RENORBIO
Programa de Pós-graduação em Biotecnologia

# Expressão de Genes Codificantes de Proteínas Transportadoras de Zinco, Metalotioneína e Interleucina-6 em Modelo Experimental de Doença Renal Crônica

**Amanda de Castro Amorim Serpa Brandão**

Teresina-PI
2014

Amanda de Castro Amorim Serpa Brandão

# Expressão de Genes Codificantes de Proteínas Transportadoras de Zinco, Metalotioneína e Interleucina-6 em Modelo Experimental de Doença Renal Crônica

Tese de Doutorado apresentada ao Programa de Pós-Graduação da Rede Nordeste de Biotecnologia (RENORBIO) / UFPI, como requisito para obtenção do título de doutora em Biotecnologia em Saúde.

Orientadora: Prof.ª Dr.ª Semiramis Jamil Hadad do Monte

Teresina-PI
2014

FICHA CATALOGRÁFICA
Universidade Federal do Piauí
Biblioteca Comunitária Jornalista Carlos Castello Branco
Serviço de Processamento Técnico

B817e Brandão, Amanda de Castro Amorim Serpa.
Expressão de genes codificantes de proteínas transportadoras de zinco, metalotioneína e interleucina-6 em modelo experimental de doença renal crônica / Amanda de Castro Amorim Serpa Brandão. -- 2014.
102 f. : il.

Tese (Doutorado em Biotecnologia) – Rede Nordeste de Biotecnologia (RENORBIO), Universidade Federal do Piauí, Teresina, 2014.
"Orientação: Prof.ª Dr.ª Semiramis Jamil Hadad do Monte."

1. Doença renal crônica. 2. Transportadores de zinco. 3. Expressão gênica. 4. Zinco. I. Título.

CDD 616.61

UNIVERSIDADE FEDERAL DO PIAUÍ
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA
*Campus Universitário "Ministro Petrônio Portella" – Bairro Ininga – Bloco da PREX – Anexo I*
*Telefone: (86) 3217-3331 – e-mail: renorbio@ufpi.edu.br*
*CEP 64049-550 – Teresina–PI*

# FOLHA DE APROVAÇÃO – DEFESA DE TESE

**ALUNO**: AMANDA DE CASTRO AMORIM

**TÍTULO DO PROJETO:** "*Expressão de genes codificantes de proteínas transportadoras de zinco, metalotioneína e IL-6 em um modelo experimental de Doença Renal Crônica*".

**PROFESSOR ORIENTADOR:** Profª. Drª. Semiramis Jamil Hadad do Monte

| **BANCA EXAMINADORA:** | **CONCEITO** | **ASSINATURA** |
|---|---|---|
| Profª. Drª. Semiramis Jamil Hadad do Monte, UFPI (Orientadora - Presidente) | APROVADA | |
| Prof. Dr. Joaquim Soares da Costa Júnior - IFPI (Examinador) | APROVADA | |
| Prof. Dr. José Adail Fonseca de Castro - UESPI (Examinador) | APROVADA | |
| Prof. Dr. Benedito Borges da Silva – UFPI (Examinador) | APROVADA | |
| Prof. Dr. José Ribeiro dos Santos Júnior - UFPI (Examinador) | APROVADA | |

**DATA DA AVALIAÇÃO:** 06 de Março de 2014.
**HORÁRIO:** 15h00
**LOCAL:** Auditório da Pró-Reitoria de Extensão, PREX/UFPI.

# DEDICATÓRIAS

Este trabalho é dedicado:

Ao meu filho **Daniel**, razão pela qual eu tive forças para continuar esta jornada, mesmo com diversos obstáculos pelo caminho.

Aos meus pais, **Tarcízio Amorim de Santana e Francisca de Castro Amorim** que sempre me apoiaram nesta jornada.

Em especial ao meu esposo **Rafael Brandão**, que participou ativamente de todas as etapas deste trabalho.

## AGRADECIMENTOS

À **Deus**, por ter me ajudado a vencer mais esta batalha.

À minha orientadora, **Professora Dra. Semiramis Jamil Hadad do Monte**, pelos ensinamentos, incentivo e apoio. E, também pela companhia nesta trajetória.

Agradeço à **Dra. Mirian Boim** e ao **Dr. Nestor Schor** pela receptividade e auxílio, no período em que passei na UNIFESP. Agradeço também aos técnicos da nefrologia, em especial à Luciana que foi de suma importância para o desenvolvimento do modelo experimental de Doença Renal Crônica.

Ao Professor **Dr. Marcelo Lima Ribeiro**, da Universidade São Francisco por me ajudar mesmo à distância na padronização das extrações de RNA dos tecidos para as reações de qPCR.

Ao técnico e colega de laboratório, **Antônio Vanildo de Sousa Lima**, pela colaboração nas extrações de RNA.

Ao estimado Professor **Dr. José Adail Fonseca de Castro**, que apesar do seu tempo corrido, sempre esteve presente nas horas em que precisei.

Ao Professor **Dr. Adalberto Socorro da Silva**, pela ajuda no processo de elaboração do artigo.

À **Universidade Federal do Piauí**, pelos incentivos ao programa de Doutorado da Rede Nordeste de Biotecnologia – RENORBIO.

À coordenação na pessoa da Prof. **Dra. Maria Acelina Martins de Carvalho**, ao secretário **Rhuan Marques** pela paciência e disponibilidade e aos professores do Renorbio.

Aos meus familiares e aos meus sogros, que me apoiaram e me incentivaram.

Enfim, meus sinceros agradecimentos a todos que, direta ou indiretamente, contribuíram para a realização deste trabalho.

## RESUMO

O Zinco é um elemento essencial à saúde humana, mas potencialmente tóxico. Sua homeostase é regulada principalmente por proteínas transportadoras de zinco (ZIP e ZnT) e metalotioneína (MT). O fato da Doença Renal Crônica (DRC) apresentar uma alta ocorrência de inflamação asséptica e hipozincemia, aliado a inexistência de estudos sobre a expressão gênica dessas proteínas e sua regulação nesta condição clínica, motivou o presente estudo, cujo objetivo foi verificar a expressão gênica de membros das famílias de genes que codificam proteínas transportadoras de zinco (ZIPS e ZnTs), metalotioneína e interleucina-6 em PBMC`s, intestino (ZIPs, ZnTs e MT) e fígado (ZIP14, ZnT1, MT e IL-6) de ratos submetidos a um protocolo de nefrectomia 5/6 (modelo DRC) e ratos controles saudáveis, ambos submetidos a dieta depletada, adequada ou repletada em zinco durante 15 dias. O RNA foi extraído usando o reagente Trizol. A fluorescência do SYBR green foi lida usando a ciclagem padrão do equipamento de PCR em tempo real. A expressão dos genes estudados em PBMC`s de ratos com DRC em relação aos controles saudáveis antes do início do protocolo dietético mostrou uma diminuição significativa na expressão do ZIP3 em ratos com DRC. Após 15 dias de dieta depletada em zinco, os genes ZIP3 e ZIP14 foram expressos em ratos DRC em níveis significativamente mais altos do que no início do estudo. Por outro lado, quando os ratos DRC foram alimentados com dieta repletada em zinco, observou-se um acentuado aumento na expressão de todos os genes da família ZnT. A comparação entre ratos DRC submetidos a dietas depletadas e repletadas em zinco após 15 dias de sua introdução mostrou mudanças significativas na expressão dos genes avaliados, incluindo a IL-6 e MT. Quando comparou-se os ratos DRC e ratos controle saudáveis após o protocolo dietético, o ZIP14 foi diferencialmente expresso nos ratos com dieta depletada em zinco, sendo regulado positivamente em ratos com DRC. A análise da expressão do mRNA mostrou que a expressão dos genes estudados no intestino para os grupos DRC e controle saudáveis apresentava comportamento semelhante, com regulação positiva dos membros da família ZIP e regulação negativa dos membros da família ZnT quando a dieta foi depletada em zinco e o oposto foi visto na repleção. Ao contrário, no fígado, a expressão destes genes não foi regulada pela concentração de zinco na dieta. Entretanto, verificou-se a regulação positiva dos genes ZIP14 e IL-6 em ratos DRC com uma dieta depletada em zinco, não havendo após a repleção, a regulação negativa destes genes. Pode-se concluir, que a expressão de genes codificantes de proteínas transportadoras de zinco, metalotioneína e interleucina-6 em PBMC`s mostrou-se altamente responsiva a alterações da ingestão dietética de zinco, podendo assim, vir a ser utilizada futuramente como um parâmetro mais condizente para definir a deficiência de zinco ou a necessidade celular deste micronutriente na DRC. Os resultados sugerem ainda, que existe um padrão de expressão gênica diferencial no intestino e no fígado. No intestino, a expressão dos genes estudados é regulada pela concentração de zinco na dieta, enquanto que, no fígado, é regulado pelo ambiente urêmico da DRC, independentemente dos níveis de zinco da dieta.

**Palavras-Chave:** Transportadores de zinco, expressão gênica, doença renal crônica, inflamação, zinco.

*ABSTRACT*

Zinc is an essential element to human health, but potentially toxic. Its homeostasis is regulated chiefly by zinc transporter proteins (ZIP and ZnT) and metallothionein (MT). The fact of Chronic Kidney Disease (CKD) have a high occurrence of aseptic inflammation and hypozincemia, coupled with the lack of studies on gene expression of these proteins and their regulation in this clinical condition, induced this study aimed to verify the gene expression of members of the zinc transporter proteins families (ZIPs and ZnTs), metallothionein and interleukin-6 in PBMC`s, intestine (ZIP, ZnTs and MT) and liver (ZIP14, ZnT1, MT and IL-6) of rats subjected to 5/6 nephrectomy protocol (CKD model) and healthy controls rats undergoing zinc depleted, adequate or repleted diet for 15 days. RNA was extracted using TRIzol reagent. SYBR green fluorescence was read using standard cycling mode of the real-time PCR equipment. The baseline expression of the studied genes in PBMC`s of CKD rats in relation to healthy controls, showed a significant decrease in expression of ZIP3 in CKD rats. After 15 days of diet depleted in zinc, ZIP3 and ZIP14 were expressed in CKD rats at significantly higher levels than baseline. On the other hand, when CKD rats were fed diet with zinc in excess, we observed a dramatic increase in expression of all ZnT genes. Comparison between CKD rats undergoing zinc diets Zn- and Zn+ 15 days post their introduction has shown significant changes in expression of assessed genes, including IL-6 e MT. When we compared CKD rats to healthy control rats after Zn diet protocols, ZIP14 was differentially expressed in CKD zinc depleted rats, being upregulated in CKD rats. The mRNA expression analysis showed that the expression of the genes studied in the intestine of CKD and healthy control groups behaved similarly, with upregulation of ZIP members and downregulation of ZnTs when the diet was depleted in zinc and the opposite was seen in the repletion. Contrariwise, in the liver, the expression of these genes was not regulated by the zinc concentration in the diet. However, there were upregulation of the gene ZIP14 and IL-6 in CKD rats with a depleted zinc diet and after repletion, there was no downregulation of these genes. It can be concluded that the expression of genes that encode proteins zinc carriers, interleukin-6 and metallothionein in PBMC`s was highly responsive to dietary intake of zinc alterations, may thus come to be used in the future as a parameter more suited to define zinc deficiency or cell need of this micronutrient in the DRC. The results also suggest that there is a differential tissue pattern of gene expression in the intestine and in the liver. In the intestine, the expression of these genes is regulated by the concentration of zinc in the diet, whereas in the liver, it is regulated by the uremic environment of CKD independently of the dietary zinc levels.

**Keywords:** Zinc transporters, gene expression, Chronic Kidney Disease, inflammation, zinc.

# LISTA DE ILUSTRAÇÕES E TABELAS

## ARTIGO 1



## ARTIGO 2

# LISTA DE ABREVIATURAS E SIGLAS

| Siglas | Descrição |
|---|---|
| CKD | *Chronic Kidney Disease* |
| cDNA | *Complementary Deoxyribonucleic Acid* |
| DRC | Doença Renal Crônica |
| IL-6 | Interleucina-6 |
| mRNA | *Messenger Ribonucleic Acid* |
| MT | Metalotioneina |
| NF-kB | Nuclear Factor kappa B |
| PBMC | *Peripheral Blood Mononucleated Cell* |
| qPCR | *Quantitative* Polymerase Chain Reaction |
| RNA | *Ribonucleic Acid* |
| ZIP | *Zrt- and Irt-like proteins (Slc39a)* |
| Zn | Zinco |
| ZnT | *solute-linked carrier 30 (Slc30a)* |

## SUMÁRIO

Pág.

# 1. INTRODUÇÃO

O Zinco é um elemento traço essencial à vida, pois desempenha um papel vital na manutenção de muitos processos biológicos indispensáveis para a homeostase celular. Por sua vez, a homeostase celular, requer mecanismos que controlam rigidamente a captação, armazenamento e distribuição de zinco (INSTITUTE OF MEDICINE, 2001).

Duas famílias de proteínas estão implicadas no transporte de zinco. A família ZnT que retira o zinco do meio intracelular, fazendo o efluxo de zinco da células ou influxo de zinco em vesículas intracelulares (COUSINS; LIUZZI; LICHTEN, 2006; LIUZZI; COUSINS, 2004; PALMITER; HUANG, 2004; EIDE, 2004; LICHTEN; COUSINS, 2009). Já a família Zip, faz o oposto, promovendo o transporte de zinco a partir do líquido extracelular para o meio intracelular ou a partir de vesículas intracelulares (EIDE, 2004; LICHTEN; COUSINS, 2009; ANDREINI, 2006). A coordenação do pool de zinco citoplasmático parece ser realizado por uma proteína rica em cisteína, denominada metalotioneína (ANDREINI, 2006).

Apesar deste rígido controle, alguns fatores, principalmente o dietético, podem levar à deficiência do mineral. As manifestações da deficiência de zinco incluem hipogeusia, lesões de pele, atrofia testicular, e dificuldade de cicatrização. Uma vez que estas anormalidades são também comuns na uremia, o metabolismo de zinco na Doença renal Crônica (DRC) tem sido estudado (PRASAD, 1996).

A deficiência de zinco é relatada desde os estágios iniciais da doença renal crônica até em pacientes renais em terapia de reposição (MAFRA et al.,

2004). Amorim (2008), em estudo sobre o estado nutricional relativo ao zinco de pacientes renais crônicos em programa de hemodiálise, verificou prevalência de 49% de hipozincemia plasmática em pacientes de um centro especializado doenças renais crônicas de Teresina, Piauí.

A ingestão alimentar insuficiente, a má nutrição, a interação droga-nutrientes, o estado inflamatório subclínico e a má absorção são geralmente apontados como os principais fatores que levam a uma redução nas concentrações de zinco plasmática (PRASAD, 2003). No entanto, a homeostase de zinco na presença de disfunção renal é pouco compreendida. Estudos apontam para a ocorrência de uma redistribuição tecidual de zinco nesta condição clínica, sendo a responsável pela hipozincemia (MAFRA & COZZOLINO, 2004; YONOVA; VAZELOV; TZATCHEV, 2012).

Assim, a fim de melhor compreender a homeostase de zinco na Doença Renal Crônica, torna-se apropriado estudar a expressão de genes transportadores de zinco, metalotioneína e interleucina-6 em um modelo experimental de DRC.

# 2. REVISÃO DA LITERATURA

## 2.1. Importância do Zinco

O zinco (Zn) é um metal de transição que difere dos demais por conter a camada eletrônica "d" completa. Com isso, não participa de reações redox, mas age como ácido de Lewis para aceitar um par de elétrons, tornando-se um íon estável. É encontrado na forma de 5 isótopos estáveis, que são eles: $^{64}Zn$, $^{66}Zn$, $^{67}Zn$, $^{68}Zn$, e $^{70}Zn$. Tem afinidade com grupos tióis e hidrogênio e, geralmente se complexa com aminoácidos, peptídeos e nucleotídeos (MCCALL; HUANG; FIERKE, 2000).

É um elemento essencial para a vida e necessário para o crescimento, pois participa de todas as etapas do ciclo celular, mantém a integridade estrutural das proteínas, regula a expressão gênica e é o componente essencial de aproximadamente 300 enzimas que participam de diferentes vias metabólicas (INSTITUTE OF MEDICINE, 2001).

O zinco teve sua essencialidade primeiramente demonstrada por Raulin em 1869, para o crescimento de *Aspergillus niger*. A partir daí, esse micronutriente tem-se mostrado em diversos tipos de estudo ser essencial para o crescimento, desenvolvimento e diferenciação de todos os tipos de vida, incluindo microrganismos, plantas, animais e humanos (SANDSTEAD, 1994). O reconhecimento do zinco como um micronutriente de grande importância na nutrição humana ocorreu em 1961 (HAMBIDGE, 2000). Contudo, ao contrário do ferro, que possui papéis fisiológicos determinados e distribuição celular conhecida, o zinco ainda não possui essas características bem determinadas. Apesar disso, o papel do zinco na biologia pode ser definido por três funções gerais, que são elas: catalíticas, estruturais e regulatórias (COUSINS, 1996).

## 2.2. Biodisponibilidade

A biodisponibilidade do zinco diz respeito à quantidade desse alimento que é ingerida, que fica retida e que é utilizada para a realização das funções fisiológicas, podendo ser determinada em indivíduos sadios por três fatores: o

estado nutricional do indivíduo relativo ao mineral, o conteúdo total de zinco da dieta e a disponibilidade do mineral na forma solúvel a partir dos componentes da dieta (LONNERDAL, 2000).

