UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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CENTRO DE BIOTECNOLOGIA DO ESTADO DO RIO GRANDE DO SUL
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

ESTRESSE OXIDATIVO E HORMÔNIOS ESTEROIDES NA ASSOCIAÇÃO ENTRE DISTÚRBIOS RESPIRATÓRIOS DO SONO E DOENÇA ATEROSCLERÓTICA CORONARIANA

Dissertação de Mestrado

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Dissertação submetida ao programa de Pós-Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da UFRGS como um dos requisitos para obtenção do grau de Mestre em Biologia Celular e Molecular.

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ABREVIATURAS

8-OHdG: 8-oxo-7,8-dihidro-2’-desoxiguanosina

AOS: apneia obstrutiva do sono;

CAT: catalase;

DAC: doença aterosclerótica coronariana;

DHT: dihidrotestosterona;

DRS: distúrbios respiratórios do sono;

eNOS: óxido nitrico sintase endotelial;

ER-α: recepetor de estrógeno alfa;

EROs: espécies reativas de oxigênio;

ERNs: espécies reativas de nitrogênio;

GPx: glutationa peroxidase;

GSH: glutationa reduzida;

GSSG: glutationa oxidada;

GsT: glutationa S-transferase;

HDL: lipoproteínas de alta densidade;

HIF: fator induzido por hipóxia;

IAH: índice de apneia-hipopneia;

IL: interleucina;

LDL: lipoproteínas de baixa densidade;

MDA: malondialdeído;

Ox-LDL: lipoproteínas de baixa densidade oxidadas;

SOD: superóxido dismutase;

TNF: fator de necrose tumoral;

TBARS: ácido tiobarbitúrico.

RESUMO

Título: Estresse oxidativo e hormônios esteróides na associação entre Distúrbios Respiratórios do Sono e Doença Aterosclerótica Coronariana

Introdução: Estudos epidemiológicos mostram a existência de associação entre a Doença Aterosclerótica Coronariana (DAC) e os Distúrbios Respiratórios do Sono (DRS). Evidencias sugerem que o estresse oxidativo gerado pela hipóxia intermitente sofrida pelos pacientes com DRS pode estar relacionado à progressão da DAC. Os hormônios esteróides testosterona, progesterona e estradiol estão relacionados ao estresse oxidativo, e podem ter papel em ambas as doenças. A enzima glutationa S-tranferase utiliza a molécula antioxidante glutationa na detoxificação de compostos que podem ser formados neste processo. A enzima paraoxonase-1 hidrolisa peróxidos lipídicos, atuando sobre as lipoproteínas de baixa densidade oxidadas (ox-LDL). Ox-LDL são marcadores de peroxidação lipídica, e são importantes na formação da placa aterosclerótica. O vaso dilatador óxido nítrico ($NO^{\bullet}$) é considerado ateroprotetor e pode estar reduzido, agravando a DAC.

Objetivos: Estudar o estresse oxidativo e as alterações fisiopatológicas decorrentes da associação entre DRS e DAC, e avaliar a participação dos hormônios esteroides neste processo.

Material e Métodos: 56 pacientes com prévio diagnóstico para Doença Aterosclerótica Coronariana (DAC) e avaliação do Índice de Apneias-hipopneias (IAH) para diagnóstico de Distúrbio Respiratório do sono (DRS) foram divididos em dois grupos, 29 pacientes controles e 27 pacientes com DAC, definidos por apresentarem obstrução coronariana >30%. Foram quantificadas as concentrações séricas dos triglicerídeos, HDL, LDL, ferritina, tranferrina e ferro disponível, assim como dos níveis séricos dos hormônios testosterona, estradiol e progesterona, das enzimas paraoxonase-1 e glutationa S-transferase, e das ox-LDL. Foram quantificadas as concentrações de glutationa total, glutationa reduzida, glutationa oxidada e nitritos e nitratos (medida indireta de $NO^{\bullet}$) em eritrócitos. A concentração do marcador de dano oxidativo em DNA 8-oxo-7,8-dihidro-2'-desoxiguanosina foi obtida em leucócitos.

Resultados: Pacientes com DAC possuem reduzida concentração de nitritos e nitratos. A concentração de 8-OHdG, a atividade da GsT, os níveis de glutationa total, glutationa reduzida e glutationa oxidada, assim com o estradiol e a progesterona, não apresentaram relação com DAC ou DRS. Além do IAH, a redução da testosterona e do ferro disponível estão relacionados a DAC. A redução da atividade da paraoxonase-1 e a maior concentração de ox-LDL são preditores de DAC. A testosterona está relacionada à concentração de ferritina, transferrina e ferro disponível nestes pacientes. A ferritina correlacionou-se positivamente ao dano oxidativo em proteínas e com o IAH, negativamente aos níveis de nitritos e nitratos, e é maior nos pacientes com DAC.

Conclusão: Baixos níveis de testosterona e ferro disponível, assim com o aumento da ferritina podem estar relacionados à fisiopatologia da associação entre DRS e DAC. Paraoxonase-1 e ox-LDL são importantes preditores de DAC, mas parecem não estar diretamente relacionados ao IAH nestes pacientes.

Palavras-chave: apneia obstrutiva do sono, aterosclerose, testosterona, ferritina, paraoxonase-1, ox-LDL.

ABSTRACT

Title: Oxidative stress and steroid hormones in the association between Sleep Disordered Breathing and Coronary Artery Disease

Introduction: Epidemiological studies have shown a possible association between Coronary Artery Disease (CAD) and Sleep Disordered Breathing (SDB). Evidences suggest that oxidative stress generated by the intermittent hypoxia experienced by patients with sleep disorders may be related to progression of CAD.

The steroid hormones testosterone, progesterone and estradiol are related to oxidative stress, and may have a role in both diseases. Glutathione S-transferase uses the antioxidant molecule glutathione in the detoxification of compounds that can be formed in this process. The enzyme paraoxonase-1 hydrolyzes lipid peroxides, acting on oxidized low-density lipoproteins (ox-LDL). Ox-LDL are lipid peroxidation markers, being important for the atherosclerotic plaque formation. The vasodilator nitric oxide ($NO^{\bullet}$) is considered atheroprotective and can be reduced, aggravating DAC.

Objective: Evaluate the oxidative stress and the pathophysiological changes arising from the association between SDB and CAD, and the role of steroid hormones in this process.

Material and Methods: 56 patients with prior Coronary Artery Disease (CAD) diagnosis and apnea-hypopnea index (AHI) evaluation for diagnosis of sleep-disordered breathing (SDB) were divided into two groups, 29 control patients and 27 patients with CAD, defined by present a coronary obstruction > 30%. The serum concentration of triglycerides, HDL, LDL, ferritin, transferrin and available iron was obtained, as well as the serum levels of the hormones testosterone, estradiol and progesterone, enzymes paraoxonase-1 and glutathione S-transferase, and ox-LDL. Were measured concentrations of total glutathione, reduced glutathione, glutathione disulfide and nitrites and nitrates ($NO^{\bullet}$ indirect measure) in erythrocytes. The concentration of the 8-oxo-7,8-dihydro-2'-deoxyguanosine, oxidative DNA damage marker, was obtained from leukocytes.

