universitario de Valencia mediante un ensayo estandarizado por inmunonefelometría en un nefelómetro *BNII (Siemens Healthcare Diagnostics)* (Figura 14).

Figura 14. Nefelómetro BNII (*Siemens Healthcare Diagnostics*)

La inmunonefelometría es una técnica automatizada que se utiliza para cuantificar proteínas del suero y otros líquidos biológicos con mayor sensibilidad y precisión que las técnicas tradicionales de inmunodifusión radial y turbidimetría.

La reacción de inmunoprecipitación producida por el complejo antígeno-anticuerpo es detectada por un haz de luz que se dispersa en un ángulo determinado entre 10 a 70º en relación al rayo incidente (Figura 15). La cantidad de luz dispersada es proporcional a la concentración de los complejos antígeno-anticuerpo presentes en la muestra en un amplio intervalo de concentración. La sensibilidad se puede incrementar mediante las partículas de látex recubiertas con anticuerpos o antígenos. Esta tecnología hace posible medir pequeñas moléculas, tales como la Homocisteína y la Cistatina-C o compuestos que requieren una óptima sensibilidad a bajas concentraciones, como PCR de alta sensibilidad o las subclases de IgG3/IgG4.

Figura 15. Principio de la nefelometría
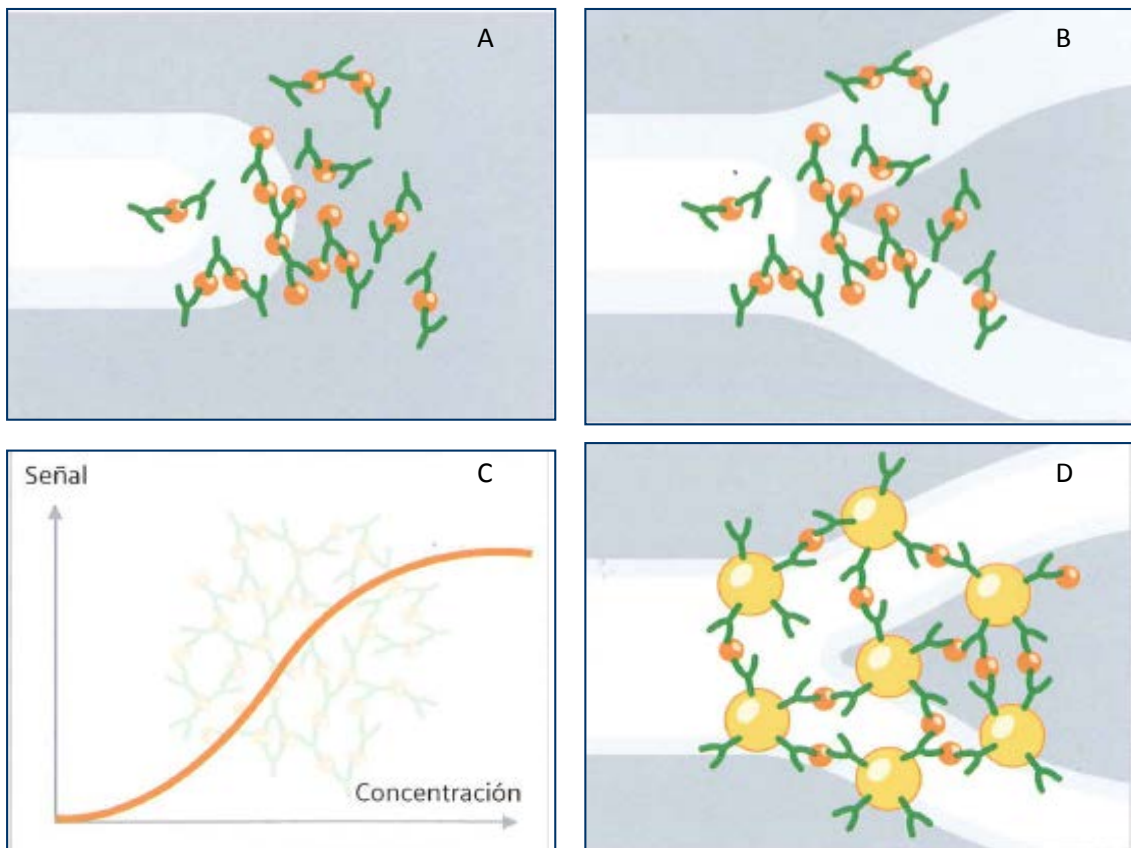
A. Un haz de luz con una longitud de onda de 840 nm pasa a través de una cubeta que contiene el complejo antígeno-anticuerpo. **B.** La nefelometría mide la cantidad de luz dispersada por el complejo antígeno-anticuerpo. **C.** La cantidad de luz dispersada es proporcional a la concentración de los complejos antígeno-anticuerpo presentes en la muestra en un amplio intervalo de concentración. **D.** La sensibilidad se puede incrementar mediante las partículas de látex recubiertas con anticuerpos o antígenos.

4. Análisis estadístico

Los resultados se analizaron utilizando los programas SPSS 19.0 y GraphPad PRISM 4.0.

Para la comparación de medias de más de dos variables, se realizó un ANOVA de un

factor con análisis post-hoc de Bonferroni y para comparación de dos medias el test de

Student o el test de Mann-Whitney en el caso de distribución no normal. Para estudiar

la relación entre las diferentes variables se realizaron correlaciones de Pearson. El nivel

de probabilidad aceptado como significativo fue de p <0.05. La sensibilidad y especificidad de los distintos marcadores se evaluó mediante curvas ROC (*Receiver Operating Characteristic*). Un AUC-ROC > 0.70 definió un predictor como útil [131].

Las correlaciones entre la presencia de FRA y los niveles de Klotho en orina se realizaron mediante correlación de Spearman. Las correlaciones entre los niveles de Klotho medidos con los dos ensayos ELISA y entre el nivel de Klotho (ng/mg creatinina) y el incremento de creatinina sérica (creatinina máxima en suero – creatinina basal) se realizaron mediante correlación bivariada de Pearson. El nivel de significación se estableció en p < 0.05.

RESULTADOS

1. Primera fase del estudio. IL-18 en orina, NGAL en orina y cistatina-C en suero para el diagnóstico precoz de fracaso renal agudo tras angiografía coronaria en pacientes con síndrome coronario agudo o fallo cardiaco

1.1. Características de los pacientes

Las características clínicas y demográficas de los pacientes sometidos a angiografía coronaria se exponen en la tabla 2 y las de pacientes sometidos a cirugía cardiaca en la tabla 3. En el grupo de angiografía coronaria, los valores de creatinina previa a la intervención en los pacientes FRA eran significativamente más altos que en los pacientes sin FRA (p <0,001) (Tabla 2), y no se observaron diferencias significativas en el grupo de pacientes tratados con cirugía cardiaca.

Ventiséis pacientes desarrollaron FRA, de los cuales 12 pertenecían al grupo de angiografía (13%, 12/89) y 14 al grupo de cirugía cardíaca (30%, 14/46). Respecto a la mortalidad, seis pacientes sometidos a angiografía fallecieron, de los cuales tres habían dearrollado un FRA, mientras que los cuatro pacientes fallecidos del grupo de cirugía cardiaca pertenecían al grupo con FRA.

Tabla 2. Primera fase del estudio. Características clínicas y demográficas de los pacientes sometidos a angiografía coronaria

	PACIENTES NO FRA	PACIENTES FRA
Nº de sujetos	77	12
Edad	61 ± 13	73 ± 9
Sexo (H/M)	59/18	8/4
eFG (MDRD) previo (ml/min/1.73m^2)	81 ± 24	58 ± 18**
Creatinina previa (mg/dl)	0.94 ± 0.22	1.2 ± 0.3***
Creatinina máxima (mg/dl)	1.0 ± 0.23	2.03 ± 0.56***
RIFLE (R/I/F)		8/3/1
FRA (día)		4 ± 3
Éxitus	3.9%	25%
Estancia (días)	10 ± 6	21 ± 15

FRA: Fracaso renal agudo; eFG (MDRD) previo: Filtrado glomerular estimado por MDRD antes de la intervención. Los valores de los pacientes FRA que se diferencian significativamente de los pacientes NO FRA se expresan con **p<0.01; ***p<0.001.

Tabla 3. Primera fase del estudio. Características clínicas y demográficas de los pacientes sometidos a cirugía cardiaca

	PACIENTES NO FRA	PACIENTES FRA
Nº de sujetos	32	14
Edad	70 ± 9	66 ± 15
Sexo (H/M)	22/10	12/2
eFG (MDRD) previo (ml/min/1.73m^2)	70 ± 32	64 ± 28
Creatinina previa (mg/dl)	1.1 ± 0.4	1.23 ± 0.5
Creatinina máxima (mg/dl)	1.2 ± 0.4	2.12 ± 0.7***
RIFLE (R/I/F)		9/5/0
FRA (día)		3 ± 1
Éxitus	0	29%
Tipo de Cirugía:		
- Bypass	19	4
- Valvular	13	9
- Bypass y valvular	1	0
Tiempo CEC (min)	65 ± 25	91 ± 59
Estancia (días)	14 ± 8	30 ± 24

FRA: Fracaso renal agudo; eFG (MDRD) previo: Filtrado glomerular estimado por MDRD antes de la intervención. Tiempo CEC: Tiempo de Circulación extra corpórea en minutos. Los valores de los pacientes FRA que se diferencian significativamente de los pacientes NO FRA se expresan con ***p<0.001.

1.2. Valores de NGAL (*Neutrophil Gelatinase-Associated Lipocalin*) en orina de controles sanos y pacientes

Los resultados obtenidos mostraron una diferencia significativa (p <0,001) de NGAL en orina entre los pacientes que desarrollaron FRA y los pacientes sin FRA, lo que permite distinguir claramente los dos grupos (Tabla 4 y Figura 16).

Los valores de NGAL en orina en el grupo control (voluntarios sanos) eran de 18 ± 5 ng/ml. Se observaba una diferencia significativa (p <0,001) de NGAL en los pacientes con FRA (angiografía: 96 ± 24; cirugía cardíaca: 129 ± 28) con respecto a los valores del grupo control (Figura 16, A y B). Los valores de NGAL de los pacientes sin FRA no diferían significativamente de los del grupo control (angiografía: 17,4 ± 1,5; cirugía cardíaca: 27,4 ± 3,7) (Figura 16, A y B).

Tabla 4. Valores de NGAL, cistatina-C e IL-18 en los grupos de estudio

	ANGIOGRAFÍA		CIRUGIA CARDIACA	
	NO FRA	FRA	NO FRA	FRA
NGAL (ng/ml)	17.4 ± 1.5	95.8 ± 24[b]	27.4 ± 3.7	129 ± 28[b]
CISTATINA C (mg/ml)	0.71 ± 0.02	1.22 ± 0.16[b]	0.78 ± 0.04	1.01 ± 0.12[b]
IL-18 (pg/ml)	196 ± 18	292 ± 39[a]	217 ± 20	319 ± 38[a]

NO FRA: Pacientes que no desarrollaron fracaso renal agudo; FRA: pacientes que desarrollaron fracaso renal agudo. NGAL: Neutrophil Gelatinase-Associated Lipocalin; IL-18: interleucina 18. Cistatina C se midió en suero; NGAL e IL-18 se midieron en orina. Los valores se expresan como media ± SEM. Los valores que son diferentes significativamente en los pacientes FRA con respecto a los NO FRA se representan con a p<0.05, b p<0.001.

Figura 16. Determinación de los valores de NGAL en orina de controles, pacientes sin fracaso renal (NO FRA) y pacientes con fracaso renal (FRA) en los grupos de estudio
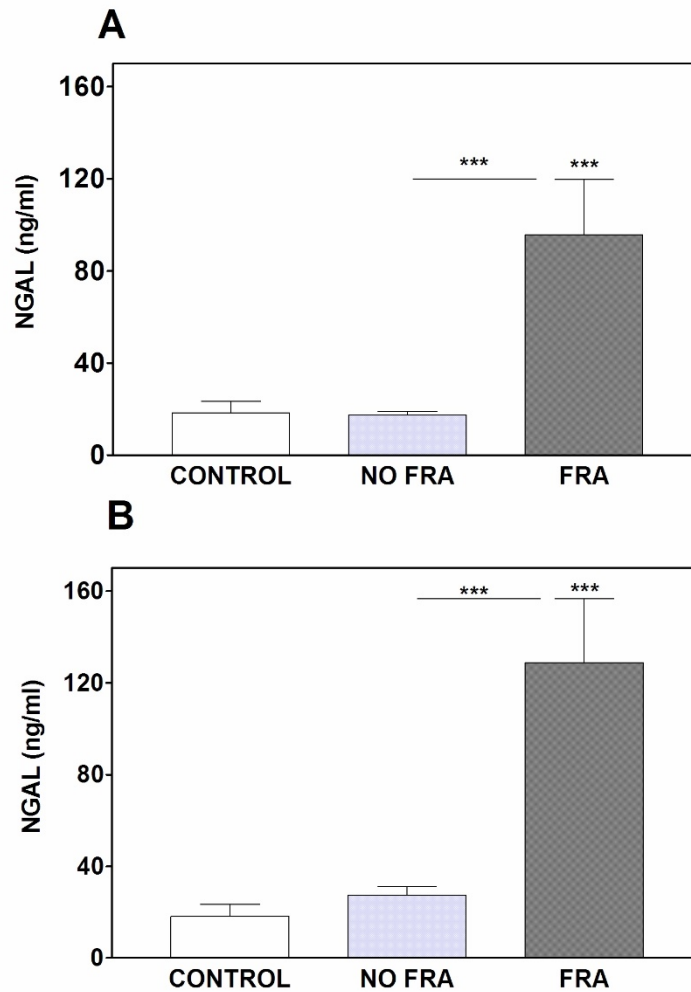
A: pacientes sometidos a angiografía coronaria; **B:** pacientes sometidos a cirugía cardiaca. Los valores se dan como mediana y rango intercuartil. Los asteriscos (***) representan que la diferencia entre los grupos es significativa (p<0.001). NGAL: Neutrophil Gelatinase-Associated Lipocalin.

1.3. Valores de cistatina-C en suero

Los valores de cistatina-C en suero diferenciaban significativamente (p <0,001) los pacientes FRA de los que no tenían FRA. En angiografía la media de los pacientes con FRA fue de 1,22 ± 0,16 mg/dl y de los que no tenían FRA de 0,71 ± 0,02 mg/dl. En cirugía

cardiaca los valores de los pacientes con FRA y sin FRA fueron de 1.01 ± 0,12 mg/dl y

0,78 ± 0,04 mg/dl, respectivamente (Figura 17 y Tabla 4).

Figura 17. Determinación de los valores de Cistatina-C en suero de pacientes sin fracaso renal (NO FRA) y pacientes con fracaso renal (FRA) en los grupos de estudio
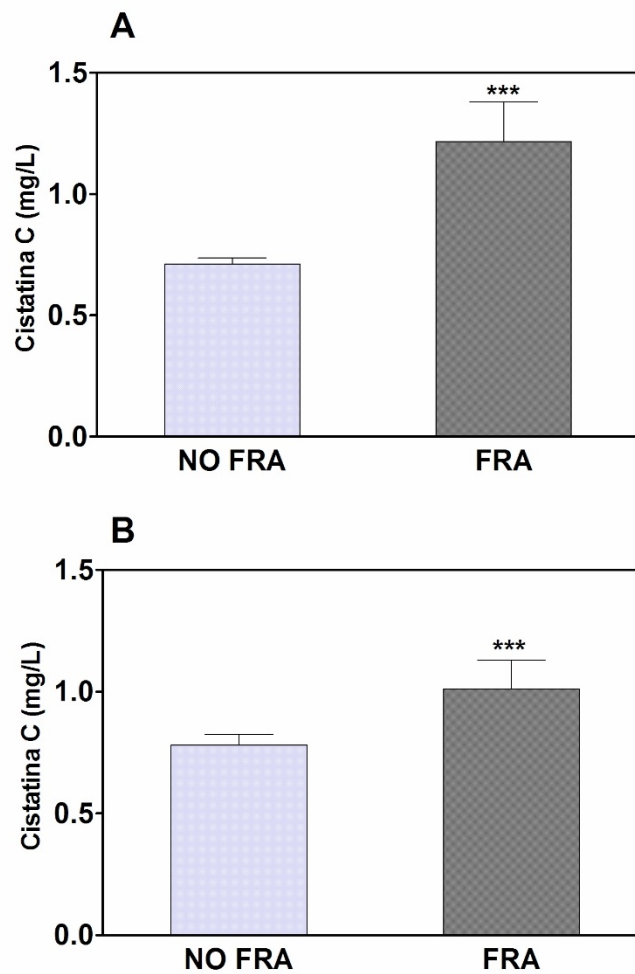
A: pacientes sometidos a angiografía coronaria; **B:** pacientes sometidos a cirugía cardiaca. Los valores se dan como mediana y rango intercuartil. Los asteriscos (***) representan que la diferencia entre los grupos es significativa ($p < 0.001$).

1.4. Valores de IL (interleucina 18) en orina

En el caso de IL-18, los pacientes FRA presentaban niveles significativamente más elevados que los pacientes sin FRA (Figura 18 y Tabla 4). Tanto en angiografía como en cirugía cardiaca se observaba una diferencia significativa entre los pacientes con FRA y sin FRA (p <0,05).

Figura 18. Determinación de los valores de IL-18 en orina de pacientes sin fracaso renal (NO FRA) y pacientes con fracaso renal (FRA) en los grupos de estudio
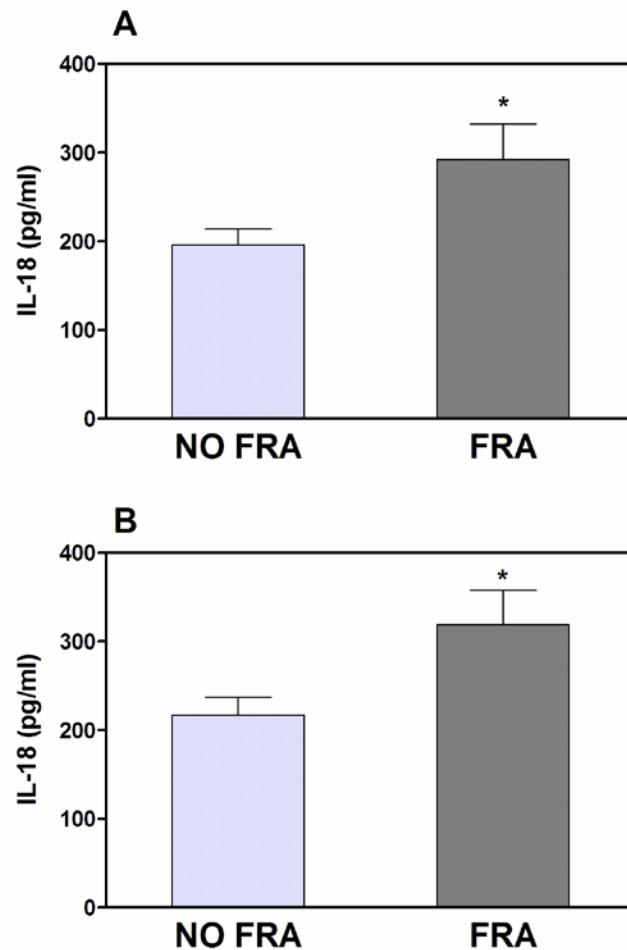
A: pacientes sometidos a angiografía coronaria; **B:** pacientes sometidos a cirugía cardiaca. Los valores se dan como mediana y rango intercuartil. Los asteriscos representan que la diferencia entre los grupos es significativa: * p<0.05. IL-18: interleucina 18.

1.5. Asociación entre cistatina-C, IL-18 y NGAL

Se han realizado correlaciones entre estos parámetros y se observa una correlación significativa entre NGAL en orina y cistatina-C en suero (r = 0,311; p = 0,001) y entre NGAL e IL-18 en orina (r = 0,448; p <0,001).

1.6. Curvas ROC de NGAL, cistatina-C e IL-18

Para determinar si estos marcadores son buenos predictores de la aparición de FRA en pacientes sometidos a angiografía o a cirugía cardiaca, se hizo un análisis de sensibilidad y especificidad utilizando curvas ROC (Figura 19). En la figura 19A se expone la curva ROC de los tres marcadores analizados en el grupo de angiografía. NGAL, cistatina-C e IL-18 son marcadores útiles de FRA, siendo el NGAL el más predictivo, con un área bajo la curva (AUC) de 0,983, respecto a la cistatina-C e IL-18, con AUC de 0,869 y 0,727, respectivamente (Tabla 5). Para un punto de corte de NGAL en orina de 31,9 ng/ml, la sensibilidad fue del 100% y la especificidad del 91%. Para la cistatina-C, un punto de corte de 0,8 mg/l en suero ofreció una sensibilidad del 89% y una especificidad del 76%. En el caso de la IL-18 se obtuvo una sensibilidad del 67% y especificidad del 73% para un punto de corte de 202 pg/ml. La curva ROC de los pacientes sometidos a cirugía cardiaca se expone en la figura 19B. En estos pacientes, aunque la incidencia de FRA era más alta que en el grupo de pacientes con angiografía (Tablas 2 y 3), las AUC ROC eran menores para los tres marcadores (Tabla 5). NGAL seguía siendo el mejor predictor (AUC: 0,773), con valores de sensibilidad y especificidad del 64% y del 80%, respectivamente, considerando un punto de corte de 31,9 ng/ml de NGAL en orina. La Cistatina-C presentaba un AUC de 0,675, con sensibilidad y especificidad del 64% para un punto de corte de 0,8 mg/l, y en la curva ROC para IL-18 se observaba un AUC de 0,676, siendo el

mejor punto de corte de 249 pg/ml de IL-18 en orina, con sensibilidad del 64% y especificidad del 60% (Tabla 5).

Figura 19. Curvas ROC de NGAL, Cistatina-C e IL-18 en cada grupo de sujetos
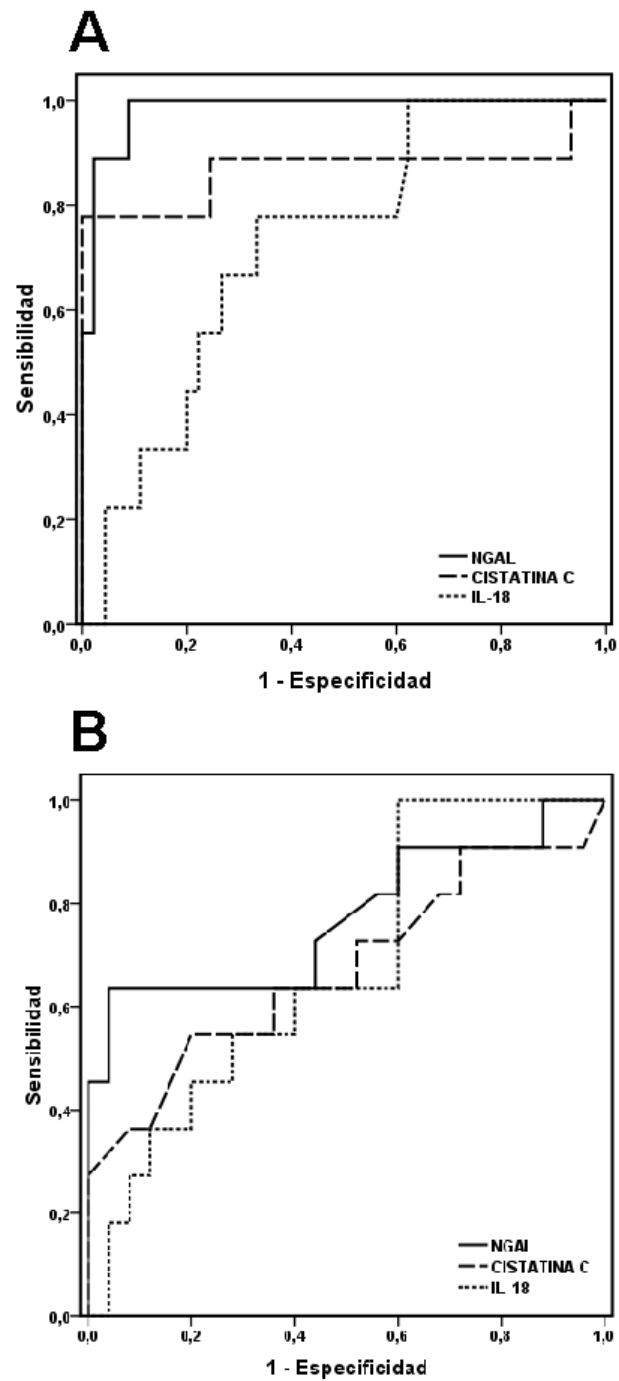
A: pacientes sometidos a angiografía coronaria (AUC para NGAL: 0.983; Cistatina C: 0.869; IL-18: 0.727); B: pacientes sometidos a cirugía cardiaca (AUC para NGAL: 0.773; Cistatina-C: 0.675; IL-18: 0.676). ROC: Receiver Operating Characteristic; AUC: area under the curve; NGAL: Neutrophil Gelatinase-Associated Lipocalin; IL-18: interleucina 18.

Tabla 5. Capacidad diagnóstica de NGAL, cistatina-C e IL-18 para la detección del fracaso renal agudo en los diferentes grupos de pacientes

Grupo de estudio	NGAL		CISTATINA-C		IL-18	
	Punto de corte[a] (S-E)[b]	AUC (IC 95%)[c]	Punto de corte[a] (S-E)[b]	AUC (IC 95%)	Punto de corte[a] (S-E)[b]	AUC (IC 95%)
ANGIOGRAFIA	31.9 (100-91)	0.983 (0.954-1.012)	0.8 (89-76)	0.869 (0.676-1.06)	202 (67-73)	0.727 (0.566-0.888)
CIRUGÍA CARDIACA	31.9 (64-80)	0.773 (0.580-0.96)	0.8 (64-64)	0.675 (0.46-0.88)	249 (64-60)	0.676 (0.49-0.86)

[a] Los puntos de corte para cada marcador se expresan en ng/ml para NGAL, mg/l para Cistatina-C y pg/ml para IL-18.
[b] S-E: Sensibilidad (%) y Especificidad (%), respectivamente, en el punto de corte considerado. AUC: Área bajo la curva ROC.
[c] IC 95%: Intervalo de confianza del 95%.
AUC: area under the curve; NGAL: Neutrophil Gelatinase-Associated Lipocalin; IL-18: interleucina 18.

2. Segunda fase del estudio. KIM-1, NGAL y L-FABP urinarios para el diagnóstico precoz de fracaso renal agudo tras angiografía coronaria en pacientes con síndrome coronario o fallo cardiaco agudo

2.1 Características de los pacientes

Las tabla 6 y 7 muestran las características clínicas y demográficas de los pacientes. Treinta y cinco pacientes desarrollaron FRA, 20 en el grupo de angiografía (14 %) y 15 en el de cirugía cardiaca (30 %). El diagnóstico de FRA por creatinina necesitó 3 ± 1 días en los pacientes de cirugía cardiaca y 4 ± 3 días en el de angiografía. En el grupo de angiografía coronaria, los valores de creatinina basales eran significativamente más altos en los pacientes con FRA (p <0,001) (Tabla 6), mientras que no se observaron diferencias significativas en el grupo de pacientes tratados con cirugía cardíaca. En el grupo de angiografía 8 pacientes fallecieron, de los cuales 4 habían desarrollado un FRA. Todas las muertes se debieron a complicaciones relacionadas con la enfermedad cardiaca y no estaban directamente relacionadas con el FRA en la angiografía. Cuatro pacientes fallecieron del grupo de cirugía cardíaca habiendo presentado todos ellos FRA.

Tabla 6. Segunda fase del estudio. Características clínicas y demográficas de los pacientes sometidos a angiografía coronaria

	PACIENTES NO FRA	PACIENTES FRA
Nº de sujetos	124	20
Edad	62 ± 13	72 ± 10
Sexo (H/M)	94/30	16/4
eFG (MDRD) previo (ml/min/1.73m^2)	84 ± 41	62 ± 18*
Creatinina previa (mg/dl)	0.94 ± 0.26	1.14 ± 0.2**
Creatinina máxima (mg/dl)	1.01 ± 0.28	1.91 ± 0.6***
RIFLE (R/I/F)		14/5/1
FRA (día)		4 ± 3
Éxitus	4	4
Estancia (días)	10 ± 5	17 ± 12

FRA: Fracaso renal agudo; eFG (MDRD) previo: Filtrado glomerular estimado por MDRD antes de la intervención. Los valores de los pacientes FRA que se diferencian significativamente de los pacientes NO FRA se expresan con *p<0.05; **p<0.01; ***p<0.001.