Este processo vai depender da forma ou estado em que se encontra o elemento químico. Minerais, tais como cobre, ferro, manganês e zinco, podem apresentar-se nas formas elementar, iônica, quelada ou ainda na forma de colóides e todas podem afetar a taxa de absorção. Alguns destes micronutrientes são alterados pelo conteúdo intestinal, assim, componentes da alimentação pode interagir com os minerais e aumentar, reduzir ou atrasar absorção dos mesmos (ANDERSON, 2004). Podem ocorrer também interações entre eles, ainda no lúmen intestinal, como as interações entre cátions divalentes, provocando a redução da absorção de alguns destes elementos, como é o caso do cálcio que diminui a absorção do zinco e do ferro, e deste último que pode também influenciar a biodisponibilidade do zinco (LONNERDAL, 2000).

Essas interações não ocorrem apenas com outros minerais, elementos presentes em produtos de origem vegetal, como os fitatos (hexafosfato de mioinositol), encontrados principalmente em cereais e legumes, quando em contato com o zinco, no lúmen intestinal, ligam-se de modo irreversível e são responsáveis pela menor eficiência da absorção do mesmo (LONNERDAL, 2000; MILLER; KREBS; HAMBIDGE, 2007).

## 2.3. Deficiência

A deficiência de zinco pode ser gerada a partir de causas isoladas ou combinadas, que incluem a ingestão inadequada do mineral, aumento das necessidades, má-absorção, aumento das perdas, entre outras. Porém, a deficiência primária de zinco (induzida pela dieta), ocorre quando há uma ingestão inadequada de zinco absorvível (SOLOMONS; JACOB, 1981).

A deficiência do micronutriente em animais e humanos pode causar dano tecidual, desde a peroxidação, citotoxicidade, apoptose, proliferação

tecidual diminuída, déficit imunológico incluindo desequilíbrios de citocinas e até alterações de desenvolvimento (FRAKER et al., 2000; KONDOH et al., 2002; KOSKI; SCOTT, 2001; RINK; KIRCHNER, 2000).

Grande parte de deficiência de zinco que acomete os seres humanos corresponde a uma deficiência marginal e responde à suplementação de zinco. A partir de estudos realizados em vários países, chegou-se a um consenso de que o crescimento de crianças em alguns grupos populacionais se beneficia da suplementação deste micronutriente. Estudos apontam ainda para uma melhoria do desempenho cognitivo e neuropsicológico mediante a suplementação com o mineral, porém, em outros estudos, a suplementação com o mineral não produziu reduções consideráveis nas taxas de morbidade e mortalidade infantis (BLACK, M. M., 2003; BLACK, R. E., 2003).

## 2.4. Absorção e Proteínas transportadoras de zinco

O zinco da dieta é absorvido principalmente no intestino delgado e excretado do organismo via secreções pancreática e intestinal (KING; SHAMES; WOODHOUSE, 2000; KREBS, 2000). Essa absorção ocorre a favor de um gradiente de concentração a partir da concentração luminal (relativamente alta) após uma refeição (EIDE, 2006; HOADLEY; LEINART; COUSINS, 1987). É considerável, o aumento da velocidade de absorção de zinco durante sua deficiência, sugerindo uma regulação aumentada do transporte do mineral quando o consumo do mesmo na dieta se apresenta menor do que as necessidades fisiológicas (LEE et al., 1993).

A captação de zinco pelo enterócito pode ocorrer de duas formas, por cinética saturável e/ou insaturável, sendo este último um processo mediado por carreadores, que são as proteínas transportadoras de zinco e que fazem tanto este transporte para dentro dos enterócitos quanto a compartimentalização vesicular do mineral dentro dos mesmos (DUFNER-BEATTIE et al. 2003a; FORD, 2004; STEEL; COUSINS, 1985).

Palmiter e Findley (1995) descreveram o primeiro gene relacionado com o transporte de zinco, em estudo com células renais, o ZnT1. Este, encontra-se localizado no cromossomo 1. Sua funcionalidade é expressivamente alterada de acordo com o *pool* de zinco intracelular. Assim, quando a concentração do micronutriente no citoplasma está aumentada, ocorre uma alta expressão do mesmo e consequentemente, a saída de zinco da célula, reduzindo sua concentração intracelular (MCMAHON; COUSINS, 1998).

Essas proteínas estão agrupadas em duas famílias, denominadas ZnT e ZIP e possuem vários membros (EIDE, 2004; PALMITER; HUANG, 2004). As proteínas ZnTs facilitam o efluxo do zinco ou através da membrana celular ou para dentro de vesículas intracelulares. Há pelo menos 10 proteínas transportadoras ZnT identificadas (ZnT1-ZnT10). As proteínas ZIPs, fazem o oposto, facilitando o influxo de zinco, ou para o interior das células ou a partir das vesículas. São conhecidas 15 proteínas transportadoras ZIP. Os genes de ambas as famílias exibem expressão tecidual específica. A localização subcelular não parece uniforme e sim dependente das condições fisiológicas e do estado nutricional relativo ao zinco (BOSOMWORTH, H. J. et al. 2012; DUFNER-BEATTIE et al., 2003a; LIUZZI et al., 2003).

As proteínas mais estudadas da família ZIP são a ZIP1, ZIP2, ZIP3 e ZIP4. A proteína ZIP1 encontra-se tanto na membrana plasmática quanto nas vesículas. Já as demais, parecem localizar-se apenas na membrana plasmática, porém, todas atuam na captação de zinco pela célula, sendo a ZIP1 capaz de fazer o efluxo de zinco das vesículas celulares (LIUZZI; COUSINS, 2004).

A descoberta das proteínas transportadoras de zinco proporcionou o conhecimento de que o metabolismo celular do mineral, em humanos, envolve a regulação dos genes referentes a essas proteínas por alguns fatores, como a dieta e hormônios/citocinas, especificidade tecidual de expressão e interações, bem como pelas mutações e polimorfismos nesses genes e suas consequências fenotípicas (EIDE, 2004).

O metabolismo do zinco é regulado principalmente através do controle intracelular e por meio dos transportadores. Análises em ratos, cujos genes dos transportadores de zinco foram geneticamente modificados, demonstraram que este fato fez com que os ratos apresentassem sintomas relacionados ao metabolismo anormal de zinco. Isto tem fornecido provas de que algumas dessas proteínas desempenham papéis cruciais na homeostase do mineral (KAMBE et al., 2008).

A proteína ZIP4 é descrita como a mais importante proteína em situações de baixas ingestões de zinco (LIUZZI et al., 2004). Sua localização celular é apical e foi demonstrado em um modelo murino, que a transfecção celular do gene ZIP4 aumentou a captação de zinco saturável, ao passo que o RNAm e a expressão da proteína encontravam-se supra-regulados em casos de deficiência murina de zinco (DUFNER-BEATTIE et al. 2003b). Esse quadro é compatível com o aumento homeostático no componente saturável da absorção de zinco durante as condições de deficiência (KURY et al., 2002; WANG et al., 2002).

Uma mutação no gene ZIP4 humano é responsável pelo surgimento de uma doença grave, denominada acrodermatite enteropática, caracterizada pela perda da capacidade de absorção de zinco (EIDE, 2006; KURY et al., 2002; WANG et al., 2002) Assim, a mutação leva a disfunções imunológicas e cognitivas responsivas ao zinco, demonstrando a importância da proteína ZIP4 na captação de zinco pelo organismo (ANDREWS, 2008). Porém, outros genes que codificam proteínas transportadoras de zinco são também expressos no intestino, indicando a participação de múltiplos transportadores na compartimentalização e/ou na liberação de zinco pelos enterócitos (LIUZZI et al., 2004).

Liuzzi et al. (2004), em estudo para verificar a importância do intestino e pâncreas na homeostase do zinco, quantificaram os níveis de mRNA das proteínas transportadoras de zinco e da metalotioneína, em camundongos alimentados com dietas com conteúdos de zinco reduzido, normal e aumentado. No intestino, observaram que, os níveis de mRNA do ZnT2, ZnT4,

ZnT5 e ZnT6 modificaram-se diretamente em função das concentrações de Zn da dieta. Além disso, verificaram que os níveis de mRNA do ZnT2 e ZnT4 e da metalotioneína foram os que mais se modificaram quando a dieta era reduzida em Zn. O estudo revelou ainda uma correlação negativa entre a expressão gênica do ZIP4 e a restrição do mineral na dieta.

A expressão do gene da metalotioneína no intestino também é responsiva ao zinco da dieta, influenciando a fase transcelular de absorção do zinco (COYLE; PHILCOX; ROFE, 1999). A metalotioneína atua como um tampão induzível de zinco que exibe rápida troca de ligante e pode influenciar o processo de transferência do mineral. Assim, como vários dos genes codificantes de proteínas transportadoras de zinco expressos em enterócitos demonstram responsividade à ingestão do micronutriente pela dieta, acredita-se na existência de um processo regulatório complexo para absorção do mineral (LIUZZI et al., 2004).

A expressão gênica dessas proteínas é também regulada por fatores não dependentes de zinco, do tipo inflamação (LIUZZI et al., 2005). De acordo com Vasto et al. (2006), este processo é sustentado pelo estado de hipozincemia gerado que por sua vez mantém ativada a via NF-kB e portanto, a inflamação. A reposição do micronutriente faz com que ocorra inibição desta via e desvia a resposta em direção anti-inflamatória.

Em 2005, Liuzzi et al., estudando o ZIP14 em camundongos, relataram que este transportador está relacionado com a hipozincemia e hipoferremia produzidas pela inflamação e infecção. O estudo mostrou também, tanto *in vivo* quanto *in vitro*, que a expressão aumentada do ZIP14 no fígado, em resposta à estas duas situações, é regulada pela interleucina-6, mostrando ser esta proteína transportadora de zinco, a maior responsável pela hipozincemia que acompanha a resposta de fase aguda para inflamação e infecção.

Aydemir et al. (2006) em estudo com humanos, observaram que a expressão das proteínas transportadoras de zinco e metalotioneína foi modificada nos três subgrupos de células brancas do sangue quando suplementou um grupo de adultos jovens sadios.

Foi demonstrado que as proteínas transportadoras de zinco são componentes essenciais do sistema que influencia a captação de zinco em situações de ingestão dietética reduzida do mineral ou em excesso, e também durante o estresse fisiológico agudo e crônico (LIUZZI et al., 2004).

## 2.5. Regulação homeostática

A manutenção do controle do zinco corporal total está intimamente relacionada com a eficiência da absorção, sendo esta, a principal forma de controla-la. A fração de zinco não absorvida e o zinco excretado constituem o débito fecal de zinco que está diretamente relacionado ao conteúdo desse elemento químico na dieta. Esse débito corresponde ao controle total sobre a eficiência de absorção e a regulação da secreção no trato gastrointestinal. Assim, quando a ingestão zinco encontra-se reduzida, a concentração intracelular reduzida aumenta a taxa de absorção, resultando em uma absorção fracional mais elevada (FUNG et al., 1997). Concomitantemente, há um declínio na secreção de Zn para o lúmen intestinal via secreções pancreáticas e a partir do intestino via fluxo transepitelial da serosa para a mucosa. Como a absorção adapta-se às necessidades fisiológicas, algumas condições de estresse, como doenças infecciosas, podem alterar a eficiência da absorção (BAER; KING, 1984).

As concentrações plasmáticas de zinco normalmente são mantidas dentro de limites estreitos. A redução no estado nutricional de zinco em seres humanos produz um declínio nesses níveis, mas os resultados não são constantes (KING, 1990). A doença aguda pode reduzir o Zn plasmático acentuadamente como resultado da resposta de fase aguda, porém a inanição aguda pode aumentar os níveis acima do normal em consequência da liberação tecidual de zinco devido o catabolismo (GORDON et al., 1982). No entanto, quando o consumo é maior que a ingestão diária recomendada (RDA), ocorre um aumento transitório no Zn plasmático (CAO; COUSINS, 2000).

## 2.6. Distribuição

O Zn plasmático encontra-se distribuído também entre proteínas. A albumina representa até 70% do Zn total no plasma. Em sua concentração plasmática normal, a albumina tem uma relação molar com o Zn de 40:1. A $\alpha_2$-macroglobulina também se liga ao Zn e representa grande parte do Zn ligado ao restante de proteína no plasma (LOWE et al., 1997).

Com relação ao zinco encontrado em células sanguíneas, este exibe uma concentração maior em leucócitos do que em eritrócitos. O zinco eritrocitário é encontrado principalmente na anidrase carbônica (>85%), na Cu-Zn superóxido dismutase e em outras proteínas, inclusive metalotioneína (GRIDER; BAILEY; COUSINS, 1990). As concentrações da proteína metalotioneína em populações de leucócitos humanos são mais baixos do que as dos eritrócitos, porém, os níveis de RNAm de MT podem ser medidos nos leucócitos. Esta abordagem tem permitido a medição direta da abundância do mRNA da MT em monócitos purificados (o tipo de leucócitos que tem a maior expressão MT), bem como em células mononucleares do sangue periférico (PBMC's) e em leucócitos obtidos a partir de indivíduos suplementados com zinco (CAO; COUSINS, 2000; COUSINS et al., 2003a). Os níveis de mRNA de MT e de outros genes relacionados ao metabolismo do zinco são bastante sensíveis a suplementação de zinco, sugerindo que leucócitos, principalmente monócitos, são um modelo atraente em estudos de suplementação com este mineral. Cousins et al. (2003a), demonstraram que os genes de PBMC's são muito sensíveis ao zinco e que os genes expressos em leucócitos circulantes podem responder aos níveis plasmáticos de zinco.

Acredita-se que o zinco que mantem as funções zinco-dependentes é mobilizado a partir de *pools* de zinco, pequenos e rapidamente modificáveis, encontrados principalmente no plasma e no fígado (MILLER et al., 1994).

O metabolismo do zinco é também influenciado por uma regulação hormonal, que faz com que ocorram flutuações transitórias nas concentrações de zinco plasmático, como a redução das concentrações no período pós-

prandial, provavelmente devido alterações na insulina e em outros hormônios induzidos pela refeição (KING et al., 1994). Os aumentos nos níveis de zinco durante o jejum intenso provavelmente são causados pelo catabolismo muscular influenciado também por mecanismos hormonais, com consequente liberação do mineral. O zinco plasmático também sofre uma redução transitória após o estresse agudo, como o que ocorre na presença de infecção, traumatismo e cirurgia, como mencionado anteriormente (HENKIN et al., 1984).

A hipozincemia associada ao estresse possui mecanismos parcialmente estabelecidos. A metalotioneína é induzível por muitos mediadores reduzindo os níveis de zinco plasmático. As citocinas, principalmente a interleucina-6, que desencadeia a resposta hepática de fase aguda, constituem os principais reguladores da expressão da metalotioneína. De modo semelhante, os genes responsivos de proteínas transportadoras de zinco complementam essa adaptação metabólica (HUBER; COUSINS, 1993).

O zinco não possui um local de armazenamento específico. Uma quantidade variável de zinco citosólico pode localizar-se em vesículas. Acredita-se que a quantidade de zinco livre nas células seja muito baixa (TRUONG-TRAN et al., 2000). A alta afinidade de ligação do zinco a ácidos nucléicos, grupos tióis proteicos e ligantes nitrogenados são responsáveis por essa baixa concentração (OUTTEN; O'HALLORAN, 2001). No entanto, os mecanismos de conservação são tão eficientes que os níveis de zinco são mantidos durante a restrição leve do mineral. Além dos mecanismos diretos de retenção de zinco pelo organismo, essa retenção também é realizada por mecanismos reciclagem (EMMERT; BAKER, 1995). Como a reciclagem do zinco ocorre por meio das hemácias à semelhança do ferro, o organismo necessita de suprimento diário de uma quantidade significativa de zinco para somar ao *pool* eritrocitário (DIAZ-GOMEZ et al., 2003).

A principal via de excreção de zinco no organismo é a secreção no trato gastrintestinal, processo este realizado pela secreção pancreática, pela descamação das células da mucosa do lúmen intestinal e pelo fluxo transepitelial de zinco da serosa para a mucosa intestinal (BAER; KING, 1984).

O Zn perdido nas secreções pancreáticas encontra-se ligado às metaloenzimas zinco-dependentes e é estimulado pelas refeições, estando a excreção gastrintestinal de zinco diretamente relacionada à ingestão de do minera pela dieta (BAER; KING, 1984). Já o débito urinário de Zn é baixo (< 1 mg/dia), sendo refratário à alteração em uma ampla variação de ingestão (4 a 25 mg/dia) e não traduz apenas a ingestão do mineral pela dieta, visto que a ocorrência de condições como inanição ou traumatismo e de outras condições que aumentam o catabolismo proteico muscular, aumenta excreção de zinco pela urina à medida que a carga de aminoácidos filtrados pelo rim aumenta (BAER; KING, 1984; JACKSON et al., 1984).

## 2.7. Avaliação do estado nutricional relativo ao zinco

Avaliar o real estado nutricional relativo ao zinco, ainda é um desafio, visto que a eficiente regulação da homeostasia do mesmo dificulta o diagnóstico dos casos de deficiência, além da não existência de um indicador confiável e reconhecido do estado nutricional de zinco em humanos, tornando diagnóstico definitivo possível apenas quando a deficiência de zinco é mais grave e os sintomas ficam aparentes. Assim, a possibilidade da deficiência de zinco em um indivíduo é geralmente avaliada a partir de relatos de baixa ingestão pela dieta, biodisponibilidade insatisfatória do mineral e sinais clínicos sugestivos, como retardo no crescimento, atraso na maturidade sexual, dermatite, déficit imunológico, entre outros. Aliado a isso, a mensuração de uma resposta funcional à suplementação com zinco confirma a deficiência, mas essa abordagem é limitada pelo custo da avaliação e pela necessidade de até vários meses para o registro seguro dessa resposta funcional, como é o caso do crescimento em crianças (INSTITUTE OF MEDICINE, 2001).