Results: CAD patients have reduced concentrations of nitrates and nitrites. The concentration of 8-OHdG, GST activity, levels of total glutathione, reduced glutathione and glutathione disulfide, and estradiol and progesterone, showed no relationship with CAD or SDB. In addition to AHI, the reduction of testosterone and iron available are related to CAD. The reduced activity of paraoxonase-1 and the highest concentration of ox-LDL are CAD predictors. Testosterone is related to the concentration of ferritin, transferrin and iron available in these patients. Ferritin was positively correlated to oxidative damage in protein and with the AHI, and negatively to the levels of nitrites and nitrates, and is higher in CAD patients.

Conclusion: Low testosterone levels and iron available, as well as the increase ferritin may be related to the pathophysiology of the association between SDB and CAD. Paraoxonase-1 and ox-LDL are important CAD predictors, but do not seem to be directly related to AHI in these patients.

Keywords: obstructive sleep apnea, atherosclerosis, testosterone, ferritin, paraoxonase-1, ox-LDL.

INTRODUÇÃO

Estresse Oxidativo

Diferentes processos metabólicos dos organismos aeróbios utilizam o oxigênio molecular (O_2) em reações de oxidação e redução. Um importante e clássico exemplo é a cadeia transportadora de elétrons mitocondrial, onde ocorre a formação de água (H_2O) a partir da captura de elétrons pelo oxigênio. Durante este processo, as espécies reativas de oxigênio (EROs) podem ser produzidas, dentre elas o peróxido de hidrogênio (H_2O_2), o radical superóxido ($O_2^{\bullet -}$), o radical hidroperoxila ($HO_2^{\bullet}$), o radical hidroxila ($OH^{\bullet}$), sendo este o mais reativo em sistemas biológicos, devido a sua facilidade em ligar-se a metais, outros radicais ou qualquer molécula biológica. A geração de EROs pode levar ao estresse oxidativo, porém possui grande importância em diversos processos biológicos como fagocitose, regulação do crescimento celular e sinalização intra e intercelular (HALLIWELL & GUTTERIDGE, 2007).

O estresse oxidativo, em sistemas biológicos, é caracterizado por um desequilíbrio entre EROs e espécies reativas de nitrogênio (ERNs) e seus respectivos agentes antioxidantes (HALLIWELL & GUTTERIDGE, 2007). O aumento de espécies reativas ou redução de defesas antioxidantes está envolvido na peroxidação lipídica, oxidação de proteínas e ácidos nucleicos (SUZUKI *et al*., 2006). É necessário um equilíbrio entre espécies reativas e antioxidantes e, por isso, os organismos desenvolveram, ao longo da evolução, mecanismos de defesas antioxidantes enzimáticos e não-enzimáticos. As moléculas antioxidantes não-enzimáticas podem ser de origem endógena ou exógena (HALLIWELL &

GUTTERIDGE, 2007).

As principais defesas antioxidantes enzimáticas são a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx). O $O_2^{\bullet -}$ sofre ação da SOD, formando H_2O_2, produto não-radicalar, menos reativo, pela reação $O_2^{\bullet -} + e^- + 2H^+ \rightarrow H_2O_2$. Em mamíferos, existem três diferentes tipos: a MnSOD (mitocondrial), a CuZnSOD (citoplasmática) e ainda a EC-SOD (uma CuZnSOD extracelular) (HALLIWELL & GUTTERIDGE, 2007).

Existem defesas antioxidantes não-enzimáticas endógenas e exógenas. O ácido úrico, a glutationa (VIÑA *et al*., 2005), a progesterona e o estradiol (MOORTHY *et al*., 2005, VIÑA *et al*., 2005) são importantes exemplos de antioxidantes não-enzimáticos endógenos.

A glutationa (GSH), um oligopeptídeo endógeno, além de ser substrato da GPx, também é o principal composto não-enzimático antioxidante intracelular (VIÑA *et al*., 1978), estando presente em concentrações semelhantes a da glicose em hepatócitos (VIÑA *et al*., 1978). A GSH é um sequestrador de radicais livres em condições fisiológicas ou sob ação de xenobióticos, além de participar na regeneração dos antioxidantes ácido ascórbico e tocoferol. Sua ação como antioxidante não-enzimático está mais diretamente vinculada a sua ação sobre o H_2O_2 e peróxidos orgânicos (MONOSTORI *et al*., 2009). A GSH forma adutos com diferentes xenobióticos (clorofórmio, nitratos orgânicos, bromobenzeno, aflatoxina, DDT, naftaleno e paracetamol), reação esta que pode ocorrer espontaneamente ou catalisada pela família de enzimas glutationa S-transferases (GsT), através da reação $RX^{\bullet} + GSH \rightarrow RSG + HX$. Em eucariotos, existem diversas GsT citoplasmáticas, bem como ligadas a membranas. Além de apresentarem papel na

detoxificação, também funcionam com carreadoras, ligando-se de forma não-enzimática a proteínas com grupos heme, bilirrubina e hormônios esteroides e da tireoide. Interessantemente, um resíduo de tirosina é importante na ligação da GsT a GSH, porém este pode sofrer nitração pelo peroxinitrito ($ONOO^-$), inativando a enzima (HALLIWELL & GUTTERIDGE, 2007).

Existem importantes antioxidantes de origem exógena, como carotenoides, flavonoides, ácido ascórbico (vitamina C) e tocoferol (vitamina E). O tocoferol é um dos mais importantes antioxidantes lipofílicos, possuindo importante atuação na proteção contra a peroxidação lipídica e é reduzido pelo ácido ascórbico, recuperando sua atividade antioxidante (HALLIWELL & GUTTERIDGE, 2007).

Os marcadores do dano em biomoléculas têm sido utilizados para inferir as consequências do estresse oxidativo. A mensuração de grupos carbonila é utilizada para inferir o dano oxidativo em proteínas (HALLIWELL & GUTTERIDGE, 2007). O malondialdeído (MDA) é um dos subprodutos da peroxidação lipídica e importante marcador desta (HALLIWELL& GUTTERIDGE, 2007). A LDL oxidada (ox-LDL – lipoproteínas de baixa densidade oxidadas) é um marcador da peroxidação das lipoproteínas de baixa densidade (LDL), de grande importância no estudo da aterosclerose (LEVITAN *et al*., 2010). As consequências mutagênicas da ação do estresse oxidativo podem ser inferidas pela utilização do marcador, 8-oxo-7,8-dihidro-2'-desoxiguanosina (8-OHdG), resultante da oxidação dos resíduos de guanosina (HALLIWELL & GUTTERIDGE, 2007).