Tabla 7. Segunda fase del estudio. Características clínicas y demográficas de los pacientes sometidos a cirugía cardiaca

	PACIENTES NO FRA	PACIENTES FRA
Nº de sujetos	34	15
Edad	69 ± 9	66 ± 14
Sexo (H/M)	24/10	13/2
eFG (MDRD) previo (ml/min/1.73m^2)	70 ± 32	64 ± 28
Creatinina previa (mg/dl)	1.03 ± 0.31	1.18 ± 0.5
Creatinina máxima (mg/dl)	1.13 ± 0.33	2.14 ± 0.7***
RIFLE (R/I/F)		10/5/0
FRA (día)		3 ± 1
Éxitus	0	4
Tipo de Cirugía:		
- *By-pass*	19	4
- Valvular	13	9
- *By-pass* y valvular	1	1
Tiempo CEC (min)	65 ± 25	91 ± 59
Estancia (días)	14 ± 8	30 ± 24

FRA: Fracaso renal agudo; eFG (MDRD) previo: Filtrado glomerular estimado por MDRD antes de la intervención. Tiempo CEC: Tiempo de circulación extracorpórea en minutos. Los valores de los pacientes FRA que se diferencian significativamente de los pacientes NO FRA se expresan con ***p<0.001.

2.2. Valores de KIM-1 (*Kidney Injury Molecule-1*), L-FABP (*Liver-type fatty acid binding protein*) y NGAL (*Neutrophil Gelatinase-Associated Lipocalin*) en orina de controles sanos y pacientes

Los pacientes con FRA mostraron un incremento significativo en todos los biomarcadores comparados con los pacientes sin FRA y con los controles (Figura 20, Tabla 8). Se encontró una diferencia significativa ($p < 0.001$) en ambos grupos en los niveles de NGAL entre los pacientes que desarrollaron FRA y aquellos que no, lo que permite distinguir ambos grupos (Figura 20 A, B; Tabla 8). Los niveles de KIM-1 también estaban significativamente aumentados en los pacientes con FRA comparados con los pacientes que no lo desarrollaron ($p < 0.001$ para angiografía, $p < 0.01$ para cirugía cardiaca) (Figura 20 C, D; Tabla 8), pero L-FABP sólo fue capaz de discriminar entre los pacientes con y sin FRA en el grupo de cirugía cardiaca ($p < 0.01$) (Figura 20 E, F; Tabla 8). Los pacientes sin FRA en ambos grupos no diferían significativamente de los sujetos controles en NGAL o KIM-1 urinarios (Figura 20 A-D; Tabla 8), pero se encontró un incremento significativo de los niveles de L-FABP en los pacientes sin FRA al compararlos tanto con los sujetos controles como con los niveles pre-intervención en los pacientes sometidos a angiografía (Figura 20 E, F; Tabla 8). Los pacientes que desarrollaron AKI tras angiografía mostraron un incremento significativo de los tres biomarcadores al compararlos con sus niveles pre-angiografía (Figura 20 A, C, E; Tabla 8).

Figura 20. Determinación de los valores de NGAL, KIM-1 y L-FABP en muestras de orina de controles, pacientes sin fracaso renal (NO FRA) y pacientes con fracaso renal (FRA) en los grupos de estudio
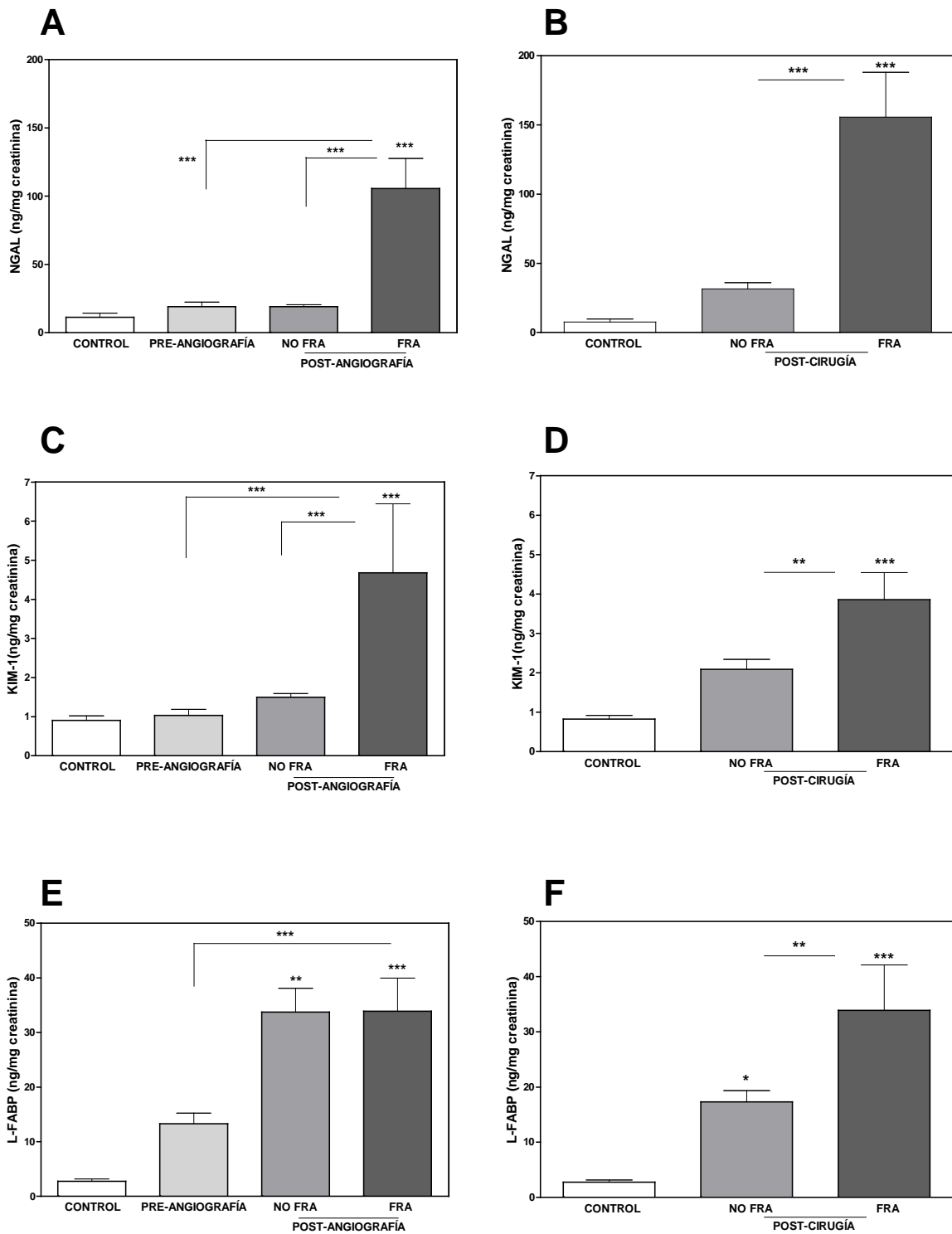
A, C, E angiografía coronaria; **B, D, F** cirugía cardiaca**.** Los valores de los biomarcadores se expresan como ng/ml de creatinina urinaria y se dan como media ± SEM. Las diferencia entre grupos se indican por asteriscos: *p<0.05; **p<0.01; ***p<0.001. NGAL: Neutrophil Gelatinase-Associated Lipocalin. KIM-1: Kidney Injury Molecule-1; L-FABP: Liver-type Fatty Acid–Binding Protein. Pre-angiografía: grupo de pacientes antes de la angiografía.

Tabla 8. Valores de NGAL, KIM-1 y L-FABP corregidos por creatinina urinaria en los grupos de estudio

	CONTROL (n=20)	ANGIOGRAFÍA			CIRUGÍA CARDIACA	
		PRE-INTERVENCIÓN (n=28)	NO FRA (n=124)	FRA (n=20)	NO FRA (n=32)	FRA (n=15)
NGAL (ng/mg creatinina)	11 ± 3	18.9 ± 3.3	19 ± 1.4	105 ± 22[b,d,f]	31.5 ± 4.5	156 ± 32[b,d]
KIM-1 (ng/mg creatinina)	0.9 ± 0.1	1.03 ± 0.15	1.5 ± 0.09	4.7 ± 1.7[b,d,f]	2.1 ± 0.2	3.8 ± 0.7[a,d]
L-FABP (ng/mg creatinina)	3 ± 0.4	15.5 ± 2.4	33.7 ± 4.3[c]	33.9 ± 6[d,f]	17.3 ± 2[c]	33.9 ± 8.2[a,d]

Los valores se expresan como media ± SEM. Los valores que son significativamente diferentes en los pacientes con FRA con respecto a los NO FRA se indican como [a] $p<0.01$, [b] $p<0.001$. Los valores que son significativamente diferentes de los del grupo control se indican con [c] $p<0.05$, [d] $p<0.001$. Los valores diferentes significativamente entre pre y post angiografía se indican con [e] $p<0.05$, [f] $p<0.001$.
NO FRA: Pacientes que no desarrollaron fracaso renal agudo; FRA: pacientes que desarrollaron fracaso renal agudo. NGAL: Neutrophil Gelatinase-Associated Lipocaline; KIM-1: Kidney Injury Molecule 1; L-FABP: Liver Fatty Acid-Binding Protein.

2.3. Curvas ROC de KIM-1, NGAL y L-FABP

El análisis de sensibilidad y especificidad usando curvas ROC mostró que NGAL en orina era el mejor predictor de FRA en ambos grupos de pacientes, con un AUC de 0.958 (95 % CI 0.909–1.007; p0.0001) para el grupo de angiografía y un AUC de 0.916 (95 % CI 0.834–0.998; p<0.0001) para el grupo de cirugía cardiaca (Figura 21 A, B; tabla 9). KIM-1 también fue útil para predecir el FRA en ambos grupos de pacientes con un AUC de 0.713 (95 % CI 0.551–0.876; p = 0.01) para el grupo de angiografía y un AUC de 0.716 (95 % CI 0.556–0.875; p = 0.02) para el de cirugía cardiaca (Figura 21 A, B; Tabla 9). L-FABP no fue capaz de predecir el FRA en el grupo de angiografía (Tabla 9) pero sí fue un predictor significativo de FRA en el grupo de cirugía cardiaca (AUC:0.743; 95 %CI 0.588–0.898; p = 0.015).

Figura 21. Curvas ROC de NGAL, KIM-1 Y L-FABP en cada grupo de sujetos
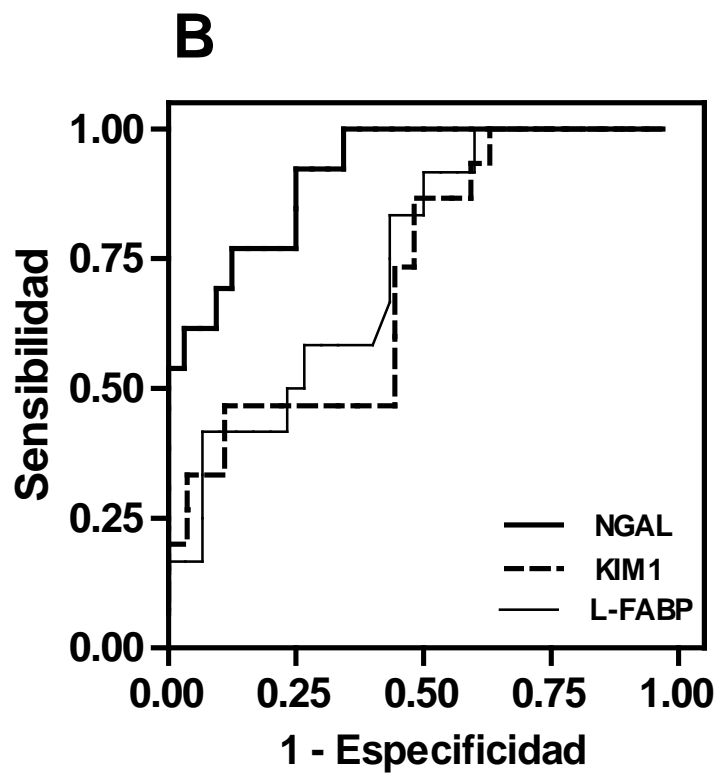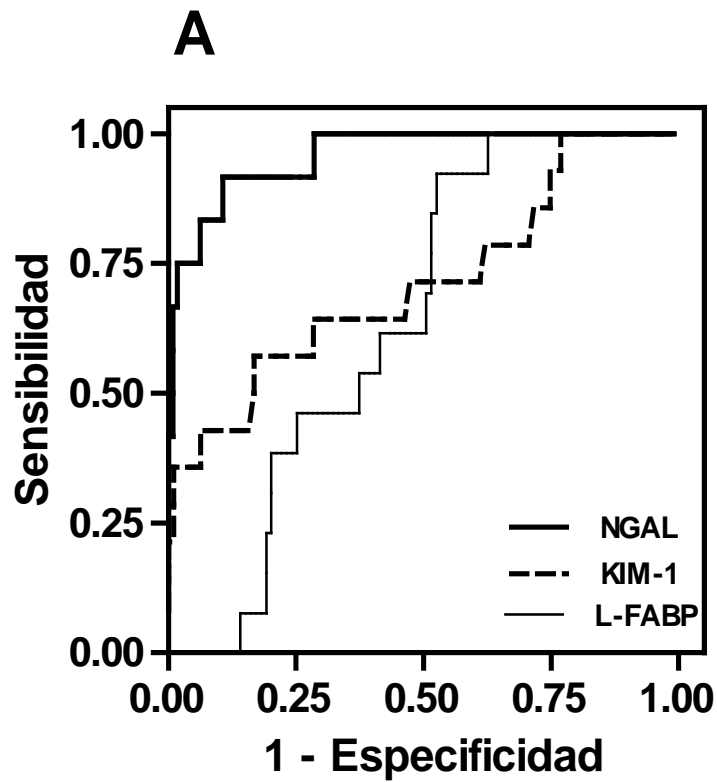
A: pacientes sometidos a angiografía coronaria (AUC para NGAL: 0.958; KIM-1: 0.713; L-FABP: 0.642); **B:** pacientes sometidos a cirugía cardiaca (AUC para NGAL: 0.916; KIM-1: 0.716; L-FABP: 0.743); ROC: Receiver Operating Characteristic; AUC: area under the curve; NGAL: Neutrophil Gelatinase-Associated Lipocalin; KIM-1: Kidney Injury Molecule-1; L-FABP: Liver-type Fatty Acid–Binding Protein.

Tabla 9. Capacidad diagnóstica de NGAL, KIM-1 y L-FABP para la detección del fracaso renal agudo en los diferentes grupos de pacientes

Grupo de estudio	NGAL		KIM-1		L-FABP	
	Punto de corte[a] (S-E)[b]	AUC (IC 95%)[c]	Punto de corte[a] (S-E)[b]	AUC (IC 95%)	Punto de corte[a] (S-E)[b]	AUC (IC 95%)
ANGIOGRAFIA	41.7 (89-92)	0.958 (0.909-1.007) p<0.0001	1.73 (71.6-64)	0.713 (0.551-0.876) p=0.01	20.7 (54-62)	0.642 (0.527-0.757) p=0.097
CIRUGÍA CARDIACA	35 (75-92)	0.916 (0.834-0.998) p<0.0001	2.11 (55-73)	0.716 (0.556-0.875) p=0.02	17.86 (56.7-83)	0.743 (0.588-0.898) p=0.015

[a] Los puntos de corte para cada marcador se expresan en ng/mg de creatinina en orina
[b] S-E: Sensibilidad (%) y Especificidad (%), respectivamente, en el punto de corte considerado. AUC: Área bajo la curva ROC.
[c] IC 95%: Intervalo de confianza del 95%.
NGAL: Neutrophil Gelatinase-Associated Lipocaline; KIM-1: Kidney Injury Molecule 1; L-FABP: Liver Fatty Acid-Binding Protein.

2.4. Correlaciones entre KIM-1, NGAL y L-FABP

Encontramos correlaciones significativas entre KIM-1 y NGAL en el grupo de angiografía (r = 0.221; p = 0.021) y en el de cirugía cardiaca (r = 0.454; p = 0.002). L-FABP sólo se correlacionó de manera significativa con NGAL en el grupo de cirugía cardiaca (r = 0.348; p = 0.019). No hubo correlación significativa entre KIM-1 y L-FABP.

3. Tercera fase del estudio. Klotho urinario determinado por ELISA como biomarcador precoz de fracaso renal agudo en pacientes sometidos a cirugía cardiaca o angiografía coronaria

3.1. Características de los pacientes

En la tabla 10 se muestran las características clínicas y demográficas de los pacientes. El diagnóstico de FRA por creatinina necesitó 3 ± 1 días en los pacientes de cirugía cardiaca y 4 ± 2 días en el de angiografía. Respecto a la mortalidad, ocho pacientes fallecieron, 4 de ellos del grupo de cirugía cardiaca y los otros 4 del grupo de angiografía; todos ellos habían desarrollado FRA. En el grupo de angiografía la creatinina sérica pre-intervención era significativamente mayor ($p < 0,05$) en los pacientes que desarrollaron FRA que en los que no lo desarrollaron, mientras que el filtrado glomerular estimado basal (eGFR) era significativamente menor en los pacientes con FRA ($p < 0,01$). No se encontraron diferencias significativas en estos valores en el grupo de pacientes sometidos a cirugía cardiaca (tabla 10). En este grupo, los pacientes que desarrollaron FRA habían estado sometidos a mayor tiempo de circulación extracorpórea (CEC) ($p < 0,05$) que los que no lo desarrollaron (tabla 10).

Tabla 10. Tercera fase del estudio. Características clínicas y demográficas de los pacientes sometidos a angiografía coronaria y cirugía cardiaca

ANGIOGRAFÍA CORONARIA	Pacientes sin FRA	Pacientes con FRA
Nº pacientes	12	18
Edad (años)	63 ± 15	72 ± 10
Sexo (M/F)	8/4	14/4
eGFR basal (MDRD) (ml/min/1.73m^2)	78 ± 15	60 ± 18**
Creatinina basal (mg/dl)	0.93 ± 0.23	1.16 ± 0.27*
Creatinina máxima (mg/dl)	0.94 ± 0.23	1.99 ± 0.61***
FRA (día)		4 ± 2
RIFLE (R/I/F)		12/5/1
Estancia hospital (días)	13 ± 11	17 ± 13
Muertes	0	4

CIRUGÍA CARDIACA	Pacientes sin FRA	Pacientes con FRA
Nº pacientes	15	15
Edad (años)	68 ± 9	67 ± 15
Sexo (M/F)	10/5	13/2
eGFR basal (MDRD) (ml/min/1.73m2)	60 ± 16	62 ± 29
Creatinina basal (mg/dl)	1.15 ± 0.37	1.18 ± 0.51
Creatinina máxima (mg/dl)	1.26 ± 0.35	2.14 ± 0.71***
FRA (día)		3 ± 1
RIFLE (R/I/F)		10/5/0
Estancia hospital (días)	17 ± 10	27 ± 24
Muertes	0	4
Tipo de cirugía:		
- By-pass	10	4
- Valvular	4	10
- By-pass y valvular	1	1
Tiempo en CEC (min)	62 ± 19	98 ± 57*

FRA: Fracaso Renal Agudo; eGFR basal (MDRD): filtrado glomerular estimado por MDRD antes de la intervención. FRA (día): día de diagnóstico del FRA por creatinina en suero. Tiempo en CEC: tiempo de circulación extracorpórea en minutos. Los valores se dan como media ± DE. Los valores de los pacientes con FRA que se diferencian significativamente de los pacientes sin FRA se expresan con *p<0.05; **p<0.01; ***p<0.001.

3.2. Valores de Klotho urinario

Los niveles de proteína Klotho en orina estaban significativamente elevados en los pacientes con FRA tanto en el grupo de angiografía como en el de cirugía cardiaca, cuando se comparan con el grupo control ($p < 0,05$) (tabla 11). Al corregir las concentraciones de Klotho por los niveles de creatinina en orina solo los pacientes con FRA del grupo de cirugía diferían de los controles ($p < 0,01$), pero no había diferencias significativas respecto a los pacientes sin FRA (tabla 11). Cuando se realizó la prueba de Spearman tampoco se halló correlación entre los valores de Klotho y la presencia de FRA ($r = 0,182$, $p = 0,67$). Además se estudió si había correlación entre el nivel de Klotho (ng/mg creatinina en orina) y el delta de creatinina sérica (creatinina máxima en suero – creatinina basal) para ambos ensayos ELISA, no encontrándose correlaciones significativas en ninguno de los grupos estudiados, independientemente del ensayo utilizado. No había correlación significativa entre los niveles de Klotho urinario (ng/ml) medido en ambos ensayos ELISA ($r = –0,079$; $p = 0,614$), ni tras corregir con la creatinina en orina ($r = –0,043$; $p = 0,792$).

Tabla 11. Valores de Klotho urinario utilizando dos kits ELISA comerciales

PARÁMETRO	CONTROL (n=10)	ANGIOGRAFÍA		CIRUGÍA CARDIACA	
		NO FRA (n=12)	FRA (n=18)	NO FRA (n=15)	FRA (n=15)
A. Klotho (ng/ml)	1.80± 0.04	1.87 ± 0.05	1.94 ± 0.05*	1.80± 0.08	1.97 ± 0.04*
Klotho (ng/mg creatinina)	1.37± 0.24	2.12 ± 0.38	2.40± 0.45	2.00± 0.23	2.51 ± 0.21**
B. Klotho (ng/ml)	0.77 ± 0.18	1.24 ± 0.34	1.51 ± 0.25	1.34 ± 0.36	1.25 ± 0.33
Klotho (ng/mg creatinina)	0.62 ± 0.05	1.23 ± 0.20	1.53 ± 0.30	1.25 ± 0.25	1.70± 0.30*

A: Kit ELISA Sun Red Biotechnologies. B: Kit ELISA IBL International. Los valores se expresan como media ± SEM. Los resultados se analizaron mediante ANOVA de un factor con análisis post-hoc de Bonferroni. Los valores en pacientes que son significativamente diferentes del grupo control se indican como: *$p<0.05$; **$p<0.01$. NO FRA: Pacientes que no desarrollaron fracaso renal agudo. FRA: Pacientes que desarrollaron fracaso renal agudo.

DISCUSIÓN

DISCUSIÓN

En este estudio se ha evaluado la utilidad de distintos biomarcadores precoces de fracaso renal agudo en pacientes con síndrome coronario o fallo cardiaco agudo a los que se les realizó una angiografía coronaria o una cirugía cardiaca y de entre todos ellos, NGAL en orina es el marcador que mayor utilidad ha mostrado.

El FRA sigue siendo actualmente una causa significativa de morbilidad y mortalidad en numerosos contextos clínicos para la que no se dispone de intervenciones efectivas[57,132] . Las causas por las que las medidas que han demostrado ser eficaces en los estudios animales no lo han sido en ensayos clínicos con humanos incluyen la complejidad de la patogénesis y la patobiología del FRA, la heterogeneidad de las poblaciones estudiadas, tanto en las causas del FRA como en las características y comorbilidades de los pacientes y el retraso en el inicio de las intervenciones[57,132]. Este retraso en las intervenciones es consecuencia de la imposibilidad de detectar precozmente el FRA utilizando la creatinina sérica como biomarcador y ha sido el motor fundamental de la búsqueda de nuevos biomarcadores precoces. Desde el primer momento el objetivo ha sido encontrar un biomarcador similar a la troponina cardiaca que permitiera diagnosticar el FRA de manera precoz y con una elevada sensibilidad y especificidad.

Podemos situar el inicio de la carrera por encontrar el biomarcador ideal en 2005, cuando Mishra y colaboradores, en un trabajo publicado en *Lancet*[71] demostraron, en una población de pacientes pediátricos sometidos a cirugía cardíaca con circulación extracorpórea, que la determinación de NGAL tanto en suero como en orina era útil para

detectar precozmente (en las primeras horas del postoperatorio) y con una altísima sensibilidad y especificidad, a aquellos pacientes que desarrollarían en los días posteriores un FRA.

Desde entonces numerosos grupos han publicado sus resultados en población pediátrica o adultos, tanto con NGAL como con otros biomarcadores, fundamentalmente IL- 18, cistatina-C, KIM-1 y L-FABP, aunque se han investigado muchos otros. Desafortunadamente la utilidad clínica de los nuevos biomarcadores sigue sin estar bien establecida, los múltiples trabajos realizados no han demostrado una capacidad predictiva comparable a la de la troponina y por tanto no se ha conseguido aún encontrar la "troponina renal". Existen diferencias fundamentales entre la patobiología del FRA y la del infarto agudo de miocardio y por tanto los biomarcadores del primero son necesariamente distintos de los del segundo. En contraste con el mecanismo relativamente sencillo del infarto de miocardio, el FRA no es una entidad simple sino un síndrome clínico que puede ser el resultado de múltiples insultos renales, actuando a distintos niveles y su origen es además, a menudo, multifactorial. Los nuevos biomarcadores renales son el reflejo de distintos procesos fisiopatológicos en el tejido lesionado mientras que el nivel de troponina en suero refleja la liberación de la proteína preformada desde los cardiomiocitos lesionados[65]. Esto explicaría por qué diferentes marcadores alcanzan su pico de concentración máxima en diferentes momentos. En la figura 22 se muestra la evolución de la concentración de los distintos biomarcadores en orina en el estudio TRIBE-AKI en pacientes adultos sometidos a cirugía cardiaca[65,72,73,99].

Figura 22. Cambios en la concentración de los biomarcadores de FRA a lo largo del tiempo tras la lesión renal
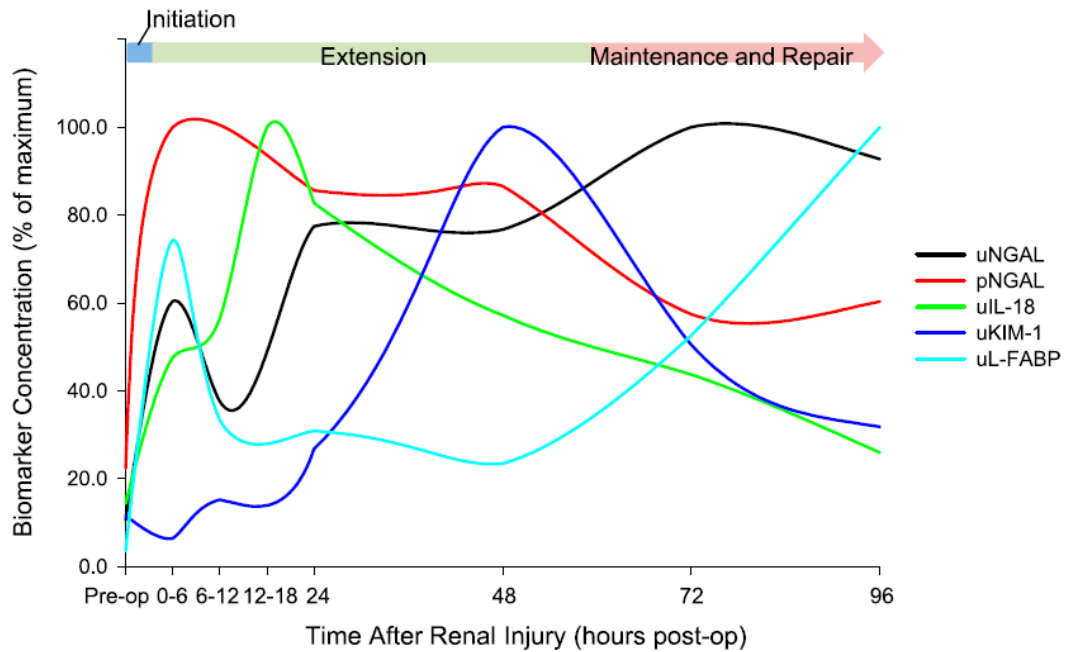
Alge et al. [65] han propuesto recientemente un modelo integrado de biomarcadores de FRA (Figura 23) en el que muestran como los nuevos biomarcadores son determinantes moleculares del curso del FRA enmarcándolos en el contexto de las distintas fases clínicas del FRA. En la primera fase, de iniciación, se produce el insulto original que desencadena una disminución del FG[22,23] (Figura 3). En la fase de extensión el FG continúa cayendo porque aumenta el daño renal por las alteraciones hemodinámicas y la inflamación, apoptosis y necrosis de las células epiteliales tubulares[22,23]. Es durante esta fase en la que el FRA pasa de leve a grave y cuando la IL-18, que exacerba el daño renal a través de sus efectos proinflamatorios, alcanza su máximo nivel[65,72,73]. A diferencia de IL-18, el aumento de los niveles de NGAL y l-FABP durante la fase de

extensión representa una respuesta al insulto renal, porque estos marcadores atenúan el daño renal mediante mecanismos antiapoptóticos y antoxidantes. La fase de mantenimiento se caracteriza por la estabilización del filtrado glomerular y la proliferación y migración de las células epiteliales supervivientes para reparar el epitelio dañado[22,23] y la de reparación implica el restablecimiento de la función celular normal. Estas dos fases no comienzan hasta 2 o 3 días tras el inicio del daño renal y se prolongan más allá de una semana[22,23]. La elevación mantenida de NGAL y L-FABP[65,72,73,99] sugiere que ambas podrían de alguna manera estar implicadas en la reparación del daño. Se ha demostrado que NGAL tiene efectos proliferativos en las células epiteliales tubulares[68,69]. De modo similar, KIM-1, que alcanza su máxima concentración a las 48 horas de inicio de la lesión, justo antes del inicio de la fase de mantenimiento[65,99] podría actuar favoreciendo la recuperación, porque aumenta la fagocitosis de los cuerpos apoptóticos y los restos celulares.