Como o zinco não possui um índice bem estabelecido que possa ser utilizado no laboratório clínico para avaliação do estado nutricional, torna-se atrativa a facilidade relativa das mensurações plasmáticas do mesmo. Contudo, como já citado anteriormente que o controle homeostático do zinco é eficiente,

os níveis plasmáticos desse elemento são mantidos dentro de limites estreitos em uma ampla variedade de níveis de ingestão (INSTITUTE OF MEDICINE, 2001).

A concentração plasmática de zinco varia entre 70 e 110 µg/dL. Sob condições experimentais, a restrição aguda de zinco na dieta em seres humanos reduz significativamente o zinco plasmático (GIBSON, 2005). Os efeitos pós-prandiais sobre os níveis plasmáticos de zinco e aqueles associados com doença aguda e crônica são fatores que limitam esse parâmetro como indicador (KING, 1990). Apesar da responsividade limitada desse índice, inúmeros estudos de intervenção utilizaram o zinco plasmático ou sérico.

Assim, pela inexistência de um marcador ideal para avaliar o estado nutricional de zinco e pelo fato da expressão de genes codificantes de proteínas transportadoras de zinco (ZIPs e ZnTs) e metalotioneína ser responsiva a esse elemento, Cousins et al. (2003b) sugeriu a utilização da expressão desses genes como abordagem para avaliar o estado nutricional deste mineral.

## 2.8. Doença Renal Crônica

A Doença Renal Crônica (DRC) é uma condição clínica que se caracteriza pela perda progressiva e irreversível da função renal, culminando no estágio 5 (último estágio), dependente de suporte dialítico e ou transplante renal (BRENNER; LAZARUS, 1992).

De acordo com o senso realizado anualmente pela Sociedade Brasileira de Nefrologia, o total de pacientes renais crônicos em tratamento dialítico vem crescendo. Em 2002, estimava-se em 54.523 a população de pacientes em diálise no Brasil, cerca de 19% desse total (10.285) encontrava-se na Região Nordeste. Já em 2012, o total de pacientes em diálise estava em torno de 100 mil (SOCIEDADE BRASILEIRA DE NEFROLOGIA, 2012).

Apesar dos avanços no tratamento da DRC, a morbimortalidade continua elevada. Por conseguinte, a sobrevida no primeiro ano não ultrapassa 79%, e estes índices caem de forma expressiva atingindo 41% no quinto ano de tratamento (CABRAL, 2005).

A inflamação da DRC acompanha a perda progressiva da filtração glomerular, tornando-se mais intensa ao atingir o estágio 5 da doença. Atualmente a DRC é considerada como a principal causa de doença inflamatória não infecciosa (NAVARRO et al., 2007).

Esse processo inflamatório leva a distúrbios no metabolismo de micronutrientes (GALLI, 2007). O ferro é o elemento mais estudado, com biodisponibilidade bem definida e com marcadores laboratoriais capazes de auxiliar na identificação de suas alterações (FLEMING; BACON, 2005).

Segundo Mafra e Cozzolino (1999), trabalhos realizados em pacientes renais crônicos indicam que o estado nutricional relativo ao zinco está inadequado, estando o organismo depletado deste mineral, ocorrendo, em alguns casos, os sintomas associados à deficiência deste micronutriente. Portanto, o conhecimento do metabolismo do zinco nesta enfermidade deve ser cada vez mais explorado, visto ser este mineral essencial para muitas atividades metabólicas do organismo, podendo ser responsável por complicações da doença em questão.

Dentro desse contexto, a literatura tem demonstrado que pacientes renais crônicos apresentam concentrações plasmáticas de zinco variando da normalidade à hipozincemia (KIMMEL et al., 1988; MARUMO et al., 1984). Estudos com pacientes em programa de hemodiálise demonstram a alta prevalência de hipozincemia de até 82% (AMORIM, 2008; ERTEN et al., 1998; KAMINSKA-GALWA et al., 1994; LEE et al., 2000; MAHAJAN et al., 1989; MENDES et al., 1988).

As baixas concentrações de zinco sérico detectadas nesses pacientes têm sido atribuídas à desnutrição, resultando na redução da ingestão do mineral, porém outros fatores parecem estar associados a este quadro, dentre estes, a habilidade do zinco em se ligar a proteínas que pode estar alterada na

uremia, resultando numa redução de zinco ionizado nesses pacientes (ERTEN et al., 1998).

Com base no exposto, torna-se interessante a realização de estudos com a finalidade de caracterizar o perfil de expressão de genes envolvidos no metabolismo do zinco em modelo experimental de Doença Renal Crônica a fim de tentar elucidar os mecanismos envolvidos na homeostase do mineral nesta condição. E, com isso, a possibilidade futura de alternativas terapêuticas para estes pacientes.

# 3. REFERÊNCIAS BIBLIOGRÁFICAS

AMORIM, A.C. **Espressão de genes codificantes de proteínas transportadoras de zinco e metalotioneína em pacientes hemodialisados**. Teresina: MCS-UFPI, 2008.

ANDERSON, J. J. B. Minerals. In: MAHAN, L. K.; ESCOTT-STUMP, S. **Krause's Food, Nutrition, & Diet Therapy**. 11th ed. Philadelphia: Saunders, 2004, p. 120–163.

ANDREINI, C. et al. Counting the zinc-proteins encoded in the human genome. **J Proteome Res,** v. 5, p. 196-201, 2006.

ANDREWS, G. K. Regulation and function of Zip4, the acrodermatitis enteropathica gene. **Biochem Soc Trans**, v. 36, p. 1242-6, 2008.

AYDEMIR, T. B.; BLANCHARD, R. K.; COUSINS, R. J. Zinc supplementation of young men alters metallothionein, zinc transporter, and cytokine gene expression in leukocyte populations. **Proc Natl Acad Sci U S A,** v. 103, n. 6, p. 1699-704, 2006.

BAER, M. T.; KING, J. C. Tissue zinc levels and zinc excretion during experimental zinc depletion in young men. **Am J Clin Nutr,** v. 39, n. 4, p. 556-70, 1984.

BLACK, M. M. The evidence linking zinc deficiency with children's cognitive and motor functioning. **J Nutr,** v. 133, n. 5 Suppl 1, p. 1473S-6S, 2003.

BLACK, R. E. Zinc deficiency, infectious disease and mortality in the developing world. **J Nutr,** v. 133, n. 5 Suppl 1, p. 1485S-9S, 2003.

BOSOMWORTH, H. J. et al. Efflux function, tissue-specific expression and intracellular trafficking of the Zn transporter ZnT10 indicate roles in adult Zn homeostasis. **Metallomics**, v. 4, n. 8, 2012.

BRENNER, B. M.; LAZARUS, J. M. Insuficiência renal crônica. In: WILSON, J. D.; BRAUNWALD, E. et al. eds. **Medicina Interna,** 12 ed. Rio de Janeiro: Guanabara Koogan, v. 8, p. 20-6, 1992.

CABRAL, P. C.; DINIZ, A. S.; ARRUDA, I. K. G. Avaliação nutricional de pacientes em hemodiálise. **Rev Nutr**, Campinas, v. 18, n. 1, p. 29-40, 2005.

CAO, J.; COUSINS, R. J. Metallothionein mRNA in monocytes and peripheral blood mononuclear cells and in cells from dried blood spots increases after zinc supplementation of men. **J Nutr,** v. 130, n. 9, p. 2180-7, 2000.

COUSINS, R. J. Zinc. In: FILER, L. J.; ZIEGLER, E. E. eds. Present Knowledge in Nutrition. 7th ed. **Intl Life Sci Inst** – Nutrition Foundation, Washington, DC, p. 293-306, 1996.

COUSINS, R. J. et al. A global view of the selectivity of zinc deprivation and excess on genes expressed in human THP-1 mononuclear cells. **Proc Natl Acad Sci U S A,** v. 100, n. 12, p. 6952-7, 2003a.

COUSINS, R. J. et al. Regulation of zinc metabolism and genomic outcomes. **J Nutr,** v. 133, n. 5 Suppl 1, p. 1521S-6S, 2003b.

COUSINS, R.J.; LIUZZI, J.P.; LICHTEN, L.A. Mammalian zinc transport, trafficking, and signals. **J Biol Chem**, v.281, p. 24085-9, 2006.

COYLE, P.; PHILCOX, J. C.; ROFE, A. M. Metallothionein-null mice absorb less Zn from an egg-white diet, but a similar amount from solutions, although with altered intertissue Zn distribution. **J Nutr,** v. 129, n. 2, p. 372-9, 1999.

DIAZ-GOMEZ, N. M. et al. The effect of zinc supplementation on linear growth, body composition, and growth factors in preterm infants. **Pediatrics,** v. 111, n. 5 Pt 1, p. 1002-9, 2003.

DUFNER-BEATTIE, J. et al. Structure, function, and regulation of a subfamily of mouse zinc transporter genes. **J Biol Chem,** v. 278, n. 50, p. 50142-50, 2003a.

DUFNER-BEATTIE, J. et al. The acrodermatitis enteropathica gene ZIP4 encodes a tissue-specific, zinc-regulated zinc transporter in mice. **J Biol Chem,** v. 278, n. 35, p. 33474-81, 2003b.

EIDE, D. J. The SLC39 family of metal ion transporters. **Pflugers Arch,** v. 447, n. 5, p. 796-800, 2004.

EIDE, D. J. Zinc transporters and the cellular trafficking of zinc. **Biochim Biophys Acta**, v. 1763, n. 7, p. 711-22, 2006.

EMMERT, J. L.; BAKER, D. H. Zinc stores in chickens delay the onset of zinc deficiency symptoms. **Poult Sci,** v. 74, n. 6, p. 1011-21, 1995.

ERTEN, Y. et al. Zinc deficiency: prevalence and causes in hemodialysis patients and effect on cellular immune response. **Transplant Proc,** v. 30, n. 3, p. 850-1, 1998.

FLEMING, R. E.; BACON, B. R. Orchestration of iron homeostasis. **N Engl J Med**, v.352, n.17, Apr 28, p.1741-4, 2005.

FORD, D. Intestinal and placental zinc transport pathways. **Proc Nutr Soc**, v. 63, n. 1, p. 21-9, 2004.

FRAKER, P. J. et al. The dynamic link between the integrity of the immune system and zinc status. **J Nutr,** v. 130, n. 5S Suppl, p. 1399S-406S, 2000.

FUNG, E. B. et al. Zinc absorption in women during pregnancy and lactation: a longitudinal study. **Am J Clin Nutr,** v. 66, n. 1, p. 80-8, 1997.

GALLI, F. Protein damage and inflammation in uraemia and dialysis patients. **Nephrol Dial Transplant**, v.22 Suppl 5, Jul, p.v20-36, 2007.

GIBSON, R. S. Assessment of chromium, copper and zinc status. In: GIBSON, R. S. **Principles of Nutritional Assessment**. 2nd ed. New York: Oxford University Press, p. 711-30, 2005.

GORDON, P. R. et al. Effect of acute zinc deprivation on plasma zinc and platelet aggregation in adult males. **Am J Clin Nutr,** v. 35, n. 1, p. 113-9, 1982.

GRIDER, A.; BAILEY, L. B.; COUSINS, R. J. Erythrocyte metallothionein as an index of zinc status in humans. **Proc Natl Acad Sci U S A,** v. 87, n. 4, p. 1259-62, 1990.

HAMBIDGE, M. Human zinc deficiency. **J Nutr,** v. 130, n. 5S Suppl, p. 1344S-9S, 2000.

HENKIN, R. I. et al. Zinc metabolism in adrenal cortical insufficiency: effects of carbohydrate-active steroids. **Metabolism,** v. 33, n. 6, p. 491-501, 1984.

HOADLEY, J. E.; LEINART, A. S.; COUSINS, R. J. Kinetic analysis of zinc uptake and serosal transfer by vascularly perfused rat intestine. **Am J Physiol,** v. 252, n. 6 Pt 1, p. G825-31, 1987.

HUBER, K. L.; COUSINS, R. J. Metallothionein expression in rat bone marrow is dependent on dietary zinc but not dependent on interleukin-1 or interleukin-6. **J Nutr,** v. 123, n. 4, p. 642-8, 1993.

INSTITUTE OF MEDICINE. Zinc. In: Dietary reference intakes for vitamin A, vitamin K, Arsenic, Boron, Chromium, cooper, iodine, iron, manganese, molybdenum, nickel, silicon, candium, and zinc. **Natl Aca Press**, p. 442-501, 2001.

JACKSON, M. J. et al. Zinc homeostasis in man: studies using a new stable isotope-dilution technique. **Br J Nutr,** v. 51, n. 2, p. 199-208, 1984.

KAMBE, T. et al. Slc39a1 to 3 (subfamily II) Zip genes in mice have unique cell-specific functions during adaptation to zinc deficiency. **Am J Physiol Regul Integr Comp Physiol,** v. 294, n. 5, p. R1474-81, 2008.

KAMINSKA-GALWA, B. et al. Influence of long-term hemodialysis on serum trace elements concentration in patients with chronic renal failure. **Przegl Lek,** v. 51, n. 1, p. 9-14, 1994.

KIMMEL, P. L. et al. Zinc balance in combined zinc deficiency and uremia. **Kidney Int,** v. 33, n. 6, p. 1091-9, 1988.

KING, J. C. Assessment of zinc status. **J Nutr,** v. 120 Suppl 11, p. 1474-9, 1990.

KING, J. C. et al. Daily variation in plasma zinc concentrations in women fed meals at six-hour intervals. **J Nutr,** v. 124, n. 4, p. 508-16, 1994.

KING, J. C.; SHAMES, D. M.; WOODHOUSE, L. R. Zinc homeostasis in humans. **J Nutr**, v. 130, n. 5S Suppl, p. 1360S-6S, 2000.

KONDOH, M. et al. Requirement of caspase and p38MAPK activation in zinc-induced apoptosis in human leukemia HL-60 cells. **Eur J Biochem,** v. 269, n. 24, p. 6204-11, 2002.

KOSKI, K. G.; SCOTT, M. E. Gastrointestinal nematodes, nutrition and immunity: breaking the negative spiral. **Annu Rev Nutr,** v. 21, p. 297-321, 2001.

KREBS, N. F. Overview of zinc absorption and ecretion in the human gastrointestinal tract. **J Nutr**, v. 130, n. 5S Suppl, p. 1374S-7S, 2000.

KURY, S. et al. Identification of SLC39A4, a gene involved in acrodermatitis enteropathica. **Nat Genet,** v. 31, n. 3, p. 239-40, 2002.

LEE, D. Y. et al. Homeostasis of zinc in marginal human zinc deficiency: role of absorption and endogenous excretion of zinc. **J Lab Clin Med,** v. 122, n. 5, p. 549-56, 1993.

LEE, S. H. et al. Trace Metals' abnormalities in hemodialysis patients: relationship with medications. **Artif Organs,** v. 24, n. 11, p. 841-4, 2000.

LICHTEN, P.; COUSINS, R. J. Mammalian zinc transporters: nutritional and physiologic regulation. **Annu Rev Nutr,** v. 29, p. 153-76, 2009.

LIUZZI, J. P. et al. Zinc transporters 1, 2 and 4 are differentially expressed and localized in rats during pregnancy and lactation. **J Nutr,** v. 133, n. 2, p. 342-51, 2003.

LIUZZI, J. P. et al. Responsive transporter genes within the murine intestinal-pancreatic axis form a basis of zinc homeostasis. **Proc Natl Acad Sci U S A,** v. 101, n. 40, p. 14355-60, 2004.

LIUZZI, J. P.; COUSINS, R. J. Mammalian zinc transporters. **Annu Rev Nutr,** v. 24, p. 151-72, 2004.

LIUZZI, J. P. et al. Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response. **Proc Natl Acad Sci U S A,** v. 102, n. 19, p. 6843-8, 2005.

LONNERDAL, B. Dietary factors influencing zinc absorption. **J Nutr,** v. 130, n. 5S Suppl, p. 1378S-83S, 2000.

LOWE, N. M. et al. A compartmental model of zinc metabolism in healthy women using oral and intravenous stable isotope tracers. **Am J Clin Nutr,** v. 65, n. 6, p. 1810-9, 1997.

MAFRA, D.; COZZOLINO, S. M. F. **Avaliação do estado nutricional relativo ao zinco em pacientes com insuficiência renal crônica.** São Paulo: FCF-USP, 1999, 95 p.

MAFRA, D.; COZZOLINO, S. M. F. The importance of zinc in human nutrition. **Rev Nutr**, v. 17, p. 79-87, 2004.

MAFRA, D. et al. Zinc levels after iron supplementation in patients with chronic kidney disease. **J Ren Nutr,** v. 14, p.164-9, 2004.

MAHAJAN, S. K. et al. Factors underlying abnormal zinc metabolism in uremia. **Kidney Int Suppl,** v. 27, p. S269-73, 1989.

MARUMO, F. et al. Trace element concentrations in hair, fingernails and plasma of patients with chronic renal failure on hemodialysis and hemofiltration. **Nephron,** v. 38, n. 4, p. 267-72, 1984.

MCCALL, K. A., HUANG, C., FIERKE, C. A. Function and mechanism of zinc metalloenzymes. **J Nutr**, v. 130, n. 5S Suppl, p. 1437S-6S, 2000.

MCMAHON, R. J.; COUSINS, R. J. Mammalian zinc transporters. **J Nutr,** v. 128, n. 4, p. 667-70, 1998.

MENDES, V. et al. [Serum zinc in patients undergoing chronic hemodialysis]. **Rev Hosp Clin Fac Med Sao Paulo,** v. 43, n. 5, p. 225-7, 1988.

MILLER, L. V. et al. Size of the zinc pools that exchange rapidly with plasma zinc in humans: alternative techniques for measuring and relation to dietary zinc intake. **J Nutr,** v. 124, n. 2, p. 268-76, 1994.

MILLER, L. V., KREBS, N. F., HAMBIDGE, K. M. A mathematical model of zinc absorption in humans as a function of dietary zinc and phyate. **J Nutr**, v. 137, n. 1, p. 135-41, 2007.