Hormônios Sexuais e Estresse Oxidativo

Grande parte da literatura sobre estresse oxidativo e hormônios sexuais tem apontado os hormônios andrógenos como pró-oxidantes. A testosterona aumenta o estresse oxidativo e, ainda, homens castrados vivem mais (HALLIWELL & GUTTERIDGE, 2007), mostrando que a queda fisiológica nas concentrações de tesosterona, que ocorre ao longo do envelheciemento, pode ter um efeito benéfico. Dentre os poucos estudos com andrógenos, a orquiectomização (procedimento de remoção dos testículos) em ratos causou aumento do estresse oxidativo, porém a administração de testosterona ou dihidrotestosterona (DHT) não modificam a produção de EROs, nem alteram a atividade de MnSOD (apesar de, interessantemente, a testosterona ter aumentado sua expressão). Além disso, ocorre um aumento considerável do peso corporal em ratos sob administração de testosterona ou DHT (RAMARA *et al*., 2007). Porém, este mesmo estudo demonstra que o estradiol, em ambos os sexos, reduz o estresse oxidativo e a produção de $O_2^{\bullet -}$, diminuindo ainda o peso corporal.

Os estrógenos possuem reconhecido efeito antioxidante. Estradiol, estrona e estriol reduzem EROs e inibem peroxidação lipídica, incluindo a oxidação de LDL (HALLIWELL & GUTTERIDGE, 2007). Em ratos, mitocôndrias de fêmeas produzem aproximadamente metade do H_2O_2 produzido pelos machos e seus níveis de 8-OHdG são um quarto menores (VIÑA *et al*., 2005). Os estrógenos reduzem a produção de H_2O_2 mitocondrial, além de aumentar a expressão de diversas proteínas da cadeia transportadora de elétrons mitocondrial, como o citocromo C e as subunidades do complexo IV, aumentando também a atividade do último e da citrato sintase (STIRONE *et al*., 2005). Além disso, sua característica antioxidante está relacionada à modulação da atividade de

diferentes enzimas antioxidantes. O tratamento com estrógenos aumenta a atividade da MnSOD em tecido vascular tanto em testes *in vitro* como *in vivo* (PEDRAM *et al*., 2006, STREHLOW *et al*., 2003) e em quantidades fisiológicas aumenta a atividade desta e da GPx-1 (HALLIWELL & GUTTERIDGE, 2007).

Os estrógenos, antes apontados como antioxidantes quimicamente ativos, possuem sua ação principalmente vinculada à ativação dos genes de enzimas antioxidantes, como a MnSOD e a GPx, através da ligação do estrógeno ao seu receptor por cascatas de sinalização que incluem a MAP-cinase e NFκα (VIÑA *et al*., 2005). Receptores de estrógeno são encontrados em diferentes tecidos e, por este motivo, a ação deste hormônio se torna importante não apenas nos ovários, útero e glândulas mamárias, mas também em linfócitos B e T, macrófagos, estroma tímico, estroma da medula óssea e células endoteliais (CUTOLO *et al*., 1995, SHAMES, 2002). Os estrógenos possuem dois receptores nucleares, as isoformas ER-α e ER-β (COUSE & KORACH, 1999).

Aterosclerose e Estresse Oxidativo

O estresse oxidativo tem importante papel na formação da placa aterosclerótica. A aterosclerose é caracterizada pelo desenvolvimento de uma lesão aterosclerótica em grandes artérias (SCHWENKE, 1998). O processo inicial da aterosclerose ocorre através da modificação da superfície do endotélio vascular através do dano oxidativo, onde as células danificadas recrutam monócitos e linfócitos-T para a parede do vaso. A alteração endotelial leva à indução de células endoteliais, moléculas de adesão e de células inflamatórias

(SUZUKI *et al*., 2006). Na parede arterial, os macrófagos (antes monócitos) fagocitam lipoproteínas de baixa densidade (LDL) peroxidadas presentes no tecido (MADAMANCHI *et al*., 2005). A peroxidação lipídica das LDL, formando LDL oxidadas (ox-LDL) e sua importância no processo inflamatório de formação da placa aterosclerótica reforçam a importância do estresse oxidativo na aterosclerose.

As EROs liberadas pelo próprio macrófago sobre ox-LDL formam moléculas hiperoxidadas, causando sua transformação em células espumosas que, associadas aos leucócitos, formam as estrias de gordura arteriais, secretam fatores de crescimento que induzem a migração e proliferação das células de músculo liso para a camada íntima (MADAMANCHI *et al*., 2005). Há amplificação do recrutamento de leucócitos e agregação plaquetária e progressivo acúmulo de depósitos lipídicos, levando a maior ativação da cascata inflamatória. Ocorre então o revestimento fibrótico desta região e possível calcificação (LIBBY & THEROUX, 2005). Em síndromes agudas coronarianas, como o infarto do miocárdio, ocorre rompimento da placa e formação de trombo, que pode causar a obstrução de vasos (MADAMANCHI *et al*., 2005). A participação das EROs na formação da placa aterosclerótica está esquematizado na figura 1:

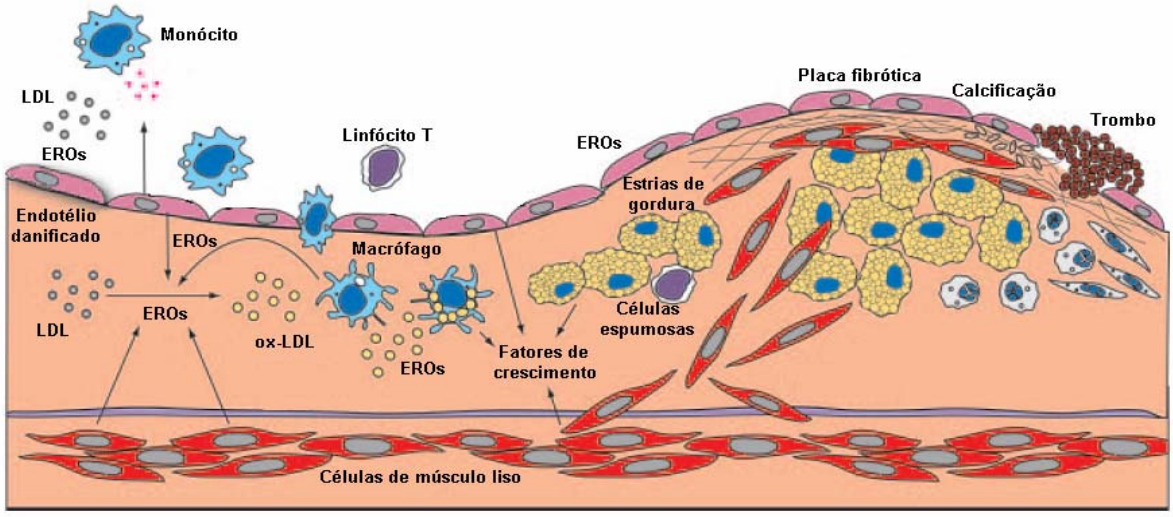

Figura 1: representação esquemática da formação da placa aterosclerótica e a participação das EROs neste processo. Traduzida e modificada de Madamanchi *et al*., 2005.