Figura 23. Modelo integrado de biomarcadores de FRA
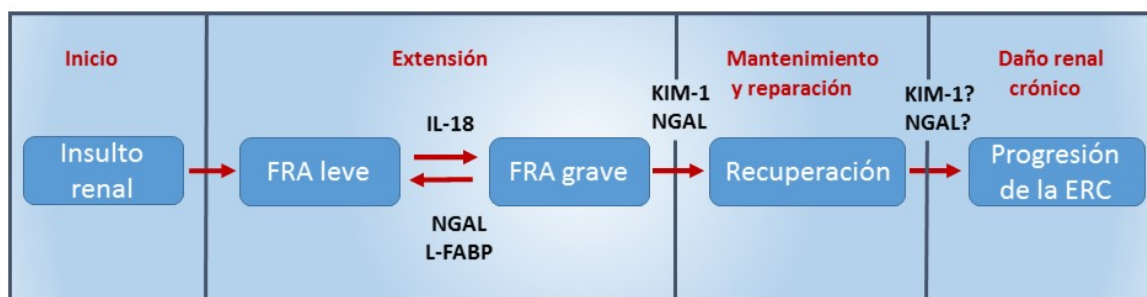
Modificado de Alge JL et al. Biomarkers of AKI: a review of mechanistic relevance and potential therapeutic implications. Clin J Am Soc Nephrol 2015; 10:147-155

El grupo más numeroso de trabajos sobre biomarcadores precoces de FRA se ha realizado en pacientes sometidos a cirugía cardiaca, pero también se han publicado estudios tras angiografía, shock séptico, trasplante renal y otras situaciones con FRA acompañante. Los resultados obtenidos son dispares aunque en adultos son inferiores a los publicados en población pediátrica. Probablemente la causa de los peores resultados obtenidos en adultos que en niños esté relacionada con la existencia de otras condiciones acompañantes. Así, se ha comunicado, por ejemplo, que los valores de NGAL son superiores en hipertensos[133] y en diabéticos[134] y además aumentan con la edad[135]. También la presencia de sepsis puede afectar a sus valores[136].

A pesar de los años transcurridos y los numerosos trabajos publicados, el diagnóstico precoz del FRA sigue sin estar resuelto y la búsqueda del biomarcador ideal prosigue. En el trabajo presentado hemos intentado ayudar a aclarar algunas de las cuestiones que quedan aún pendientes.

1. NGAL es superior a IL-18, cistatina-C, KIM-1 o L-FABP para el diagnóstico precoz del FRA tras angiografía coronaria o cirugía cardíaca en pacientes con síndrome coronario o fallo cardiaco agudo

En este estudio se ha evaluado la utilidad de la determinación de NGAL, cistatina-C, IL-18, KIM-1, L-FABP y Klotho en orina para la detección precoz (12 horas tras la intervención) del FRA en pacientes ingresados en la unidad de cuidados intensivos con síndrome coronario o fallo cardiaco agudo a los que se les realizó una angiografía coronaria con o sin angioplastia y/o implantación de endoprótesis o una cirugía cardiaca. El trabajo se desarrolló en tres fases, en la primera se evaluó NGAL e IL-18 en orina y cistatina-C en suero en una cohorte de 135 pacientes. En la segunda se seleccionó el

biomarcador que había mostrado mayor capacidad discriminativa en la primera (NGAL) y se comparó con KIM-1 y L-FABP. Para este estudio se añadió a la población inicial 58 pacientes más, hasta un total de 193. En la última fase, que se discute en el apartado siguiente, se seleccionó una parte de la cohorte para realizar un estudio preliminar sobre un posible nuevo biomarcador, la proteína Klotho en orina.

De los 144 pacientes del grupo de angiografía coronaria incluidos en total, 20 (13.9%) presentaron FRA. Este porcentaje se corresponde bien con los datos publicados en la literatura. De los 49 pacientes de cirugía cardíaca 15 (30.6%) presentaron FRA, una cifra también dentro de lo esperable.

Cuando se evaluó en la primera fase del estudio los biomarcadores NGAL, IL-18 y cistatina-C, NGAL en orina demostró ser útil para la detección precoz del FRA en ambos grupos de pacientes, mientras que cistatina-C en suero e IL-18 en orina mostraron ser moderadamente útiles tan sólo en el grupo de angiografía coronaria aunque con un AUC claramente inferior a la de NGAL.

En la segunda fase, KIM-1 urinario demostró ser un marcador útil para la detección temprana del FRA en ambos grupos de pacientes pero NGAL mostró una mayor sensibilidad y especificidad. L-FABP en orina mostró una menor capacidad de discriminación en el grupo de angiografía coronaria que en el de cirugía cardiaca.

De entre todos los biomarcadores de FRA que se están explorando, NGAL es el que probablemente más interés ha despertado. Desde la publicación original de Mishra et al. en 2005[71] en pacientes pediátricos, NGAL ha sido objeto de investigación en numerosos estudios y ha demostrado ser útil para la detección precoz del FRA tras la cirugía cardiaca, tanto en niños como en adultos[72,73,137-143], aunque los resultados obtenidos son inferiores en el segundo grupo. Como ya se ha dicho, probablemente la

causa de la menor especificidad observada en adultos se relaciona con la presencia de otras condiciones asociadas[133-136]. Se ha descrito así mismo, que la relación entre NGAL y FRA tras la cirugía cardiaca varía en función del filtrado glomerular basal, desapareciendo la capacidad discriminatoria de NGAL en pacientes con filtrado glomerular estimado (eGFR) por debajo 60 ml/min [142,144].

También se ha demostrado que NGAL es útil para el diagnóstico del FRA que aparece tras la angiografía coronaria[134,145-149] pero se han publicado menos trabajos en este campo y su poder predictivo, los puntos de corte y el momento óptimo para la recolección de las muestras no son conocidos.

En el presente estudio el AUC de NGAL en orina es superior a lo descrito en otros trabajos. En un metaanálisis publicado por Haase et al.[150] el AUC media fue 0.894 tras angiografía coronaria y 0.775 tras cirugía cardiaca, frente a los valores de 0.958 y 0.916 obtenidos en nuestro trabajo. En la gran serie de pacientes de Parikh et al.[73] que incluyó 1219 pacientes de cirugía cardiaca, destaca el valor del AUC para NGAL en orina obtenida entre 6 y 12 horas tras la cirugía, que era aún menor (0.7). La razón para este rendimiento mayor en el trabajo aquí presentado no es clara pero debe tenerse en cuenta que existen importantes diferencias en los valores del AUC entre los distintos estudios, que se justifican, al menos en parte, por diferencias metodológicas, como el momento de la recogida de la muestra o la normalización o no a creatinina urinaria, diferencias en las características de las poblaciones y la definición de FRA empleada. En el presente trabajo los resultados obtenidos para NGAL en orina en la primera y segunda fase tampoco son directamente comparables puesto que las poblaciones estudiadas no son las mismas y en la segunda los resultados de NGAL en orina se normalizaron para creatinina urinaria, mientras que en la primera no.

La IL-18 urinaria también ha sido ampliamente estudiada como posible marcador precoz de FRA en pacientes sometidos a cirugía cardiaca[72,73,138,151-156] y en menor medida a angiografía coronaria[146,157]. En 2013 se publicó un metaanálisis que incluyó 18 estudios en los que se había analizado la capacidad de IL-18 determinada en orina para predecir precozmente el FRA en distintos contextos clínicos, tanto en niños como en adultos[158]. El AUC fue 0.7 incluyendo el total de los pacientes. Considerando sólo el FRA tras cirugía cardiaca el AUC fue 0.72, mientras que el valor descendió a 0.66 para pacientes ingresados en la unidad de cuidados intensivos o la unidad coronaria. Tras estratificación por edad, la capacidad predictiva de IL-18 era mejor en niños que en adultos.

Los resultados obtenidos en el presente estudio son relativamente superponibles a los presentados en el metaanálisis (0.727 para los pacientes de angiografía coronaria y 0.676 para cirugía cardiaca), mostrando que IL-18 tiene un valor predictivo claramente inferior a NGAL para el diagnóstico precoz del FRA.

A diferencia de NGAL e IL-18, cistatina-C no es un marcador de daño renal sino de filtrado glomerular. En los últimos años se ha explorado su utilidad como posible sustituto de la creatinina para la determinación del filtrado glomerular, tanto en ERC como en FRA, por su menor dependencia de la masa muscular[91,93].

Se han publicado múltiples estudios en pacientes sometidos a cirugía cardíaca, tanto en suero[159-163] como en orina[144,153,164,165]. El valor de cistatina-C tras angiografía coronaria ha sido objeto de menos atención[147,166-168]. En 2011 se publicó una revisión sistemática sobre la capacidad predictiva de cistatina-C en orina o suero[169], en la que se incluyó 13 estudios en contextos clínicos muy diversos de FRA (pacientes críticos, trasplante hepático, cirugía cardiaca y otros), tanto niños como adultos. El AUC para el grupo total fue 0.87 (cistatina-C en suero) y 0.67 (orina). Tan sólo un estudio incluía pacientes tras

angiografía coronaria y se trataba además de pacientes con ERC[167]. El AUC para cistatina-C en suero en los estudios que incluían pacientes adultos sometidos a cirugía cardiaca era muy variable, desde 0.66 a 0.99, aunque las diferencias metodológicas y la definición de FRA también lo eran.

Recientemente se ha publicado un estudio en el que la definición de FRA basada en cistatina-C se asociaba de manera más intensa con IL-18 y KIM-1 en orina que la basada en creatinina en niños sometidos a cirugía cardiaca, por lo que proponen que en futuros estudios y en la práctica clínica se debería considerar definir el FRA por cistatina-C[170].

En el estudio aquí presentado, el AUC para cistatina-C fue 0,869 para el grupo de angiografía coronaria y 0,675 para el grupo de cirugía cardíaca, mostrándose por tanto cistatina-C útil para la predicción del FRA en el primero pero no así en el segundo. Como ya se ha comentado las diferencias a todos los niveles entre los distintos estudios hace muy difícil comparar los resultados.

Diversos estudios han evaluado los niveles de KIM-1 en orina en pacientes sometidos a cirugía cardiaca[99,153,154,171,172]. En la serie más grande publicada[99] el AUC para KIM-1 en orina fue 0.71 en las primeras 6 horas y 0.64 en las siguientes 6 horas, un resultado parecido al obtenido en este estudio, que fue 0.716. En un metaanálisis de 2014[173], en el que se incluía FRA en distintos contextos clínicos, el AUC media era 0.85. A diferencia de lo que ocurre con el FRA que aparece tras cirugía cardiaca, se dispone hasta el momento de muy escasa información sobre la utilidad de KIM-1 para el diagnóstico precoz del FRA tras angiografía coronaria.

En el presente estudio KIM-1 se mostró como un biomarcador moderadamente útil para la predicción del FRA que aparece tras angiografía coronaria, con un AUC de 0.713, similar a la obtenida tras cirugía cardiaca (AUC 0.716).

La utilidad de L-FABP para el diagnóstico precoz del FRA también ha sido investigada de manera extensa, tanto tras cirugía cardiaca[99,106,155,174,175] como tras angiografía [108,134,148,176-179]. En un metaanálisis publicado en 2013[180] la sensibilidad y especificidad estimadas de L-FABP para el diagnóstico del FRA en diferentes situaciones clínicas fueron 74.5 and 77.6 % respectivamente, siendo su rendimiento mejor en pacientes de cirugía cardiaca que tras angiografía coronaria. Los resultados aquí presentados muestran también una menor capacidad discriminativa para el FRA en el grupo de angiografía (AUC 0.642 para angiografía vs 0.743 para cirugía cardiaca).

Otro hallazgo importante es que los pacientes sin FRA de ambos grupos no diferían significativamente de los sujetos control respecto a NGAL ni KIM-1 en orina. Sin embargo, se encontró un aumento significativo de L-FABP en los pacientes que no desarrollaron FRA comparados con los valores obtenidos en los controles y los obtenidos antes de la intervención en el grupo de angiografía coronaria. Es probable que ese incremento en L-FABP en los pacientes sin FRA en el grupo de angiografía sea la causa de la menor utilidad del biomarcador en esta población. Por otro lado, teniendo en cuenta que la definición de FRA se basó en el incremento del valor de la creatinina sérica, podría suceder que L-FABP fuera más sensible para detectar FRA subclínico. Sería necesario realizar estudios histológicos para aclarar esta cuestión.

Por tanto, y a pesar de los esfuerzos realizados en esta materia y el gran número de estudios publicados durante los últimos años, la utilidad real de los diferentes biomarcadores precoces de FRA aún está por determinar. En la búsqueda del biomarcador ideal aún quedan importantes cuestiones por resolver: el mejor biomarcador (o combinación de biomarcadores), el punto de corte que ofrece mejor sensibilidad y especificidad para cada uno de ellos, los factores extrínsecos que pueden

influir en los resultados, la eficiencia en diferentes poblaciones y situaciones clínicas, la conveniencia de determinarlos en sangre o en orina, el mejor momento para obtener las muestras y su capacidad para diferenciar entre FRA funcional y estructural y para establecer un pronóstico además del diagnóstico. En este estudio se ha intentado contribuir a responder algunas de estas cuestiones.

Las muestras se recogieron 12 horas tras la intervención, porque se consideró que era un momento que facilitaba la recolección de las mismas en un contexto clínico normal. Así si la intervención de realizaba durante la tarde la muestra podía ser recogida a primera hora de la mañana, con el resto de las analíticas habituales.

Como puede apreciarse en la figura 22 en la actualidad sabemos que cada biomarcador tiene un comportamiento distinto, alcanzando su concentración máxima en orina entre pocas horas y varios días tras el insulto original. Sin embargo, si el objetivo es el diagnóstico (y la intervención) precoz, es necesario conocer cómo se comportan en un momento temprano de la evolución. Aunque cuando se inició el estudio no se disponía de dicha información, 12 horas es un momento en el que los biomarcadores que ascienden más tempranamente aún muestran niveles elevados y los que lo hacen más tardíamente ya han iniciado su ascenso (Figura 22).

No fue posible recoger muestras para la determinación de los biomarcadores antes de la intervención en todos los pacientes pero debe tenerse en cuenta que el objetivo último es disponer de un marcador fiable en una situación determinada, independientemente de sus valores basales y que en la práctica clínica, cuando se determinan marcadores como troponina o dímero D no se dispone de niveles basales.

Se incluyó la determinación de biomarcadores en una población de individuos sanos con función renal normal (NGAL en la primera fase del estudio y NGAL, KIM-1 y LFABP en la

segunda) para disponer de valores de referencia, así como en una parte de los pacientes del grupo de angiografía coronaria antes de la intervención y no se encontraron diferencias en los niveles de los biomarcadores entre estos dos grupos.

En la gran mayoría de los trabajos, los marcadores de daño tubular se han medido en orina. Sin embargo en el caso de NGAL no existe evidencia clara acerca de si es mejor determinarlos en suero o en orina. En este trabajo, dado que los marcadores se expresan en el FRA en el túbulo renal, se decidió determinarlos todos en orina. Como ya se ha dicho cistatina-C no es en realidad un biomarcador de daño tubular sino de filtrado glomerular y por tanto, a diferencia del resto, se midió en suero.

Si bien en la primera fase del estudio, al comparar NGAL, cistatina-C e IL-18 se utilizaron los resultados de los biomarcadores en orina expresados como concentración absoluta y no se normalizaron para la creatinina urinaria, en la segunda fase se decidió normalizarlos porque la evidencia disponible en ese momento así lo aconsejaba.

Ralib et al.[181] exploraron esta cuestión. Compararon la capacidad diagnóstica y pronóstica de tres métodos de cuantificar los biomarcadores: concentración absoluta, concentración normalizada a creatinina urinaria y ritmo de excreción del biomarcador en pacientes en UCI. La concentración absoluta se correlacionaba mejor con la presencia de FRA en el momento de la admisión pero las concentraciones normalizadas predecían mejor el desarrollo posterior de FRA, la necesidad de diálisis o la muerte. Los autores concluían que la normalización a creatinina urinaria mejoraba la predicción del FRA incipiente pero no ofrecía ventajas para diagnosticar el FRA establecido, por lo que el método ideal para cuantificar los biomarcadores en orina depende del objetivo buscado.

Otra cuestión que queda por dilucidar es la capacidad de los distintos biomarcadores para distinguir entre FRA funcional y estructural. Se ha comunicado que NGAL podría ser

capaz de diferenciar entre fracaso renal funcional e intrínseco o estructural[182]. En este estudio no se ha explorado esta cuestión pero dos trabajos previos muestran que incluso en casos de FRA transitorio pueden estar elevados los marcadores de daño tubular, sugiriendo que el FRA funcional y estructural pueden corresponder en realidad a fases evolutivas de un mismo proceso[183,184].

Un aspecto importante que no se ha explorado en este estudio es la posibilidad de combinar marcadores para aumentar la capacidad discriminativa. Parikh et al. obtuvieron mejores resultados combinando KIM-1 en orina recogida entre 6 y 12 horas tras la cirugía cardiaca con IL-18 en orina y NGAL en plasma a los dos días de la cirugía cardiaca[99]. Obviamente el precio de esta estrategia es una marcada disminución de la utilidad práctica en la clínica por la complejidad añadida y sería necesaria una combinación más sencilla de marcadores obtenidos además en la misma muestra (orina o suero) y en el mismo momento.

Para finalizar con esta primera parte de la discusión podemos decir que a pesar de los resultados dispares publicados en la literatura, especialmente en situaciones clínicas complejas como el FRA secundario a la sepsis que aparece en pacientes críticos, en el cual se encadenan múltiples insultos y se acompaña de una marcada reacción inflamatoria sistémica y a menudo de fallo multiorgánico, y en las que la capacidad discriminativa de los marcadores no es clara, los biomarcadores precoces podrían ser útiles en determinadas situaciones, especialmente en aquellas en las que el momento y la causa del daño renal son bien conocidos y el insulto es recortado en el tiempo, como es el caso del FRA que aparece tras la cirugía cardiaca o la angiografía coronaria. En este estudio NGAL en orina se mostró superior a los otros biomarcadores evaluados. El poder disponer de un diagnóstico temprano de FRA en estas poblaciones, antes de que la

creatinina sérica comience a subir, puede suponer una oportunidad valiosa para implementar medidas precoces y minimizar los riesgos añadidos que el FRA conlleva.

2. Klotho urinario determinado por ELISA no parece un biomarcador precoz prometedor de FRA tras angiografía coronaria o cirugía cardíaca en pacientes con síndrome coronario o fallo cardiaco agudo

En esta última parte del estudio pretendimos contribuir a la búsqueda de nuevos biomarcadores. Para ello evaluamos la utilidad de la determinación por ELISA de proteína Klotho en orina en una parte de los pacientes incluidos en los estudios anteriores y no encontramos diferencias significativas en los niveles de Klotho entre los pacientes que presentaron FRA y aquellos que no lo desarrollaron. Sin embargo sí encontramos un pequeño pero significativo aumento en los niveles de Klotho urinario en los pacientes con FRA tras cirugía cardiaca en comparación con los controles sanos. Hasta donde sabemos no se ha publicado hasta el momento ningún otro estudio en el que se haya probado el Klotho urinario como biomarcador precoz de FRA en humanos. Existe un único estudio en el que se midió Klotho en orina en 17 pacientes con FRA por inmunoblot usando anticuerpos anti-Klotho, y los autores comunicaron una disminución de Klotho en los pacientes con FRA en comparación con 14 controles sanos, pero las muestras de orina se recogieron tarde en la evolución del fracaso renal [125].

Se ha demostrado en modelos animales que el Klotho está infraexpresado en tejido renal en FRA secundario a isquemia-reperfusión, obstrucción ureteral, sepsis o agentes nefrotóxicos [122-129] y que la sobrexpresión de la proteína transmembrana o la administración de la proteína soluble tiene un efecto protector contra el daño renal inducido por isquemia y reperfusión [124,125] o nefrotoxinas[129].

En 2010 Hu et al.[125] midieron la expresión de Klotho en tejido renal así como los niveles de proteína Klotho en plasma y orina en un modelo animal de FRA inducido por isquemia y reperfusión, y encontraron que Klotho estaba disminuido a todos los niveles.

Basándonos en la evidencia obtenida en los modelos animales de FRA, esperábamos encontrar una disminución en los niveles de proteína Klotho en orina en los pacientes con FRA, pero no obtuvimos dicho resultado.

Hay varias explicaciones para estos resultados divergentes. En primer lugar, todos los experimentos han sido realizados en ratas y ratones. Es obvio que las condiciones en las que se desarrolla un experimento animal no son comparables con lo que sucede en un contexto clínico y que los resultados obtenidos no pueden ser directamente extrapolados. Más aún, es posible que la proteína Klotho no se comporte igual en humanos que en los modelos animales. Otra posible explicación radica en el momento de la recolección de la muestra. Hu et al.[125] encontraron niveles disminuidos de Klotho en orina un día tras la isquemia-reperfusión, pero en nuestro estudio las muestras se recogieron 12 horas tras la intervención. También midieron Klotho en orina de 17 pacientes con FRA y encontraron que los niveles eran muy inferiores a los obtenidos de voluntarios sanos, pero en este estudio la población con FRA era muy heterogénea (incluyendo FRA funcional y asociado a sepsis, lupus, obstrucción, fallo hepático, trasplante, síndrome hemolítico urémico y embarazo) y no se mostraban los datos acerca del momento de recogida de las muestras. De cualquier modo, cuando se obtuvieron estas, la creatinina sérica era 3,76 ± 0,58 mg/dl, por lo que fueron recogidas muy tardíamente. También es posible que los niveles de Klotho urinario no reflejen bien la expresión de Klotho a nivel del tejido renal. Sabemos que los niveles circulantes no se relacionan con la expresión en tejido renal en ERC. Los datos de experimentación animal

indican claramente que existe déficit de Klotho a nivel renal en la ERC [185]. Sin embargo, la determinación de la proteína Klotho en plasma ofrece resultados divergentes. Hu et al.[185] encontraron niveles muy bajos de Klotho en tejido renal, plasma y orina en ratones con ERC mientras que Sugiura et al.[186] hallaron niveles elevados en plasma obtenido de pacientes con ERC. Devaraj et al.[187] comunicaron niveles disminuidos en pacientes diabéticos y elevados en pacientes con ERC y además estos valores se correlacionaban con la creatinina plasmática. Propusieron la hipótesis de que la proteína Klotho se podría estar sintetizando a nivel extrarrenal para proveer protección renal por mecanismos anti-oxidantes y antiinflamatorios. Sin embargo Seiler et al.[121] determinaron niveles de Klotho plasmático en una cohorte de 312 pacientes con ERC estadio 2-4 y no encontraron correlación con el filtrado glomerular. Los niveles plasmáticos de Klotho no se relacionaban con la función renal y no predecían la evolución en pacientes con ERC.

Es posible que en los pacientes con FRA la proteína Klotho se esté sintetizando a nivel extrarrenal también para proveer protección renal y que, debido a la filtración glomerular, esté apareciendo en orina. Hu et al.[125] y Moreno et al.[127] encontraron niveles disminuidos de Klotho en plasma en modelos animales de FRA. Hasta ahora, no hay estudios en los que se haya determinado Klotho en plasma en humanos con FRA. Incluso aunque los niveles no estuvieran aumentados en plasma, pudiera ser que se estuviera sintetizando a nivel extrarrenal y pasando a la orina. Por tanto, los niveles en plasma u orina pueden no reflejar lo que está sucediendo a nivel tisular. Se requerirían más estudios para clarificar esta cuestión.

Otra posible explicación para los resultados es obviamente la existencia de diferencias metodológicas. Para dotar de mayor robustez a los resultados se utilizaron dos kits ELISA

humanos distintos, procedentes de distintos fabricantes para medir los niveles de Klotho urinario, no encontrando con ninguno de los dos kits diferencias entre los pacientes con y sin fracaso renal agudo. Destaca además la escasa concordancia obtenida entre ambos ensayos. Es posible que distintos ensayos reconozcan diferentes partes de la molécula y que algunos reconozcan la molécula entera y otros reconozcan fragmentos. Heijboer et al. [188] evaluaron tres ensayos ELISA distintos, entre ellos el de IBL, encontrando importantes diferencias en calidad entre ellos, siendo claramente superior el de IBL a los otros dos evaluados.

CONCLUSIONES

CONCLUSIONES

1. De entre todos los biomarcadores estudiados para el diagnóstico precoz del fracaso renal agudo en pacientes adultos con síndrome coronario o fallo cardiaco agudo y sometidos a angiografía coronaria o cirugía cardíaca, NGAL en orina a las 12 horas de la intervención es el que ha demostrado una mayor utilidad, con un capacidad predictiva superior a cistatina-C en suero e IL-18, KIM-1 y L-FABP en orina.

2. La heterogeneidad y complejidad del fracaso renal agudo hacen difícil -o quizá imposible- la obtención de un biomarcador que ofrezca una elevada sensibilidad y especificidad en todo tipo de poblaciones y contextos clínicos.

3. Los resultados obtenidos en los distintos trabajos que han evaluado la capacidad predictiva de los nuevos biomarcadores de fracaso renal agudo son dispares, probablemente en relación con diferencias en la población estudiada, metodológicas y en la definición de fracaso renal agudo empleada.

4. NGAL en orina ha mostrado en este estudio una capacidad predictiva para el diagnóstico del fracaso renal agudo en pacientes sometidos a angiografía coronaria o cirugía cardiaca superior a la descrita en la mayoría de los trabajos publicados. Esta mayor utilidad, comparable a la comunicada en los estudios que han obtenido mejores resultados, es atribuible a los factores citados en el punto 3.

5. Interleucina 18 en orina a las 12 horas de la intervención muestra una utilidad sólo moderada para el diagnóstico precoz del fracaso renal agudo tras angiografía coronaria pero no es predictiva de FRA tras la cirugía cardiaca en la población de pacientes estudiada.

6. Cistatina-C en suero a las 12 horas de la intervención también mostró ser útil para el diagnóstico precoz del fracaso renal agudo tras angiografía coronaria pero no tras cirugía cardiaca, en la población estudiada.

7. KIM-1 en orina a las 12 horas de la intervención se mostró como un biomarcador moderadamente útil para el diagnóstico precoz del fracaso renal agudo en pacientes adultos tanto en angiografía coronaria como en cirugía cardiaca.

8. L-FABP en orina a las 12 horas de la intervención fue un predictor de fracaso renal agudo moderadamente útil en cirugía cardiaca pero no tras angiografía coronaria.

9. Klotho en orina medido por ELISA no parece ser un buen candidato para ser usado como biomarcador precoz de fracaso renal agudo.

10. Se requieren más estudios para resolver importantes cuestiones sobre la utilidad clínica de los biomarcadores precoces de fracaso renal agudo en diferentes poblaciones y situaciones clínicas.

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ANEXO. PUBLICACIONES DERIVADAS DE ESTA TESIS

ANEXO. PUBLICACIONES DERIVADAS DE ESTA TESIS

1. Introducción a los artículos publicados

El primer artículo ***"Early biomarkers of acute kidney failure after heart angiography or heart surgery in patients with acute coronary syndrome or acute heart failure"*** es el resultado de la primera fase del estudio en la que se evaluó la utilidad para el diagnóstico precoz del FRA de NGAL e IL-18 en orina y cistatina-C en suero. Se publicó en la revista *Nefrología* en 2012.

En el segundo, ***"Urinary KIM-1, NGAL and L-FABP for the diagnosis of AKI in patients with acute coronary syndrome or heart failure undergoing coronary angiography"*** se muestran los resultados obtenidos en la segunda fase del estudio, en la que los marcadores investigados fueron NGAL, KIM-1 y L-FABP en orina. Se publicó *on line* en la revista *Heart and Vessels* en julio de 2014.

El tercero artículo, ***"Urinary Klotho measured by ELISA as an early biomarker of acute kidney injury in patients after cardiac surgery or coronary angiography"*** es la publicación correspondiente a la tercera parte del estudio y ha aparecido en la revista *Nefrología* en abril de 2015.

La última publicación, ***"Blocking NMDA Receptors Delays Death in Rats with Acute Liver Failure by Dual Protective Mechanisms in Kidney and Brain"***, apareció en la revista *Neuromolecular Medicine* en 2014 y se diferencia de los tres artículos anteriores en que el estudio presentado no deriva directamente de los datos obtenidos durante la elaboración de la tesis, sino que es el resultado de la aplicación de los conocimientos

adquiridos en la misma sobre la determinación de biomarcadores en fracaso renal agudo a un modelo de experimentación animal.

Se trata de un estudio diseñado con el objetivo de conocer los mecanismos por los que el bloqueo de los receptores NMDA puede retrasar o prevenir la muerte de ratas con fallo hepático agudo.

El fallo hepático agudo se indujo en los animales de experimentación con una inyección de galactosamina y los receptores NMDA se bloquearon mediante la administración continua de un antagonista de los receptores NMDA, el MK-801. El edema y el flujo sanguíneo cerebral se evaluaron por resonancia magnética. Se midió también la evolución temporal de los niveles de amonio en cerebro, músculo, sangre y orina, de los niveles de glutamina, lactato y agua en cerebro, del filtrado glomerular y de los biomarcadores de daño renal y de la encefalopatía hepática y la presión intracraneal.