NAVARRO, M. D. et al. Bacterial DNA prolongs the survival of inflamed mononuclear cells in haemodialysis patients. **Nephrol Dial Transplant**, v.22, n.12, Dec, p.3580-5, 2007.

OUTTEN, C. E.; O'HALLORAN, T. V. Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. **Science,** v. 292, n. 5526, p. 2488-92, 2001.

PALMITER, R. D.; FINDLEY, S. D. Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. **EMBO J,** v. 14, n. 4, p. 639-49, 1995.

PALMITER, R. D.; HUANG, L. Efflux and compartmentalization of zinc by members of the SLC30 family of solute carriers. **Pflugers Arch,** v. 447, n. 5, p. 744-51, 2004.

PRASAD, A. S. Zinc deficiency in women, infants and children. **J Am Coll Nutr**, v.15, n.2, p.113-20, 1996.

PRASAD, A. S. Zinc deficiency. **Bmj,** v. 326, p. 409-10, 2003.

RINK, L.; KIRCHNER, H. Zinc-altered immune function and cytokine production. **J Nutr,** v. 130, n. 5S Suppl, p. 1407S-11S, 2000.

SANDSTEAD, H. H. Understanding zinc: recent observations and interpretations. **J Lab Clin Med**, v. 124, n. 3, p.322-7, 1994.

SOCIEDADE BRASILEIRA DE NEFROLOGIA. IN: Censo de dialise SBN 2012. Disponível em: www.sbn.org.br. Acesso em 13/01/2014.

SOLOMONS, N. W.; JACOB, R. A. Studies on the bioavailability of zinc in humans: effects of heme and nonheme iron on the absorption of zinc. **Am J Clin Nutr,** v. 34, n. 4, p. 475-82, 1981.

STEEL, L.; COUSINS, R. J. Kinetics of zinc absorption by luminally and vascularly perfused rat intestine. **Am J Physiol**, v. 248, p. G46-53, 1985.

TRUONG-TRAN, A. Q. et al. Cellular zinc fluxes and the regulation of apoptosis/gene-directed cell death. **J Nutr,** v. 130, n. 5S Suppl, p. 1459S-66S, 2000.

VASTO, S. et al. Inflammation, genes and zinc in ageing and age-related diseases. **Biogerontology,** v. 7, n. 5-6, p. 315-27, 2006.

WANG, K. et al. A novel member of a zinc transporter family is defective in acrodermatitis enteropathica. **Am J Hum Genet,** v. 71, n. 1, p. 66-73, 2002.

YONOVA, D.; VAZELOV, E.; TZATCHEV, K. Zinc status in patients with chronic renal failure on conservative and peritoneal dialysis treatment. **Hippokratia**, v. 16 p. 356-9, 2012.

# 4. OBJETIVOS

GERAL:

- Caracterizar o perfil de expressão de genes que codificam proteínas transportadoras de zinco, metalotioneína e interleucina-6 em tecidos de ratos renais crônicos submetidos a dietas com diferentes níveis de zinco.

ESPECÍFICOS:

- Verificar a expressão de genes que codificam proteínas transportadoras de zinco, metalotioneína e interleucina-6 em células mononucleares (PBMC's) de ratos renais crônicos submetidos a dietas depletada, normal e repletada em zinco.
- Determinar o perfil de expressão de genes que codificam proteínas transportadoras de zinco, metalotioneína e interleucina-6 no intestino e no fígado de ratos renais crônicos submetidos a dietas depletada, normal e repletada em zinco.

# 5. ARTIGOS

## 5.1. Artigo 1

THE JOURNAL OF NUTRITION
Official Publication of the American Society for Nutrition

The Journal of Nutrition
NUTRITION/2014/191544
Version 1

# Expression of zinc transporters genes, metallothionein and IL-6 in an experimental model of Chronic Kidney Disease

Amanda Castro Amorim, Juan P Liuzzi, Rafael Melo Santos de Serpa Brandão, Anatalia Labilloy, Mirian Aparecida Boim, Maria Helena Bellini, Jose Machado Moita Neto, Adalberto Socorro Silva, and Semiramis Jamil Hadad Monte

Artigo Submetido: **The Journal of Nutrition**

Fator de Impacto – **JCR 2012: 4,2**

# Expression of zinc transporters genes, metallothionein and IL-6 in an experimental model of Chronic Kidney Disease

Amanda C Amorim [1*], Juan P Liuzzi[2], Rafael MSS Brandão[1], Anatalia Labilloy[3], Miriam A Boim[4], Maria H Bellini[4], Jose M Moita Neto[5], Adalberto S Silva[1], Semiramis JH Monte[1]

1. Immunogenetics and Molecular Biology Laboratory, Federal University of Piauí, Teresina, PI, Brazil

2. Department of Dietetics and Nutrition, Florida International University, Miami, FI, USA

3. Renal-Electrolyte Division, University of Pittsburgh, Pittsburgh, PA, USA

4. Federal University of Sao Paulo – UNIFESP, São Paulo, SP, Brazil

5. Departament of Chemistry, Federal University of Piaui, Teresina, PI, Brazil

Correspondingauthor*

Amanda de Castro Amorim

Laboratório de Imunogenética e Biologia Molecular – LIB - SG-16

Campus Universitário Ministro Petrônio Portella

Bairro Ininga, Teresina – PI

CEP: 64049-550

Phone: 55 86 32155691

Fax: 55 86 32155690

Email: amandacastronut@yahoo.com.br

List of authors: Amorim AC, Liuzzi JP, Brandão RMSS, Labilloy A, Boim MA, Bellini MH, Moita Neto JM, Silva AS, Monte SJH.

Word count: 2747 words

Numbers of figures: 02

Numbers of Table: 01

Running Title: mRNA of zinc transporters, MT and IL-6 in CKD

Footnotes:

(a) This work was supported only by the Immunogenetics and Molecular Biology Laboratory of the Federal University of Piauí

(b) Amorim AC, Liuzzi JP, Brandão RMSS, Labilloy A, Boim MA, Bellini MH, Moita Neto JM, Silva AS, Monte SJH, no conflicts of interest.

Abstract

Zinc (Zn) is a trace element that plays a key role in the chemistry of life. Given its indispensable but potentially toxic nature, it is not surprising that the levels of intracellular zinc are tightly controlled. Zinc deficiency is a highly prevalent disorder among early stage Chronic Kidney Disease (CKD) and/or in renal replacement therapy patients, but micronutrient supplementation for CKD patients remains controversial. We determined the mRNA expression of the zinc transporters Zip1, 3, 8 and 14, ZnT1, 4, 5 and 6 and metallothionein (MT) as well as of IL-6 in PBMC's tissue from healthy control rats and CKD rats before and after 15 days of diet depleted (Zn- ; <1 ppm), normal (ZnN; 50 ppm), or repleted (Zn+; normal Zn diet + Zn 5mg/kg). The baseline expression of the studied genes in CKD rats in relation to healthy controls, showed a significant decrease in expression of Zip3 in CKD rats. After 15 days of diet depleted in zinc, Zip3 and Zip14 were expressed in CKD rats at significantly higher levels than baseline. On the other hand, when CKD rats were fed diet with zinc in excess, we observed a dramatic increase in expression of all ZnT genes. Comparison between CKD rats undergoing zinc diets Zn- and Zn+ 15 days post their introduction has shown significant changes in expression of assessed genes, including IL-6 e MT. When we compared CKD rats to healthy control rats after Zn diet protocols, Zip14 was differentially expressed in CKD zinc depleted rats, being upregulated in CKD rats. Since CKD is a inflammatory condition, it becomes evident that zinc plays a key role in regulating such condition.

## INTRODUCTION

Zinc (Zn) is a trace element that plays a key role in the chemistry of life, being a cofactor for several numerous enzymes and transcription factors and working as an intracellular mediator (1,2). Given its indispensable but potentially toxic nature, it is not surprising that the levels of intracellular zinc are tightly controlled (3). Physiologically, free zinc is mainly regulated at the single cellular level, and the cells employ an intricate homeostasis that involves several proteins (2,4).

Two main protein families that have been implicated in Zn homeostasis include the ZnT and Zip transporters. ZnT [solute-linked carrier 30 (Slc30a)] proteins reduce the intracellular zinc levels by mediating zinc efflux from cells or influx into intracellular vesicles. Zip [Zrt- and Irt-like proteins (Slc39a)] proteins promote zinc transport from the extracellular fluid or intracellular vesicles into the cytoplasm (4-8). Chelation of the cytoplasmic pool of zinc is performed by the cysteine-rich protein metallothionein (MT) (9). Spikes in the concentration of Zn, which can be driven by acute or chronic variations in dietary Zn intake or systemic conditions (10,11), induces changes in the activation pattern of Zn-responsive transcription factors, culminating into modified expression of Zinc transporters and intracellular chelators (12). This mechanism works as a protective tool to deleterious variations in intracellular Zn (13).

Zinc deficiency is a highly prevalent disorder among early stage Chronic Kidney Disease (CKD) and/or in renal replacement therapy patients (14-19). Insufficient dietary intake, malnourishment, drug-nutrient interaction, subclinical

inflammatory state and mal-absorption are usually pointed as the main factors leading to this hypozincemia (15,20). However zinc homeostasis under condition of kidney dysfunction is still not well understood (21,22).

Micronutrient supplementation for CKD patients remains controversial. While some studies show that zinc supplementation improves symptoms related to hypozincemia (17,21,23), other authors point to a potential risk of toxicity induced by mineral supplementation (24), since excess intracellular zinc can be present despite hypozincemia. Thus in order to better understand zinc homeostasis under these conditions, it is appropriate to study the expression of zinc transporter genes, MT and IL-6 in a non-dialysis CKD experimental model.

## METHODS

### Animals

All experimental procedures were conducted according to the National Institutes of Health guidelines for the use and care of animals, and the study protocol was approved by the Institutional Review Board at the Federal University of Piauí (UFPI) (process N°. 004/13). Fourty-two male adult Wistar rats were group housed, given access to rat chow and water *ad libitum* and maintained in a temperature-controlled environment (23 °C) on a 12-hour light/dark cycle.

### Experimental Protocols

After a seven-day adaptation period, 30 male adult rats were randomly submitted to a five-sixths nephrectomy (5/6Nx) under anesthesia with ketamine (100 mg/Kg i.p.) plus xylazine (10 mg/Kg i.p.). After ventral laparotomy, the right

kidney was removed and two branches of the left renal artery were ligated in order to achieve infarction of two-thirds of the left kidney. CKD status was confirmed by increased serum creatinine levels and the presence of proteinuria. Dietary protocols were initiated thereafter.

**Dietary protocols**

CKD and healthy control rats were allocated into three different groups according to zinc supplementation: [Zn- (<1 ppm), n=4; Zn normal (ZnN; 50 ppm), n=3; or $Zn^{+}$ (normal Zn diet + Zn 5mg/kg by gavage), n=4], which represent depleted, adequate, or repleted intakes of Zn, respectively. Rats were fed individually during 15 days.

**RNA extraction**

Peripheral blood mononuclear cells (PBMCs) were collected by puncture of the retro-orbital plexus and total cellular RNA was extracted using TRIzol reagent according to the manufacturer's protocol (Gibco BRL, Life Technologies, Roskilde, Denmark). RNA was quantified by NanoDrop spectrophotometer and only samples with a purity of 1.8 or higher in the 260 nm and 280 nm scale were accepted. Single-stranded cDNA synthesis was performed using 100 ng of total RNA through using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems), according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA).

**Real-Time PCR**

Oligonucleotide primers sequences for the genes Zip1, Zip3, Zip8, Zip14, IL-6, ZnT1, ZnT4, ZnT5, ZnT6 and MT are shown in **Table 1**. Power SYBR green PCR master mix (Applied Biosystems) was added to experimental wells in triplicate containing cDNA and primers according to the manufacturer's protocol. SYBR green fluorescence was read using an ABI prism 7500 real-time PCR system (Applied Biosystems) under standard cycling mode. Cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min and 45 cycles of 95°C for 15s, followed by 60°C for 1 min. Relative amount of copies was calculated using the comparative Ct method, having β-actin as the reference gene.

**Table 1.** Primers used in this study.

| Genes | Foward | Reverse |
|---|---|---|
| ZIP1 | 5'- AAGCCTAGTGAGCTGCTTCG -3' | 5'-ATGGCCAGGATGAACTCTTG-3' |
| ZIP3 | 5'-CGTCTTCCTGGCTACATGCT-3' | 5'-TCCACGAACACAGTGAGGAA-3' |
| ZIP8 | 5'-CCAGATAACCAGCTCGAACTTCA-3' | 5'-TGTGGATCCTCACAGGGATGA-3' |
| ZIP14 | 5'-CCTCACGAGCTGGGAGACTTC-3' | 5'-AGAGGGCCTGCTGGATACTCA-3' |
| ZnT1 | 5'-ATCCAGCCTGAATTCGCTAGC-3' | 5'-CGTGTCCCACAGCACTGCT-3' |
| ZnT4 | 5'-GCTGAAGCAGAGGAAGGTGAA-3' | 5'-TCTCCGATCATGAAAAGCAAGTAG-3' |
| ZnT5 | 5'-TGGCTAAGATGGCCGAACAC-3' | 5'-CCAGGAAGGCGATAGCTGTATAAA-3' |
| ZnT6 | 5'-TCCCAGGACTCAGCAGTATCTTC-3' | 5'-GCCCCAGCAAGATCGATCAG-3' |
| IL-6 | 5'-CTTCCAGCCAGTTGCCTTCTTG-3' | 5'-GGTCTGTTGTGGGTGGTATCCTC-3' |
| MT | 5'-TGCAAGAAAAGCTGCTGTTCC-3' | 5'-GTCCGAAGCCTCTTTGCAGAT-3' |
| β-actin | 5'-TGCCCTAGACTTCGAGCAAG-3' | 5'-GGCAGCTCATAGCTCTTCTCC-3' |

## StatisticalAnalysis

Data are expressed as mean ± SEM. Normality was tested using Kolmogorov-Smirnov. For normally distributed samples, intra-group comparison was performed using paired t-test, while intergroup comparison was done with independent samples t-test. For parameters that did not follow normality, Mann-Whitney was used. For analysis of normalized fold change differences, confidence interval test was used. The significance level was set at $p < 0.05$.

## RESULTS

We investigated the mRNA expression of the zinc transporters Zip1, 3, 8 and 14, ZnT1, 4, 5 and 6 and metallothionein as well as of IL-6 in PBMC's tissue from health control rats as well as rats that underwent 5/6 nephrectomy-ligation (CKD model) before and after 15 days of diet depleted (Zn- ; <1 ppm), normal (ZnN; 50 ppm), or repleted ($Zn^{+}$; normal Zn diet + Zn 5mg/kg). These zinc transporters genes were selected due to their role in zinc transport and homeostasis and/or because they had been previously shown to be responsive to changes in dietary zinc.

### ***Baseline expression of ZIPs, ZnTs, MT and IL6***

All examined genes were expressed at detectable levels in PBMC's tissue. We induced CKD state in previously healthy rats through the 5/6 nephrectomy-ligation model. When comparing relative baseline expression of the studied genes in CKD rats in relation to healthy controls, we saw a significant decrease in expression of Zip3 in CKD rats. ($p<0.05$).

***Expression ofZIPs, ZnTs, MT and IL6 in healthy rats under zinc dietary protocols***

We examined expression of the studied genes after 15 days of introduction of zinc dietary protocols, and normalized gene expression to values of rats under diet adequate in zinc. Healthy rats under a zinc depleted diet showed increased expression of Zip1 and Zip8. No significant changes were seen in the expression pattern of genes of the ZnT family nor in MT. On the other hand, regarding healthy rats receiving a zinc repleted diet, we observed a decreased expression of Zip1 and Zip14 and an increased expression of ZnT1, 5 and 6. (**Figure 1**) When assessing differences in expression of the studied genes between zinc dietary protocols (i.e. Zn- vs Zn+) we observed a similar pattern of changes in gene expression as seen between baseline and 15 days after dietary protocols, with significant difference in expression of Zip1, Zip8, ZnT1, ZnT5 and ZnT6 (**Figure 2**).

***Expression of Zips, ZnTs, MT and IL-6 in CKD rats under zinc dietary protocols***

In addition, we assessed the expression pattern of the zinc transporters and the inflammatory marker IL-6 in CKD rats undergoing the same zinc dietary protocols. After 15 days of diet depleted in zinc, Zip3 and Zip14 were expressed at significantly higher levels than baseline. On the other hand, when CKD rats were fed diet with zinc in excess for 15 days, we observed a dramatic increase in expression of all ZnT genes (Figure 1). Strikingly, comparison between rats undergoing zinc diets Zn- and Zn+ 15 days post their introduction

has shown significant changes in expression of all assessed genes, with exception of Zip8 (Figure 2). Interestingly, when we compared CKD rats to healthy control rats after Zn diet protocols, Zip14 was differentially expressed in CKD zinc depleted rats compared to healthy controls following the same dietary protocol, being upregulated in CKD rats (data not shown, p=0.036).

## DISCUSSION

While regulation of zinc homeostasis related to zinc intake is well described in rodents under physiological conditions (8), to the best of our knowledge, this is the first study to assess variations in expression of zinc transporters in CKD.

The major difference we found between healthy controls and CKD rats when comparing mRNA levels of zinc transporters under baseline conditions was a downregulation of ZIP3. ZIP3 encodes for a membrane protein involved in zinc transport from the extracellular space to the cytoplasm, and its expression is tightly regulated by intracellular zinc levels. (25,26) Thus, we postulate that such decrease in mRNA levels could reflect increased zinc in cytoplasm.