O 8-OHdG encontra-se aumentado em doenças cardíacas (COLLINS *et al*., 1998) e é possível estar também relacionado à aterosclerose (GACKOWSKI *et al*., 2001), já que o 8-OHdG está associado ao encurtamento dos telômeros em células progenitoras endoteliais em pacientes com DAC (SATOH *et al*., 2008).

As lipoproteínas de baixa densidade (HDL) são consideradas antiaterogênicas (JAYAKUMARI & THEJASEEBAI, 2009, TAN *et al*., 2006) graças às suas propriedades antiinflamatórias e antioxidantes (TAN *et al*., 2006). A maior parte de sua ação antioxidante está relacionada à enzima paraoxonase-1 (PON-1), uma esterase componente de sua estrutura. A PON-1 hidrolisa lipídeos peroxidados e sua ação sobre as ox-LDL pode explicar a característica antiaterogênica das HDL (JAYAKUMARI & THEJASEEBAI, 2009). As HDL previnem o acúmulo de peróxidos lipídicos nas paredes dos vasos sanguíneos

através da atividade de PON-1 (AVIRAM *et al*., 2000), evitando o acúmulo de ox-LDL (DURRINGTON *et al*., 2001). Corroborando com estes dados, um estudo prévio em pacientes com DAC mostrou que estes apresentam menor atividade da PON-1, comparados a um grupo controle (JAYAKUMARI & THEJASEEBAI, 2009). Sua concentração está relacionada à quantidade de HDL circulante, já que é transportada por estes complexos. Na resposta inflamatória em sepse, foi demonstrado que o estresse oxidativo reduz as concentrações de HDL e, consequentemente, a atividade da PON-1 (NOVAK *et al*., 2010), mostrando que o processo inflamatório pode estar envolvido nesta diminuição (DURRINGTON *et al*., 2001, NOVAK *et al*., 2010).

Quanto à relação entre os compostos antioxidantes e a aterosclerose, estudos em adultos saudáveis mostram uma relação inversamente proporcional entre espessamento arterial e GSH (ASHFAQ *et al*., 2006). Em cultura de células endoteliais de *Coturnix coturnix japonica* (codorna japonesa) foi demonstrado que um grupo suscetível à aterosclerose apresenta reduzida concentração de glutationa total (GSH +GSSG) em relação ao resistente (HOEKSTRA *et al*., 2003). Estes estudos mostram uma tendência do organismo a aumentar a expressão deste antioxidante durante o processo aterosclerótico.

Aterosclerose e Hormônios Sexuais

A participação dos hormônios esteroides tem sido discutida da fisiopatologia da DAC. Apesar de muitos estudos avaliarem seu efeito através da administração destes em concentrações preestabelecidas, as concentrações endógenas podem ter importante participação na predisposição de um indivíduo a

desenvolver uma patologia. Como pode ser visto na Tabela 1, indivíduos normais apresentam grande variação nas concentrações séricas dos hormônios testosterona, progesterona e estradiol. Além disso, modificações consequentes de uma determinada doença podem também alterar as concentrações normais dos hormônios circulantes.

Tabela 1: valores de clínicos utilizado como referência em análises por eletroquimioluminescência, para a concentração dos hormônios esteroides testosterona, progesterona e estradiol em indivíduos normais.

Valores de referência	
Testosterona (ng/mL)	homens: 3 a 10 mulheres: fase folicular e luteal: 0,2 a 0,8 pós-menopausa: 0,08 a 0,35
Progesterona (ng/mL)	homens: 0,2 a 1,4 mulheres: fase folicular: 0,2 a 1,5 fase ovulatória: 0,8 a 3,0 fase luteal: 1,7 a 27 pós-menopausa: 0,1 a 0,8
Estradiol (pg/mL)	homens: 7,63 a 42,6 mulheres: fase folicular: 12,5 a 166 pico ovulatório: 85,8 a 498 fase luteal: 43,8 a 211 pós-menopausa: 5,0 a 54,7

Os efeitos dos estrógenos têm sido amplamente discutidos na aterosclerose. A administração de estradiol em mulheres antes da menopausa tem demonstrado efeito ateroprotetor, porém, sua administração pós-menopausa causa considerável efeito deletério, aumentando a placa de ateroma (ARNAL *et al*., 2006, GOURDY *et al*., 2008). Em um estudo realizado utilizando coelhos hipercolesterolêmicos, o efeito protetor da administração do estradiol

demonstrado nos controles foi abolido pela injúria da artéria provocada por cateter-balão, sugerindo que a integridade do epitélio é necessária para que a administração do estradiol apresente efeito ateroprotetor (HOLM *et al*., 1997).

Em ratos, o estradiol exógeno previne a formação de estrias de gordura arteriais e ainda, por influenciar a formação de HDL e LDL, reduzindo colesteróis no soro (BOURASSA *et al*., 1996, ELHAGE *et al*., 1997). As células inflamatórias também são influenciadas pela ação dos estrógenos (CUTOLO *et al*., 1995, SHAMES, 2002) e, consequentemente, seu estudo pode elucidar a ação destes e talvez de outros hormônios na formação da placa de ateroma. Porém, esta relação é ainda pouco compreendida e controversa (GOURDY *et al*., 2008, SELI & ARICI, 2002).

Estudos em células progenitoras epiteliais da circulação, em pacientes, mostram que andrógenos não possuem influência na proliferação e adesão, mas não descartam a possível importância do estradiol nestas células (FADINI *et al*., 2009). A frequência com que baixos níveis de testosterona são encontrados em pacientes com DAC (TRAISH *et al*., 2009, AKISHITA *et al*., 2010, WU & VON ECKARDSTEIN, 2003) torna a concentração deste hormônio preditora de DAC (KAUSHIK *et al*., 2010, FADINI *et al*., 2009).