El fallo hepático agudo disminuyó el filtrado glomerular tal y como se demostró por la reducción del aclaramiento de inulina. Esta disminución del filtrado glomerular se debió tanto a una disminución de la perfusión renal como a daño tubular, que se pudo apreciar por la elevación de KIM-1 en orina y en el análisis histológico de los riñones. El bloqueo de los receptores NMDA retrasó el daño renal permitiendo una menor disminución del filtrado glomerular y una mayor eliminación urinaria de amonio, lo que retrasó la hiperamonemia y los cambios cerebrales asociados. El bloqueo de los receptores NMDA no previno el edema cerebral o las alteraciones de la permeabilidad de la barrera hematoencefálica pero redujo o previno los cambios en el flujo sanguíneo y el lactato cerebral. Los datos mostraron que los efectos positivos duales que MK-801 ejerce sobre riñón y cerebro retrasan las alteraciones cerebrales, la encefalopatía hepática, la presión intracraneal y la muerte. Los antagonistas de los receptores NMDA podrían aumentar la

supervivencia de los pacientes con fallo hepático agudo al ofrecer un periodo de tiempo

adicional para el trasplante hepático o la regeneración.

2. "Early biomarkers of acute kidney failure after heart angiography or heart surgery in patients with acute coronary syndrome or acute heart failure". Torregrosa I, Montoliu C, Urios A, Elmlili N, Juan I, Puchades MJ, Solís MA, Sanjuán R, Blasco ML, Ramos C, Tomás P, Ribés J, Carratalá A, Miguel A. Nefrologia 2012; 32:44-52

Early biomarkers of acute kidney failure after heart angiography or heart surgery in patients with acute coronary syndrome or acute heart failure

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ABSTRACT

Background: Acute kidney injury (AKI) is a common complication in cardiac surgery and coronary angiography, which worsens patients' prognosis. The diagnosis is based on the increase in serum creatinine, which is delayed. It is necessary to identify and validate new biomarkers that allow for early and effective interventions. **Aims:** To assess the sensitivity and specificity of neutrophil gelatinase-associated lipocalin in urine (uNGAL), interleukin-18 (IL-18) in urine and cystatin C in serum for the early detection of AKI in patients with acute coronary syndrome or heart failure, and who underwent cardiac surgery or catheterization. **Methods:** The study included 135 patients admitted to the intensive care unit for acute coronary syndrome or heart failure due to coronary or valvular pathology and who underwent coronary angiography or cardiac bypass surgery or valvular replacement. The biomarkers were determined 12 hours after surgery and serum creatinine was monitored during the next six days for the diagnosis of AKI. **Results:** The area under the ROC curve (AUC) for NGAL was 0.983, and for cystatin C and IL-18 the AUCs were 0.869 and 0.727, respectively. At a cut-off of 31.9ng/ml for uNGAL the sensitivity was 100% and the specificity was 91%. **Conclusions:** uNGAL is an early marker of AKI in patients with acute coronary syndrome or heart failure and undergoing cardiac surgery and coronary angiography, with a higher predictive value than cystatin C or IL-18.

Keywords: Acute kidney injury. NGAL. Interleukin-18. Cystatin C. Cardiac surgery. Coronary angiography. Acute coronary syndrome. Heart failure.

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Biomarcadores precoces de fracaso renal agudo tras angiografía coronaria o cirugía cardíaca en pacientes con síndrome coronario o fallo cardíaco agudos

RESUMEN

Antecedentes: El fracaso renal agudo (FRA) es una complicación frecuente tras la cirugía cardíaca y la angiografía coronaria que ensombrece el pronóstico de estos pacientes. El diagnóstico se basa en el ascenso de la creatinina sérica, que es tardío. Es necesaria la identificación y validación de nuevos biomarcadores precoces que permitan intervenciones más tempranas y eficaces. Objetivos: Evaluar la sensibilidad y especificidad de interleuquina-18 (IL-18) en orina, neutrophil gelatinase-associated lipocalin en orina (uNGAL) y cistatina C en suero para la detección precoz del FRA en una población de pacientes con síndrome coronario agudo o fallo cardíaco y sometidos a cirugía cardíaca o cateterismo. Métodos: Se incluyeron en el estudio 135 pacientes ingresados en una unidad de cuidados intensivos por síndrome coronario agudo o fallo cardíaco por patología coronaria o valvular y a los que se realizaron una angiografía cardíaca o una cirugía cardíaca de revascularización o recambio valvular. Se determinaron los biomarcadores a las 12 horas de la intervención y se monitorizó la creatinina sérica durante los siguientes seis días para el diagnóstico del FRA. Resultados: Para NGAL se obtuvo un área bajo la curva ROC (AUC) de 0,983 y para cistatina C e IL-18 de 0,869 y 0,727, respectivamente. Para un punto de corte de NGAL en orina de 31,9 ng/ml la sensibilidad fue del 100% y la especificidad del 91%. Conclusiones: uNGAL es un marcador precoz de FRA en pacientes con síndrome coronario o fallo cardíaco agudo y sometidos a cirugía cardíaca y angiografía cardíaca, con una capacidad predictiva superior a cistatina o a IL-18.

Palabras clave: *Fracaso renal agudo. NGAL. Interleukina-18. Cistatina-C. Cirugía cardíaca. Angiografía coronaria. Síndrome coronario agudo. Fallo cardíaco.*

INTRODUCTION

Acute kidney injury (AKI) is a common complication following cardiovascular surgery, with varying incidence rates evidenced

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in the medical literature that depend both on the definition of AKI used and the population studied, but most estimates are close to 30%.[1-3] AKI prolongs the duration of hospital stays and increases the risk of mortality by three to nine times, depending on its severity.[2,3] Even small increases in creatinine levels worsen the situation for these patients.[4] The mechanisms that contribute to kidney injury include exogenous and endogenous toxins, metabolic factors, ischaemia and reperfusion, neurohumoral activation, inflammation, and oxidative stress.[5] The diagnosis of AKI is based on the detection of increased serum creatinine levels, which is delayed and does not properly show glomerular filtration rates in acute patients.[6] However, experimental studies have demonstrated that although AKI can be prevented or treated with several different therapies, it should be started immediately after the renal damage is produced.[7] The ineffectiveness of these interventions in clinical trials with humans has been attributed to significant delays in diagnosing AKI. As such, it is clear that in order to be effective, the treatment of AKI must start as early as possible.

The incidence of AKI following percutaneous coronary operations can be placed between 5% and 20%, according to the definition of AKI and the population studied, and constitutes a potentially serious complication.[8,9] Peak serum creatinine values tend to arise within the first 5 days following the surgery, and return to baseline values within 3 weeks (although permanent damage can be caused). As in AKI following heart surgery, increased serum creatinine values are late markers of kidney damage.

To try and resolve these problems, different research groups have attempted in recent years to identify new markers for the early diagnosis and stratification of the AKI risk. Despite the effort invested and the progress made, the actual usefulness of these markers has yet to be established in the different clinical contexts of AKI.[10,11] The most commonly studied markers are cystatin-C, which is not a marker of kidney damage but rather glomerular filtration rate, neutrophil gelatinase-associated lipocalin (NGAL), interleukin-18 (IL-18), kidney injury molecule-1 (KIM-1), and liver fatty acid-binding protein (L-FABP), in serum or urine samples.

The objective of this study is to evaluate the sensitivity and specificity of IL-18 in urine, NGAL in urine (uNGAL), and serum cystatin for the early detection of acute kidney injury in a population of patients with acute coronary syndrome or heart failure and undergoing heart surgery or catheterization.

MATERIAL AND METHOD

Study sample

Our study included 135 patients admitted between May 2008 and December 2009 to the intensive care unit of the Valencia University Clinical Hospital for acute coronary syndrome or heart failure due to coronary or valvular pathologies, and who underwent a heart angiography with or without angioplasty and/or an implanted endoprosthesis (group 1, n=89), or cardiac surgery involving revascularization or valve replacement (group 2, n=46). The exclusion criteria were: being younger than 18 years of age, pre-existing chronic kidney disease on renal replacement therapy with dialysis or transplant, and acute renal failure due to cardiogenic shock upon hospitalisation. We also excluded patients that had consecutively undergone both angiography and surgery in order to avoid confusion.

All patients were monitored prospectively starting at their inclusion in the study. We monitored creatinine levels from the day prior to the procedure to six days after, and followed the clinical evolution of each patient until they were discharged from the hospital. We also took serum and urine samples 12 hours after the procedure for assessing the biomarkers. Baseline creatinine levels were established as the value measured the day before the intervention.

Blood and urine samples were centrifuged for 10 minutes at 1500g. Several 0.5ml aliquots were taken from each serum and urine sample for storage at -80ºC for later analysis. We took urine samples from 20 healthy volunteers in order to obtain normal urine NGAL values.

Table 1. Clinical and demographic characteristics of patients that underwent coronary angiography

	Patients without AKI	Patients with AKI
No. patients	77	12
Age	61 (13)	73 (9)
Sex (M/F)	59/18	8/4
Previous eGFR (MDRD) (ml/min/1.73m²)	81 (24)	58 (18)[a]
Previous creatinine (mg/dl)	0.94 (0.22)	1.2 (0.3)[a]
Maximum creatinine (mg/dl)	1.0 (0.23)	2.03 (0.56)[a]
RIFLE (R/I/F)		6/2/1
AKI (day)		4 (3)
Deaths	3.9%	25%
Hospital stay (days)	10 (6)	21 (15)

AKI: acute kidney injury; previous eGFR (MDRD): estimated glomerular filtration rate using MDRD before intervention. AKI (day): day of the diagnosis of AKI using creatinine levels. Values are given as mean (SD). The values for patients with AKI that were significantly different from those of patients without AKI had P-values of [a] P<.01; [b] P<.001.

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The following information was also collected from each patient: demographic variables and comorbidities, parameters of the surgical procedure, and complications during or after the catheter placement or heart surgery (Table 1 and Table 2). The endpoint used for evaluating our patients was the appearance of AKI, defined as creatinine levels increasing by 50% or more, in keeping with the RIFLE (Risk, Injury, Failure, Loss, End-stage renal disease) classification system.[12] The secondary criteria were in-hospital mortality rates and duration of stays in the ICU and hospital.

Measurements of biomarkers

NGAL and IL-18 in urine

NGAL was measured from urine samples using ELISA kits (Human NGAL ELISA, Hycult biotechnology b.v.). The urine samples were diluted at 1:20 in a dilution buffer provided by the manufacturing laboratory, and 0.1ml aliquots were taken for analysis. The minimum detection level for this test is 0.4ng/ml.

We measured IL-18 levels from urine samples using ELISA kits (Human IL-18 ELISA, Bender MedSystems), and used 50μl of each urine sample for the analysis. The detection limit for this test was 9pg/ml.

Cystatin-C in serum

We measured cystatin-C in serum samples using a standardised immunonephelometry analysis using a BNII nephelometer (Siemens Healthcare Diagnostics).

Statistical analysis

We analysed the results using SPSS (version 17.0) and GraphPad PRISM (version 4.0) software. The Kolmogorov-Smirnov tests were used for verifying that the variables had a normal distribution. We compared the means of more than two variables using a one-way ANOVA test with post-hoc Bonferroni analysis, and used Student's t-tests for comparing two means, or Mann-Whitney U-tests in the case of non-normal distributions. We used Pearson's correlation coefficients to analyse the relationship between the different variables. The significance level was set at $P<.05$. We evaluated the sensitivity and specificity of each marker using ROC (Receiver Operating Characteristic) curves.

RESULTS

Clinical characteristics

The clinical and demographic characteristics of our study patients are summarised in Table 1 and Table 2. A total of 26 patients developed AKI, 12 of which were in the angiography group (13%, 12/89) and 14 were from the cardiac surgery group (30%, 14/46). Six patients that underwent catheterization died, three of which had developed AKI, and four patients died from the cardiac surgery group, all of which had AKI. The patients with AKI had a longer mean hospital stay than those that did not in both groups of patients.

In the group of patients that underwent catheterization, creatinine values prior to the procedure were significantly higher in patients with AKI ($P<.001$) (Table 1), and we did not observe any significant differences in the group of patients that underwent cardiac surgery.

NGAL values in urine samples from healthy controls and patients

We observed a significant difference in the values of NGAL from urine samples between patients that developed AKI and

Table 2. Clinical and demographic characteristics of patients that underwent cardiac surgery

	Patients without AKI	Patients with AKI
No. patients	32	14
Age	70 (9)	66 (15)
Sex (M/F)	22/10	12/2
Previous eGFR (MDRD)		
(ml/min/1.73m²)	70 (32)	64 (28)
Previous creatinine (mg/dl)	1.1 (0.4)	1.23 (0.5)
Maximum creatinine (mg/dl)	1.2 (0.4)	2.12 (0.7)ª
RIFLE (R/I/F)		9/5/0
AKI (day)		3 (1)
Deaths	0	29%
Type of surgery:		
- By-pass	19	4
- Valvular	13	9
- By-pass and valvular	1	0
Time on ECC (min)	65 (25)	91 (59)
Hospital stay (days)	14 (8)	30 (24)

AKI: acute kidney injury; previous eGFR (MDRD): estimated glomerular filtration rate using MDRD before intervention. ECC: time on extracorporeal circulation (minutes). AKI (day): day of the diagnosis of AKI using creatinine levels. Values are given as mean (SD). The values for patients with AKI that were significantly different from those of patients without AKI had a P-value of ª $P<.001$.

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those that did not, allowing us to clearly distinguish between the two groups (*P*<.001) (Figure 1).

The mean urine level of NGAL in the control group (healthy volunteers) was 18 (5) ng/ml. We observed a significant difference in NGAL (*P*<.001) between patients with AKI and the control group (catheters: 96 [24]; cardiac surgery: 129 [28]) (Figure 1A, Figure 1B, and Table 3).

The NGAL values in the group of patients without AKI were not significantly different from the control group (catheter: 17.4 [1.5]; cardiac surgery: 27.4 [3.7]) (Figure 1A, Figure 1B, and Table 3). Figure 1C shows how patients without AKI did not have significantly different urine NGAL values from the control group.

Cystatin-C serum values

The cystatin-C serum values were significantly different (*P*<.001) between AKI and non-AKI patients. In the catheter group, the mean value for patients with AKI was 1.22 (0.16) mg/dl, and 0.71 (0.02) mg/dl in patients without AKI. In the cardiac surgery group, the values for patients with and without AKI were 1 (0.1) mg/dl and 0.78 (0.04) mg/dl, respectively. The overall results were 1.1 (0.09) mg/dl and 0.7 (0.02) mg/dl in patients with and without AKI, respectively (Table 3).

Urine IL-18 values

In the case of the IL-18 inflammatory marker, AKI patients had significantly higher values than those without AKI (Table 3).

In both the catheter and cardiac surgery groups, we observed a significant difference between patients with and without AKI (*P*<.05). This significant difference was greater when we analysed all values together (*P*<.001).

Relationship between cystatin, IL-18, and NGAL

We tested for correlations between these parameters and observed a significant correlation between NGAL in urine samples and cystatin in serum samples (r=0.311; *P*=.001) and between NGAL and IL-18 in urine samples (r=0.448; *P*<.001).

ROC curves for NGAL, cystatin-C, and IL-18

In order to determine whether these markers are good predictors for the appearance of AKI in patients that undergo catheterization or cardiac revascularisation surgery, we

A: patients undergoing catheterization; B: patients that underwent cardiac surgery; C: graph corresponding to both study groups together (catheter + cardiac surgery). Values are given as mean (SD). Asterisks (***) indicate that the difference between the two groups is significant (*P*<.001). NGAL: Neutrophil Gelatinase-Associated Lipocalin.

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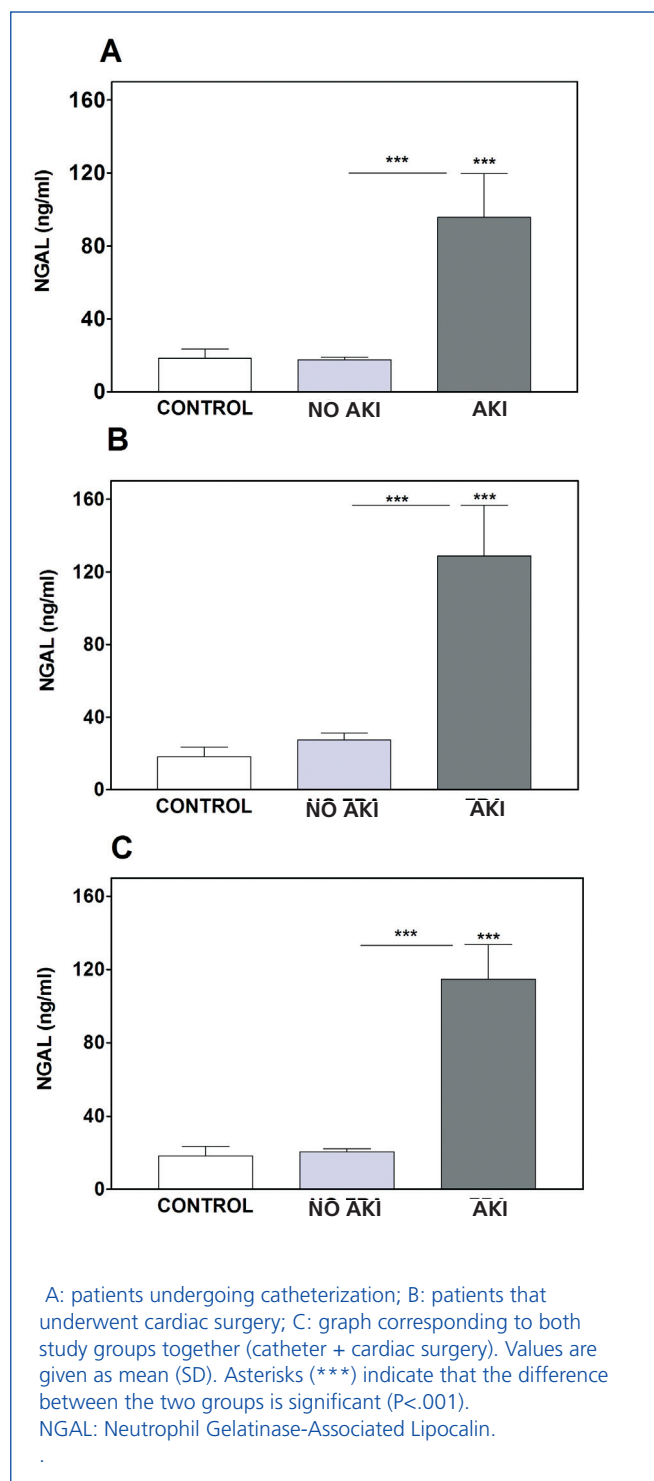**Figure 1.** Measurements of NGAL in urine samples from controls, patients without acute kidney injury (no AKI), and patients with acute kidney injury (AKI) in the different study groups

carried out a sensitivity/specificity analysis using ROC curves (Figure 2). Figure 2A shows the ROC curve of the three markers that were analysed in the catheter group. NGAL, cystatin-C, and IL-18 were good markers for AKI,

and NGAL was the best predictor, with an area under the curve (AUC) of 0.983, followed by cystatin-C (0.869) and IL-18 (0.727) (Table 4). For a cut-off point of urine NGAL at 31.9ng/ml, sensitivity was 100% and specificity was 91%. For cystatin-C, the cut-off point of 0.8mg/l in serum samples yielded a sensitivity of 89% and specificity of 76%. The cut-off point of 202pg/ml for IL-18 yielded a sensitivity of 67% and a specificity of 73%.

The ROC curve for patients that underwent cardiac surgery is displayed in Figure 2B. In these patients, although the incidence of AKI was higher than in the catheter group (Table 1 and Table 2), the ROC AUC were lower for all three markers (Table 4). NGAL continued to be the best predictor (AUC: 0.773) with sensitivity and specificity values of 64% and 80% respectively, considering 31.9ng/ml as the cut-off point for urine NGAL. Cystatin-C produced an AUC of 0.675, with sensitivity and specificity of 64% for the cut-off point of 0.8mg/l, and the ROC curve for IL-18 produced an AUC of 0.676, with the best cut-off point for urine IL-18 at 249pg/ml, with a sensitivity of 64% and specificity of 60% (Table 4).

Figure 2C shows the ROC curve for the three markers, considering the two groups of patients studied. NGAL produced an AUC of 0.881, and was capable of detecting 80% of AKI cases with a specificity of 86%, considering the cut-off point for urine NGAL to be 31.9ng/ml. Cystatin-C produced an AUC of 0.774, detecting 75% of AKI cases with a specificity of 71% for a cut-off point of 0.8mg/l of cystatin-C in serum samples. The cut-off point for IL-18 was 201pg/ml, with sensitivity and specificity values lower than the other markers (70% and 61%, respectively) and an AUC of 0.722 (Table 4).

DISCUSSION

In this study we evaluated the usefulness of NGAL and IL-18 in urine samples and cystatin-C in serum samples for the early (within 12 hours of the intervention) detection of AKI in a group of emergency patients in the ICU with acute coronary syndrome or heart failure, who underwent cardiac surgery or coronary angiography with or without angioplasty or endoprosthesis implantation with the following results:

uNGAL is useful for the early detection of AKI with an AUC of 0.881.

Cystatin in serum samples and IL-18 in urine samples also offer good options, although not quite as strong (AUC of 0.774 and 0.722, respectively).

Of the 46 patients that underwent cardiac surgery, 14 (30%) developed AKI, 9 of which had a status of R on the RIFLE classification system, and five of which were I. This 30% is

A: patients undergoing catheterization (AUC for NGAL: 0.983; cystatin: 0.869; IL-18: 0.727); B: patients that underwent cardiac surgery (AUC for NGAL: 0.773; cystatin: 0.675; IL-18: 0.676); C: ROC curve corresponding to both study groups together (catheter + cardiac surgery) (AUC for NGAL: 0.881; cystatin: 0.774; IL-18: 0.722).
ROC: Receiver Operating Characteristic; AUC: area under the curve; NGAL: Neutrophil Gelatinase-Associated Lipocalin.

Figure 2. ROC curves for the different markers analysed in each patient group

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Table 3. Values for the three markers analysed

	Catheter		Cardiac surgery		Catheterization + cardiac surgery	
	No AKI	**AKI**	**No AKI**	**AKI**	**No AKI**	**AKI**
NGAL (ng/ml)	17.4 (1.5)	95.8 (24)[b]	27.4 (3.7)	129 (28)[b]	20.5 (1.6)	115 (19)[b]
Cystatin-C (mg/ml)	0.71 (0.02)	1.22 (0.16)[b]	0.78 (0.04)	1.01 (0.12)[b]	0.73 (0.02)	1.10 (0.09)[b]
IL-18 (pg/ml)	196 (18)	292 (39)[a]	217 (20)	319 (38)[a]	203 (14)	305 (27)[b]

No AKI: patients that do not develop acute kidney injury; AKI: patients that do develop acute kidney injury. Cystatin-C was measured in serum samples; NGAL and IL-18 were measured in urine samples. Values are given as mean (SDM). The values for patients with AKI that were significantly different from those of patients without AKI had P-values of [a] P<.05; [b] P<.001.

in accordance with the data previously published on the subject. Of the 89 patients that underwent angiography, 12 (13%) developed AKI, six of which were stage R, two were I, and one was F. These results were also within expected ranges. The delay for AKI diagnosis using creatinine took 3 (1) days in the cardiac surgery group and 4 (3) days in the angiography group.

Of all the biomarker for AKI that are being studied, NGAL has probably inspired the greatest amount of interest. NGAL is a 25 kD protein that is covalently bound to neutrophil gelatinase. It is normally expressed in very low concentrations in several different tissues such as the kidneys, lungs, stomach, and colon, but is found at very high levels in kidneys with ischaemic or toxic damage.[13-15] It has been most heavily researched as a marker for AKI following cardiac surgery. In a study published in *The Lancet* in 2005,[16] Mishra et al carried out a study using paediatric patients that underwent cardiac surgery with extracorporeal circulation, and showed that NGAL is useful both in serum and urine samples for the early (in the first few hours following the

procedure) detection of patients that will develop AKI in the coming days, with extremely high sensitivity and specificity levels. The usefulness of NGAL (in blood or urine samples) for the early detection of AKI following cardiac surgery was later confirmed both in children[17,18] and adults,[19-24] although the results are more varied and are clearly worse in adults. In other studies, however, NGAL was predictive for AKI in urine samples, but not in serum,[25,26] and some studies have even produced negative results from using urine NGAL.[27] Probably, the reason for the lower specificity observed in adults than in children is related to the existence of other associated conditions. For instance, it has been shown that NGAL values are higher in hypertensive[28] and diabetic[29] patients, and that they also increase with age.[30] Sepsis can also affect NGAL values.[31] It has also been shown that the relationship between NGAL and AKI after cardiac surgery varies according to baseline glomerular filtration rate, and the predictive capacity of NGAL is lost in patients with an estimated glomerular filtration rate (eGFR) below 60ml/min.[32] Although the strength of the results are variable in both urine and blood tests, it appears that urine samples

Table 4. Diagnostic capacity of the markers analysed for the detection of acute kidney injury in the different study groups

	N-GAL		Cystatin C		IL-18	
Study group	**Cut-off point[a]**	**AUC**	**Cut-off point[a]**	**AUC**	**Cut-off point[a]**	**AUC**
	(S-s)[b]	**(95% CI)[c]**	**(S-s)[b]**	**(95% CI)**	**(S-s)[b]**	**(95% CI)**
Angiography	31.9	0.983	0.8	0.869	202	0.727
	(100-91)	(0.954-1.012)	(89-76)	(0.676-1.06)	(67-73)	(0.566-0.888)
Cardiac surgery	31.9	0.773	0.8	0.675	249	0.676
	(64-80)	(0.580-0.96)	(64-64)	(0.46-0.88)	(64-60)	(0.49-0.86)
Angiography + cardiac surgery	31.9	0.881	0.8	0.774	201	0.722
	(80-86)	(0.784-0.977)	(75-71)	(0.626-0.92)	(70-61)	(0.609-0.835)

[a] Cut-off points for each marker are expressed in ng/ml for NGAL, mg/l for cystatin-C, and pg/ml for IL-18.
[b] S-s: sensitivity (%) specificity (%), respectively, for the cut-off point indicated. AUC: area under the ROC curve.
[c] 95% CI: 95% confidence interval

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are generally superior.[25,26] The best time to obtain and analyse the samples has not been defined yet.

NGAL has also been shown to be useful for the early diagnosis of AKI following coronary angiography,[33-38] although fewer studies have been published in support, and the predictive power of this analysis, the cut-off point, and the optimal time for measurement are not well established.

In our study, the results from the overall group of patients were very positive for the measurement of uNGAL 12 hours after the procedure, with an AUC of 0.881. In the cardiac surgery group, AUC was 0.773, somewhat higher than the results published in other studies with adult cardiac surgery patients (0.62-0.72).[19-25] The AUC for patients that underwent coronary angiography was higher too (0.983). The cut-off point for better sensitivity and specificity in the overall group was 31.9ng/ml, with a sensitivity of 80% and a specificity of 86%.

Urine IL-18 has also been studied as a possible early marker for the detection of AKI in patients that undergo cardiac surgery, yielding both positive[39] and negative[40] results, as well as following coronary angiography,[36] although this measurement has inspired less interest as a possible biomarker for AKI.

In contrast to NGAL and IL-18, cystatin is not a marker of kidney damage, but rather glomerular filtration rate. Cystatin-C is a small endogenous protein that is freely filtered in the glomerulus, and is reabsorbed and catabolised in the proximal tubule, such that only a small quantity is excreted in the urine. Its usefulness as a possible substitute for creatinine as a method of measuring glomerular filtration rate has been examined in recent years, both in chronic and acute kidney disease, because of its lower dependence on muscle mass. However, its value does depend on other variables such as age, sex, race, and the presence of diabetes or inflammation.[41] The results using this measure are also variable in patients that undergo cardiac surgery, with positive,[21] modest,[42] and negative results,[26] although in this latter study, urine cystatin was capable of predicting the appearance of AKI, compared to serum samples. The published studies with cardiac catheters are also inconsistent, with varying and occasionally contrasting results.[35, 43-45] In our study, the AUC for cystatin was 0.774 in all patients, 0.869 in the coronary angiography group, and 0.675 in the cardiac surgery group. The results for IL-18 were quite similar (Table 4).

Therefore, the search for the ideal biological marker for the early detection of AKI still has the following important questions pending:

1. The best biomarker (or combination of biomarkers) and cut-off point that offers optimal sensitivity and specificity.

2. Whether blood or urine samples should be used, and the optimal moment for obtaining the sample.

3. Extrinsic factors that may influence the results and viability of the measurements in different clinical contexts.

4. The prognostic power in addition to the diagnostic capacity.

In this study, we have attempted to contribute a response to some of these questions. This study differs from those previously published by including both cardiac surgery and catheter patients, as well as the fact that all patients were admitted to intensive care units for coronary syndrome or acute heart failure, making them unstable patients. This is important because we need to know how these biomarkers work in large groups of the population with different characteristics, not just in homogeneous groups, and also because of the need to observe how other factors influence these measurements.