The fact that feeding CKD rats with a diet poor in zinc reverted ZIP3 mRNA expression pattern corroborates with our hypothesis. Accordingly, CKD rats undergoing a diet with excess zinc showed increased expression of Znts. Since the Znt gene family encodes for proteins involved in zinc transport from the cytoplasm to the extracellular space and organelles, (8) their upregulation might reflect additional proof that CKD rats need to diminish cytoplasmic Zinc.

It was interesting to observe that dietary zinc privation lead to augmented IL-6 mRNA levels, specially by the fact that IL-6 appears to regulate the expression for both ZIP14 (27,28) and MT (29,30) genes. Here, augmented IL-6 mRNA levels was followed by in the increase of ZIP14 mRNA levels in rats with a zinc depleted dietary, when compared to zinc repleted ones. On the other hand, MT mRNA levels were higher in zinc repleted than in zinc depleted CKD rats.

At the end of the dietary protocol it was possible to observe a difference of the expression of ZIP14 gene in rats with diet depleted in zinc between CKD rats and healthy controls. Here, it seems that the zinc depleted diet was the stress factor or at least an additive factor for the inflammation be evidenced in CKD. Since CKD is a inflammatory condition, it becomes evident that zinc plays a key role in regulating such condition, as happens for others inflammatory conditions.

It is suggested to evaluate the expression of zinc transporters genes as a new approach for assessing the nutritional status of zinc (31). However, more studies should be conducted using the CKD model to see how the expression of zinc transporter genes, MT and inflammation markers behaves in important tissues for the regulation of zinc homeostasis, such as the intestine and liver in an attempt to confirm this differential expression found here for CKD.

**Acknowledgments**

The authors would like to sincerely thank Luciana Cristina Teixeira for your help in conducting the rats nephrectomy. The authors also thank the Renorbio

Postgraduate Program and the Federal University of Piauí for their research incentive in the northeastern region of Brazil.

**Statement of author's contributions to manuscript**

A.C.A. and S.J.H.M designed research; A.C.A., R.M.S.S.B., M.A.B., M.H.B. conducted research; A.C.A., J.P.L., R.M.S.S.B., A.L., J.M.M.N., A.S.S. and S.J.H.M. analyzed data; ; A.C.A., R.M.S.S.B., A.L., A.S.S. and S.J.H.M. wrote the paper; S.J.H.M. had primary responsibility for final content. All authors read anda pproved the final manuscript.

## LITERATURE CITED

1. Prasad AS. Zinc deficiency in human subjects. ProgClinBiol Res. 1983;129:1-33.

2. Rink L, Haase H. Zinc homeostasis and immunity. TrendsImmunol. 2007;28:1-4.

3. Outten CE, O'halloran TV. Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science. 2001;292:2488-92.

4. Cousins RJ, Liuzzi JP, Lichten LA. Mammalian zinc transport, trafficking, and signals. J BiolChem. 2006;281:24085-9.

5. Liuzzi JP, Cousins RJ. Mammalian zinc transporters. AnnuRev Nutr. 2004;24:151-72.

6. Palmiter RD, Huang L. Efflux and compartmentalization of zinc by members of the SLC30 family of solute carriers. PflugersArchiv. 2004;447:744-51.

7. Eide DJ. The SLC39 family of metal ion transporters. PflugersArchiv. 2004;447:796-800.

8. Lichten LA, Cousins RJ. Mammalian zinc transporters: nutritional and physiologic regulation. AnnuRev Nutr.2009;29:153-76.

9. Andreini C, Banci L, Bertini I, Rosato A. Counting the zinc-proteins encoded in the human genome. J Proteome Res. 2006;5: 196-201.

10. Stefanidou M, Maravelias C, Dona A, Spiliopoulou C. Zinc: a multipurpose trace element. ArchToxicol.2009; 80:1-9.

11. Maret W, Li Y. Coordination dynamics of zinc in proteins. Chem Rev. 2009;109:4682-707.

12. Thirumoorthy N, Shyam Sunder A, Manisenthil Kumar K, Senthil Kumar M, Ganesh G, Chatterjee M. A review of metallothionein isoforms and their role in pathophysiology. World J SurgOncol. 2011;9:54.

13. Wright RO, Baccarelli A. Metals and neurotoxicology. J Nutr. 2007;137:2809-13.

14. Aggett PJ. Zinc metabolism in chronic renal insufficiency with or without dialysis therapy. ContribNephrol. 1984;38:95-102.

15. Sandstead HH. Trace elements in uremia and hemodialysis. Am J Clin Nutr. 1980;33:1501-8.

16. Smythe WR, Alfrey AC, Craswell PW, Crouch CA, Ibels LS, Kubo H, Nunnelley LL, Rudolph H. Trace element abnormalities in chronic uremia. Ann Intern Med. 1982;96:302-10.

17. Atkin-Thor E, Goddard BW, O'nion J, Stephen RL, Kolff WJ. Hypogeusia and zinc depletion in chronic dialysis patients. AmJClin Nutr. 1978;31:1948-51.

18. Antoniou LD, Shalhoub RJ, Sudhakar T, Smith Jr. JC. Reversal of uraemic impotence by zinc. Lancet. 1977;2:895-8.

19. Mafra D, Cuppari L, Favaro DI, Cozzolino SM. Zinc levels after iron supplementation in patients with chronic kidney disease. J Ren Nutr. 2004;14:164-9.

20. Prasad AS. Zinc deficiency. Bmj. 2003;326:409-410.

21. Mahajan SK, Prasad AS, Rabbani P, Briggs WA, Mcdonald FD. Zinc deficiency: a reversible complication of uremia. Am J Clin Nutr.1982;36:1177-83.

22. Antoniou LD, Shalhoub RJ, Schechter GP. The effect of zinc on cellular immunity in chronic uremia. Am J Clin Nutr. 1981;34:1912-7.

23. Mahajan SK, Abbasi AA, Prasad AS, Rabbani P, Briggs WA, Mcdonald FD. Effect of oral zinc therapy on gonadal function in hemodialysis patients. A double-blindstudy. Ann Intern Med. 1982;97:357-61.

24. Maret W, Sandstead HH. Zinc requirements and the risks and benefits of zinc supplementation. J Trace ElemMed Biol. 2006;20:3-18.

25. Eide DJ. Zinc transporters and the cellular trafficking of zinc. BiochimBiophys Acta. 2006; 1763:711-22.

26. Wang F, Dufner-Beattie J, Kim BE, Petris MJ, Andrews G, Eide DJ. Zinc-stimulated endocytosis controls activity of the mouse ZIP1 and ZIP3 zinc uptake transporters. J BiolChem. 2004;279:24631-9.

27. Liuzzi JP, Lichten LA, Rivera S, Blanchard RK, Aydemir TB, Knutson MD, Ganz T, Cousins RJ. Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response. ProcNatlAcadSci U S A. 2005;102;6843-8.

28. Liuzzi JP, Aydemir F, Nam H, Knutson MD, Cousins RJ. Zip14 (Slc39a14) mediates non-transferrin-bound iron uptake into cells. ProcNatlAcadSci U S A. 2006;103:13612-7.

29. Kobayashi K, Kuroda J, Shibata N, Hasegawa T, Seko Y, Satoh M, Tohyama C, Takano H, Imura N, Sakabe K, et al. Induction of metallothionein by manganese is completely dependent on interleukin-6 production. J PharmacolExpTher.2007;320:721-7.

30. Hernandez J, Carrasco J, Belloso E, Giralt M, Bluethmann H, Kee Lee D, Andrews GK, Hidalgo J. Metallothionein induction by restraint stress: role of glucocorticoids and IL-6. Cytokine. 2000;12:791-6.

31. Cousins RJ, Blanchard RK, Moore JB, Cui L, Green CL, Liuzzi JP, Cao J, Bobo JA. Regulation of zinc metabolism and genomic outcomes. J Nutr. 2003;133;1521S-6S.

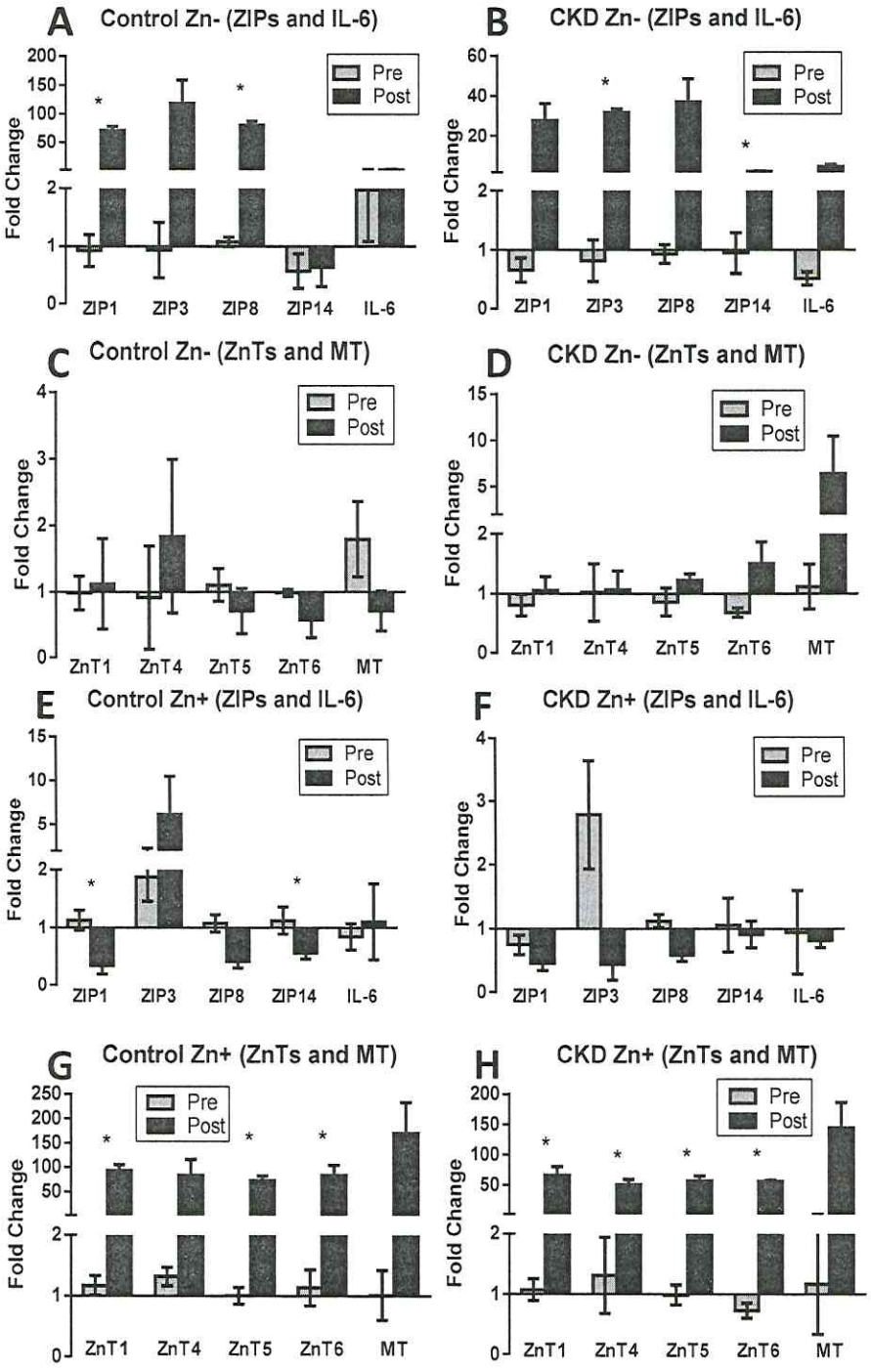



**Figure 1.Zinc transporters and IL-6 are differentially expressed upon changes in zinc diet.** Expression ofZnTs, MT, ZIPs, IL-6 in healthy control and CKD rats before (pre) and after 15 days (post) of diet depleted (Zn-) or repleted (Zn+) in zinc using Real-time PCR. Values are expressed in induced fold change normalized against adequate zinc diet.* $p<0.05$.

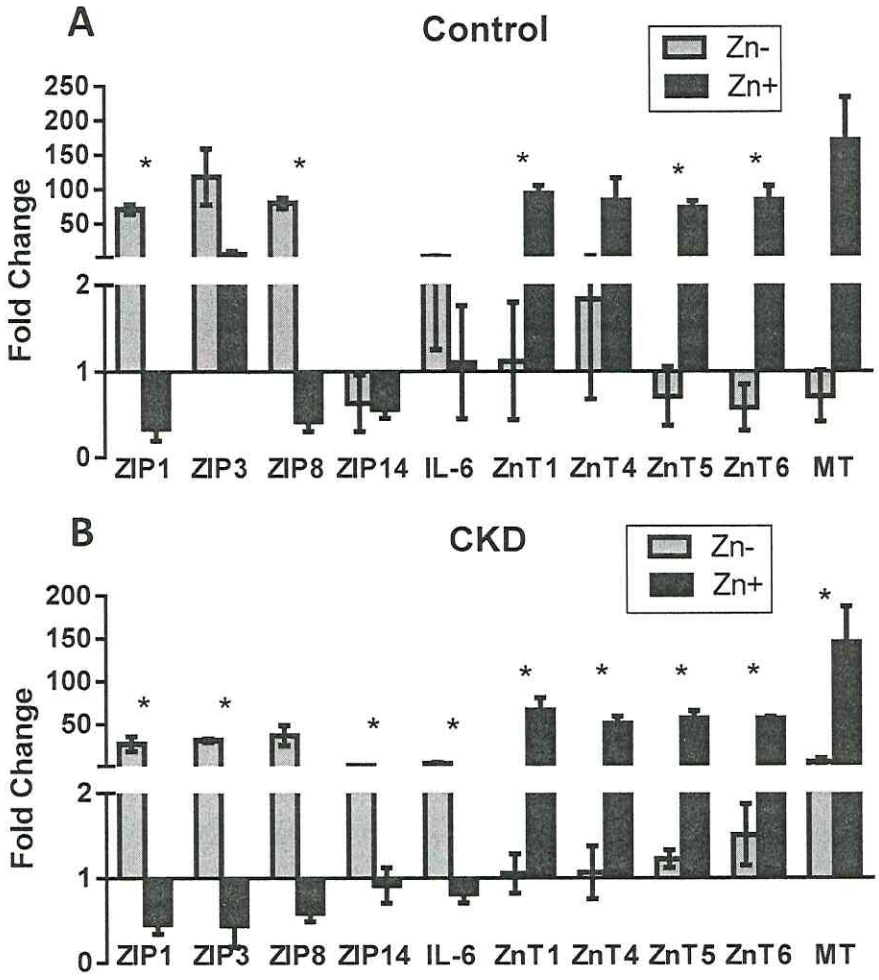



**Figure 2.ZIP14, ZnT4 and IL-6 are differentially expressed in CKD rats undergoing zinc depleted diet.** Relative expression of ZIPs, IL-6, ZnTs and MT in healthy control and CKD rats that underwent 15 days of diet depleted (Zn-) or repleted (Zn+) in zinc normalized to normal zinc diet (ZnN) using Real-Time PCR. Values are expressed in inducedfoldchange. * $p<0.05$.

## 5.2. Artigo 2

Artigo Submetido: **Genes and Nutrition**

Fator de Impacto – **JCR 2012: 3,3**

# Differential tissue-specific expression of zinc transporters, MT and IL-6 genes in an experimental Chronic Kidney Disease model.

Amanda de Castro Amorim [1*], Rafael Melo Santos de Serpa Brandão[1], Antonio Vanildo de Sousa Lima[1], Miriam Aparecida Boim[2], Maria Helena Bellini[2], Jose Machado Moita Neto[3], Adalberto Socorro Silva[1], Semiramis Jamil Hadad do Monte[1]

1. Immunogenetics and Molecular Biology Laboratory, Federal University of Piauí, Teresina, PI, Brazil

2. Federal University of Sao Paulo – UNIFESP, São Paulo, SP, Brazil

3. Departament of Chemistry, Federal University of Piaui, Teresina, PI, Brazil

Amanda de Castro Amorim

Address: Laboratório de Imunogenética e Biologia Molecular – LIB - SG-16

Campus Universitário Ministro Petrônio Portella

Bairro Ininga, Teresina – PI

CEP: 64049-550

Rafael Melo Santos de Serpa Brandão

Address: Laboratório de Imunogenética e Biologia Molecular – LIB - SG-16

Campus Universitário Ministro Petrônio Portella

Bairro Ininga, Teresina – PI

CEP: 64049-550

Antonio Vanildo de Sousa Lima

Address: Laboratório de Imunogenética e Biologia Molecular – LIB - SG-16

Campus Universitário Ministro Petrônio Portella

Bairro Ininga, Teresina – PI

CEP: 64049-550

Mirian Aparecida Boim

Address: Rua Pedro de Toledo, 781 - 13º andar

Vila Clementino - São Paulo

CEP:04039-032

Maria Helena Bellini

Address: Rua Pedro de Toledo, 720 – 2º andar

Vila Clementino - São Paulo

CEP 04039-032

Jose Machado Moita Neto

Address: Departamento de Quimica SG-02

Campus Universitário Ministro Petrônio Portella

Bairro Ininga, Teresina – PI

CEP: 64049-550

Adalberto Socorro Silva

Address: Laboratório de Imunogenética e Biologia Molecular – LIB - SG-16

Campus Universitário Ministro Petrônio Portella

Bairro Ininga, Teresina – PI

CEP: 64049-550

Semiramis Jamil Hadad do Monte

Address: Laboratório de Imunogenética e Biologia Molecular – LIB - SG-16

Campus Universitário Ministro Petrônio Portella

Bairro Ininga, Teresina – PI

CEP: 64049-550

Corresponding author*

Amanda de Castro Amorim

Laboratório de Imunogenética e Biologia Molecular – LIB - SG-16

Campus Universitário Ministro Petrônio Portella

Bairro Ininga, Teresina – PI

CEP: 64049-550

Phone: 55 86 32155691

Fax: 55 86 32155690

Email: amandacastronut@yahoo.com.br

**Abstract**

Zinc is an essential element. Its homeostasis is regulated chiefly by zinc transporter proteins (Zip and ZnT) and metallothionein (MT). The Chronic Kidney Disease presents a high occurrence of aseptic inflammation and zinc dyshomeostasis, with no knowledge on the genic regulation of these transporters. Here we reported the gene expression of Zip and ZnT members and MT in the intestine, and Zip14, ZnT1, MT and IL-6 in the liver of rats subjected to the 5/6 nephrectomy protocol (CKD model) and healthy controls, after 15 days of the depleted, adequate or repleted diet in zinc. RNA was extracted using TRIzol reagent. SYBR green fluorescence was read using standard cycling mode. The mRNA expression analysis showed that the expression of the genes studied in the intestine of CKD and healthy control groups behaved similarly, with upregulation of Zip members and downregulation of ZnTs when the diet was depleted in zinc and the opposite was seen in the repletion. Contrariwise, in the liver, the expression of these genes was not regulated by the zinc concentration in the diet. However, there was upregulation of the gene Zip14 and IL-6 in CKD rats with a depleted zinc diet and after repletion, there was no downregulation of these genes. Our findings suggest that there is a differential tissue pattern of gene expression in the intestine and in the liver. In the intestine, the expression of these genes is regulated by the concentration of zinc in the diet, whereas in the liver, it is regulated by the uremic environment of CKD *per se* independently of the dietary zinc levels.