Os estrógenos exercem ainda influência sobre mensageiros endoteliais como o óxido nítrico ($NO^{\bullet}$), que possui ação vasodilatadora. O estradiol aumenta a atividade da enzima NO-sintase (NOS) responsável pela produção endotelial de $NO^{\bullet}$ (MENDELSOHN, 2000). Ainda, a inibição da eNOS (NOS endotelial) resulta na redução da ação ateroprotetora do estradiol (HOLM *et al*., 1997). O ER-α de estrógeno mostrou ser a única isoforma estritamente necessária nesta regulação

da produção de $NO^{\bullet}$ endotelial (PENDARIES *et al*., 2002). O $NO^{\bullet}$ é considerado um radical livre antiaterosclerótico por inibir a adesão de monócitos às células endoteliais, inibição da proliferação de células de músculo liso, além de sua ação como vasodilatador e inibidor da agregação plaquetária (VANHOUTTE, 2009).

O $NO^{\bullet}$ produzido pela oxido nítrico sintase endotelial (eNOS) é conhecido por alterar o fluxo sanguíneo, alterando processos envolvidos na aterosclerose (VANHOUTTE, 2009), sendo considerado um importante ateroprotetor.

A atividade da eNOS é alterada pelo estresse oxidativo. Em condições normais, a enzima produz $NO^{\bullet}$, mas em condições de hipóxia passa a produzir grandes quantidades de $O_2^{\bullet -}$. Desta forma, o $NO^{\bullet}$ terá maior probabilidade de reagir com o $O_2^{\bullet -}$ formando o peroxinitrito ($ONOO^-$), molécula reativa, importante no dano oxidativo a proteínas (YAMAUCHI & KIMURA, 2008).

Distúrbios Respiratórios do Sono e Estresse Oxidativo

Os distúrbios respiratórios do sono vão desde sintomas leves, como o ronco até a apneia do sono graves (ARENS & MARCUS, 2004). A Síndrome das Apneias-Hipopneias Obstrutivas do Sono, ou mais comumente chamada Apneia Obstrutiva do Sono (AOS), é uma doença cujos principais sintomas são roncos e sonolência diurna alta, tendo seu diagnóstico confirmado por polissonografia (MCNICHOLAS *et al*., 2002). É caracteriza pela obstrução repetitiva completa da via aérea superior por ao menos 10 segundos. A sonolência é consequência dos despertares ocorridos pela queda do oxigênio sanguíneo devido à obstrução. Esta obstrução é consequente da obstrução mecânica das vias aéreas superiores. As

causas mais comuns são a hipertrofia da adenoide, a obesidade, assim como distúrbios craniofaciais e neuromusculares (BRADLEY & FLORAS, 2009, KERSTEIN *et al*., 2009).

Esta síndrome é mais frequente em homens, idosos e obesos e está presente em torno de 1% a 10% da população de meia-idade (ARENS & MARCUS, 2004, RONALD *et al*., 1999), porém estas porcentagens variam de forma significativa de acordo com a população estudada (BRADLEY & FLORAS, 2009). Pacientes que sofrem ganho de peso de 10% têm seis vezes mais chance de desenvolver apneia do sono (BRADLEY & FLORAS, 2009).

As apneias e hipopneias repetitivas causam hipóxia, hipercapnia, aumento de frequência e arritmia cardíacas, como resultado do aumento da ativação simpática. Os eventos de hipóxia/reoxigenação, semelhantes aos já estudados eventos de hipóxia/reperfusão (SUZUKI *et al*., 2006, YAMAUCHI & KIMURA, 2008), ocasionados pela AOS causam estresse oxidativo pelo aumento na formação de EROs, principalmente $O_2^{\bullet-}$, $OH^{\bullet}$ e H_2O_2, provocando alteração do equilíbrio redox (MCNICHOLAS *et al*., 2002, SCHULZ *et al*., 2000, YAMAUCHI *et al*., 2005). O aumento de EROs em células endoteliais expostas à hipóxia já foi evidenciado. Dentre as possíveis fontes de EROs devido à hipóxia estão as mitôcondrias, leucócitos (através da enzima NADPH-oxidase) e enzimas dos tecidos epiteliais, como xantina oxidase, ciclooxigenase, lipooxigenase, NO-sintase e heme-oxigenases (LAVIE, 2003).

A AOS está relacionada a uma resposta inflamatória aumentada, sugerindo outra possibilidade para explicar o estresse oxidativo aumentado nesses pacientes (SUZUKI *et al*., 2006). Além disso, ocorre maior expressão de

moléculas de adesão e correlação com o aumento de EROs em monócitos de pacientes com apneia, podendo levar a complicações vasculares como a DAC (LAVIE, 2003).

O fator hipóxia-indutível 1 (HIF-1) regula a resposta celular à hipóxia, através da manutenção do estado redox, reduzindo o estresse oxidativo (GUO *et al*., 2009).

O dano oxidativo já foi evidenciado na AOS. Pacientes com apneia do sono apresentam maior peroxidação lipídica, aumento de ox-LDL, maior dano oxidativo ao DNA e reduzida capacidade antioxidante total (SUZUKI *et al*., 2006). Ainda, o $NO^{\bullet}$ circulante está reduzido na apneia, porém este processo é revertido com o uso do CPAP (IP *et al*., 2000).

Em pacientes com apneia obstrutiva do sono, as HDL têm menor habilidade de proteger as LDL da oxidação e os níveis de ox-LDL são maiores (TAN *et al*., 2006). Desta forma, o risco vascular associado aos DRS pode estar associado à interação LDL/HDL e ao estresse oxidativo. Um exemplo claro desta relação mostra que as HDL inibem a oxidação de LDL, reduzem a resposta inflamatória em células endoteliais, inibem cascatas de coagulação e aumentam a disponibilidade do vaso dilatador e radical livre óxido nítrico ($NO^{\bullet}$) (TAN *et al*., 2006).

Dentre os poucos estudos enfatizando as moléculas antioxidantes nos eventos de hipóxia, foram demonstrados níveis de GSH aumentados e de GSSG reduzidos durante a queda de saturação de oxigênio (LLORET *et al*., 2007). Porém, existe uma correlação negativa entre status antioxidante total (TAS) e apneia do sono severa (CHRISTOU *et al*., 2003).

Estudos em AOS já mostraram que o aumento dos níveis urinários de 8-OHdG nos pacientes está associado ao aumento da gravidade da síndrome (YAMAUCHI *et al*., 2005) e à duração das dessaturações noturnas (JORDAN *et al*., 2006).

Distúrbios Respiratórios do Sono e Hormônios Sexuais

Os efeitos dos hormônios sexuais têm sido avaliados em diferentes doenças. Porém, poucos trabalhos relacionam hormônios aos DRS. Dentre estes poucos, a influência da progesterona na apneia foi evidenciada. Experimentos realizados em ratos mostraram que este hormônio reduz a ocorrência de apneias em machos adultos (YAMAZAKI *et al*., 2005) e, ainda, reduz eventos de apneia e a resposta ventilatória a hipóxia em filhotes, apesar da duração média das apneias manter-se constante (LEFTER *et al*., 2007). A administração em ratos de 17-beta-estradiol reduz rotas inflamatórias e disfunções cardíacas relacionadas a isquemia/reperfusão (WANG *et al*., 2008).