We took all of our samples 12 hours after the procedure. We were interested in testing the usefulness of samples at this time since it is the simplest option for collecting and processing samples in a normal clinical context (if the procedure takes place in the afternoon, the samples would be taken first thing the next morning).

We decided not to take samples for the biomarkers before the procedure, since the ultimate objective is to have a reliable marker in a specific situation, regardless of baseline values. For instance, when troponin or D-dimer are measured in clinical practice, it is without a baseline value for comparison. In any case, NGAL was measured in a population of healthy individuals with normal renal function in order to provide a reference value for comparison with the study groups.

The results from the markers in urine samples were not adjusted to urinary creatinine levels since, although some authors do defend this normalisation, the majority of studies have preferred not to do so because the evidence is not clear.

We preferred to measure NGAL in urine samples over serum samples since, although currently there are rapid methods for determining NGAL in blood samples with a good correlation with the results obtained in blood samples by ELISA,[16] the results from previously published studies show a better yield in urine samples.

In conclusion, urine NGAL taken 12 hours after the procedure is a good marker for the early detection of AKI in adult patients with acute heart failure or coronary syndrome that undergo cardiac surgery or coronary angiography, with a predictive power superior to cystatin and IL-18.

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Conflicts of interest

The authors declare they have no potential conflicts of interest related to the contents of this article.

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Urinary KIM-1, NGAL and L-FABP for the diagnosis of AKI in patients with acute coronary syndrome or heart failure undergoing coronary angiography

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ORIGINAL ARTICLE

Urinary KIM-1, NGAL and L-FABP for the diagnosis of AKI in patients with acute coronary syndrome or heart failure undergoing coronary angiography

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Abstract Acute kidney injury (AKI) is a common complication after coronary angiography. Early biomarkers of this disease are needed since increase in serum creatinine levels is a late marker. To assess the usefulness of urinary kidney injury molecule-1 (uKIM-1), neutrophil gelatinase-associated lipocalin (uNGAL) and liver-type fatty acid-binding protein (uL-FABP) for early detection of AKI in these patients, comparing their performance with another group of cardiac surgery patients. Biomarkers were measured in 193 patients, 12 h after intervention. In the ROC analysis, AUC for KIM-1, NGAL and L-FABP was 0.713, 0.958 and 0.642, respectively, in the coronary angiography group, and 0.716, 0.916 and 0.743 in the cardiac surgery group. Urinary KIM-1 12 h after intervention is predictive of AKI in adult patients undergoing coronary angiography, but NGAL shows higher sensitivity and specificity. L-FABP provides inferior discrimination for AKI than KIM-1 or NGAL in contrast to its performance after cardiac surgery. This is the first study showing the predictive capacity of KIM-1 for AKI after coronary angiography. Further studies are still needed to answer relevant questions about the clinical utility of biomarkers for AKI in different clinical settings.

Keywords Acute kidney injury · Biomarkers · Coronary angiography · Cardiac surgery · KIM-1 · NGAL · L-FABP

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Introduction

Acute kidney injury (AKI) is a common event after percutaneous coronary interventions. Its reported incidence varies between 5 and 20 %, depending on AKI definition and the studied population, and it constitutes a potentially serious complication [1–4]. Deterioration of renal function on the long term after percutaneous coronary intervention has also been associated with adverse cardiac events [5]. In patients with AKI, serum creatinine usually reaches its highest level in the first 5 days after the surgery, and it returns to its basal value (although a permanent lesion could remain) after 1–3 weeks.

AKI is also very common after cardiac surgery, with a reported incidence of nearly 30 % [6–9]. AKI prolongs hospital stay length and, depending on its severity, a three- to ninefold increase may be seen in mortality risk [7, 8].

Even low increases in serum creatinine levels worsen the prognosis of these patients [10]. As also seen in the AKI which may appear after coronary angiography, serum creatinine level increase is a late marker of kidney injury in these cases [11].

To solve this problem, different groups have tried to identify new markers in the last years in order to get an early diagnosis and risk stratification of AKI. Many studies have already reported the performance of early AKI biomarkers in cardiac surgery patients, but there is much less information regarding coronary angiography-related AKI. Beyond other different biomarkers, NGAL has probably deserved the greatest interest. NGAL is expressed in very low concentrations in several different tissues, but is found at very high levels in kidneys after ischaemic or toxic damage [12]. It has been most heavily researched as a marker for AKI following cardiac surgery [13–19], but also after coronary angiography [14, 18, 20–22]. Kidney injury molecule-1(KIM-1) is not expressed in normal kidney, but is upregulated in proximal tubular cells in renal cancer and after ischemic or nephrotoxic injury. It has been studied in patients undergoing cardiac surgery [23, 24], but not after coronary angiography. Liver-type fatty acid-binding protein (L-FABP) is expressed in the tubules. It has been tested as a predictor for AKI after cardiac surgery and coronary angiography [23, 25] with variable results. Baseline urinary L-FABP has shown to be a predictor of contrast-induced AKI in chronic kidney disease (CKD) patients [26]. Finally, measurement of urinary FABP levels 48 h after a coronary angiography has shown to predict 1-year renal outcome [27].

In spite of this extensive research, the real usefulness of different markers in several clinical settings remains to be established [28]. The aim of this work was to assess the usefulness of urinary determinations of KIM-1, NGAL and L-FABP for the early detection of AKI in patients with acute coronary syndrome or heart failure undergoing coronary angiography. We have also compared the results with data from another group of patients with acute coronary syndrome or heart failure who underwent cardiac surgery.

Materials and methods

Patients

Our study included 193 patients admitted to the Intensive Care Unit (ICU) of Hospital Clínico Universitario de Valencia between May 2008–December 2009 and January–June 2011. All patients had acute coronary syndrome or heart failure due to coronary or valvular pathologies and underwent coronary angiography with or without angioplasty and/or stenting (144 patients), cardiac by-pass surgery or heart valve replacement (49 patients). The exclusion criteria were: age below 18 years, pre-existing chronic kidney disease on renal replacement therapy (dialysis or transplant) and acute renal failure due to cardiogenic shock upon hospitalization. In order to avoid confusion, we also excluded patients that had consecutively undergone both angiography and surgery.

Before angiography, isotonic saline was infused for AKI prevention. The choice of the radio-contrast agent was made by the treating physician. The volume of contrast media was highly variable, as needed. All patients were prospectively monitored starting at their inclusion in the study. We monitored serum creatinine levels from the day prior to the procedure to 6 days after, and followed the clinical evolution of all patients until they were discharged from the hospital. The serum creatinine value measured the day before the intervention was established as the baseline creatinine level. Urine samples were collected 12 h after surgery for determination of biomarkers. Urine samples were also randomly collected before intervention in the group of patients with coronary angiography.

In order to obtain normal urine values for the different biomarkers, we also analyzed urine samples from 20 healthy volunteers. Demographic variables and co-morbidities, parameters of the surgical procedure and complications during or after the catheter placement or heart surgery were registered in all cases (Table 1). The endpoint used for evaluating our patients was the appearance of AKI, defined as an increase in serum creatinine beyond 50 % according to the RIFLE (risk, injury, failure, loss, end-stage renal disease) classification system [29]. This study was approved by the Clinical Studies Committee and Ethical Committee of Hospital Clínico Universitario de Valencia. All the subjects were thoroughly informed and gave their informed consent [30].

Processing of urine samples

Urine samples were centrifuged for 10 min at $1500 \times g$ and the supernatant was stored in 0.5 ml aliquots at -80 °C for subsequent analysis.

Measurements of biomarkers

KIM-1, L-FABP and NGAL were measured in urine samples using ELISA kits (Human NGAL ELISA and Human L-FABP HK404 ELISA, from Hycult Biotech; Human TIM-1/KIM-1/HAVCR from R&D Systems Europe Ltd. UK). For NGAL and L-FABP measurements, the urine samples were diluted at 1:20 in a dilution buffer

Table 1 Clinical and demographic characteristics of patients that underwent coronary angiography or cardiac surgery

Coronary angiography	Patients without AKI	Patients with AKI
No. patients	124	20
Age	62 ± 13	72 ± 10
Sex (M/F)	94/30	16/4
ACS/HF	118/6	19/1
Previous eGFR (MDRD) (ml/min/1.73 m^2)	84 ± 41	62 ± 18[a]
Previous creatinine (mg/dl)	0.94 ± 0.26	1.14 ± 0.27[b]
Maximum creatinine (mg/dl)	1.01 ± 0.28	1.91 ± 0.61[c]
RIFLE (R/I/F)		14/5/1
AKI (day)		4 ± 3
PCI	93	10
Deaths	4	4
Hospital stay (days)	10 ± 5	17 ± 12
Cardiac surgery		
No. patients	34	15
Age	69 ± 9	66 ± 14
Sex (M/F)	24/10	13/2
ACS/HF	18/16	5/10
Previous eGFR (MDRD) (ml/min/1.73 m^2)	70 ± 32	64 ± 28
Previous creatinine (mg/dl)	1.03 ± 0.31	1.18 ± 0.51
Maximum creatinine (mg/dl)	1.13 ± 0.33	2.14 ± 0.71[c]
RIFLE (R/I/F)		10/5/0
AKI (day)		3 ± 1
Deaths	0	4
Type of surgery		
By-pass	19	4
Valvular	13	9
By-pass and valvular	1	1
Time on ECC (min)	65 ± 25	91 ± 59
Hospital stay (days)	14 ± 8	30 ± 24

Values are given as mean ±SD. The values for patients with AKI that were significantly different from those of patients without AKI had a P value of [a] $p < 0.05$; [b] $p < 0.01$, [c] $p < 0.001$

AKI acute kidney injury, *ACS/HF* acute coronary syndrome/heart failure, *Previous eGFR (MDRD)* estimated glomerular filtration rate using MDRD before intervention, *AKI (day)* day of the diagnosis of AKI using creatinine levels, *PCI* percutaneous coronary intervention, *ECC* time on extracorporeal circulation (min)

provided by the manufacturing laboratory, and 0.1 ml aliquots were taken for analysis. The minimum detectable level for these tests was 0.4 ng/ml for NGAL and 102 pg/ml for L-FABP. For KIM-1 determinations, 50 µl aliquots of urine samples were taken for analysis. The minimum detectable dose was 0.009 ng/mL. To correct for urine dilution, values of biomarkers were adjusted for urinary creatinine. Serum and urinary creatinine levels were measured using standard techniques.

Statistical analysis

The results were analyzed using GraphPad PRISM (version 4.0) software. The Kolmogorov–Smirnov tests were used for verifying that the variables had a normal distribution. A one-way ANOVA test with post hoc Bonferroni analysis was used when comparing the means of more than two variables. Student's *t* tests were used for comparing two means as well as Mann–Whitney *U* tests in the case of non-normal distributions. The significance level was set at $p < 0.05$. Bivariate correlations among variables were evaluated using the Pearson correlation test. We evaluated the sensitivity and specificity of each marker using receiver operating characteristic (ROC) curves. Data were processed with the software package SPSS Version 17.0 (SPSS Inc., Chicago, USA).

Results

Patient characteristics

Table 1 shows the clinical and demographic characteristics of our patients. Thirty-five patients developed AKI, 20 in the angiography group (14 %) and 15 in the cardiac surgery group (30 %). AKI diagnosis as defined by serum creatinine level criteria could only be established after a mean of 3 ± 1 days in the cardiac surgery group and 4 ± 3 days in the angiography group. For both groups, patients with AKI had a longer mean hospital stay than those that did not develop AKI. In the angiography group, baseline serum creatinine values were significantly higher in patients with AKI ($P < 0.01$) (Table 1), while no significant differences were seen in the group of patients who underwent cardiac surgery. In the angiography group, 4 AKI patients and 4 NO AKI patients died. All deaths were due complications related to the heart disease, and were not directly related to AKI nor angiography.

Values of KIM-1, L-FABP and NGAL in urine samples from healthy controls and patients

Patients with AKI showed a significant increase in all biomarkers compared to patients without AKI and to control subjects (Fig. 1; Table 2). There was a significant difference ($p < 0.001$ for both angiography and cardiac surgery patients) in NGAL levels between patients developing AKI and those without AKI, which allows to distinguish both groups (Fig. 1a, b; Table 2). KIM-1 levels were also significantly increased in patients with AKI compared to patients without AKI ($p < 0.001$ for angiography, $p < 0.01$ for cardiac surgery) (Fig. 1c, d; Table 2), but L-FABP was able to discriminate between AKI vs NO

AKI patients only in the cardiac surgery group ($p < 0.01$) (Fig. 1e, f; Table 2).

Patients without AKI from both groups did not significantly differ from control subjects in urinary NGAL and KIM-1 (Fig. 1a–d; Table 2), but a significant increase in L-FABP in NO AKI patients was found when compared to both controls ($p < 0.01$ for angiography, $p < 0.05$ for cardiac surgery) and L-FABP levels before intervention in patients who underwent angiography (Fig. 1e, f; Table 2).

Patients who developed AKI after angiography showed increased levels of the three biomarkers when compared to their pre-angiography levels (Fig. 1a, c, e; Table 2).

ROC analysis

The analysis of sensitivity and specificity using ROC curves showed that urinary NGAL was the best predictor of AKI in both groups of patients, with an area under the ROC curve (AUC) of 0.958 (95 % CI 0.909–1.007; $p < 0.0001$) for the angiography group and an AUC of 0.916 (95 % CI 0.834–0.998; $p < 0.0001$) for the cardiac surgery group (Fig. 2a, b; Table 3).

KIM-1 was also a sensitive and specific predictor of AKI in both groups of patients, with an AUC of 0.713 (95 % CI 0.551–0.876; $p = 0.01$) for the angiography group and an AUC of 0.716 (95 % CI 0.556–0.875; $p = 0.02$) for the cardiac surgery group (Fig. 2a, b; Table 3).

L-FABP did not significantly predict AKI in the group of angiography (Table 3), but this marker was a significant predictor of AKI in the group of cardiac surgery (AUC: 0.743; 95 %CI 0.588–0.898; $p = 0.015$).

Correlations between KIM-1, NGAL and L-FABP

There were significant correlations between KIM-1 and NGAL in the angiography group ($r = 0.221$; $p = 0.021$) and in cardiac surgery patients ($r = 0.454$; $p = 0.002$).

L-FABP biomarker only correlated significantly with NGAL in the cardiac surgery group ($r = 0.348$; $p = 0.019$). There were no significant correlations between KIM-1 and L-FABP.

Discussion

In this study, we evaluated the usefulness of KIM-1, NGAL and L-FABP determinations in urine samples for the early (12 h following intervention) detection of AKI in a group of ICU patients with acute coronary syndrome or heart failure who underwent coronary angiography and compared their performance with another group of cardiac surgery patients. Urinary KIM-1 was useful for the early detection of AKI in both groups of patients but NGAL showed superior sensitivity and specificity. Urinary L-FABP provided inferior discrimination for AKI in patients undergoing coronary angiography but its utility increased in the cardiac surgery group.

NGAL has been extensively researched and it has been shown to be useful for the early detection of AKI following cardiac surgery both in children and adults [13–19], although results are more varied and were clearly worse in this second group. Probably, the reason for the lower specificity observed in adults is related to the existence of other associated conditions [21, 31–33]. It has also been shown that the relationship between NGAL and AKI after cardiac surgery varies according to baseline glomerular filtration rate [34]. The best time to obtain and analyze the samples has not been defined.

NGAL has also been shown to be useful for the early diagnosis of AKI following coronary angiography [14, 20–22], but fewer studies have been published in support of this idea and its predictive power, the cut-off point and the optimal time for measurement are not well established. We had previously tested NGAL and found that it was an accurate early biomarker of AKI for both cardiac surgery

Table 2 Values of markers studied corrected by urinary creatinine

Parameter	Control ($n = 20$)	Angiography			Cardiac surgery	
		Pre- angiography ($n = 28$)	No AKI ($n = 124$)	AKI ($n = 20$)	No AKI ($n = 34$)	AKI ($n = 15$)
NGAL (ng/mg creatinine)	11 ± 3	18.9 ± 3.3	19 ± 1.4	$105 \pm 22^{b,d,f}$	31.5 ± 4.5	$156 \pm 32^{b,d}$
KIM-1 (ng/mg creatinine)	0.9 ± 0.1	1.03 ± 0.15	1.5 ± 0.09	$4.7 \pm 1.7^{b,d,f}$	2.1 ± 0.2	$3.8 \pm 0.7^{a,d}$
L-FABP (ng/mg creatinine)	3 ± 0.4	15.5 ± 2.4	33.7 ± 4.3^c	$33.9 \pm 6^{d,f}$	17.3 ± 2^c	$33.9 \pm 8.2^{a,d}$

Values are given as mean $\pm$ SEM. The values for patients with AKI that were significantly different from those of patients without AKI had p values of [a] $p < 0.01$, [b] $p < 0.001$. Values for patients that are significantly different from control group are indicated by [c] $p < 0.05$, [d] $p < 0.001$. Values significantly different between pre- and post-angiography are indicated by [e] $p < 0.05$, [f] $p < 0.001$

NO AKI patients without acute kidney injury, *AKI* patients that developed acute kidney injury, *NGAL* neutrophil gelatinase-associated lipocalin; *KIM-1* kidney injury molecule-1; *L-FABP* liver fatty acid-binding protein
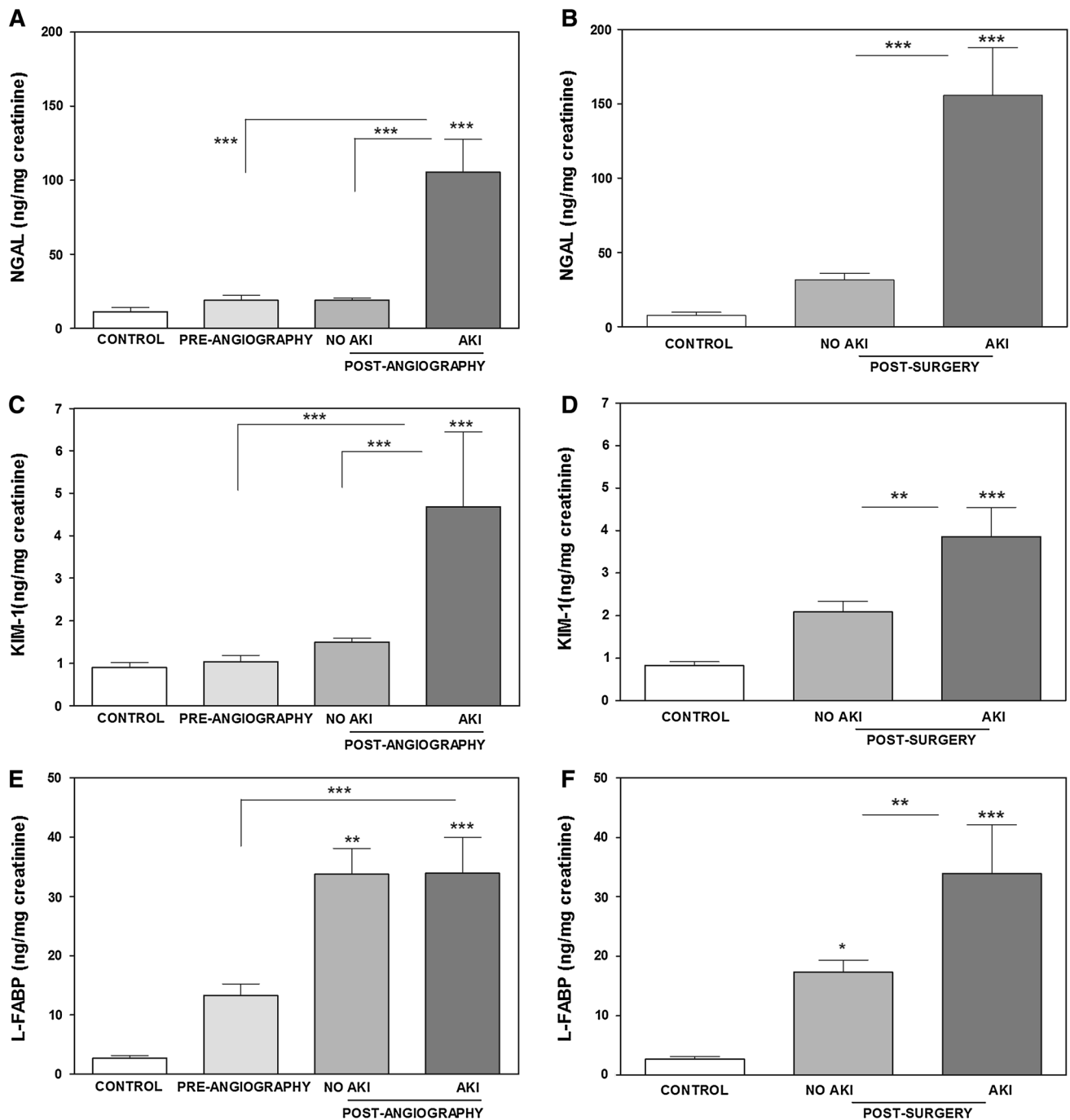
Fig. 1 Measurements of NGAL, KIM-1 and L-FABP in urine samples from controls, patients without acute kidney injury (NO AKI), and patients with acute kidney injury (AKI) of the different study groups. **a**, **c**, **e** Patients undergoing angiography; **b**, **d**, **f** patients that underwent cardiac surgery. Values of biomarkers are expressed as ng/mg of urinary creatinine and are given as mean ±SEM. Differences between groups are indicated by *asterisks* *$p < 0.05$;

$**p < 0.01$; $***p < 0.001$. *NGAL* neutrophil gelatinase-associated lipocalin, *KIM-1* kidney injury molecule-1, *L-FABP* liver-type fatty acid-binding protein. Pre-angiography: group of patients of angiography before the intervention. Post-angiography: group of patients undergoing angiography. Post-surgery: patients that underwent cardiac surgery

and coronary angiography patients, with a higher predictive value than cystatin C or Interleukin-18 [18]. In our study, the AUC of uNGAL is higher than in previous reports. In a meta-analysis by Haase et al. [14], the average AUC was

0.775 in cardiac surgery and 0.894 in coronary angiography patients. The reason for this better performance is not clear but there are also important differences in performance between studies, which are at least in part justified by

Fig. 2 ROC curves for the different markers analyzed in each patient group. **a** Patients undergoing angiography (AUC for NGAL: 0.958; KIM-1: 0.713; L-FABP: 0.642); **b** patients that underwent cardiac surgery (AUC for NGAL: 0.916; KIM-1: 0.716; L-FABP: 0.743). *ROC* receiver operating characteristic, *AUC* area under the curve, *NGAL* neutrophil gelatinase-associated lipocalin, *KIM-1* kidney injury molecule-1, *L-FABP* liver-type fatty acid-binding protein
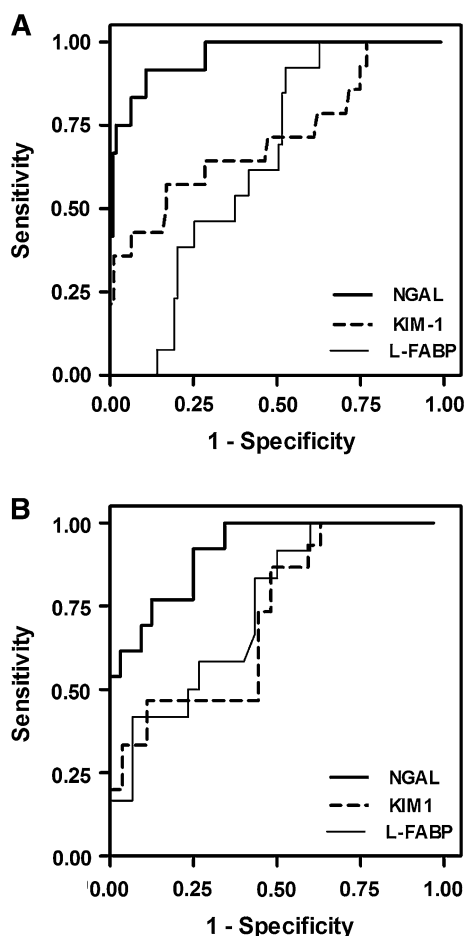
differences in population characteristics, AKI definition and sample collection methods.

Several studies have tested KIM-1 levels in patients undergoing cardiac surgery with variable results [23, 24]. In a meta-analysis published in 2014, the average AUC for cardiac surgery patients was 0.85 [24], while in our study was 0.716. We found KIM-1 to be moderately useful for the prediction of AKI after coronary angiography (AUC 0.713), similar to its performance after cardiac surgery. To the best of our knowledge, this is the first study about the predictive capacity of KIM-1 for AKI detection after coronary angiography in humans. The only previous study reporting the utility of KIM-1 for AKI detection was performed in rats [35].

The utility of L-FABP for the early diagnosis of AKI has also been extensively researched in these populations [23, 25] and the published results usually show less predictive power than the one seen with NGAL or KIM-1. In a meta-analysis published in 2013 [25], the estimated sensitivity and specificity of urinary L-FABP for the diagnosis of AKI in different clinical settings were 74.5 and 77.6 %, but its performance was better in cardiac surgery patients than in those undergoing coronary angiography. Anyway, there are few studies addressing the performance of L-FABP after coronary angiography. Our results also show inferior discrimination for AKI in the angiography group. Another interesting finding is that patients without AKI from both groups of patients did not significantly differ from control subjects in urinary NGAL and KIM-1. A significant increase in L-FABP in NO AKI patients was found when compared to controls and to L-FABP levels before intervention in patients who underwent angiography. We think that this increase in L-FABP in NO AKI patients in the angiography group is the cause of the poorer performance of the biomarker in this population. Together with this, and taking into account that the definition of AKI was established based in the serum creatinine level increase, it is possible that uL-FABP could be more sensitive in detecting subclinical AKI. It would be necessary to perform histological studies to address this issue.

Therefore, in spite of the efforts performed on this matter and the great number of related studies published in the last years, the real usefulness of different early biomarkers for AKI remains to be better established. In the search of the ideal biomarker for the early detection of AKI, several questions remain to be solved: the best biomarker (or combination of biomarkers), the cut-off point with better sensitivity and specificity, the extrinsic factors which can influence the results, the efficiency in different populations and clinical settings, the convenience of determining biomarkers in blood or urine, the best moment to obtain the samples and the ability to differentiate between functional and structural AKI.

In this study, we have tried to answer some of these questions. We consider that, in spite of the disappointing results in complex situations, such as those cases of AKI developed after sepsis, early biomarkers are useful in some clinical settings, especially those in which the time and the cause of renal injury are known. In our study, the efficiency of uNGAL for the prediction of AKI appearance outperformed KIM-1 and L-FABP.

The samples were collected 12 h after intervention, a time in which it was easy to collect and process samples in a normal clinical context. If the intervention was performed in the evening, the sample was collected at first time in the morning.

It was not possible to collect pre-intervention samples since basal levels are not usually available in clinical practice. Moreover, the aim of the study was to have a reliable marker in a given situation, regardless of baseline

Table 3 Diagnostic capacity of the markers analyzed for the detection of acute kidney injury in the different study groups

Study group	NGAL		KIM-1		L-FABP	
	Cut-off point[a] (S–s)[b]	AUC (95 % CI)[c]	Cut-off point[a] (S–s)[b]	AUC (95 % CI)[c]	Cut-off point[a] (S–s)[b]	AUC (95 % CI)[c]
Coronary angiography	**41.7** (89–92)	**0.958** (0.909–1.007) $p < 0.0001$	**1.73** (71.6–64)	**0.713** (0.551–0.876) $p = 0.01$	**20.7** (54–62)	**0.642** (0.527–0.757) $p = 0.097$
Cardiac surgery	**35** (75–92)	**0.916** (0.834–0.998) $p < 0.0001$	**2.11** (55–73)	**0.716** (0.556–0.875) $p = 0.02$	**17.86** (56.7–83)	**0.743** (0.588–0.898) $p = 0.015$

AUC area under the ROC curve, *NGAL* neutrophil gelatinase-associated lipocalin, *KIM-1* kidney injury molecule-1, *L-FABP* liver fatty acid-binding protein

[a] Cut-off points for each marker are expressed in ng/mg creatinine in urine

[b] *S–s* sensitivity (%) and specificity (%), respectively, for the cut-off point indicated

[c] *95 % CI* 95 % confidence interval

condition. We included the determination of the biomarkers in a population of healthy individuals with normal renal function, which were taken as reference values, as well as in a part of patients from the coronary angiography group before the intervention was performed, and no differences in the biomarkers levels were seen between these two groups.

Since the renal tubules express these markers, we chose to determine biomarkers in urine, although the evidence on whether serum or urine is better to determine them is again scarce. The results of the markers in urine have been normalized to urinary creatinine. Recently, Ralib et al. [36] have explored this question. They compared the diagnostic and prognostic performance of three methods of biomarker quantitation: absolute concentration, biomarker normalized to urinary creatinine concentration, and biomarker excretion rate in patients in the ICU. Absolute concentration best diagnosed AKI on admission, but normalized concentrations best predicted death, dialysis, or subsequent development of AKI. They concluded that normalization to urinary creatinine concentration improves the prediction of incipient AKI and outcome but provides no advantage in diagnosing established AKI, so the ideal method for quantitating biomarkers of urinary AKI depends on the outcome of interest.