**Keywords:** zinc transporter, gene expression, chronic kidney disease, inflammation, zinc.

## INTRODUCTION

Zinc is an essential micronutrient, potentially toxic, whose intracellular levels are tightly regulated by means of active mechanisms in eukaryotic organisms (Laity and Andrews 2007; Outten and O'halloran 2001). Such mechanisms include a complex interplay of uptake (ZIP) and efflux transporter proteins (ZnT family), coupled with metal-dependent transcriptional control of selected transport and storage proteins (Metallothioneins) (Rink and Haase 2007; Cousins et al. 2006).

The ZnT and ZIP families (Slc30a and Slc39a, respectively) are distributed in the organism as a tissue-specific pattern. The ZnT family reduces the intracellular zinc levels by mediating zinc efflux from cells or influx into intracellular vesicles. ZIP family proteins (Zrt- and Irt-like proteins), on the other hand, promotes zinc transport from the extracellular fluid or intracellular vesicles into the cytoplasm (Cousins et al. 2006; Liuzzi and Cousins 2004; Palmiter and Huang 2004; Eide 2004; Lichten and Cousins 2009).

In addition to the ZIPs and ZnTs, another protein called metallothionein (MT) is also involved in the homeostasis of zinc (Hasse and Rink 2009). Metallothionein is a protein that binds metal ions, in particular zinc (Maret 2000). The affinities of the sites for zinc vary, providing picomolar to nanomolar concentrations of free zinc ions to the cytosol (Kresel and Maret 2007). This free or mobile pool can reversibly bind to regulatory sites in signaling proteins acting as zinc signals, e.g. the metal-response element-binding transcription

factor (MTF-1) (Laity and Andrews 2007), which regulates transcription of genes whose promoters contain metal- response elements (Radtke et al. 1993).

The intestine and kidney tissues may provide important information for the understanding of the expression pattern of genes in a physiological and a pathological situation. The gastrointestinal tract has a fundamental role in the zinc homeostatic control, as, despite the absorption of this element depends on factors such as its abundance in the diet, the zinc excretion through the intestine seems to be more relevant to the homeostatic control of that micronutrient. Likely, it occurs not only because of the amount of zinc absorbed by the intestine, but because of the organism's nutritional status as regards zinc (Krebs and Hambidge 2001). The liver, on the other hand, is the site where zinc is carried to after its absorption and whence it is redistributed to the remainder tissues through plasmatic albumin (57%), *a-2* macroglobulin (40%) and amino acids (Krebs and Hambidge 2001).

Once inside the cells, the zinc homeostatic control is carried out by zinc transporter proteins; those of the ZnT family standing out (ZnT1 to ZnT4) (Henriques et al. 2003).

Remarkable changes in zinc homeostasis occur during an inflammatory immune response, where the pro-inflammatory cytokine IL-6 leads to upregulation of the zinc-import protein ZIP14 in mouse liver (Liuzzi et al. 2005). In rat hepatocytes, the zinc-binding protein MT and cellular zinc content are upregulated by IL- 6 (Coyle et al. 1995; Schroeder and Cousins 1990), and experiments in MT knockout mice showed that this protein is required for

hepatic accumulation of zinc, indicating that zinc is stored as an MT complex (Philcox et al. 1995).

Although the first zinc transporter protein, ZnT1, has been discovered in kidney cells (Palmiter and Findley 1995), there is no report in the literature of a study with this or the remainder zinc transporter proteins in renal disease.

Zinc deficiency is a highly prevalent disorder among early stage Chronic Kidney Disease (CKD) and/or in renal replacement therapy patients (Aggett 1984; Antoniou et al. 1977; Atkin-Thor et al. 1978; Mafra et al. 2004; Sandstead 1980; Smythe et al. 1982). Insufficient dietary intake, malnourishment, drug-nutrient interaction, subclinical inflammatory state and mal-absorption are usually pointed as the main factors leading to this hypozincemia (Prasad 2003). However, zinc homeostasis under condition of kidney dysfunction is still not well understood (Yonova et al. 2012). Nevertheless, studies point out to a dyshomeostasis of the mineral in the CKD (Yonova 2012, Mafra and Cozzolino 2004) both in pre-dialysis and dialysis stage, and with renal transplant .

Micronutrient supplementation for CKD patients remains controversial. While some studies show that zinc supplementation improves symptoms related to hypozincemia (Atkin-Thor et al. 1978; Mahajan et al. 1982 a,b), other authors point to a potential risk of toxicity induced by mineral supplementation (Maret and Sandstead 2006), since excess intracellular zinc can be present despite hypozincemia.

Therefore, we analyzed the gene expression of ZIP, ZnT, MT and IL-6 mRNA in small intestine and liver in a non-dialysis CKD experimental model.

## METHODS

### Animals

All experimental procedures were conducted according to the National Institutes of Health guidelines for the use and care of animals, and the study protocol was approved by the Institutional Review Board at the Federal University of Piauí (UFPI) (process No. 019/13). Forty-two male adult Wistar rats were group housed, given access to rat food and water *ad libitum* and maintained in a temperature-controlled environment (23 °C) on a 12-hour light/dark cycle.

### Experimental Protocols

After a seven-day adaptation period, 30 male adult rats were randomly submitted to a five-sixths nephrectomy (5/6Nx) under anesthesia with ketamine (100 mg/Kg i.p.) plus xylazine (10 mg/Kg i.p.). After ventral laparotomy, the right kidney was removed and two branches of the left renal artery were ligated in order to achieve infarction of two-thirds of the left kidney. CKD status was confirmed by increased serum creatinine levels and the presence of proteinuria. Dietary protocols were initiated thereafter.

### Dietary protocols

CKD and healthy control rats were allocated into three different groups according to zinc supplementation: Zn- (<1 ppm), Zn normal (ZnN; 50 ppm), or

$Zn^+$ (normal Zn diet + Zn 5mg/kg by gavage), which represent depleted, adequate, or repleted intakes of Zn, respectively. Rats were fed individually during 15 days.

**Tissues**

After 15 days of the dietary protocol, the rats were anesthetized with ketamine + xylazine. After this procedure, the intestine and the liver were quickly removed and stored in Allprotect Tissue Reagent™ at -80 °C. The part of the intestine removed corresponded to the small intestine (7 cm, starting from the tail to the pyloric sphincter).

**RNA extraction**

RNA was extracted using TRIzol reagent according to the manufacturer's protocol (Gibco BRL, Life Technologies, Roskilde, Denmark). RNA was quantified by NanoDrop spectrophotometer and only samples with a purity of 1.8 or higher in the 260 nm and 280 nm scale were accepted. Single-stranded cDNA synthesis was performed using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems), according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA).

**Real-Time PCR**

Oligonucleotide primers sequences for the genes (ZIPs, ZnTs, MT and IL-6) are shown in **Table 1**. Power SYBR green PCR master mix (Applied Biosystems) was added to experimental wells in triplicate containing cDNA and primers according to the manufacturer's protocol. SYBR green fluorescence

was read using an ABI prism, 7500 real-time PCR system (Applied Biosystems) under standard cycling mode. Cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min and 45 cycles of 95 °C for 15s, followed by 60 °C for 1 min. Relative amount of copies was calculated using the comparative Ct method, having β-actin as the reference gene.

**Statistical Analysis**

Data are expressed as mean ± SEM. Normality was tested using Kolmogorov-Smirnov. Intragroup and intergroup comparison was carried out with independent samples t-test. The significance level was set at $p < 0.05$.

**RESULTS**

We investigated the mRNA expression of the zinc transporters ZIP1, 3, 4, 5, 6, 7, 8, 10 and 14, ZnT1, 2, 4, 5, 6, 7, 10 and MT in small intestine tissue. In the liver, we investigated the mRNA expression of the ZIP14, ZnT1, MT and IL-6. Both the small intestine and liver came from healthy control rats as well as CKD rats after 15 days of depleted, adequate or repleted diet. These zinc transporters genes were selected due to their role in zinc transport and homeostasis and/or because they had been previously shown to be responsive to changes in dietary zinc.

**Intestine expression in healthy control rats (zinc depleted diet versus zinc repleted diet)**

We examined expression of the studied genes after 15 days of introduction of zinc dietary protocols, and normalized gene expression to values

of rats under adequate diet in zinc. We observed differences in the expression of the genes ZIP3, ZIP4 and ZnT1, with ZIP3 and ZIP4 upexpressed and the ZnT1 downexpressed in rats that received zinc depleted diet as regards the rats that received zinc repleted diet (**Fig. 1A** and **1B**).

**Intestine expression in CKD rats (zinc depleted diet versus zinc repleted diet)**

We assessed the expression of the studied genes in CKD rats undergoing the same zinc dietary protocols. The comparison between rats that received zinc depleted diet and rats that received zinc repleted diet showed upregulation of ZIP3 and ZIP4 and downregulation of ZnT1, ZnT2 and MT in rats that received zinc depleted diet (**Fig. 1C** and **1D**)

**Intestine expression genes in rats with depleted diet in zinc (healthy control rats versus CKD rats)**

When comparing relative expression of the genes in CKD in relation to healthy controls, both with zinc depleted diet, no difference in expression was seen in the ZIP genes between the groups studied fed with this diet (Fig. 2A). There was downregulation of nearly all ZnTs, with a significant higher downregulation in ZnT4 in healthy control rats (**Fig. 2B**)

**Intestine expression genes in rats with repleted diet in zinc (Healthy control rats versus CKD rats)**

In addition, when comparing the relative expression of the genes in CKD rats as regards the healthy control rats - both with zinc repleted diet - we

observed down-regulation of nearly all the ZIP family genes for both groups, except for ZIP6 for the CKD group and ZIP5 and 10 for the healthy control group (**Fig. 2C** and **2D**). As regards the genes ZnTs, there was upexpression of genes ZnT1, ZnT2 and MT with downexpression of genes ZnT4-10 for both groups. The comparison showed a difference in expression between the groups for the genes ZIP4, ZIP8, ZIP14, ZnT4, ZnT10 and MT (**Fig. 2D**).

**Expression of ZIP14, ZnT1, MT and IL-6 in the liver**

In the CKD group, the genes ZIP14, MT and IL-6 were shown upexpressed, whereas the ZnT1 was downexpressed; however, when the expression of these genes was compared according to the diet, a statistical difference was not found in the genes expression between the rats fed with zinc depleted diet and those with zinc repleted diet (**Fig. 3A**). For the group of healthy control rats, all the genes are found down-expressed, except the ZnT1 for the group that received zinc repleted diet (**Fig. 3B**).

**Hepatic gene expression in rats with zinc depleted diet (CKD *versus* Healthy Controls)**

Interestingly, when we compared CKD to healthy control rats after zinc depleted diet protocol, IL-6 and ZIP14 genes were differently expressed in CKD rats, being up-regulated in these (**Fig. 4A**).

**Hepatic gene expression in rats with zinc repleted diet (CKD *versus* Healthy Controls)**

In addition, when the genes expression between CKD and healthy control rats was compared - both groups fed with zinc repleted diet - we could observe a genic expression different from the ZIP14, which was up-regulated in the CKD group (**Fig. 4B**)

## DISCUSSION

This study showed that the CKD experimental model has a tissue-specific zinc transporter, MT and IL6 genes expression pattern, reflecting a direct relationship with the level of zinc in the diet for the intestine and with inflammatory pathways for the liver.

The limited intake of zinc led to an upregulation of the mRNA of genes ZIP3 and ZIP4 and to a concomitant downregulation of ZnT1, ZnT2 and MT in the CKD model (**Fig. 1C** and **1D**). It is well-established that both the subcellular distribution and the ZIP3 activity are zinc-regulated. However, this gene shows a small regulation by zinc status, in comparison with the ZIP4 (Wang et al. 2004). Our data show that the gene presents larger downregulation in the presence of an excessive zinc diet than upregulation in the presence of low zinc intakes, and this expression was significantly different between the diets for both groups (CKD and control). Although we have not assessed the intracellular changes caused by zinc levels herein, it is possible that these changes, including the post-translational degradation, be responsible for downregulating the expression de of zinc transporter proteins, as shown elsewhere (Gitan et al. 1998, Gitan et al. 2003).

ZIP4 is considered the primary zinc importer responsible for intestinal zinc absorption, suggesting regulation due to reduced levels of this micronutrient intake (Jou et al. 2009). Studies showed upregulation under zinc deficiency (Liuzzi et al. 2004; Jou et al. 2009) and downregulation under increased zinc concentration (Dufner-Beattie et al. 2003) at the ZIP4 mRNA levels. The importance of protein ZIP4 in the adequate intestinal uptake of zinc to keep the mineral's homeostasis and cell functions is shown in knockout genetic models of the gene ZIP4, mimicking the Enteropathic Acrodermatitis (Dufner-Beattie et al. 2007; Andrews 2008).

Concomitantly, the expression of genes ZnT1 and ZnT2 was related to the amount of Zn in the diet for the intestine (**Fig. 1D**), as demonstrated in other studies (Liuzzi et al. 2004; Liuzzi et al. 2001). The function of ZnT1 and ZnT2 transporters in the enterocyte is well known. The former transports zinc to the extracellular medium (*lumen*) and the latter, to the organelles, decreasing, thus, the intracellular levels of zinc (Palmiter et al. 1996; Palmiter and Findley 1995). As for MT, changes in mRNA levels of ZnT1 and ZnT2 can be Zn-induced, suggesting that zinc is a similar way for regulation of these zinc transporters (Liuzzi et al. 2001). ZnT1 and ZIP4 are intertwined in such a way that ZnT1 knockout in mice (Walsh et al.1994) recapitulates the lethality phenotype of ZIP4 -/- on embryos (Dufner-Beattie et al. 2007).

The high expression of intestinal MT in the presence of a zinc-rich diet (**Fig. 1B** and **1D**) shown herein corroborates the findings of Liuzzi et al. (2004). The synthesis induction of MT occurs at the transcription level and it is

regulated by MREs (Metal-Response Elements). The gene is activated by MTF-1 after zinc occupancy, and this MTF-1 functions as a sensor of free pools of zinc in the cell. Therefore, when there is an increase in the intracellular free zinc, the MTF-1 activates the MT to sequestrate part of this free zinc (Davis and Cousins 2000).

The upexpression of genes ZIP14, IL-6 and MT in the liver tissue of the CKD group occurred independently of the dietary zinc levels, once the repletion with the mineral did not cause downregulation of the genes (**Fig. 3A**). The expression profile found herein for these genes was differentiated between the CKD group and the control group, and for the CKD group it was similar to the one found in sterile inflammation models (Liuzzi et al. 2005; Lichten et al. 2009). In these models, the inflammation induction leads to an increase in the genes expression that codify pro-inflammatory cytokines, chiefly IL-6 (Liuzzi et al. 2005) and IL-1B (Lichten et al. 2009), followed by upregulation of genes ZIP14 and MT.

The end-stage renal disease is characterized by immune deficiency and a remarkable systemic inflammation (Carrero and Stenvinkel 2010; Girndt et al. 1999). The synergy between these two abnormalities is responsible for a wide range of morbidity and mortality in CKD patients (Vaziri et al. 2012). In fact, whereas the systemic inflammation contributes to homeostasis changes of nutrients (Vaziri et al. 2012), especially trace elements (Shenkin 1995), the immune deficiency weakens the responses to pathogens and to vaccination (Vaziri et al. 2012).

Studies identified that from the genes codifying zinc transporter proteins in the liver, ZIP14 was the most deeply upregulated in inflammation models, both in mice treated with turpentine (to create a sterile abscess), and those treated with lipopolysaccharide (LPS, mimicking the beginning of innate immunity). This upregulation was induced via IL-6 and other mediators, e.g. IL-1B (Liuzzi et al. 2005; Lichten et al. 2009).

The upregulation of ZIP14 is regarded as responsible for the zinc tissue redistribution, with the accumulation of zinc in the liver and consequent hypozincemia shown in these experimental inflammation models (Liuzzi et al. 2005; Lichten et al. 2009). However, the mechanisms involved in this redistribution, the role and the biological meaning of hypozincemia in response to inflammation still remain elusive.

Authors suggest the occurrence of a tissue redistribution of the mineral in CKD, causing hypozincemia, but this was not confirmed yet (Yonova et al. 2012; Mafra and Cozzolino 2004). Herein, according to the profile of the genes studied in the liver, we verified that there was an orchestration at the molecular level that confirms this hypothesis.