A maior parte da literatura científica mostra os efeitos da administração desses hormônios, dificilmente retratando modificações nas concentrações endógenas. Alguns estudos epidemiológicos têm tentado relacionar modificações hormonais endógenas a distúrbios respiratórios do sono, porém os mecanismos pelos quais esta relação ocorre ainda são desconhecidos (SHAHAR *et al*., 2003, YOUNG *et al*., 2003). KIRBAS (2007) mostrou concentrações significantemente menores de testosterona em pacientes com AOS (KIRBAS *et al*., 2007), assim como tem sido evidenciado na DAC.

Homeostase do ferro, DRS e Aterosclerose

A ferritina é um complexo de proteínas responsável pelo estoque intracelular do ferro (YOU & WANG, 2005). A concentração de ferritina no soro está diretamente relacionada ao estoque de ferro corporal (YOU & WANG, 2005), e níveis elevados de ferritina estão associados com maior risco de DAC (AHLUWALIA *et al.*, 2010; TORTI & TORTI, 2002; YOU & WANG, 2005).

Condições de hipoxia regulam positivamente a ferritina (TORTI & TORTI, 2002) e o estresse oxidativo gerado pela hipóxia/reoxigenação participa em sua regulação (HOWER *et al*., 2009). Por exemplo, células em cultura tratadas com H_2O_2 apresentam um aumento contínuo de transcrição e tradução da ferritina (TSUJI *et al.*, 2000). Ainda, a inflamação dependente de hipóxia pode participar na sua regulação, já que também sua transcrição e tradução são induzidas por citocinas inflamatórias, como IL-1alfa, IL-6 e TNF-alfa (TORTI & TORTI, 2002; YOU & WANG, 2005).

A transferrina é um complexo de proteínas responsável pelo transporte do ferro na circulação para os tecidos (HOWER *et al.*, 2009). A soma do ferro ligado à transferrina e o ferro ligado a outras moléculas, como a albumina e o citrato, representa o ferro disponível para o uso metabólico, chamado de ferro livre (HALLIWELL & GUTTERIDGE, 2007; HOWER *et al.*, 2009). O ferro livre, assim como a concentração de ferritina, é frequentemente inverso aos níveis de ferritina (HOWER *et al.*, 2009; LEE & JACOBS, 2004; YOU & WANG, 2005). Baixas concentrações de ferro livre estão associadas à DAC (PATEL *et al.*, 2008). Existe uma relação entre deficiência de ferro e DRS em pacientes com falha cardíaca

crônica e ainda, a incidência clínica e os efeitos dos DRS são reduzidos pela suplementação do ferro (KERSTEIN *et al.*, 2009). Os mecanismos envolvidos na redução do ferro livre nos DRS e DAC não são claros, e podem estar relacionados ao aumento da ferritina pela hipóxia, inflamação e estresse oxidativo.

A influência da testosterona na homeostase do ferro ainda está em discussão. Sabe-se que a testosterona exógena e endógena aumentam a eritropoiese e os níveis de hemoglobina (COVIELLO *et al*., 2008; RUSHTON & BARTH, 2010), porém, não existem dados referentes ao ferro livre e a ferritina (RUSHTON & BARTH, 2010).

Distúrbios Respiratórios do Sono e Aterosclerose

Os DRS induzem modificações biológicas através de diversos processos citados acima, porém a hipóxia/reoxigenação é considerada a maior causa de alterações cardiovasculares (SUZUKI *et al*., 2006). DRS e DAC apresentam quadro de inflamação e de estresse oxidativo (JELIC *et al*., 2008).

Diversos trabalhos têm demonstrado a associação dos DRS com diversos problemas cardiovasculares. A apneia do sono também aumenta o risco de ataque cardíaco e morte (YAGGI *et al*., 2005). Pacientes com SAHOS têm maior espessamento da camada íntima-média da carótida (MINOGUCHI *et al*., 2005, SUZUKI *et al*., 2006, SUZUKI *et al*., 2004) e apresentam 4,6 vezes mais chance de desenvolver DAC (DYUGOVSKAYA *et al*., 2002).

Quanto mais severas as manifestações clínicas dos DRS, como sonolência diurna, maior a probabilidade de desenvolver complicações cardiovasculares

(BRADLEY & FLORAS, 2009). Em pacientes com DAC, a concomitância com os DRS aumenta a mortalidade de 9% para 38% (PEKER *et al*., 2006). Interessantemente, a inflamação endotelial e alguns marcadores de estresse oxidativo são reduzidos pelo tratamento padrão-ouro para apneia do sono, o CPAP (*continuous positive airway pressure*) (JELIC *et al*., 2008).

Ainda, pacientes com AOS, mas sem doença cardiovascular, apresentam sinais de aterosclerose. Desta forma é possível inferir que os DRS tenham papel no desenvolvimento e progressão da aterosclerose (DRAGER *et al*., 2005). A prevalência da AOS é maior em populações que sofrem com doenças cardiovasculares, como a hipertensão (30–83%), falha cardíaca (12–53%), doença cardíaca esquêmica (30–58%) e ataque cadíaco (43–91%) (BRADLEY & FLORAS, 2009).

Apesar das evidências apresentadas, existem ainda dados contraditórios que reforçam a necessidade de estudos mais aprofundados sobre a associação entre DRS e DAC. Shahar e colaboradores (SHAHAR *et al*., 2003, SHAHAR *et al*., 2001) encontraram uma relação moderada entre IAH e DAC, porém forte entre o IAH e falha cardíaca e ataque cardíaco.

Os dados citados indicam existir associação entre estresse oxidativo, hormônios esteroides, gravidade da doença aterosclerótica obstrutiva coronariana e DRS, embora os mecanismos estejam ainda incertos. Para esclarecer aspectos destas associações, se faz necessária investigação dos possíveis mecanismos hormonais e suas relações com o estresse oxidativo, com o objetivo de elucidar o papel dos DRS na formação da placa aterosclerótica e da doença arterial coronariana.

OBJETIVOS

Objetivo Geral

Estudar o estresse oxidativo e as alterações fisiopatológicas na associação entre DRS a DAC, bem como avaliar a participação dos hormônios esteroides estradiol, progesterona e testosterona neste processo.

Objetivos específicos

I. Avaliar a relação entre os níveis de estradiol, testosterona e progesterona e diferentes parâmetros de estresse oxidativo (dano oxidativo e defesas antioxidantes) e os parâmetros de bioquímicos dos pacientes;
II. Relacionar os níveis de estradiol, testosterona e progesterona na associação entre DAC e DRS;
III. Avaliar a relação entre os parâmetros de estresse oxidativo na associação entre DAC e DRS.