Finally, it is unclear whether the biomarkers are able to discern between functional and structural AKI. It is reported that NGAL is able to differentiate between pre-renal and intrinsic renal failure [37]. We have not explored this aspect but two recent works [38, 39] show that, even in cases of transient AKI, tubular damage markers could be present, suggesting that functional and intrinsic AKI are probably gradations of an evolving spectrum of AKI.

Conclusion

Urinary KIM-1 12 h after intervention is predictive of AKI in adult patients undergoing coronary angiography, but NGAL shows higher sensitivity and specificity. L-FABP provides inferior discrimination for AKI than KIM-1 or NGAL, in contrast to its performance after cardiac surgery. This is the first study showing the predictive capacity of KIM-1 for AKI after coronary angiography. Further studies are still needed to answer relevant questions about the clinical utility of biomarkers for AKI in different clinical settings.

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Urinary Klotho measured by ELISA as an early biomarker of acute kidney injury in patients after cardiac surgery or coronary angiography

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ARTICLE INFORMATION

ABSTRACT

Background. Acute kidney injury (AKI) is a common complication after cardiac surgery and percutaneous coronary interventions which markedly worsens prognosis. In recent years, new early biomarkers of AKI have been identified, but many important aspects still remain to be solved. Klotho is a pleiotropic protein that acts as a paracrine and endocrine factor in multiple organs. Reduced renal Klotho levels have been show in several animal models of AKI. No study has been published in which Klotho was tested in humans as an early marker of AKI. The aim of this work is to assess the usefulness of measuring urinary Klotho for the early diagnosis of AKI in patients with acute coronary syndrome or heart failure undergoing cardiac surgery or coronary angiography. *Methods.* Urinary Klotho was measured 12 hours after intervention in 60 patients admitted to the Intensive Care Unit with acute coronary syndrome or heart failure secondary to coronary or valvular conditions, who underwent coronary angiography (30 patients) or cardiac bypass surgery or heart valve replacement (30 patients). The primary endpoint used was the onset of AKI according to the RIFLE classification system. Human Klotho levels were measured using an ELISA assay.

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Results. We found no differences in urinary Klotho levels between AKI patients and those who did not develop AKI. Moreover, there was not significant correlation between urinary Klotho levels and the presence of AKI. *Conclusion.* Urinary Klotho measured by ELISA does not seem to be a good candidate to be used as an early biomarker of AKI.

Klotho urinario determinado por ELISA como biomarcador precoz de fracaso renal agudo en pacientes sometidos a cirugía cardiaca o angiografía coronaria

R E S U M E N

Introducción y objetivos: El fracaso renal agudo (FRA) es una complicación frecuente tras la cirugía cardiaca y las intervenciones percutáneas coronarias cuya aparición empeora el pronóstico de manera marcada. En los últimos años se han identificado nuevos biomarcadores precoces de FRA, pero aún quedan muchos aspectos importantes por resolver. Klotho es una proteína pleiotrópica que actúa como un factor paracrino y endocrino en múltiples órganos. En diversos modelos animales de FRA se ha demostrado niveles disminuidos de Klotho renal. No se ha publicado ningún estudio en el que se haya probado Klotho como marcador precoz de FRA en humanos. El objetivo de este trabajo es investigar la utilidad de la determinación de Klotho en orina para el diagnóstico precoz del FRA en pacientes con síndrome coronario agudo o fallo cardiaco sometidos a cirugía cardiaca o angiografía coronaria.
Métodos: Se midió Klotho urinario 12 horas tras la intervención en 60 pacientes ingresados en la unidad de cuidados intensivos por síndrome coronario agudo o fallo cardiaco secundarios a enfermedad coronaria o valvular y a los que se realizó angiografía coronaria (30 pacientes) o cirugía cardiaca de recambio valvular o bypass (30 pacientes). El criterio de valoración primario fue la aparición de FRA según la clasificación RIFLE. Los niveles de Klotho humano se midieron utilizando un ensayo ELISA.
Resultados: No encontramos diferencias en los niveles de Klotho en orina entre los pacientes que desarrollaron FRA y aquellos que no. Además, no había correlación significativa entre niveles de klotho en orina y presencia de FRA.
Conclusión: Klotho urinario medido por ELISA no parece ser un buen candidato para ser usado como biomarcador precoz de FRA.

Introduction

Acute kidney injury (AKI) is a common complication following cardiac surgery and percutaneous coronary interventions, with an estimated incidence around 30% in the former[1-3] and between 5 and 20% in the latter[4-6]. The onset of AKI significantly worsens prognosis in these patients[2,3,7]. AKI increases mortality in different clinical contexts and, additionally, patients often develop chronic kidney disease (CKD) after AKI[8-9]. The mechanisms involved in AKI include both endogenous and exogenous toxins, metabolic factors, ischaemia and reperfusion, neurohumoral activation, inflammation and oxidative stress[10]. The diagnosis of AKI is based on detecting increased serum creatinine, which occurs late and does not adequately reflect the glomerular filtration in acute patients[11]. However, experimental studies show that AKI can only be prevented or treated by means of early interventions[12]. New early biomarkers of AKI, such as NGAL (Neutrophil Gelatinase-Associated Lipocaline), KIM-1 (Kidney Injury Molecule 1), L-FABP (Liver Fatty Acid-Binding Protein), Cystatin C or IL-18 (Interleukin 18), have been identified in recent years, but many important aspects remain to be solved in the search for the ideal biomarker[13]. The study of these molecules has also provided a greater understanding of AKI pathogenesis.

The Klotho gene was identified in 1997 as an anti-aging gene[14]. This gene is expressed in multiple tissues, the kidney being the organ where it is expressed more markedly,[15] especially in the distal tubule, but also in the proximal tubule[16] and the collecting duct[17,18]. Klotho is a transmembrane protein that acts as a co-receptor for fibroblast growth factor-23 (FGF-23)[19,20]. The ectodomain may be cut and released into the extracellular space by the proteases anchored to ADAM10 and ADAM17[21] membrane. This results in soluble Klotho,

which is present in blood, cerebrospinal fluid[22] and urine[23]. There is another soluble Klotho protein which is shorter, but its function is not well known[24]. Soluble Klotho is a pleiotropic protein that acts as a paracrine and endocrine hormonal factor, both in the kidney and in other organs[25]. In the renal tubule, Klotho modulates sodium-phosphate cotransporters[23], calcium channels[26] and potassium channels[27]. Finally, Klotho is also present at nuclear and cytoplasmic levels, where it functions as an anti-aging protein[28,29].

Klotho's role in aging and in phosphocalcic metabolism continues to become more understood[16,30,31]. Its implications in the progression of CKD and its non-renal complications have also been the focus of intense study in recent years[25,32-34], as well as the relationship existing among its circulating levels, glomerular filtration and CKD prognosis[35] or its potential use as an anti-fibrotic agent[36]. With respect to AKI, the presence of decreased Klotho levels has been shown in various animal models of AKI induced by ischaemia-reperfusion, ureteral obstruction or nephrotoxic agents[23,37-43]. In 2010, Hu et al.[23] measured urinary Klotho in 17 AKI patients and found decreased levels compared to the values obtained in 14 healthy volunteers. No other studies have been published about Klotho as an AKI biomarker in humans. The objective of this paper was to assess the usefulness of measuring urinary Klotho for the early detection of AKI in patients with acute coronary syndrome or heart failure undergoing cardiac surgery or coronary angiography.

Methods

Patients

Sixty patients were enrolled from a cohort of 193 patients admitted to the intensive care unit (ICU) of the Hospital Clínico Universitario de Valencia with acute coronary syndrome (ACS) or heart failure secondary to coronary or valvular disease. All patients had undergone coronary angiography with or without angioplasty or cardiac surgery. The coronary angiography group was made up of 30 patients: 18 had AKI after the intervention and 12 did not. Another 30 patients were also selected in the cardiac surgery group: 15 with AKI and 15 without AKI (Table 1).

The exclusion criteria were: age younger than 18 years; CKD on replacement therapy and AKI secondary to cardiogenic shock during hospitalisation. All patients were prospectively monitored since their enrolment in the study. Serum creatinine was measured from before the procedure up to six days after it, and the clinical progress of each patient was monitored until discharge. The first value of serum creatinine obtained on admission was used as the baseline creatinine value. Urine samples for Klotho measurement were collected 12 hours after the intervention and processed immediately thereafter. We also obtained urine samples from 10 healthy volunteers to determine the normal values. Additionally, the following information was collected from each patient: demographic variables and comorbidities, parameters of the surgical procedure and complications during or after interventions (Table 1). The primary endpoint was the onset

of AKI, defined as an increase of creatinine of 50% or more based on the RIFLE (Risk, Injury, Failure, Loss, End-stage renal disease) classification[44]. This study was approved by the Hospital Clínico Universitario de Valencia Ethics Committee and it was conducted in compliance with the principles of the Declaration of Helsinki[45].

Processing of urine samples

Urine samples were centrifuged for 10 minutes at 1500 g immediately after being collected, and the supernatant was stored in 0.5-mL aliquots at –80°C for later use.

Measurement of urinary Klotho levels

Human Klotho levels were determined in urine, both in patients and in controls, using two commercial ELISA assays: one from Shanghai Sunred Biological Technology Co., Ltd,

Table 1 – Clinical and demographic characteristics of patients		
	Non-AKI patients	AKI patients
Coronary angiography		
No. of patients	12	18
Age (years)	63 ± 15	72 ± 10
Sex (M/F)	8/4	14/4
Baseline eGFR (MDRD) (mL/min/1.73m²)	78 ± 15	60 ± 18**
Baseline creatinine (mg/dL)	0.93 ± 0.23	1.16 ± 0.27*
Maximum creatinine (mg/dL)	0.94 ± 0.23	1.99 ± 0.61***
AKI (day)		4 ± 2
RIFLE (R/I/F)		12/5/1
Hospital stay (days)	13 ± 11	17 ± 13
Deaths	0	4
CARDIAC SURGERY		
No. of patients	15	15
Age (years)	68 ± 9	67 ± 15
Sex (M/F)	10/5	13/2
Baseline eGFR (MDRD) (mL/min/1.73m2)	60 ± 16	62 ± 29
Baseline Creatinine (mg/dL)	1.15 ± 0.37	1.18 ± 0.51
Maximum creatinine (mg/dL)	1.26 ± 0.35	2.14 ± 0.71***
AKI (day)		3 ± 1
RIFLE (R/I/F)		10/5/0
Hospital stay (days)	17 ± 10	27 ± 24
Deaths	0	4
Type of surgery		
Bypass	10	4
Valvular	4	10
Bypass and valvular	1	1
Time on ECC (min)	62 ± 19	98 ± 57*

AKI: Acute kidney injury; baseline eGFR (MDRD): glomerular filtration rate estimated by MDRD before the intervention; AKI (day): day of AKI diagnosis by serum creatinine; Time on ECC: time of extracorporeal circulation in minutes; Values are expressed as mean ± SD; Values from AKI patients that differ significantly from non-AKI patients are expressed with *p < 0.05; **p < 0.01; ***p < 0.001.

Table 2 – Urinary Klotho values using two commercial ELISA kits						
Parameter	Control (n = 10)	Angiography		Cardiac surgery		Angiography + Cardiac surgery	
		Non-AKI (n = 12)	AKI (n = 18)	Non-AKI (n = 15)	AKI (n = 15)	Non-AKI (n = 27)	AKI (n = 33)
ELISA kit (Sun Red Biotechnologies)							
Klotho (ng/ml)	1.80 ± 0.04	1.87 ± 0.05	1.94 ± 0.05*	1.80 ± 0.08	1.97 ± 0.04*	1.85 ± 0.04	1.96 ± 0.03
Klotho (ng/mg creatinine)	1.37 ± 0.24	2.12 ± 0.38	2.40 ± 0.45	2.00 ± 0.23	2.51 ± 0.21**	2.04 ± 0.20	2.45 ± 0.26*
ELISA Kit (IBL International)							
Klotho (ng/ml)	0.77 ± 0.18	1.24 ± 0.34	1.51 ± 0.25	1.34 ± 0.36	1.25 ± 0.33	1.29 ± 0.25	1.38 ± 0.15
Klotho (ng/mg creatinine)	0.62 ± 0.05	1.23 ± 0.20	1.53 ± 0.30	1.25 ± 0.25	1.70 ± 0.30*	1.24 ± 0.30	1.60 ± 0.30

Values are expressed as mean ± SEM. The results are analysed by one-factor ANOVA with Bonferroni post-hoc analysis. Values from patients that are significantly different from the control group are indicated as: *$p < 0.05$; **$p < 0.01$; NON-AKI: Patients who did not develope acute kidney injury; AKI: Patients who developed acute kidney injury

with a sensitivity of 0.05 ng/mL and an assay range of 0.1–20 ng/mL, and the other assay from IBL International (Human soluble α-Klotho Assay Kit – IBL, Immuno-Biological Laboratories Co., Ltd), with a sensitivity of 0.006 ng/mL and an assay range of 0.093–6 ng/mL. The values obtained were adjusted to urine creatinine. Creatinine was measured in serum and urine using standard techniques.

Statistical analysis

We analysed our results with GraphPad PRISM (version 4.0) software. A Kolmogorov-Smirnov test was conducted to verify that the variables followed a normal distribution. For the comparison of means with more than two variables, a one-factor ANOVA was performed with a post-hoc Bonferroni analysis, and for the comparison of two means, a Student's or Mann-Whitney test was used in case of non-normal distribution. The correlations between the presence of AKI and urinary Klotho levels were conducted by Spearman's correlation. The correlations between the Klotho levels measured with the two ELISA assays, and between the Klotho level (ng/mg creatinine) and the serum delta creatinine (maximum serum creatinine – baseline creatinine), were conducted by Pearson's bivariate correlation, with the SPSS vs. 19 analysis program. The significance level was set at $p < 0.05$.

Results

Patients characteristics

The clinical and demographic characteristics of patients are shown in Table 1. The diagnosis of AKI using creatinine required 3 ± 1 days in cardiac surgery patients and 4 ± 2 days in angiography patients. With respect to mortality, eight patients died: four from the cardiac surgery group and the other four from the angiography group, all of whom had developed AKI. AKI patients had a mean hospital stay longer

than patients who did not develop AKI in both groups of patients, although the differences were non-significant. In the angiography group, pre-intervention serum creatinine was significantly higher ($p < 0.05$) in AKI patients than in non-AKI patients, whereas the baseline estimated glomerular filtration rate (eGFR) was significantly lower in AKI patients ($p < 0.01$). No significant differences were found in these values in the group of patients with cardiac surgery (Table 1). In this group, the patients who developed AKI had been on extracorporeal circulation (ECC) for a longer period ($p < 0.05$) than patients who did not develop AKI (Table 1).

Urinary Klotho values

Urinary Klotho protein levels were significantly elevated in AKI patients both in the angiography group and the cardiac surgery group, when compared to the control group ($p < 0.05$) (Table 2). When correcting Klotho concentrations based on urine creatinine levels, only the AKI patients from the surgery group differed from the controls ($p < 0.01$), but there were no significant differences with respect to non-AKI patients (Table 2). If the patients from both groups are considered together, urinary Klotho levels (corrected for creatinine) were significantly higher in AKI patients than in healthy controls ($p < 0.05$), but there were no significant differences compared to non-AKI patients (Table 2). A correlation was not found between Klotho levels and the presence of AKI ($r = 0.182$, $p = 0.67$) when the Spearman's test was conducted. Additionally, we examined whether there was a correlation between the Klotho level (ng/mg of urine creatinine) and delta serum creatinine (maximum serum creatinine – baseline creatinine) for both ELISA assays. No significant correlations were found when the patients from both groups were considered together or separately, regardless of the assay used.

There was no significant correlation between urinary Klotho levels (ng/mL) measured by the two ELISA assays ($r = -0.079$; $p = 0.614$) or after correction based on urine creatinine ($r = -0.043$; $p = 0.792$).

Discussion

In this study, we assessed the usefulness of measuring Klotho protein in urine samples by ELISA for the early (12 hours post-intervention) detection of AKI in a group of patients with acute coronary syndrome or heart failure who had undergone cardiac surgery or coronary angiography, either with or without angioplasty or stenting. We found no significant differences in Klotho levels between the patients who developed AKI and those who did not. We also found a small but significant increase in urinary Klotho levels in AKI patients following cardiac surgery compared to healthy control subjects. The Klotho protein was measured in urine with two different ELISA kits for human Klotho.

In the literature, there are no other studies testing urinary Klotho as an early biomarker of AKI in humans. There is only one study[23] in which urinary Klotho was measured in 17 AKI patients by immunoblotting using anti-Klotho antibodies, and the authors reported decreased Klotho in AKI patients compared to 14 healthy controls, although the urine samples were collected at a late stage of renal failure progression. It has been shown in animal models that Klotho is underexpressed in the renal tissue in AKI secondary to ischaemia-reperfusion, ureteral obstruction, sepsis or nephrotoxic agents[23,37-43], and that the overexpression of the transmembrane protein or the administration of soluble protein has a protective effect against the renal damage induced by ischaemia and reperfusion[23,28] or nephrotoxins[42]. In 2010, Hu et al.[23] measured Klotho expression in renal tissue as well as the levels of Klotho protein in plasma and urine in an animal model of ischaemia/reperfusion-induced AKI and found that Klotho was decreased at all levels.

We had already studied the usefulness of different biomarkers as early predictors of AKI in this cohort of patients and had shown that NGAL[46,47] was a strong predictor. Based on the evidence obtained in animal models of AKI, we expected to find decreased levels of Klotho protein in the urine of patients with AKI. There are several explanations for these dissimilar results. Firstly, all the experiments have been conducted in rats and mice. It is obvious that animal experiment conditions are not comparable to those in a clinical context and that the results obtained cannot be directly extrapolated. Moreover, it is possible that Klotho protein does not behave in the same manner in humans as in animal models. Another possible explanation lies in sample collection time. Hu et al.[23] found decreased Klotho levels in urine one day after ischaemia-reperfusion, but in our study the samples were collected 12 hours after the intervention. They also measured urinary Klotho in 17 patients with AKI and found that the levels were much lower than those obtained from healthy volunteers. However, the population in their study was very heterogeneous (including prerenal AKI and sepsis-induced AKI, lupus, obstruction, liver failure, transplantation, uremic haemolytic syndrome and pregnancy) and data about the sample collection time were not provided. Notwithstanding, when the samples were collected, serum creatinine was 3.76 ± 0.58 mg/dL, so they were collected very late. It is also possible that urinary Klotho levels

do not reflect Klotho expression well at renal tissue level. We know that the circulating levels do not relate to the expression in renal tissue in CKD. Data from animal experimentation clearly indicate the existence of Klotho deficit at renal level in CKD[48]. However, the measurement of Klotho protein in plasma provides dissimilar results. Hu et al.[48] found very low Klotho levels in renal tissue, plasma and urine in mice with CKD, whereas Sugiura et al.[49] found elevated levels in plasma from patients with CKD. Devaraj et al.[50] reported decreased levels in diabetic patients and high levels in CKD patients and, furthermore, those values correlated with plasma creatinine. They hypothesised that the Klotho protein could be synthesised at an extrarenal level to provide renal protection by anti-oxidant and anti-inflammatory mechanisms. Nevertheless, Seiler et al.[35] measured plasma Klotho levels in a cohort of 312 patients with Stage 2–4 CKD and did not find a correlation with glomerular filtration. Plasma Klotho levels did not correlate with renal function and did not predict progression in patients with CKD. It is possible that, in patients with AKI, the Klotho protein is also being synthesised at the extrarenal level to provide renal protection and that it is appearing in the urine due to the glomerular filtration. Hu et al.[23] and Moreno et al.[40] found decreased Klotho levels in plasma from animal models of AKI. So far, no study has measured Klotho in plasma from humans with AKI. Even if Klotho levels were not increased in plasma, it is possible that it could be synthesised at the extrarenal level and pass into urine. Therefore, plasma or urine levels may not reflect what is going on at tissue level. Further studies would be required to clarify this issue.

Obviously, another possible explanation for the results is the existence of methodological differences. To obtain more robust results, two different human ELISA kits from different manufacturers were used to measure urinary Klotho levels, but no differences were found in patients with or without acute kidney injury using either kit. The little consistency between both assays is also notable. It is possible that different assays recognise different parts of the molecule and that some assays recognise the entire molecule while others recognise fragments. Heijboer et al.[51] assessed three different ELISA assays, including the IBL one, and found major quality differences among them, IBL being clearly superior to the other two evaluated assays.

Conclusion

Despite the evidence in animal experimentation that Klotho is underexpressed in the kidney after either toxic or ischaemic AKI, in this study we did not find any correlation between the presence of AKI and urinary Klotho levels 12 hours after the intervention in a group of patients admitted to the ICU for acute coronary syndrome or heart failure who had undergone cardiac surgery or coronary angiography, either with or without angioplasty or stenting. Although these results need to be confirmed, urinary Klotho measured by ELISA does not seem to be a good candidate to be used as an early biomarker of AKI.

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5. *"Blocking NMDA Receptors Delays Death in Rats with Acute Liver Failure by Dual Protective Mechanisms in Kidney and Brain"* Cauli O, González-Usano A, Cabrera-Pastor A, Gimenez-Garzó C, López-Larrubia P, Ruiz-Saurí A, Hernández-Rabaza V, Duszczyk M, Malek M, Lazarewicz JW, Carratalá A, Urios A, Miguel A, Torregrosa I, Carda C, Montoliu C, Felipo V. Neuromolecular Medicine 2014; 16: 360-375

ORIGINAL PAPER

Blocking NMDA Receptors Delays Death in Rats with Acute Liver Failure by Dual Protective Mechanisms in Kidney and Brain

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Abstract Treatment of patients with acute liver failure (ALF) is unsatisfactory and mortality remains unacceptably high. Blocking NMDA receptors delays or prevents death of rats with ALF. The underlying mechanisms remain unclear. Clarifying these mechanisms will help to design more efficient treatments to increase patient's survival. The aim of this work was to shed light on the mechanisms by which blocking NMDA receptors delays rat's death in ALF. ALF was induced by galactosamine injection. NMDA receptors were blocked by continuous MK-801 administration. Edema and cerebral blood flow were assessed by magnetic resonance. The time course of ammonia levels in brain, muscle, blood, and urine; of glutamine, lactate, and water content in brain; of glomerular filtration rate and kidney damage; and of hepatic encephalopathy (HE) and intracranial pressure was assessed. ALF reduces kidney glomerular filtration rate (GFR) as reflected by reduced inulin clearance. GFR reduction is due to both reduced renal perfusion and kidney tubular damage as reflected by increased Kim-1 in urine and histological analysis. Blocking NMDA receptors delays kidney damage, allowing transient increased GFR and ammonia elimination which delays hyperammonemia and associated changes in brain. Blocking NMDA receptors does not prevent cerebral edema or blood–brain barrier permeability but reduces or prevents changes in cerebral blood flow and brain lactate. The data show that dual protective effects of MK-801 in kidney and brain delay cerebral alterations, HE, intracranial pressure increase and death. NMDA receptors antagonists may increase survival of patients with ALF by providing additional time for liver transplantation or regeneration.

Keywords Hyperammonemia · Acute liver failure · NMDA receptor · Intracranial pressure · Blood flow · Tubular injury · Glomerular filtration rate

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Introduction

Acute liver failure (ALF) leads to rapid progression of deleterious events including systemic inflammatory response, multi-organ (including renal) failure, hyperammonemia, cerebral edema, hepatic encephalopathy (HE), increased intracranial pressure (ICP), coma, and rapid death of patients. Many patients with ALF die of increased ICP and cerebral herniation.

The mechanisms responsible for HE, increased ICP, and death in ALF are still not well understood. Treatment of ALF is still unsatisfactory and mortality remains unacceptably high (Jalan 2005). Understanding these mechanisms would help to design new therapeutic strategies to improve survival of patients with ALF.

We have shown that during ALF in rats, the sequence of cerebral events leading to increased ICP and death involves an initial increase in blood–brain barrier (BBB) permeability and vasogenic edema. Ammonia and glutamine increase progressively leading in a second phase to cytotoxic edema and further increase ICP. Alterations in lactate and cerebral blood flow (CBF) are later events that further increase ICP (Cauli et al. 2011).

Inflammation plays an important role in the pathogenesis of increased ICP possibly through its effects on CBF (Jalan et al. 2004).

A main contributor to HE, ICP, and death in ALF is the increase in ammonia. Persistent arterial hyperammonemia and extracellular ammonia in brain correlate with ICP (Tofteng et al. 2006; Ytrebø et al. 2009). Arterial ammonia predicts brain herniation (Clemmesen et al. 1999) and patient's survival (Bernal et al. 2007; Bhatia et al. 2008).

Hyperammonemia contributes to the process leading to death by different mechanisms. Hyperammonemia may unlock the BBB (Jalan and Bernuau 2007) and induce cytotoxic edema (Butterworth 2003).

We showed many years ago that the main factor leading to death in acute intoxication with large doses of ammonia is activation of NMDA receptors in brain. Intraperitoneal injection of ammonia in rats leads to activation of NMDA receptors in cerebellum in vivo as assessed by microdialysis in freely moving rats. Only ammonia doses inducing neurological symptoms, coma, or death activate NMDA receptors, while lower doses did not (Hermenegildo et al. 2000). Moreover, we showed that ammonia-induced death of rats or mice is prevented nearly completely by ten different antagonists acting on three different sites of the NMDA receptor (Marcaida et al. 1992; Hermenegildo et al. 1996). This clearly indicates that ammonia-induced

activation of NMDA receptors in brain is mainly responsible for death in acute ammonia intoxication.

The mechanism by which ammonia induces activation of NMDA receptors would involve depolarization of neurons, or other cells expressing NMDA receptors (Hermenegildo et al. 2000). Under normal conditions, the ion channel of NMDA receptors is blocked by Mg^{2+} ions in a voltage-dependent manner. Depolarization of the neurons releases the blockade by Mg^{2+}, allowing increased activation of the receptors without increasing extracellular glutamate (Mayer et al. 1984). When ammonia reach concentrations enough to depolarize the cells, this would activate NMDA receptors.

In ALF, the increase in ammonia levels is lower and proceeds more slowly than in acute ammonia intoxication (Cauli et al. 2011). However, once certain threshold of ammonia is reached, NMDA receptors are also activated, contributing to the process leading to death.

In fact, blocking NMDA receptors with the NMDA receptor antagonists MK-801 or memantine delays death in strong ALF and increases survival in mild ALF in rats injected with different doses of the hepatotoxin galactosamine (Cauli et al. 2008).

However, the mechanisms by which blocking NMDA receptors affords protection against ALF remain unclear. We have shown that blocking NMDA receptors with MK-801 reduces the increases in ammonia and lactate in brain induced by acute ammonia intoxication in rats and prevents their death (Kosenko et al. 1994). As detailed in the discussion, activation of NMDA receptors modulates CBF in cerebral cortex, hippocampus, and cerebellum (Chi et al. 2003; Pelligrino et al. 1996; Weiss et al. 1996; Hara et al. 1996; Park et al. 1989). Therefore, some of the factors involved in ALF-induced death which could be modulated by activation or blocking of NMDA receptors are CBF and the increases in ammonia or lactate in brain.

The aim of this work was to shed light on the mechanisms by which blocking NMDA receptors delays death in ALF, including the possible effects on CBF and on the changes in ammonia and lactate in brain.

ALF was induced by injecting galactosamine (2.5 g/kg) as in Cauli et al. (2008). We assessed the effects of blocking NMDA receptors with MK-801 on the cerebral events leading to death, including changes in BBB permeability, CBF, vasogenic and cytotoxic edema, and the increases in ammonia, glutamine, lactate, and ICP.

One finding of these studies was that blocking NMDA receptors delays the increase in ammonia levels. We then assessed the effects of MK-801 on the time course of

hyperammonemia, on ammonia elimination, and on glomerular filtration rate and kidney function.

The results reported show that dual protective mechanisms in kidney and brain contribute to NMDA receptor antagonists-induced delay of death in ALF.

Methods

Drugs

MK-801 and D-galactosamine hydrochloride were from Sigma (St Louis, MO, USA).

MK-801 Administration

Male Wistar rats (220–270 g) were anesthetized with isoflurane and osmotic pumps (ALZET, model 2001) releasing 1 μL per hour during 7 days were implanted subcutaneously in the back. Two groups of rats were used: one with osmotic pumps filled with vehicle (0.9 % NaCl, 200 μL) and another with MK-801 (2 mg/mL) to keep NMDA receptors continuously blocked.

Animal experiments were approved by the Center and met the guidelines of the European Community for care and management of experimental animals.