In murine ZIP14-/- model, it was demonstrated that the ablation of this gene prevented the hypozincemia produced by the administration of LPS, confirming the transporter's key role in hypozincemia produced by acute-phase inflammation. The induction of MT mRNA in the liver by LPS was also demonstrated, and this was significantly reduced in ZIP14 knockout mice. In that same work, they suggested that this induction was a result of the LPS

signaling and Metal regulatory Transcription Factor-1 activation, as the expression of MT mRNA is proportional to the intracellular zinc availability (Aydemir et al. 2012). Similarly, when the CKD group was compared with the control group, independently of the diet used, the MT was shown significantly upregulated in the CKD group (data not shown), suggesting it was secondary to the increase of the free pool of cytosolic zinc, induced by the upregulation of ZIP14 with upexpression of the corresponding protein, responsible for the zinc influx. As for the control group, it showed a reduced expression of ZIP14 and IL-6 even in reduced dietary zinc offer conditions. These findings corroborate the hypothesis of redistribution of zinc to the liver via ZIP14, mediated by inflammation, in the presence of Chronic Kidney Disease.

In conclusion tissue-specific changes in Zn transporter gene in the experimental CKD modelo reflects the regulating role of the intestine in the zinc uptake to the organism; whereas the upregulation of genes ZIP14, MT and IL6 might suggest that the high occurrence of hypozincemia can be explained in part by the upexpression of these proteins in the hepatic tissue. Therefore, the liver may behave in the mineral's homeostasis as a site of a mobile pool of zinc in severe dietary deficiency conditions, as this micronutrient is essential to our life and the upexpression of ZIP4 is not supported when there is an extended intake deficiency. Simultaneously, the knowledge attained in this study opens perspectives for further studies using genetic models of genes ablation to elucidate the zinc-inflammation-CKD relationship, regarding that the chronic inflammation follows the loss of the rate glomerular filtration and the addition to

being strongly related to the high cardiovascular mortality rates of this patient population.

## Conflict of interest

Amanda C Amorim, Rafael MSS Brandão, Antonio VS Lima, Miriam A Boim, Maria H Bellini, Jose M Moita Neto, Adalberto S Silva, Semiramis JH Monte declare that they have no conflicts of interest.

## Ethical standard

All institutional and national guidelines for the care and use of laboratory animals were followed.

## Acknowledgments

The authors would like to sincerely thank João Batista de Oliveira Silva Jr. for translation of the manuscript into English. The authors also thank the Renorbio Postgraduate Program for its research incentive in the northeastern region of Brazil.

## Table Caption

**Table 1.** Primers used in this study.

## Figures Captions

**Fig. 1** Zinc transporters and MT mRNAs are differentially expressed in small intestine upon changes in zinc diet. Expression of ZIPs, ZnTs and MT mRNA in healthy control and CKD rats after 15 days of depleted (Zn-) or repleted (Zn+)

diet in zinc using Real-time PCR. Values expressed were normalized against adequate zinc diet. * p<0.05

**Fig. 2** Small intestine expression genes in rats with depleted or repleted diet in zinc (healthy controls versus CKD rats). Comparison of the relative expression of ZIPs, ZnTs and MT mRNA between the healthy control and CKD groups after 15 days of depleted (Zn-) or repleted (Zn+) diet in zinc using Real-time PCR. Values expressed were normalized against adequate zinc diet. * p<0.05

**Fig. 3** Relative expression of ZIP14, ZnT1, MT and IL-6 in the liver. Expression of ZIP14, ZnT1, MT and IL-6 mRNA in healthy control and CKD rats after 15 days of depleted (Zn-) or repleted (Zn+) diet in zinc using Real-time PCR. Values expressed were normalized against adequate zinc diet

**Fig. 4** Liver expression genes in rats with depleted or repleted diet in zinc (healthy controls versus CKD rats). Comparison of the relative expression of ZIP14, ZnT1, MT and IL-6 mRNA between the healthy control and the CKD groups after 15 days of depleted (Zn-) or repleted (Zn+) diet in zinc using Real-time PCR. Values expressed were normalized against adequate zinc diet. * p<0.05

**References**

Aggett PJ (1984) Zinc metabolism in chronic renal insufficiency with or without dialysis therapy. Contrib Nephrol 38:95-102.

Andrews GK (2008) Regulation and function of Zip4, the acrodermatitis enteropathica gene. Biochem Soc Trans 36:1242-1246.

Antoniou LD, Shalhoub RJ, Sudhakar T, Smith JC, Jr (1977) Reversal of uraemic impotence by zinc. Lancet 2:895-898.

Atkin-Thor E, Goddard BW, O'nion J, Stephen RL, Kolff WJ (1978) Hypogeusia and zinc depletion in chronic dialysis patients. Am J Clin Nutr 31:1948-1951.

Aydemir TB, Sitren HS, Cousins RJ (2012) The zinc transporter Zip14 influences c-Met phosphorylation and hepatocyte proliferation during liver regeneration in mice. Gastroenterology 142:1536-1546.

Carrero JJ, Stenvinkel P (2010) Inflammation in end-stage renal disease--what have we learned in 10 years? Semin Dial 23:498-509.

Cousins RJ, Liuzzi JP, Lichten LA (2006) Mammalian zinc transport, trafficking, and signals. J Biol Chem 34:24085-24089.

Coyle P, Philcox JC, Rofe AM (1995) Metallothionein induction in cultured rat hepatocytes by arthritic rat serum, activated macrophages, interleukin-6, interleukin-11 and leukaemia inhibitory factor. Inflamm Res 44:475-481.

Davis SR, Cousins RJ (2000) Metallothionein expression in animals: a physiological perspective on function. J Nutr 130:1085-1088.

Dufner-Beattie J, Wang F, Kuo YM, Gitschier J, Eide D, Andrews GK (2003) The acrodermatitis enteropathica gene ZIP4 encodes a tissue-specific, zinc-regulated zinc transporter in mice. J Biol Chem 278: 33474-33481.

Dufner-Beattie J, Weaver BP, Geiser J et al (2007) The mouse acrodermatitis enteropathica gene Slc39a4 (Zip4) is essential for early development and

heterozygosity causes hypersensitivity to zinc deficiency. Hum Mol Genet 16:1391-1399.

Eide DJ (2004) The SLC39 family of metal ion transporters. Pflugers Archiv 447:796-800.

Girndt M, Sester U, Sester M, Kaul H, Kohler H (1999) Impaired cellular immune function in patients with end-stage renal failure. Nephrol Dial Transpl 14:2807-2810.

Gitan RS, Luo H, Rodgers J, Broderius M, Eide D (1998) Zinc-induced inactivation of the yeast ZRT1 zinc transporter occurs through endocytosis and vacuolar degradation. J Biol Chem 273:28617-28624.

Gitan RS, Shababi M, Kramer M, Eide DJ (2003) A cytosolic domain of the yeast Zrt1 zinc transporter is required for its post-translational inactivation in response to zinc and cadmium. J Biol Chem 278:39558-39564.

Haase H, Rink L (2009) Functional significance of zinc-related signaling pathways in immune cells. Ann Rev Nutr 29:133-152.

Henriques GS, Hirata MH, Cozzolino SMF (2003) Recent aspects of zinc absorption and bioavailability and correlations with physiology of the testicular Angiotensin-converting enzyme. Rev Nutr 16:333-345.

Jou MY, Hall AG, Philipps AF, Kelleher SL, Lonnerdal B (2009) Tissue-specific alterations in zinc transporter expression in intestine and liver reflect a threshold for homeostatic compensation during dietary zinc deficiency in weanling rats. J Nutr 139:835-841.

Krebs NE, Hambidge KM (2001) Zinc metabolism and homeostasis: the application of tracer techniques to human zinc physiology. Biometals 14:397-412.

Krezel A, Maret W (2007) Dual nanomolar and picomolar Zn(II) binding properties of metallothionein. J Am Chem Soc 129:10911-10921.

Laity JH, Andrews GK (2007) Understanding the mechanisms of zinc-sensing by metal-response element binding transcription factor-1 (MTF-1). Arch Biochem Biophys 463:201-210.

Lichten LA, Cousins RJ (2009) Mammalian zinc transporters: nutritional and physiologic regulation. Ann Rev Nutr 29:153-176.

Lichten LA, Liuzzi JP, Cousins RJ (2009) Interleukin-1beta contributes via nitric oxide to the upregulation and functional activity of the zinc transporter Zip14 (Slc39a14) in murine hepatocytesAm J Physiol Gastrointest Liver Physiol 296:G860-867.

Liuzzi JP, Blanchard RK, Cousins RJ (2001) Differential regulation of zinc transporter 1, 2, and 4 mRNA expression by dietary zinc in rats. J Nutr 131:46-52.

Liuzzi JP, Bobo JA, Lichten LA, Samuelson DA, Cousins RJ (2004) Responsive transporter genes within the murine intestinal-pancreatic axis form a basis of zinc homeostasis. Proc Natl Acad Sci USA 101:14355-14360.

Liuzzi JP, Cousins RJ (2004) Mammalian zinc transporters. Ann Rev Nutr 24:151-172.

Liuzzi JP, Lichten LA, Rivera S et al (2005) Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response. Proc Natl Acad Sci USA 102:6843-6848.

Mafra D, Cozzolino SMF (2004) The importance of zinc in human nutrition. Rev Nutr 17:79-87.

Mafra D, Cuppari L, Favaro DI, Cozzolino SM (2004) Zinc levels after iron supplementation in patients with chronic kidney disease. J Ren Nutr 14:164-169.

Mahajan SK, Abbasi AA, Prasad AS, Rabbani P, Briggs WA, Mcdonald FD (1982) Effect of oral zinc therapy on gonadal function in hemodialysis patients. A double-blind study. Ann Intern Med 97:357-361.

Mahajan SK, Prasad AS, Rabbani P, Briggs WA, Mcdonald FD (1982) Zinc deficiency: a reversible complication of uremia. Am J Clin Nutr 36:1177-1183.

Maret W (2000) The function of zinc metallothionein: a link between cellular zinc and redox state. J Nutr 130:1455S-1458S.

Maret W, Sandstead HH (2006) Zinc requirements and the risks and benefits of zinc supplementation. J Trace Elem Med Biol 20:3-18.

Outten CE, O'halloran TV (2001) Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science 292:2488-2492.

Palmiter RD, Cole TB, Findley SD (1996) ZnT-2, a mammalian protein that confers resistance to zinc by facilitating vesicular sequestration. EMBO J 15:1784-1791.

Palmiter RD, Findley SD (1995) Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. EMBO J 14:639-649.

Palmiter RD, Huang L (2004) Efflux and compartmentalization of zinc by members of the SLC30 family of solute carriers. Pflugers Archiv 447:744-751.

Philcox JC, Coyle P, Michalska A, Choo KH, Rofe AM (1995) Endotoxin-induced inflammation does not cause hepatic zinc accumulation in mice lacking metallothionein gene expression. Biochem J 308:543-546.

Prasad AS (2003) Zinc deficiency. Bmj 326:409-410.

Radtke F, Heuchel R, Georgiev O et al (1993) Cloned transcription factor MTF-1 activates the mouse metallothionein I promoter. EMBO J 12:1355-1362.

Rink L, Haase H (2007) Zinc homeostasis and immunity. Trends Immunol 28:1-4.

Sandstead HH (1980) Trace elements in uremia and hemodialysis. Am J Clin Nutr 33:1501-1508.

Schroeder JJ, Cousins RJ (1990) Interleukin 6 regulates metallothionein gene expression and zinc metabolism in hepatocyte monolayer cultures. Proc Natl Acad Sci USA 87: 3137-3141.

Shenkin A (1995) Trace elements and inflammatory response: implications for nutritional support. Nutrition 11:100-105.

Smythe WR, Alfrey AC, Craswell PW et al (1982) Trace element abnormalities in chronic uremia. Ann Intern Med 96:302-310.

Vaziri ND, Pahl MV, Crum A, Norris K (2012) Effect of uremia on structure and function of immune system. J Ren Nutr 22:149-156.

Walsh CT, Sandstead HH, Prasad AS, Newberne PM, Fraker PJ (1994) Zinc: health effects and research priorities for the 1990s. Environ Health Perspect 102:5-46.

Wang F, Dufner-Beattie J, Kim BE, Petris MJ, Andrews G, Eide DJ (2004) Zinc-stimulated endocytosis controls activity of the mouse ZIP1 and ZIP3 zinc uptake transporters. J Biol Chem 279: 24631-24639.

Yonova D, Vazelov E, Tzatchev K (2012) Zinc status in patients with chronic renal failure on conservative and peritoneal dialysis treatment. Hippokratia 16:356-359.

**Table 1.** Primers used in this study.

| Genes | Foward | Reverse |
|---|---|---|
| ZIP1 | 5'- AAGCCTAGTGAGCTGCTTCG -3' | 5'-ATGGCCAGGATGAACTCTTG-3' |
| ZIP3 | 5'-CGTCTTCCTGGCTACATGCT-3' | 5'-TCCACGAACACAGTGAGGAA-3' |
| ZIP4 | 5'-GTGCGCTTCTGCTGAATCTG-3' | 5'-CGACTGCTAGAGCCACGTAGAG-3' |
| ZIP5 | 5'-GGGCAGCCTCATGTTTAGTA-3' | 5'-CCACATCAGCCATCAGGAA-3' |
| ZIP6 | 5'-GCCACAGCCAGCGCTACT-3' | 5'-ATCACCATCCAGGCCAACGT-3' |
| ZIP7 | 5'-AGGCATCAAGCACCACCTGG-3' | 5'-CGCGGAGATTAGCACTGTG-3' |
| ZIP8 | 5'-CCAGATAACCAGCTCGAACTTCA-3' | 5'-TGTGGATCCTCACAGGGATGA-3' |
| ZIP10 | 5'-TGGCTTACATAGGAATGCTCATAGG-3' | 5'-CGCGAAGATCCAGAGTGTGATG-3' |
| ZIP14 | 5'-CCTCACGAGCTGGGAGACTTC-3' | 5'-AGAGGGCCTGCTGGATACTCA-3' |
| ZnT1 | 5'-ATCCAGCCTGAATTCGCTAGC-3' | 5'-CGTGTCCCACAGCACTGCT-3' |
| ZnT2 | 5'-TGCTCGTGTACCTGGCTGTA-3' | 5'-TCCATGTCCAGACTGATGGA-3' |
| ZnT4 | 5'-GCTGAAGCAGAGGAAGGTGAA-3' | 5'-TCTCCGATCATGAAAAGCAAGTAG-3' |
| ZnT5 | 5'-TGGCTAAGATGGCCGAACAC-3' | 5'-CCAGGAAGGCGATAGCTGTATAAA-3' |
| ZnT6 | 5'-TCCCAGGACTCAGCAGTATCTTC-3' | 5'-GCCCCAGCAAGATCGATCAG-3' |
| ZnT7 | 5'-CCTCTCTTTCGCTTTTGTGGAA-3' | 5'-GTGGAACGAGTCGGAGATCAAG-3' |
| ZnT10 | 5'-GTAGCAGGTGATTCCCTGAAC-3' | 5'-GTGACAACCACAATCACGGAC-3' |
| IL-6 | 5'-CTTCCAGCCAGTTGCCTTCTTG-3' | 5'-GGTCTGTTGTGGGTGGTATCCTC-3' |
| MT | 5'-TGCAAGAAAAGCTGCTGTTCC-3' | 5'-GTCCGAAGCCTCTTTGCAGAT-3' |
| β-actin | 5'-TGCCCTAGACTTCGAGCAAG-3' | 5'-GGCAGCTCATAGCTCTTCTCC-3' |

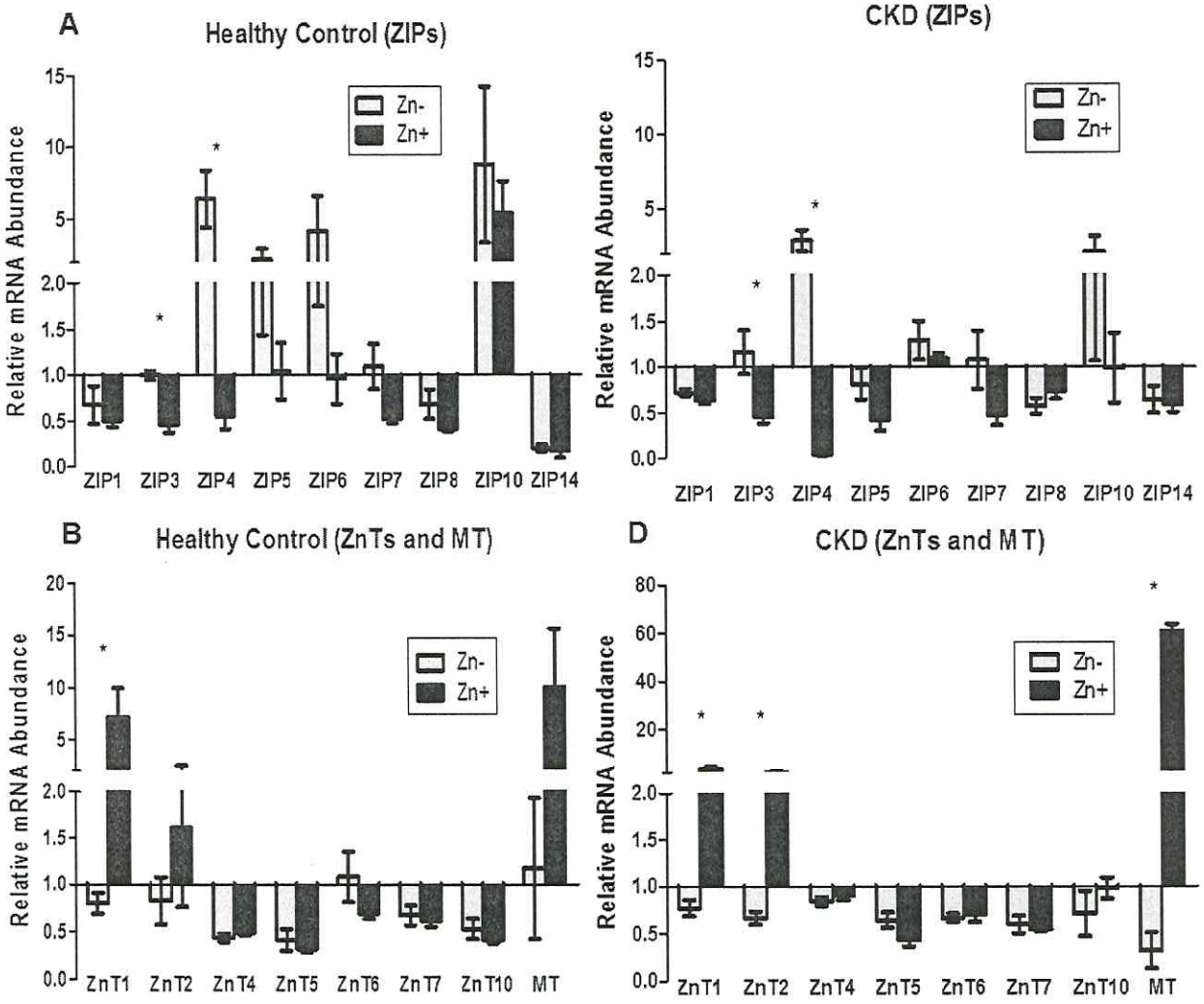



**Figure 1. Zinc transporters and MT mRNAs are differentially expressed in small intestine upon changes in zinc diet.** Expression of ZIPs, ZnTs and MT mRNA in healthy control and CKD rats after 15 days of depleted (Zn-) or repleted (Zn+) diet in zinc using Real-time PCR. Values expressed were normalized against adequate zinc diet. * $p<0.05$