CAPÍTULO 1

Artigo Científico 1

Artigo submetido à revista *Free Radical Research.*

Iron Homeostasis and Testosterone in the Association between Coronary Artery Disease and Sleep Disordered Breathing

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Keywords: Obstructive sleep apnea, atherosclerosis, oxidative stress, ferritin, nitric oxide.

Abstract

Sleep Disordered Breathing (SDB) and Coronary Artery Disease (CAD) are both considered oxidative stress-associated disorders. Obstructive sleep apnea (OSA) and hypoxia cause a reduction in $NO^{\bullet}$-dependent vasodilation, causing endothelial dysfunction and atherosclerosis progression. Hypoxic conditions upregulate ferritin. Elevated ferritin levels are associated with high CAD risk. Serum free iron and testosterone are frequently reduced under hypoxic conditions and in atherosclerosis. Testosterone is related to iron homeostasis proteins regulation. In this report we studied 29 controls and 27 cases patients with CAD (defined as > 30% coronary narrowing). We found that ferritin was increased in CAD patients and was positively correlated with the apnea-hypopnea index (AHI) and was negatively correlated with O_2 saturation. Ferritin was strongly correlated with erythrocyte carbonyl levels. Nitrites and nitrates were lower in CAD patients and were negatively correlated with ferritin. Regression analysis showed that total testosterone reduction and available iron decrease were important CAD predictors. SDB and decreased testosterone may drive the ferritin increase, consequently resulting in decreased levels of free and transferrin-bound iron. The increase in ferritin caused by hypoxia may be related to oxidative stress and $NO^{\bullet}$ decrease in CAD patients.

Abbreviations: AHI, apnea-hypopnea index; BMI, body mass index; CAD, coronary artery disease; CRP: C-reactive protein; GSH: reduced glutathione. GSSG: oxidized glutathione; GsT: glutathione-S-transferase; HIF: hypoxia-inducible factors; IL: interleukin; IRE: Iron-responsive elements; IRP: iron-

regulatory proteins; $NO^{\bullet}$: nitric oxide; OSA, obstructive sleep apnea; SDB, Sleep disordered breathing; TNF: Tumor necrosis factor.

Introduction

Morbidity and mortality due to coronary artery disease (CAD) are highly associated with sleep disordered breathing (SDB) [1-3]. SDB and CAD are both considered to be oxidative stress-associated and inflammation-associated disorders [2, 4, 5]. Obstructive sleep apnea (OSA) is an important form of SDB, characterized by repetitive upper airway obstruction resulting in intermittent hypoxia [2]. Intermittent hypoxia induces inflammatory pathways and oxidative stress, causing function impairment of the vascular endothelium [2]. *In vivo* and *in vitro* experiments have provided strong evidence of free radical formation in hypoxic conditions [6]. Also, OSA causes a reduction in $NO^{\bullet}$-dependent vasodilation [6] and may have vascular consequences.

Hypoxia-inducible factors (HIF) are transcription factors expressed as the body acclimates to hypoxic conditions [7]. HIF bind to hypoxia-response elements (HRE) in mRNA, altering translation of mRNAs related to angiogenesis, cell proliferation, cell survival, pH regulation, erythropoiesis, energy metabolism, and apoptosis [8, 9]. Iron-responsive elements (IRE) are nucleotide hairpin structures in mRNA involved in regulating the posttranscriptional response to iron concentration through binding of iron-regulatory proteins (IRP). HIF-1 alpha and HIF-2 alpha mRNA contain IRE, and in turn, HIF activity regulates iron levels by affecting the transcription of genes involved in iron homeostasis [9]. Thus, SDB

hypoxia may contribute to changes in iron regulation frequently present in CAD patients.

Ferritin is an iron stocking protein complex regulated through IRE [10]. The serum ferritin level correlates with total body iron stores [10], and elevated serum ferritin levels are associated with high CAD risk [10-12]. Hypoxic conditions upregulate ferritin [12], and oxidative stress generated from the hypoxia/reoxygenation process participates in ferritin regulation [13]. In this regard, cultured cells treated with hydrogen peroxide exhibit a sustained increase in ferritin translation and transcription [14]. Moreover, hypoxia-related inflammation may participate in ferritin regulation. Proinflammatory cytokines IL-1alpha, IL-6, and TNF-alpha also induce transcription and translation of ferritin [10, 12].

Transferrin-bound iron and non-transferrin-bound iron represent the iron available for use in cellular metabolism [13, 15], and both are frequently inversely related to ferritin levels [10, 13, 16]. Citrate and albumin are small iron-binding molecules in serum; iron complexed to these molecules is termed non-transferrin-bound iron or free iron [15]. Lower serum free iron concentrations are associated with CAD [17]. There is a relationship between iron deficiency and SDB in patients with chronic heart failure, and moreover, the clinical incidence and effects of SDB are reduced by iron supplementation [18]. The mechanisms involved in causing reduced free iron associated with SDB and CAD has to be evaluated, and may be related to the ferritin increase in hypoxic and oxidative stress conditions.

Despite controversy regarding SDB and androgens [19, 20] it is known that testosterone secretion occurs during sleep [20, 21], and that decreased testosterone levels are restored by nasal continuous positive airway pressure

(nCPAP) treatment [20]. Moreover, studies showed a reverse relationship between AHI and testosterone [21, 22]. The capacity of free radicals to decrease testosterone production has been established in cell culture [23, 24], and testosterone formation is affected by HIF-1 alpha and oxidative stress [8].

The influence of testosterone in iron homeostasis is still under discussion. It is known that endogenous and exogenous testosterone increases erythropoiesis and hemoglobin levels [25, 26]; however, no data related to ferritin or free iron have been reported [26].

A consistent inverse relationship between endogenous testosterone and adverse cardiovascular events has been shown [27-30], and low testosterone levels are considered a predictor of cardiovascular diseases [30-32]. Controversial opinions regarding testosterone abound, and both anti- and pro-atherosclerotic points of view have been propounded [30, 33]. Thus, the consequences of this hormonal change are still under discussion.

In the present work, which is a logical extension of our previous study [5], we postulated that SDB and testosterone reduction participate in increased ferritin levels; the consequent decreased transferrin-bound and free iron levels would partially explain the iron and testosterone profile frequently present in CAD patients. Moreover, increased ferritin may be related to oxidative stress and $NO^{\bullet}$ reduction in CAD patients, further implicating ferritin as a participant in the pathophysiology of CAD.