Induction of ALF and Neurological Evaluation

ALF was induced by i.p. injection of galactosamine (2.5 g/kg) 3 days after osmotic pumps implantation. The neurological status was assessed every 60 min. The grades of HE were assigned as in Cauli et al. (2008). Different groups of rats were used for the following studies: (1) ADC and spectroscopy; (2) CBF; (3) ICP; (4) brain temperature; (5) GFR and kidney damage; (6) remaining parameters (ammonia, glutamine,…). The time to reach the different grades of HE was very similar but slightly different in different studies. By this reason, slightly different times are given in the results section for different parameters. For all parameters, except for ICP, no significant differences were observed between grades I and II or between grades III and IV of HE. So that, data were pooled and expressed as grade I/II and grade III/IV.

Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) experiments were performed on a Bruker Pharmascan system (Bruker Medical Gmbh, Ettlingen, Germany) using a 7.0-T horizontal-bore superconducting magnet as in Cauli et al. (2007). Animal temperature was maintained at approx. 37 °C with a heated probe.

Apparent Diffusion Coefficient

ADC maps were obtained from diffusion weighted images and calculated using the Image J program as in Cauli et al. (2007). The brain regions analyzed (Paxinos and Watson 1996) were as follows: entorhinal cortex, motor cortex, frontal cortex, dorsal hippocampus, medial-dorsal thalamus, ventral hypothalamus, dorsal striatum–caudate–putamen, ventral striatum–nucleus accumbens, globus pallidus, substantia nigra, and cerebellar hemisphere.

In Vivo ^{1}H-MR Spectroscopy

Lactate was measured in cerebellum and frontal cortex as in Cauli et al. (2011).

Determination of Water Content by Gravimetry

After decapitation, brain hemispheres were cut into coronal slices. Samples (approximately 2 mm^3) taken from frontal cortex and cerebellum were placed into a kerosene/bromobenzene gradient column, and the equilibration point was recorded within 2 min. Brain water content was calculated as in Marmarou et al. (1978).

Cerebral Blood Flow

CBF was analyzed by MRI as in Cauli et al. (2007) and calculated as in Wittlich et al. (1995).

Intracranial Pressure

A cannula (CMA, Stockholm, Sweden) was inserted in the right lateral ventricle 2 days before galactosamine administration, at the following coordinates (AP-0.9 mm, ML-1.3 mm, DV-4 mm). ICP was measured as in Cauli et al. (2011).

In Vivo Brain Microdialysis

Rats were anesthetized using isoflurane and a microdialysis guide was implanted in the cerebellum (anteroposterior −10.2, mediolateral −1.6, and dorsoventral −1.0; Paxinos and Watson 1996). After 48 h, microdialysis probes were introduced and perfused (3 μL/min) with the following (in mM): NaCl, 145; KCl, 3.0; CaCl$_2$, 2.26; buffered at pH 7.4 with 2 mM phosphate. After 2–3 h of stabilization, 30-min samples were collected.

Telemetric Measurements of Brain Temperature

Brain temperature was monitored in freely moving rats as described previously (Duszczyk et al. 2005) using a

telemetric system (VitalView, MiniMitter Co, Oregon, USA). Two days before galactosamine injection, the rats were anesthetized with halothane, and small burr holes were drilled 2.5 mm anterior and 2.5 mm lateral from midline. A brain temperature probe holder (socket) was inserted into the hole and fixed with dental cement. The probe was inserted into the striatum, with a tip of 3.0 mm ventral to the cortical surface, approximately to the same depth as the hippocampus. Rats were placed in Plexiglas boxes resting on telemetry receivers. Temperature signals were sampled every 30 s during 105 h.

Ammonia and Glutamine Determination

Ammonia and glutamine were measured in cerebral cortex, cerebellum, muscle, microdialysis samples, blood, and urine at different grades of HE (0, I–II, III–IV). Rats were decapitated, and cerebral cortex, cerebellum, and muscle (gastrocnemius) were quickly freeze-clamped into liquid nitrogen. The tissue was powdered and deproteinized in perchloric acid–ethanol, and ammonia was measured in the supernatant (Cauli et al. 2008). Blood (150 μL) and urine (1:200 in water) were also deproteinized in perchloric acid–ethanol.

Glutamine was determined in the same supernatants by HPLC as in Cauli et al. (2008).

Urine Collection

Rats were placed in metabolic cages with free access to water and food 48 h before galactosamine injection. Thymol (5 %, 1 mL) was added as preservative. Urine was collected for 24 h before galactosamine injection (grade 0). For grades I + II of HE, urine was collected during the first 24 h after galactosamine injection. For grades III + IV of HE, urine was collected from the beginning of grade III until death (14 ± 6 h in controls and 25 ± 12 h in rats with MK-801).

Determination of Urea, Creatinine, Ions, and Transaminases

Serum was obtained from tail vein. Urea, creatinine, sodium, potassium, bicarbonate, and transaminases (GOT and GPT) were determined with a Kodak Ektachem 700 autoanalyzer (Orthoclinical Diagnostics, Rochester, NY, USA).

Blood–Brain Barrier Permeability

BBB permeability was assessed by Evans blue–albumin extravasation as in Cauli et al. (2011).

Determination of MMP-9 Content by Immunoblot

Cerebellum and cerebral cortex were homogenized and subjected to immunoblotting (Corbalán et al. 2002) using a 6 % polyacrylamide gel and anti-rat MMP-9 (1:1,000; Chemicon International). Images were captured and the intensities of the bands were measured using the Alpha-Imager 2200 program.

Glomerular Filtration Rate

Inulin clearance was analyzed as a measure of GFR. Inulin (26 mg/kg) was injected (i.v. in tail vein) and plasma samples were obtained at 5, 15, 30, 45, 60, and 120 min after injection. Samples were deproteinized with perchloric acid and inulin was determined by HPLC with UV detection and inulin clearance was calculated as dose/AUC, as described by Tseng et al. (2011).

Blood pressure was measured using an automatic blood pressure analyzer (NIPREM 564; Cibertec) attached to the rat's tail as in Pellicer et al. (2011). The mean of five data from each animal was considered to be the individual blood pressure value.

Kim-1 Determination

Kim-1 was measured in 50 μL of urine using an ELISA kit (Human TIM-1/KIM-1/HAVCR from R&D Systems Europe Ltd., UK).

Histological Analysis of Kidney Damage

Rats were anesthetized and transcardially perfused with 150 mL of saline, followed by 250 mL of 0.4 % paraformaldehyde in 0.1 M phosphate buffer pH 7.4. Kidneys were removed and immersed for 24 h at 4 °C in the same fixative. Kidneys were cut sagitally, divided into dorsal and ventral halves, and fixed in 10 % buffered formalin. Paraffin-embedded tissue sections of 5 μm were stained with hematoxylin–eosin for morphological evaluation. Changes in tubular epithelial cells, in glomeruli, and in the interstitium and vessels were evaluated, and kidney damage grade was quantified according to the scoring scale described by Duarte et al. (1997).

Statistical Analysis

The results are presented as mean ± standard error of mean (SEM). The data were analyzed by one-way ANOVA followed Dunnett T test. p values lower than 0.05 were considered statistically significant. Statistical analysis was performed using the Graph Pad Prism 4 software (Graph-Pad Software Inc., San Diego, CA, USA).

Results

MK-801 Delays the Progression of HE and Death

We first assessed whether MK-801 affords some protection against galactosamine-induced HE and death. Control rats injected with galactosamine showed HE grades I, II, III, and IV death at 12 ± 1, 20 ± 2, 25 ± 3, and 31 ± 4 h, respectively. MK-801 delayed the progression of HE and death. Rats treated with MK-801 showed HE grades I, II, III, and IV death at 15 ± 2, 26 ± 2, 37 ± 3, and 47 ± 4 h, respectively. MK-801 delayed ($p < 0.05$) the onset of grade III and grade IV death (Fig. 1a).

MK-801 Delays the Increase in ICP

As increased ICP is a main mediator in ALF-induced death, we then assessed whether blocking NMDA receptors delays the increase in ICP induced by galactosamine. Before galactosamine injection, ICP was similar in control
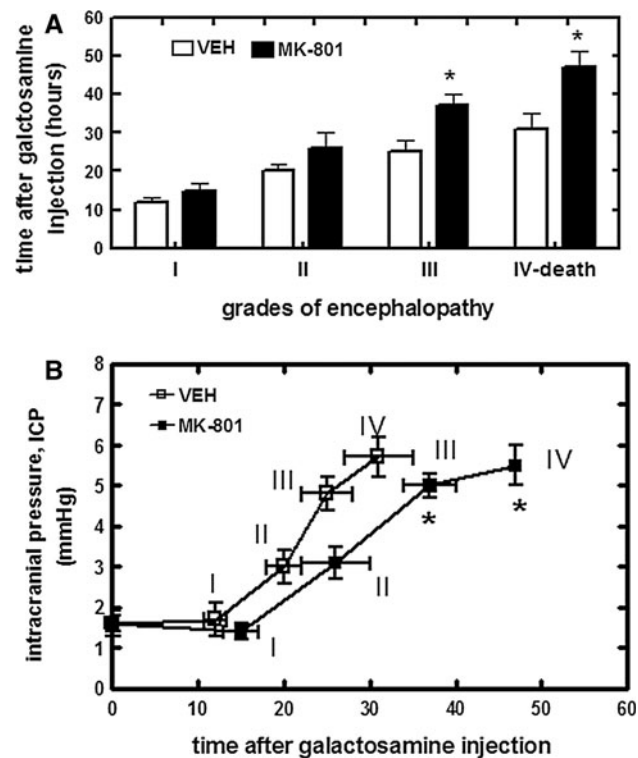

Fig. 1 MK-801 delays the progression of HE, death, and ICP induced by ALF. **a** The neurological status was examined every 60 min after galactosamine injection. The time at which each grade of encephalopathy (I–IV) occurs is shown. **b** ICP was determined in the lateral ventricle before and after galactosamine injection. Values are the mean $\pm$ SEM of 8–12 rats. Values significantly different from basal values (before galactosamine) are indicated by *asterisks*. Values significantly different in rats treated with MK-801 from controls are indicated by "a". *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; [a]$p < 0.05$

and MK-801-treated rats (1.6 ± 0.2 and 1.4 ± 0.3 mmHg, respectively).

MK-801 delays the increase in ICP (Fig. 1b). For example, in control rats ICP reaches 3.9 and 4.8 mm Hg at 20 and 25 h, respectively. In rats treated with MK-801, ICP reached 3.9 and 4.8 mm Hg later, at 26 and 37 h, respectively. 25 h after galactosamine injection, ICP was 4.8 ± 0.4 mm Hg in control rats and was lower (3.9 ± 0.4 mm Hg, $p < 0.05$) in rats with MK-801.

MK-801 Delays the Increase in Ammonia in Cerebral Cortex and in Extracellular Fluid in Cerebellum

As increased ammonia is a main mediator in ALF-induced increase in ICP and death, we then assessed whether blocking NMDA receptors delays the increase in brain ammonia induced by galactosamine. As some effects are different in cortex and cerebellum, we measured ammonia in both areas. In cerebral cortex, MK-801 delays the increase in ammonia induced by ALF. In control rats, ammonia reached 636 ± 57 nmol/g at 17 h. In rats with MK-801, ammonia remained lower (469 ± 55 nmol/g, $p < 0.05$) than in control rats at 20 h (Fig. 2a). In contrast, MK-801 did not affect the time course of ammonia in cerebellum (Fig. 2b) or of glutamine in cerebral cortex (Fig. 2c) or cerebellum (Fig. 2d).

As Ytrebø et al. (2009) showed that ICP correlates with extracellular ammonia, we assessed whether MK-801 affects it in cerebellum. MK-801 delays and reduces the increase in extracellular ammonia. In control rats, extracellular ammonia reached 414 ± 36 μM at 19 h. In rats with MK-801, ammonia remained lower (281 ± 37 μM, $p < 0.05$) than in control rats at 20 h and later on (Fig. 2e).

MK-801 Delays and Reduces the Increase in Blood Flow in Cerebral Cortex and Prevents its Decrease in Cerebellum

As changes in CBF contribute to increased ICP in ALF, we then assessed whether blocking NMDA receptors delays the changes in CBF induced by galactosamine. As CBF is modulated differently in cortex and cerebellum, we measured CBF in both areas. In cerebral cortex, blood flow (BF) before galactosamine injection was similar in control and MK-801-treated rats (0.53 ± 0.05 and 0.49 ± 0.08 mL/g s, respectively). MK-801 delayed and reduced the increase in BF. In control rats, BF increased to 1.18 ± 0.1 mL/g s at 38 h, while in rats with MK-801, BF increased only slightly (0.8 ± 0.1 mL/g s) at 51 h, remaining lower ($p < 0.05$) than in control rats (Fig. 3a).

In cerebellum, BF before galactosamine injection was similar in control and MK-801-treated rats (0.6 ± 0.1 and 0.62 ± 0.18 mL/g s, respectively). In control rats, BF in
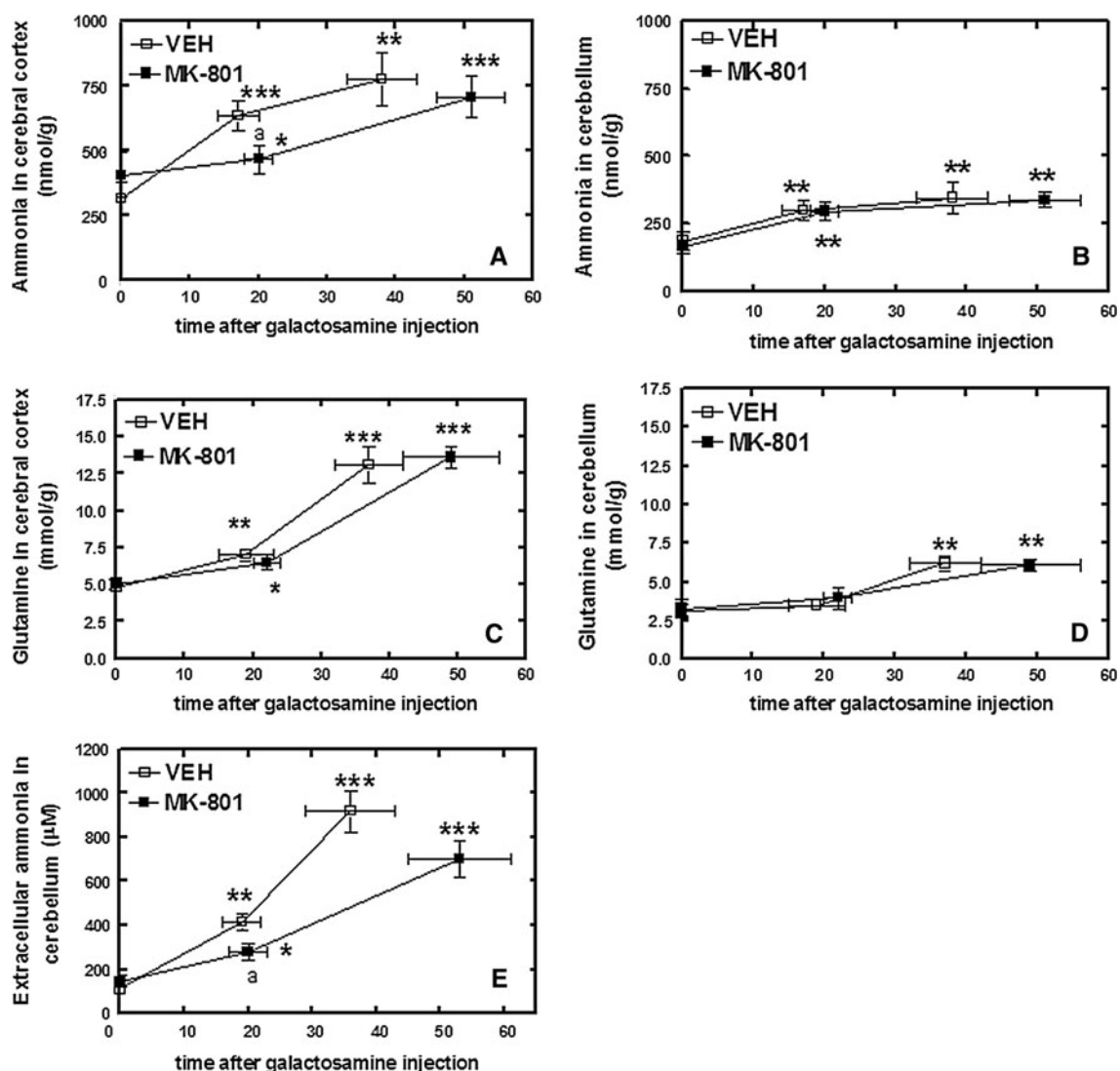
Fig. 2 MK-801 delays the increase in ammonia in cerebral cortex and in extracellular fluid in cerebellum. Ammonia (**a–b**) and glutamine (**c–d**) in cortex (**a**, **c**) and cerebellum (**b**, **d**), and ammonia in extracellular fluid in cerebellum (**e**) were measured at different grades of HE. Values are the mean ± SEM of 6–8 rats. Values significantly different from basal are indicated by *asterisks* *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Values significantly different in rats treated with MK-801 from controls are indicated by "a", $^a p < 0.05$

cerebellum decreased ($p < 0.05$) to 0.42 ± 0.02 mL/g s at 38 h. However, in rats treated with MK-801, BF in cerebellum did not decrease at any time (Fig. 3b).

MK-801 Delays and Reduces the Increase in Lactate in Cerebral Cortex and Prevents it in Cerebellum

As it has been proposed that increased lactate is a relevant contributor to ALF-induced death, we assessed whether blocking NMDA receptors affects the changes in lactate induced by galactosamine. As for the other parameters, we measured lactate in cortex and cerebellum. Lactate before ALF was similar in control and MK-801-treated rats. In cortex, MK-801 delayed and reduced the increase in lactate. Lactate in control rats increased ($p < 0.01$) to

150 ± 7 % at 37 h, while in rats treated with MK-801, it increased only slightly (132 ± 8 %, $p < 0.05$) at 49 h, remaining lower ($p < 0.05$) than in control rats (Fig. 3c).

In cerebellum, lactate in control rats increased to 158 ± 11 % at 37 h ($p < 0.01$). In rats treated with MK-801, lactate did not increase at any time, remaining lower ($p < 0.05$) than in control rats (Fig. 3d).

MK-801 Does Not Affect Changes in ADC in Different Brain Areas, Including Cortex and Cerebellum

As it has been proposed that cytotoxic edema is a relevant contributor to increased ICP and death in ALF, we assessed whether blocking NMDA receptors affects the changes in edema by measuring ADC in 11 different brain areas. MK-

801 did not affect ADC before galactosamine injection (Table 1). In prefrontal cortex, ADC values were not altered at early stages of ALF (grades I–II of HE) and were reduced (likely reflecting cytotoxic edema) at later stages

(grades III–IV). MK-801 did not affect these changes (Table 1).

In cerebellum ADC values increased early after induction of ALF and remained high (likely reflecting vasogenic
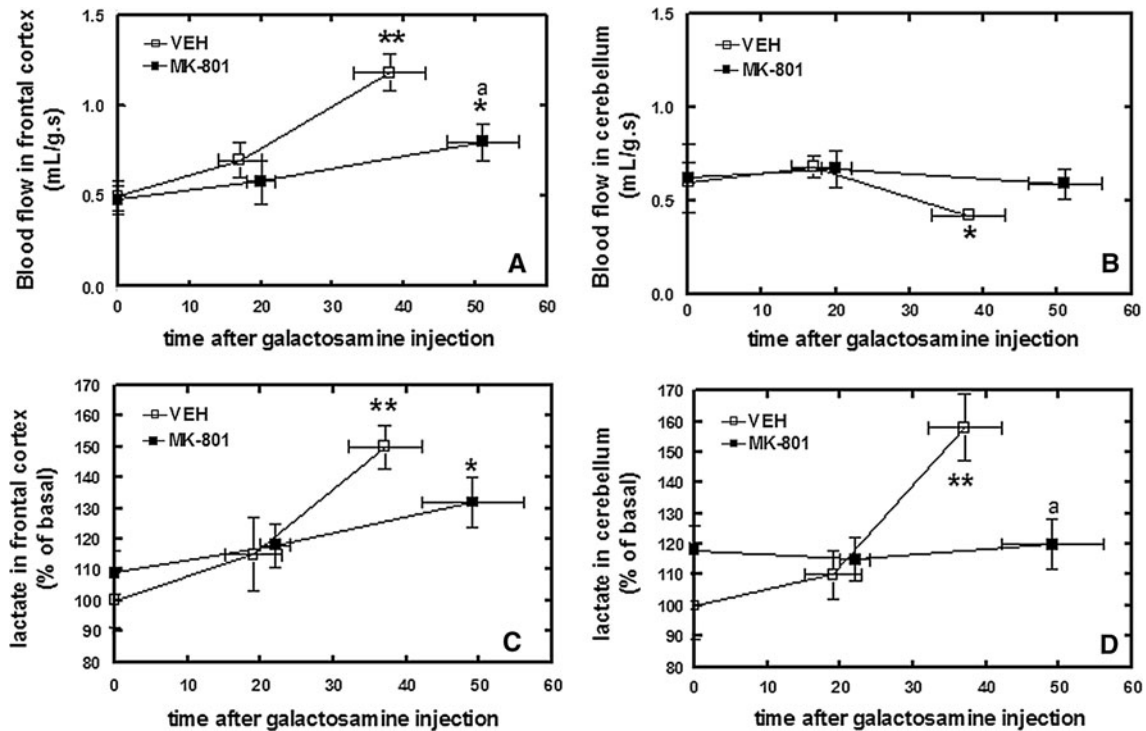

Fig. 3 MK-801 delays and reduces changes in blood flow and lactate. Blood flow (**a–b**) and lactate (**c–d**) in cortex (**a**, **c**) and cerebellum (**b**, **d**) were measured at different grades of HE. Values are the mean ± SEM of 6–8 rats. Values significantly different from basal are indicated by *asterisks* *$p < 0.05$; **$p < 0.01$; Values significantly different in rats treated with MK-801 from controls are indicated by "a", $^a p < 0.05$

Table 1 Effect of ALF and of MK-801 on apparent diffusion coefficient (ADC) in different brain areas

Brain area	VEH	MK-801	VEH + GalN I–II	MK-801 + GalN I–II	VEH + GalN III–IV	MK-801 + GalN III–IV
HIPP	623 ± 25	574 ± 46	581 ± 45	590 ± 43	**381 ± 42****	499 ± 52
MDT	601 ± 40	642 ± 61	588 ± 59	563 ± 81	**845 ± 59****	**873 ± 81***
CPu	485 ± 26	499 ± 43	**618 ± 21****	**611 ± 40***	**326 ± 42***	**388 ± 27***
NAcc	488 ± 34	462 ± 84	556 ± 61	513 ± 51	406 ± 61	413 ± 31
MCx	406 ± 45	392 ± 36	**638 ± 69****	**603 ± 36****	338 ± 69	303 ± 36
EnthCx	668 ± 36	726 ± 45	648 ± 74	624 ± 58	648 ± 78	624 ± 58
HypTh	597 ± 80	590 ± 46	**450 ± 41***	652 ± 74	**456 ± 49***	**453 ± 74***
GP	588 ± 31	562 ± 38	**744 ± 57***	**735 ± 70***	**402 ± 48***	**435 ± 36***
PFCx	531 ± 66	582 ± 69	620 ± 49	611 ± 36	**326 ± 31****	**311 ± 42***
SN	795 ± 31	805 ± 91	807 ± 70	785 ± 55	**520 ± 70***	**426 ± 55***
Cb	488 ± 52	598 ± 86	**804 ± 63****	**818 ± 76****	**866 ± 42****	**842 ± 49****

Mini-osmotic pumps containing saline or the NMDA receptor antagonist MK-801 were implanted in the rats. Two days later, all rats were injected with 2.5 g/kg of galactosamine. The neurological status was examined every 60 min. ADC was measured by magnetic resonance at different grade of HE. Values significantly different from basal are indicated by asterisks * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

edema) later on. MK-801 did not affect these changes (Table 1).

In other brain areas, ALF increased or decreased ADC values depending on the brain area and on the progression of ALF and of HE, as previously described (Cauli et al. 2011). MK-801 did not affect these changes except for a delayed reduction of ADC in hypothalamus and a prevention of it in hippocampus (Table 1).

MK-801 Does Not Affect the Increase in Water Content

To have an additional measure of edema, we also measured water content by gravimetry. This cannot be done in all brain areas, and as for the other parameters, we measured it in cortex and cerebellum. In agreement with the changes in ADC, in frontal cortex water content was not altered at early stages (grades I–II of HE) and increased at later stages (grades III–IV). MK-801 did not affect these changes (Table 2).

In cerebellum, water content increased early after induction of ALF and remained high later on. MK-801 did not affect these changes (Table 2).

MK-801 Does Not Affect the Increase in BBB Permeability and MMP-9

As increased BBB permeability may contribute to edema and increased ICP, we assessed whether blocking NMDA receptors affects the changes in BBB induced by galactosamine in cortex and cerebellum. In prefrontal cortex, BBB permeability was not altered at early stages (grades I–II of HE) and increased at later stages (grades III–IV). MK-801 did not affect these changes (Table 2).

In cerebellum, BBB permeability increased early after induction of ALF and remained high later on. MK-801 did not affect these changes (Table 2).

Similar results were obtained for the amount of MMP-9, which was increased at early stages in cerebellum but not in frontal cortex and in both areas at later stages. MK-801 did not affect these changes (Table 2).

The Effects of MK-801 Are Not Due to Changes in Brain Temperature or Reduced Liver Damage

It has been shown that hypothermia reduces ischemic brain damage (mediated by NMDA receptors) and that NMDA receptors may induce hypothermia (Warner et al. 1991). To assess whether hypothermia may play a role in the protective effects of MK-801, we measured brain temperature. We also assessed whether MK-801 could delay death by reducing the liver damage induced by galactosamine.

Galactosamine injection induced a transient drop in brain temperature followed by a rapid return to normal values and a delayed reduction of temperature to 34 °C ($p < 0.05$ compared to basal). MK-801 did not affect these changes (Fig. 4).

Liver damage, as assessed by measuring GOT and GPT or bilirubin in serum, increased in parallel with the progression of HE in rats with ALF (Table 3). MK-801 did not affect the changes in GOT, GPT, bilirubin, or urea in serum, indicating that MK-801 does not affect liver damage.

MK-801 Increases Ammonia Elimination in Urine at Early Stages of ALF and Delays the Increase in Ammonia in Blood and Muscle

The above results show that MK-801 delays the increase in ammonia in cortex and extracellular ammonia in cerebellum, which may contribute to delay some cerebral effects mediated by ammonia.

Table 2 MK-801 does not affect changes in water content, BBB permeability, or MMP-9

	Grade 0		Grade I + II		Grade III + IV	
	Vehicle	M-801	Vehicle	M-801	Vehicle	M-801
Water in frontal cortex (% of weight)	79.5 ± 0.2 (6)	79.6 ± 0.3 (6)	79.6 ± 0.2 (6)	79.5 ± 0.2 (6)	80.7 ± 0.2** (6)	81.1 ± 0.3** (6)
Water in cerebellum (% of weight)	79.2 ± 0.2 (6)	79.1 ± 0.3 (6)	79.9 ± 0.2* (6)	79.8 ± 0.2* (6)	80.0 ± 0.3* (6)	80.0 ± 0.2* (6)
BBB permeability in cerebral cortex (% of basal)	100 ± 16 (8)	100 ± 12 (8)	106 ± 11 (6)	96 ± 14 (6)	148 ± 18* (6)	140 ± 7* (6)
BBB permeability in cerebellum (% of basal)	100 ± 14 (8)	100 ± 19 (8)	163 ± 14** (6)	155 ± 8** (6)	178 ± 14** (6)	185 ± 36* (6)
MMP-9 in cerebral cortex (% of basal)	133 ± 4 (6)	137 ± 4 (6)	128 ± 5 (6)	127 ± 4 (6)	80 ± 9*** (6)	78 ± 8 *** (6)
MMP-9 in cerebellum (% of basal)	100 ± 8 (6)	100 ± 4 (6)	131 ± 13* (6)	124 ± 10* (6)	133 ± 2* (6)	130 ± 9** (6)

Water content, BBB permeability, and MMP-9 content in cortex and cerebellum were measured. Values are mean ± SEM of 6–8 rats (indicated in parenthesis). *Asterisk* significantly different from basal: * $p < 0.05$; ** $p < 0.01$

Fig. 4 MK-801 does not affect brain temperature. Mini-osmotic pumps containing saline or the NMDA receptor antagonist MK-801 were implanted in the rats as described in the "Methods" section. Rats were injected intraperitoneally with galactosamine (2.5 g/kg). Brain temperature was measured every 30 min. The graph shows the time course of brain temperature before and after galactosamine injection. Values are the mean ± SEM of 5–7 rats per group
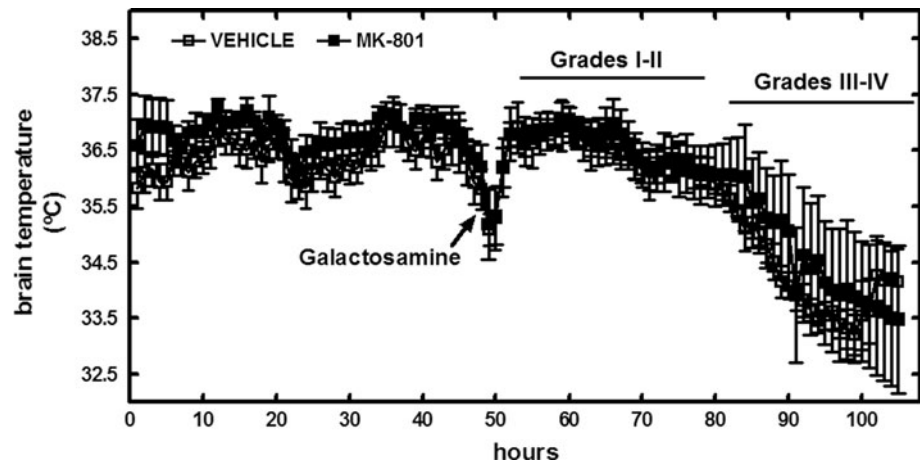
We then assessed whether MK-801 delays the induction of hyperammonemia. Before galactosamine injection, blood ammonia was similar in control and MK-801-treated rats (77 ± 5 and 83 ± 5 μM, respectively).