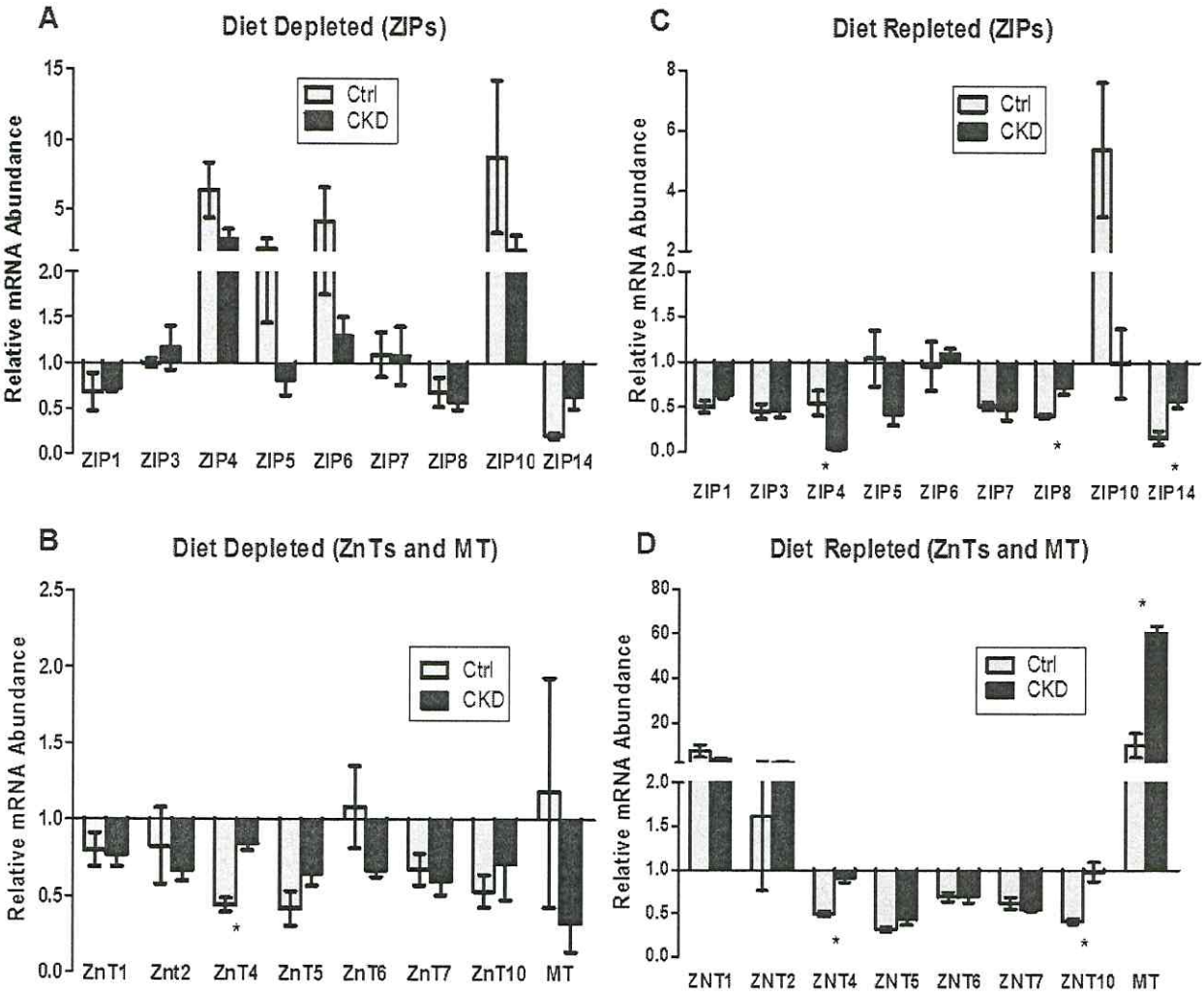



**Figure 2. Small intestine expression genes in rats with depleted or repleted diet in zinc (healthy controls versus CKD rats).** Comparison of the relative expression of ZIPs, ZnTs and MT mRNA between the healthy control and CKD groups after 15 days of depleted (Zn-) or repleted (Zn+) diet in zinc using Real-time PCR. Values expressed were normalized against adequate zinc diet. * $p<0.05$

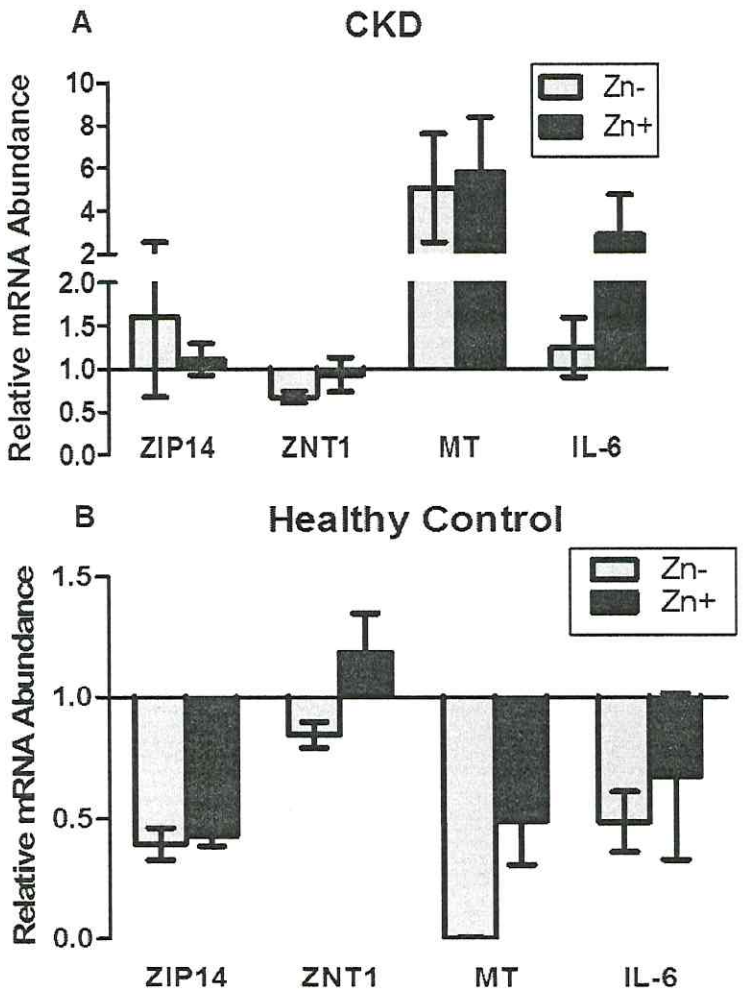



**Figure 3. Relative expression of ZIP14, ZnT1, MT and IL-6 in the liver.** Expression of ZIP14, ZnT1, MT and IL-6 mRNA in healthy control and CKD rats after 15 days of depleted (Zn-) or repleted (Zn+) diet in zinc using Real-time PCR. Values expressed were normalized against adequate zinc diet.

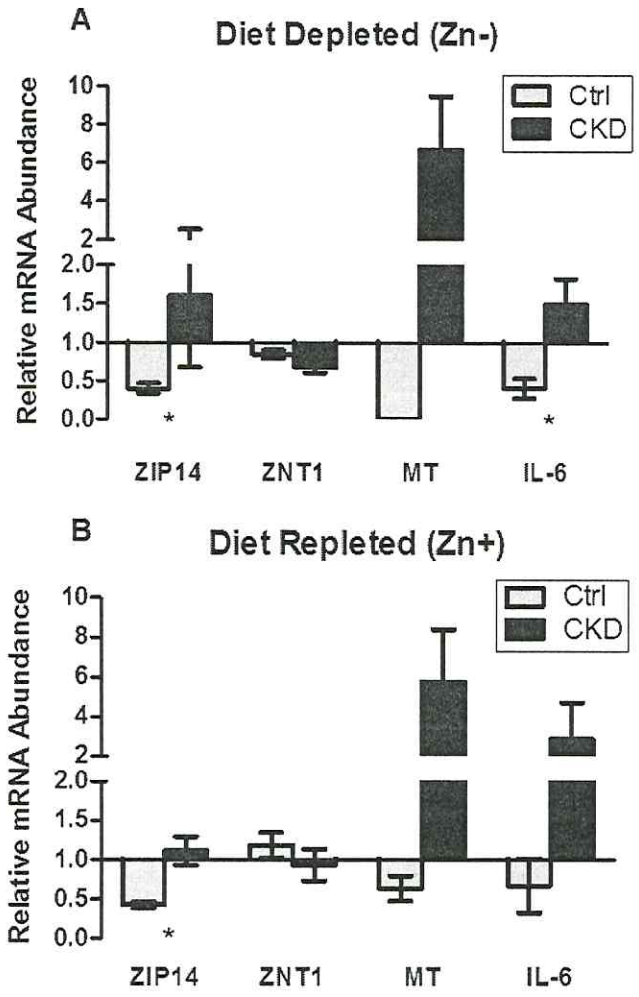



**Figure 4. Liver expression genes in rats with depleted or repleted diet in zinc (healthy controls versus CKD rats).** Comparison of the relative expression of ZIP14, ZnT1, MT and IL-6 mRNA between the healthy control and the CKD groups after 15 days of depleted (Zn-) or repleted (Zn+) diet in zinc using Real-time PCR. Values expressed were normalized against adequate zinc diet. * $p<0.05$

# 6. CONSIDERAÇÕES FINAIS

O fato do zinco participar de vários mecanismos envolvidos no metabolismo celular, sendo primordial para o bom funcionamento da célula, aliado à alta prevalência de reduzidos níveis plasmáticos na Doença Renal Crônica, além da possibilidade de uma distribuição alterada do micronutriente nesta condição, faz com que, o entendimento da homeostase do mineral se torne de suma importância. Para isto, torna-se fundamental a compreensão do comportamento de genes envolvidos no metabolismo do zinco.

A expressão de genes codificantes de proteínas transportadoras de zinco, metalotioneína e interleucina-6 em PBMC`s mostrou-se altamente responsiva a alterações da ingestão dietética de zinco, podendo assim, vir a ser utilizada futuramente como um parâmetro mais condizente para definir a deficiência de zinco ou a necessidade celular deste micronutriente na DRC. Já a expressão gênica determinada no intestino e no fígado (órgãos envolvidos na homeostase do mineral), mostrou que o grupo de ratos renais crônicos tem uma expressão tecido-específica para os genes estudados, refletindo uma relação direta entre os níveis de zinco da dieta para o intestino e com vias inflamatórias para o fígado. Estes achados permitem sugerir que a alta ocorrência de hipozincemia na Doença Renal Crônica pode ser explicada em parte pela expressão aumentada do ZIP14, IL-6 e MT no tecido hepático, corroborando a hipótese de redistribuição tecidual de zinco ao invés de deficiência do mineral.

Simultaneamente, o conhecimento obtido neste estudo abre perspectivas para estudos futuros usando modelos genéticos de ablação para

elucidar a relação zinco-inflamação-Doença Renal Crônica. E, o entendimento dos fatores envolvidos na regulação de zinco na DRC também poderá permitir novas propostas de intervenção farmacológica, objetivando reduzir os fatores de morbimortalidade nesta população.

# 7. ANEXOS

## 7.1. **Anexo A:** Parecer do Comitê de Ética em Experimentação Animal

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DO PIAUÍ
PRÓ-REITORIA DE PESQUISA
COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL
Campus Universitário Ministro Petrônio Portela, Bairro Ininga, Teresina, Piauí, Brasil; CEP: 64049-550
Telefone (86) 3215-5734 _e-mail: ceeapi@ufpi.edu.br

Teresina, 21 de Setembro de 2013.

À:

**Profa. Dra. SEMÍRAMIS JAML HADAD DO MONTE**

**Departamento: Clinica Médica_ CCS/ UFPI**

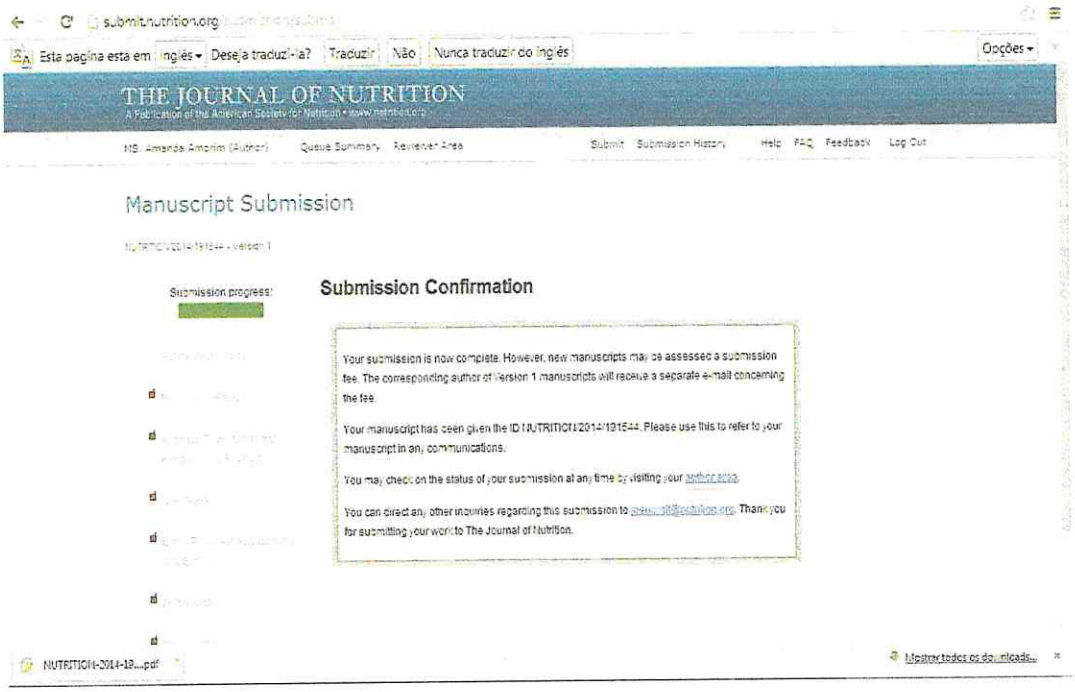
Senhora Pesquisadora,

Deciaro para os devidos fins que o projeto intitulado: **"Inflamação e Doença Renal Crônica: Aplicação da ferramenta Biotecnológica"**, foi avaliado pelo COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL – CEEA/UFPI teve parecer **APROVADO sob o Nº.019/13**. Esclarecemos que o mesmo se encontra de acordo com os requisitos exigidos para apreciação de projetos de pesquisa.

Atenciosamente,

Profª. Ivete L. de Mendonça
Comitê de Ética em Experimentação Animal-UFPI
Coordenadora

## 7.2. **Anexo B:** Comprovante de submissão do Artigo 1



## 7.3. **Anexo C:** Comprovante de submissão do Artigo 2

Esta página está em inglês Deseja traduzi-la? Traduzir Não Nunca traduzir do inglês Opções

Genes & Nutrition

Editorial Manager

HOME • LOGOUT • HELP • REGISTER • UPDATE MY INFORMATION • JOURNAL OVERVIEW
MAIN MENU • CONTACT US • SUBMIT A MANUSCRIPT • INSTRUCTIONS FOR AUTHORS

Role: Author Username: Amanda Castro

**Submissions Being Processed for Author Amanda Castro Amorim, M.D.**

Page: 1 of 1 (1 total submissions) Display 10 results per page.

| Action | Manuscript Number | Title | Initial Date Submitted | Status Date | Current Status |
|---|---|---|---|---|---|
| Action Links | | Differential tissue-specific expression of zinc transporters, MT and IL-6 genes in an experimental Chronic Kidney Disease model. | 25-02-2014 | 25-02-2014 | Submitted to Journal |

Page: 1 of 1 (1 total submissions) Display 10 results per page.

<< Author Main Menu