MK-801 delays the increase in blood ammonia. In control rats, blood ammonia reached 165 ± 11 μM at 17 h, while in rats with MK-801, ammonia remained lower (122 ± 16 μM, $p < 0.05$) at 20 h. At later stages, blood ammonia reached similar levels in control and MK-801-treated rats (208 ± 19 and 197 ± 17 μM, respectively; Fig. 5a).

MK-801 also delays the ammonia increase in muscle. In control rats, ammonia increased from 0.89 ± 0.1 to 1.22 ± 0.12 μmol/g ($p < 0.05$) at grades I–II of HE. In rats with MK-801, ammonia in muscle did not increase at these early stages but increased later to levels similar to control rats (Fig. 5b).

To assess whether the delay in hyperammonemia could be due to enhanced ammonia elimination, we measured ammonia in urine. MK-801 did not affect urine volume (Table 2). Before ALF, ammonia elimination in urine was similar in control (12 ± 2 μmol/24 h) and MK-801-treated rats (11 ± 2 μmol/24 h). However, in the first 24 h after galactosamine injection (grades I–II of HE), ammonia elimination was increased ($p < 0.05$) in rats treated with MK-801 (28 ± 5 μmol) compared to control rats (20 ± 4 μmol; Table 3). This increased ammonia elimination is transient, returning to values similar to controls during grades III–IV of HE (13 ± 2 and 15 ± 3 μmol, respectively; Table 3; Fig. 5c).

ALF Impairs Glomerular Filtration Rate: MK-801 Delays This Impairment

We assessed whether increased ammonia elimination in rats treated with MK-801 could be due to better renal function. We assessed the effects of ALF on glomerular filtration rate by measuring creatinine and inulin clearance.

In control rats, serum creatinine increased at early stages of ALF. Before galactosamine injection, creatinine levels were 35 ± 1 μM both in control rats and rats treated with MK-801. In control rats, serum creatinine increased to 43 ± 2 μM ($p < 0.01$) at grades I–II of HE (25 h after galactosamine injection), indicating impaired kidney function. However, in rats treated with MK-801, creatinine was not increased at early stages, and 27 h after galactosamine injection, it remains at 34 ± 2 μM. At later stages of ALF, serum creatinine increased further to reach 74 ± 6 μM at 42 h in control rats and 80 ± 2 μM at 56 h in rats treated with MK-801 (Table 3).

Impairment of glomerular filtration rate (GFR) following galactosamine injection was confirmed by the reduced inulin clearance. The impairment was significant at grades I–II of HE and worsened at grades III–IV (Fig. 6a). MK-801 delayed the impairment in GFR. At grades I–II, GFR was significantly ($p < 0.05$) better in rats treated with MK-801 (0.77 ± 0.02 mL/min kg) than in rats with vehicle (0.60 ± 0.01 mL/min kg). At grades III–IV, GFR was similar in rats treated with MK-801 or vehicle (0.42 ± 0.01 mL/min kg). This supports that MK-801 delays the impairment of GFR, which occurs at early stages of ALF in control rats and at later stages in rats treated with MK-801.

MK-801 Does Not Affect the Reduction in Blood Pressure in Rats with ALF

The above results show that ALF impairs GFR. This could be due to functional and/or structural damage. Reduction in blood pressure would be associated with lower renal perfusion and a functional reduction in GFR even in the absence of structural damage. We therefore measured blood pressure. Galactosamine injection induced a progressive reduction in blood pressure (Fig. 6b) which was not affected by MK-801.

Table 3 Effect of MK-801 on parameters reflecting hepatic and renal functions

	Grade 0		Grade I + II		Grade III + IV	
	Vehicle	M-801	Vehicle	M-801	Vehicle	M-801
Serum						
GOT (U.I./L)	150 ± 13 (6)	158 ± 5 (6)	6,173 ± 588 (6)***	5,426 ± 519 (6)***	9,991 ± 1,105 (6***)a	9,765 ± 973 (6)***b
GPT (U.I./L)	55 ± 4 (6)	52 ± 5 (6)	3,869 ± 380 (6)***	3,350 ± 376 (6)***	5,647 ± 486 (6)***a	5,847 ± 850 (6)***b
Bilirubin (mg/dL)	0.10 ± 0.03 (6)	0.14 ± 0.04 (6)	1.39 ± 0.40 (6)***	1.174 ± 0.60 (6)**	4.10 ± 0.33 (6)***	5.02 ± 0.40 (6)***
Urea (mM)	5.6 ± 0.3 (8)	5.8 ± 0.4 (7)	9.4 ± 0.7 (6)*	8.5 ± 0.9 (7)*	8.8 ± 0.8 (6)*	10.3 ± 0.5 (6)*
Creatinine (μM)	35 ± 1 (8)	34 ± 1 (9)	43 ± 2 (7)**	34 ± 2 (9)a	74 ± 6 (5)*** a	80 ± 2 (5)*** b
Sodium (mEq/L)	133 ± 4 (6)	137 ± 4 (6)	128 ± 5 (6)	127 ± 4 (6)	80 ± 9 (3) ***	78 ± 8 (3) ***
Urine						
Ammonia (μM)	0.27 ± 0.03 (17)	0.25 ± 0.03 (18)	0.50 ± 0.09 (6)**	0.74 ± 0.1 (10)***a	0.52 ± 0.1 (9)**	0.48 ± 0.1 (10)**b
Ammonia (total μmols)	12 ± 2	11 ± 2	20 ± 4*	28 ± 5**a	15 ± 3	13 ± 2
Creatinine (mM)	10.4 ± 1.2 (6)	12.1 ± 1.6 (6)	11.1 ± 1.1 (6)	11.4 ± 0.9 (6)	4.6 ± 0.8 (6) **	4.8 ± 0.8 (6) **
K$^+$ (mM)	166 ± 10 (6)	193 ± 13 (6)	181 ± 11 (6)	92 ± 18 (6)**a	82 ± 7 (6)**	94 ± 8 (6)**
Na$^+$ (mM)	56 ± 6 (6)	53 ± 5 (6)	68 ± 4 (6)	62 ± 5 (6)	40 ± 4 (6)*	37 ± 4 (6)*
HCO$_3^-$ (mM)	14 ± 3 (6)	10 ± 2 (6)	36 ± 7 (6)**	31 ± 6 (6)**	24 ± 3 (6)*	27 ± 5 (6)*
Urea (mM)	3.5 ± 0.3 (16)	4.2 ± 0.5 (7)	4.0 ± 0.3 (14)	3.9 ± 0.5 (10)	2.7 ± 0.3 (4)	2.9 ± 0.6 (8)
Volume (mL)	44 ± 3 (7)	43 ± 2 (7)	40 ± 2 (6)	37 ± 4 (6)	29 ± 4 (5)*	28 ± 4 (6)*
Creatinine clearance (mL/min)	0.9 ± 0.06 (6)	1.06 ± 0.1 (6)	0.72 ± 0.05 (6) *	0.88 ± 0.04 (6)	0.12 ± 0.05 (6) *** a	0.12 ± 0.08 (6) *** b
Fractional excretion sodium	0.14 ± 0.01 (6)	0.11 ± 0.1 (6)	0.20 ± 0.03 (6) *	0.14 ± 0.02 (6) a	0.79 ± 0.09 (6) ***	0.78 ± 0.08 (6) ***

Values are the mean ± SEM of the number of rats indicated in parenthesis. Values significantly different from basal are indicated by asterisks * $p < 0.05$; ** $p < 0.01$; Values significantly different in rats treated with MK-801 from controls are indicated by "a" [a] $p < 0.05$ values that are significantly different from grade I + II of HE are indicated by "b" [b] $p < 0.05$
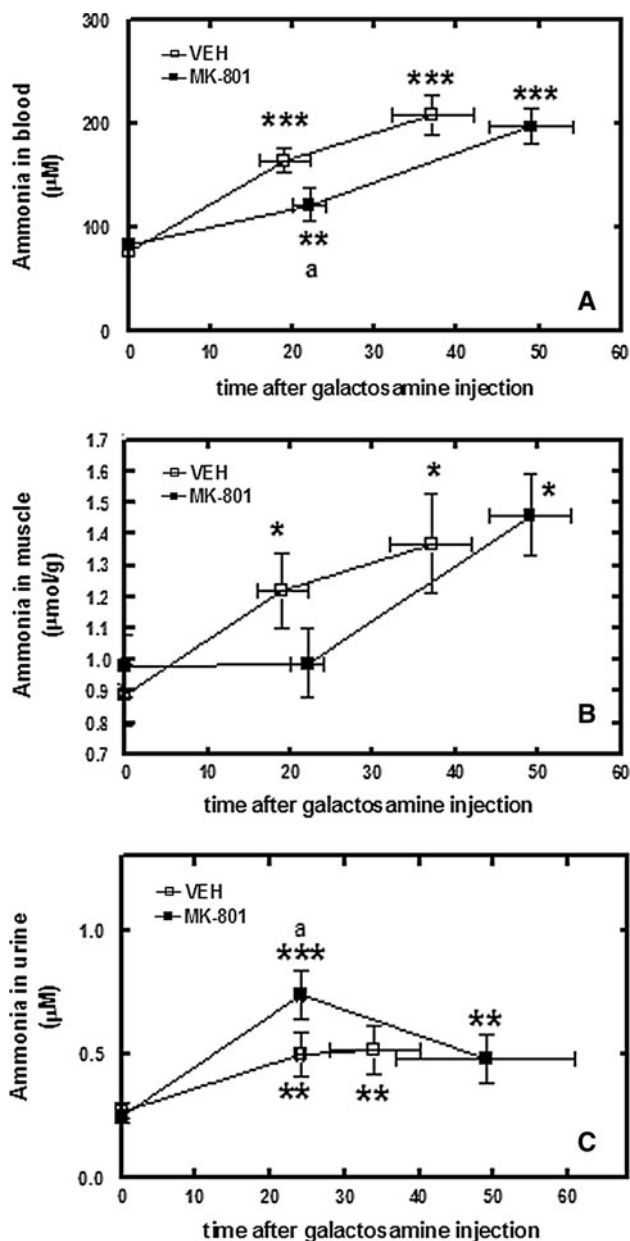
Fig. 5 MK-801 increases ammonia elimination in urine and delays the increase in ammonia in blood and muscle. Ammonia in blood (**a**), muscle (**b**), and urine (**c**) was measured at different grades of HE. Values are the mean ± SEM of 6–12 rats. Values significantly different from basal are indicated by *asterisks* *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; Values significantly different in rats treated with MK-801 from controls are indicated by "a", $^a p < 0.05$

Fig. 6 MK-801 delays tubular damage and the reduction in glomerular filtration rate. Inulin clearance (**a**), blood pressure (**b**), and Kim-1 (**c**) in urine were measured at different grades of HE. Values are the mean ± SEM of 6–9 rats. Values significantly different from grade 0 are indicated by *asterisks* *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; Values significantly different in rats treated with MK-801 from controls are indicated by "a", $^a p < 0.05$

MK-801 Reduces and Delays the Structural Tubular Damage Induced by ALF in Kidney

The above results suggest that reduced GFR in ALF must be due to structural renal damage. We therefore assessed whether galactosamine injection induces structural tubular damage in kidney and whether this is prevented or delayed by MK-801. As m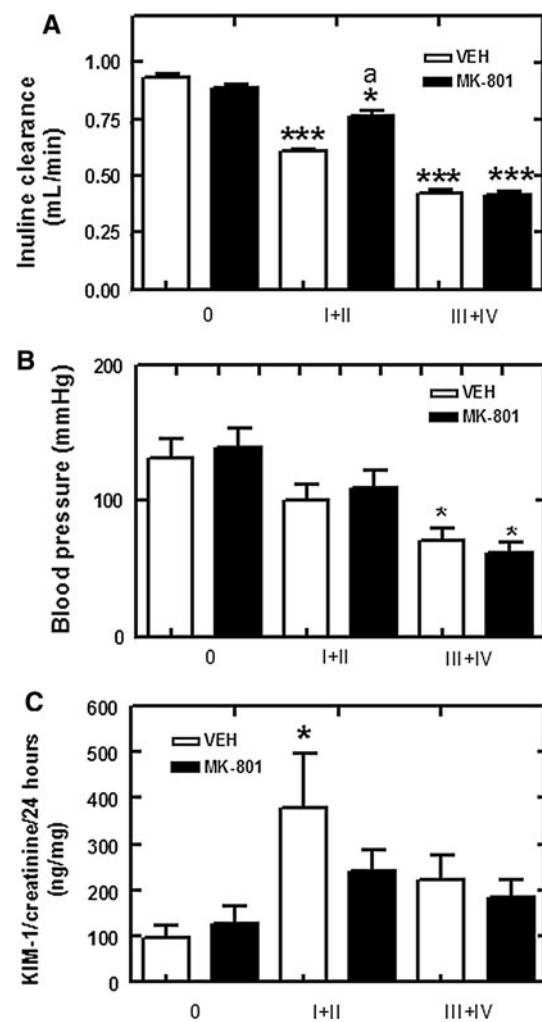arkers of structural tubular damage in kidney, we measured the effects of galactosamine injection on fractional excretion of sodium and excretion of Kim-1 in urine.

Fractional excretion of sodium was increased at grades I–II of HE in rats injected with vehicle (supporting the presence of tubular damage) but not in rats injected with MK-801. At grades III–IV, fractional excretion of sodium was strongly increased both in rats treated with MK-801 and in rats treated with vehicle (Table 3).

The induction of tubular damage in rats with ALF is also supported by the increase in Kim-1 (Fig. 6c) in urine. MK-801 reduces the increase in Kim-1 at early stages of ALF.

To confirm the damage, we performed histological analysis of kidneys. Control rats treated with vehicle did

not show any damage in proximal and distal tubules, glomeruli, or renal interstitium, with very well preserved brush border cells (Fig. 7a). Similar images were obtained for rats treated only with MK-801 (Fig. 7b).

Rats injected with galactosamine show slight renal injury at grade I of HE (Fig. 7c). Around 25 % of tubular epithelial cells showed mild degeneration with vacuoles in the cytoplasm and foci with hyaline droplets, moderate tubular brush border loss and acute tubular necrosis in some tubules, slight vascular congestion and few interstitial lymphocytic infiltrates. The damage reached grade 3 according to the scale of Duarte et al. (1997).

At grade III of HE, rats injected with galactosamine show extensive renal injury (Fig. 7e) with tubular epithelial cells showing extensive degeneration, with vacuoles and multiple foci of hyaline droplets, severe tubular brush border loss, severe acute tubular necrosis, and glomerular vacuolization. Vascular congestion and interstitial lymphocytic infiltrates were also present. Kidney damage reached grade 4 according to the scale of Duarte et al. (1997).

MK-801 afforded a significant protection against kidney damage. At grade I of HE, rats treated with MK-801 and galactosamine show proximal and distal convoluted tubules almost normal with slight loss of brush border (Fig. 7d), reaching grade 1 of kidney damage in the scale of Duarte et al. (1997). At grade III of HE, less than 25 % of tubular epithelial cells showed mild degeneration with large cytoplasmic vacuoles, a few hyaline droplets in the cytoplasm, slight changes in tubular brush border, and acute tubular necrosis in a small group of cells (Fig. 7f). Kidney damage reached grade 2 in the scale of Duarte et al.

These results indicate that ALF reduces glomerular filtration rats in kidney by two mechanisms: (a) a functional effect due to reduced blood pressure and renal perfusion, which is not affected by blocking NMDA receptors; (b) n structural tubular damage, which is delayed and reduced by blocking NMDA receptors.

Fig. 7 MK-801 delays and reduces structural tubular damage. Kidney sections were prepared and stained with hematoxylin–eosin. All images are shown at ×400 magnification. **a** Control rats treated with vehicle (*arrow* indicates well preserved brush border). **b** Control rats treated with MK-801. **c** Rats treated with galactosamine and killed at grade I of HE. **d** Rats treated with MK-801 and galactosamine and killed at grade I of HE. **e** Rats treated with galactosamine and killed at grade III of HE (*arrow* indicates vacuole). **f** Rats treated with MK-801 and galactosamine and killed at grade III of HE
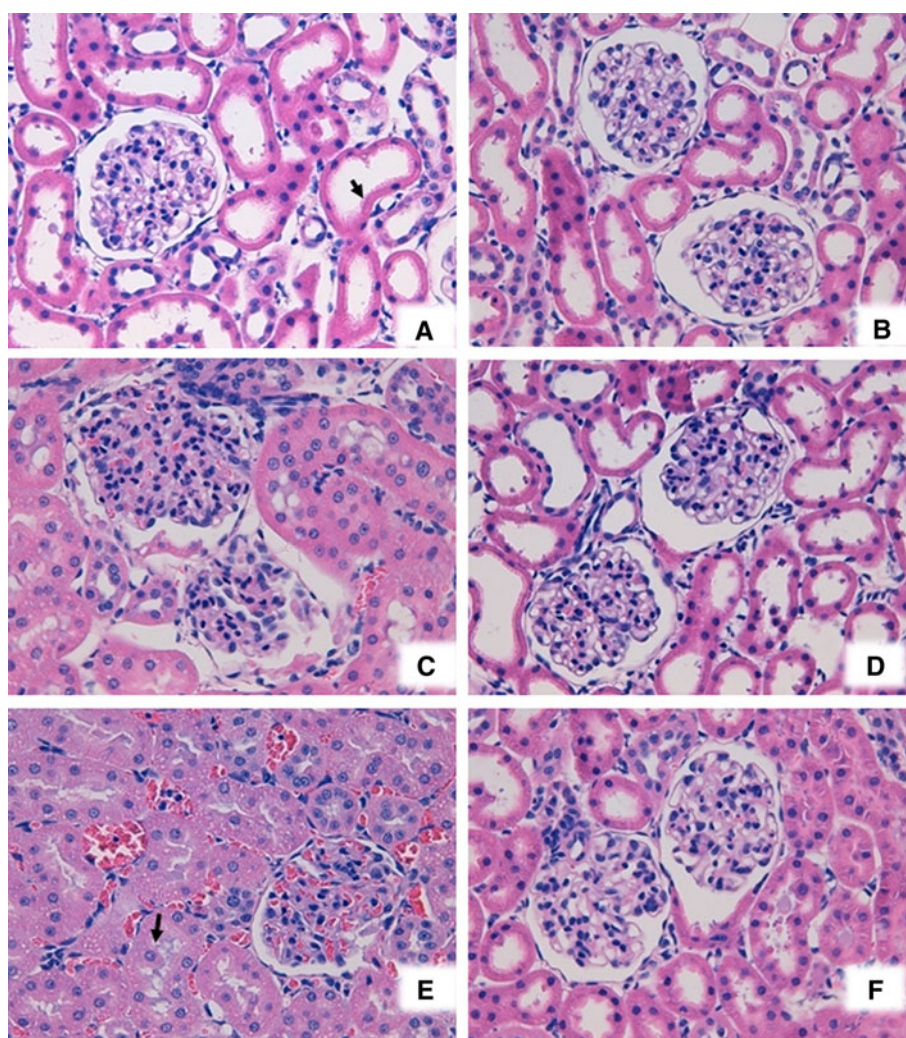
Discussion

These findings identify novel mechanisms in kidney and brain by which blocking NMDA receptors delays the progression of HE, ICP, and death in ALF. We have identified, for the first time, an interplay between liver, kidney and brain in the progression of cerebral damage in ALF. ALF induces kidney damage which is mediated, at least in part, by activation of NMDA receptors in kidney. This reduces glomerular filtration rate and contributes, together with liver failure, to increase ammonia levels in blood and brain. Blocking NMDA receptors with MK-801 delays kidney injury, reduction in GFR, the increase in ammonia in blood and brain, and, subsequently, the deleterious effects of high ammonia levels in brain.

The main new findings of this study are that blocking NMDA receptors:

1. delays kidney damage and enhances ammonia elimination in urine at early stages of ALF
2. delays the increase in ammonia in blood, muscle, cerebral cortex, and extracellular ammonia in cerebellum
3. prevents completely the changes in blood flow and lactate in cerebellum
4. delays and reduces the increases in blood flow and lactate in cortex
5. delays the progression of HE, ICP, and death

A relevant novel idea supported by the data is that effects both in kidney and brain contribute to the beneficial effects of NMDA receptor antagonists in ALF. Although NMDA receptors have been well characterized in brain, they are also present in peripheral tissues including kidney (Yang et al. 2008). The present results support that, in ALF, excessive activation of NMDA receptors in kidney and brain induces different deleterious effects which contribute to the progression of hyperammonemia, HE, ICP, and death.

Activation of NMDA receptors in kidney contributes to kidney damage and reduced GFR and ammonia elimination and their activation in brain contributes to alterations in CBF, lactate, HE, and ICP.

Blocking NMDA receptors with MK-801 delays kidney damage, the impairment of GFR, and the increase in ammonia in blood and tissues (due to better elimination) and reduces or delays changes in brain BF, lactate, HE, and ICP. Other cerebral alterations induced by ALF are not affected by blocking NMDA receptors.

We discuss these findings briefly.

The lack of effects of MK-801 on the increase in MMP-9, BBB permeability, water content, and changes in ADC (vasogenic and cytotoxic edema) indicates that activation of NMDA receptors does not play a main role in these processes, in agreement with Cauli et al. (2007), showing that in acute hyperammonemia, blocking NMDA receptors does not prevent changes in ADC (edema).

Blocking NMDA Receptors Delays Kidney Damage, Enhances Ammonia Elimination in Urine at Early Stages of ALF, and Delays the Increase in Ammonia in Blood, Muscle, and Brain

ALF induces a progressive reduction in GFR which is delayed in rats treated with MK-801. Better GFR at grades I–II of HE in rats treated with MK-801 is associated with increased ammonia elimination in urine and would be the reason for the lower ammonia levels in blood, muscle, cerebral cortex, and cerebellum compared to controls at early stages of ALF.

Both reduced renal perfusion and structural tubular injury contribute to reduce GFR in rats with ALF. Blood pressure decreases progressively in rats with ALF, resulting in renal under perfusion and functional decrease in GFR which are not prevented by MK-801.

This suggests that the delay in GFR impairment by MK-801 would be due to transient prevention of structural kidney injury. To assess this possibility, we measured Kim-1 in urine as a marker of tubular kidney injury (Han et al. 2002). Excretion of Kim-1 in urine increases transiently in rats with kidney injury induced by puromycin aminonucleoside, returning later to normal values (Sasaki et al. 2011). We found a similar transient increase followed by normalization of Kim-1 excretion. Moreover, histological analysis shows structural tubular injury in rats with ALF. This tubular injury is reduced and delayed by MK-801, as indicated by the lower increase in urinary Kim-1 and in the lower grade of kidney damage according to the scale of Duarte et al. (1997).

The fact that blocking NMDA receptors with MK-801 delays tubular injury suggests that activation of these receptors is involved in the induction of this kidney damage. NMDA receptors are present in kidney in rats (Yang et al. 2008) and seem to play a relevant role in kidney damage in gentamicin nephrotoxicity (Leung et al. 2004) and ischemia reperfusion (Yang et al. 2004). Intrarenal activation of NMDA receptors reduces glomerular filtration rate. Blocking NMDA receptors ameliorates ischemia–reperfusion-induced glomerular and tubular dysfunction in rats by restoring decreased glomerular filtration rate (Yang et al. 2004).

The results reported here support that NMDA receptors activation is involved in early steps of ALF-induced renal failure. Blocking NMDA receptors delays this damage, allowing longer maintenance of appropriate GFR and better ammonia elimination in rats treated with MK-801. The increase of 8 ± 4 μmol in ammonia elimination in urine

induced by MK-801 in the first 24 h of ALF would reduce ammonia concentration in blood and tissues around 40–50 μM in these rats, in agreement with the decrease observed in blood. This supports that the transient increased elimination of ammonia in urine at early steps of ALF in rats treated with MK-801 would be responsible for the delay in the induction of hyperammonemia and of the increase in ammonia in muscle and brain. The delayed increase in brain ammonia would contribute to delay some cerebral effects including HE, ICP, and death.

Blocking NMDA Receptors Delays and Reduces or Prevents the Changes in Blood Flow and Lactate in Cortex and Cerebellum

Activation of NMDA receptors increases CBF in cerebral cortex (Chi et al. 2003; Pelligrino et al. 1996; Weiss et al. 1996) and hippocampus (Hara et al. 1996) and blocking NMDA receptors with MK-801 reduces CBF in cerebral cortex (Park et al. 1989) This supports that the increase in CBF in cortex in ALF would be mediated by excessive activation of NMDA receptors. The delay and reduction in increased cortical CBF by MK-801 would be due to a direct effect on NMDA receptors on cortical neurons modulating the coupling between neuronal activity and CBF.

In contrast to the effect in cortex, ALF reduces CBF in cerebellum. This is not surprising.

The mechanisms coupling neuronal activity with CBF are different in cerebellum and cortex (Yang et al. 1999). Neuronal nitric oxide plays an essential role in this coupling in cerebellum but not in cortex (Hayashi et al. 2002; Yang et al. 1999). In cerebellum, CBF is mainly modulated by glutamate through AMPA but not NMDA receptors (Akgören et al. 1994; Offenhauser et al. 2005; Yang and Iadecola 1998). In fact, glutamate (likely through AMPA receptors) induces opposite vasomotor effects (contraction) than NMDA (dilation) on microvascular tone in cerebellum. This suggests that in cerebellum, NMDA receptors activation could reduce, instead of increase, blood flow (Rancillac et al. 2006). This would explain the reduced BF in cerebellum in ALF, mediated by NMDA receptors activation, and its prevention by MK-801.

The coupling between synaptic activity in neurons and energy metabolism in astrocytes may be also responsible for the increase in lactate. Glutamate release from neurons is sensed by astrocytes which synthesize and release lactate to be used as energy source by neurons (Magistretti and Pellerin 1999). ALF leads to a large increase in extracellular glutamate in grades III–IV (Cauli et al. 2008) which would be sensed by astrocytes, leading to increased lactate formation. The increase in extracellular glutamate is reduced by treatment with MK-801 (Cauli et al. 2008).

This would contribute to the reduced increase in lactate in cortex and to its prevention in cerebellum.

Alterations in CBF and lactate in ALF would be a consequence of increased synaptic activity, with a major contribution of NMDA receptors activation. Blocking NMDA receptors would reduce this synaptic activity, the increase in extracellular glutamate and lactate and changes in CBF.

Dual Protective Mechanisms in Kidney and Brain Contribute to Delay the Progression of HE, ICP, and Death in ALF by Blocking NMDA Receptors

In summary, the results reported support that treatment with MK-801 leads to delayed progression of HE, ICP, and death by dual independent protective mechanisms acting on kidney and on brain, by the following mechanisms:

1. In kidney, blocking NMDA receptors delays tubular injury, allowing a transient preservation of GFR and increased ammonia elimination in urine compared to control rats. This enhanced elimination delays the increase in ammonia in blood, muscle, and brain which contributes to delay HE, ICP, and death.
2. In brain, blocking NMDA receptors reduces or prevents changes in CBF and lactate, which contribute to delay the increase in ICP and death.

These results support that blocking NMDA receptors with selective antagonists would have beneficial effects in patients with ALF allowing delaying the progression of HE, ICP, and death. Similar beneficial effects could be obtained using other pharmacological tools that also reduce activation of NMDA receptors. This can be achieved, for example, with carnitine and choline derivatives, which potentiate activation of metabotropic glutamate receptors and prevent NMDA receptor-mediated excitotoxicity in cultured neurons and ammonia-induced death in rats (Felipo et al. 1994; Miñana et al. 1996; Montoliu et al. 1997). The possible utility of these alternative treatments in ALF remains to be tested.

It is worth noting that memantine, an antagonist of NMDA receptors, is currently being used in the treatment of Alzheimer's disease (Suzuki et al. 2013) and has been also proposed as neuroprotective drug in aging brain (Karolczak et al. 2013).

The data reported here support that any therapeutic approach leading to reduced activation of NMDA receptors, including safe use of NMDA receptor antagonists, may help to increase patient's survival, providing additional time to get an appropriate liver for transplantation or, in milder ALF, for liver regeneration.

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