Material and methods

Patients and CAD Study

The project was approved by the Hospital de Clínicas de Porto Alegre ethics committee, and all participants signed an informed consent form. A cross-sectional study was conducted by screening patients referred for diagnostic or therapeutic coronary angiography. The exclusion criteria were: age less than 35 or greater than 65 years; smoking in the previous 6 months; clinical diagnosis, dietary, or pharmacological treatment for diabetes mellitus; anginal pain in the previous week; use of anxiolytic medication; treatment for chronic pulmonary disease; use of vitamin supplement; body mass index (BMI) ≥ 40 kg/ m^2; any physical, psychological, or social issue that would interfere with conducting the home polysomnographic test; and previous coronary intervention (myocardial revascularization or angioplasty). Hypertension, past history of smoking, and medication use were not criteria for exclusion, but the numbers of these conditions was similar between controls and CAD patients. All patients were assessed by quantitative angiography using the same equipment and projection, with the table and image intensifier kept at constant height. A seven inches magnification was used for all images. Image quantification was carried out by the same investigator, who was blinded with respect to clinical and biochemical variables. Patients with luminal narrowing of 30% or higher were considered to have CAD. Patients with no narrowing or narrowing less than 30% were included in the control group.

Sleep Study

In the polysomnographic study, a Level III portable monitor (SomnoCheck, Weinmann, Germany) was used at home by the patients. A nasal cannula

connected to a pressure transducer was employed to quantify air flow and snoring. Inspiratory effort, pulse oximetry, heart rate, and sleep position were also verified. The polysomnography respiratory analyses were made by a sleep specialist who was blinded to clinical and biochemical variables. Apnea was defined as airflow reduction to 10% or less of the baseline value persisting for 10 seconds or more; hypopnea was defined as airflow reduction of 50% or more, associated with a reduction of 3% or more in oxygen saturation. The AHI was defined as apnea/hypopnea episodes per hour, calculated by dividing the sum of total apneas and hypopneas by the hours of recorded polysomnography.

Laboratory measurements

Approximately 20 mL arterial blood was collected from each patient from femoral artery puncture for catheterization. Blood was collected in vials containing coagulation inhibitor, EDTA and citrate. Immediately after collection, the samples were refrigerated to 0°C, centrifuged for 10 min at 0°C, aliquoted, and stored at -80°C. Hemolysates were prepared by lysing red blood cells with 2% ethanol (ratio 1:10) followed by centrifugation to obtain crude extracts. High-sensitivity C-reactive protein, total proteins, ferritin, transferrin, available iron, hemoglobin, and erythrocytes were quantified in routine clinical laboratory analysis, additional tests were performed at the research laboratory. In this study, conventional “free” iron will be termed “available iron,” because the routine clinical “free” iron analysis evaluates the sum of transferrin-bound and non-transferrin-bound iron (ferrozine colorimetric method). Erythrocyte carbonyl levels were measured as previously reported [5].

Steroid hormones assay

Steroid hormones including total testosterone, estradiol, and progesterone were quantified in serum by electrochemiluminescence immunoassay (*Modular E-170, Roche*)**.**

GSH assay

Total glutathione content, (the sum of reduced glutathione (GSH) and oxidized glutathione (GSSG)), was measured in hemolysates [34]. Glutathione reductase (GR) was used to reduce GSSG to 2GSH. GSH was detected through its reaction with 5,5-dithiobis(acid 2-nitrobenzoic) (DTNB), forming the 5-thio nitrobenzoate chromophore, detected at A_{412} [35]. GSH concentration was obtained through the subtraction of GSSG from total glutathione content. Briefly, 50 µL of 2 M perchloric acid, 4 mM EDTA were added to 50 µL hemolysate. After vortexing and centrifugation, 10 µL of 0.25 *N*-ethylmaleimide was added to 90 µL of supernatant (GSSG assay only). After new vortexing and centrifugation, 77 µL of 2 M KOH was added, reaching pH 6. Samples were vortexed and centrifuged to complete preparation. Samples were maintained in ice. A standard curve was prepared using 5 µM, 10 µM, 25 µM, and 50 µM GSH in 50 mM phosphate buffer, 5 mM EDTA. The microplate assay was performed adding 174 µL of 50 mM phosphate buffer, 17 µL of 4 mM NADPH (in 0.5% $NaHCO_3$), 7 µL of 6 U/mL GR (in 10 mM phosphate buffer) and 35 µL of sample or standard. Microplates were incubated at 37°C for 5 minutes and 18 µL of 4 mg/mL DTNB (in 100 mM phosphate buffer) was added. Absorbance at A_{412} was obtained after 2 minutes

incubation at 37°C.

GsT assay

The glutathione-S-transferase antioxidant assay is based on formation of S-(2,4-dinitrophenyl)-glutathione by GsT enzymatic activity through 1-chloro-2,4-dinitrobenzene (CDNB) and GSH conjugation [36]. Briefly, hemolysates were centrifuged and maintained on ice. A spectrophotometer cuvette was filled with 500 µL 0.2mM potassium phosphate (pH6.5), 100 µL 10mM GSH, 300 µL sample, and 100 µL 10mM CDNB (in 2 ethanol:3 water, at 37°C). All assays were performed in triplicate. Absorbance was determined at A_{340}. The S-(2,4-dinitrophenyl)-glutathione molar extinction coefficient at A_{340} (ε=9600$M^{-1}cm^{-1}$) was used to calculate GsT activity.

Nitrites/nitrates assay

The Griess reaction, which measure nitrites and nitrates in the sample, was employed to determine circulating $NO^{\bullet}$ concentration [37]. Hemolysates were centrifuged and maintained on ice. A standard curve was made using 1 mM sodium nitrite ($NaNO_2$) diluted in 100 mM phosphate buffer to standard curve concentrations of 2.5 nmol/mL, 5 nmol/mL, 15 nmol/mL and 30 nmol/mL. In the microplate (all wells in triplicate) 100 µL of sample or standards were add, followed by 50 µL of Griess regent (0.02 g of naphthylenediamine dichloride, 0.2 g of sulfanilamide, 500 µL of orthophosphoric acid and 10 mL of MilliQ water). The microplate was protected from light during a10 min incubation. Absorbance was read at A_{543} [38].

Statistical analysis

Categorical variables are presented as absolute values and were analyzed by chi-square test. The Kolmogorov-Smirnov test of normality was employed to verify distribution of variables. Variables with normal distribution are presented as mean ± standard error (SE); means were compared by student's t-test. Variables without normal distribution are presented as median (minimum–maximum); medians were compared by Mann-Whitney U test. Spearman coefficient was employed to test correlation between variables without normal distribution, and Pearson coefficient was used to test correlation between variables with normal distribution. Poisson Regression analysis was employed to predict CAD, using five variables, according to the number of analyzed patients. $P \leq 0.05$ was considered statistically significant.

Results

Clinical and anthropometric data in CAD groups: Table I presents anthropometric and clinical data of analyzed patients. Gender and BMI did not differ between groups (Table I). CAD patients were significantly older than controls (Table I). AHI was significantly higher in the CAD group, and lowest O_2 saturation was decreased in the CAD group (Table I). Hematocrit and hemoglobin concentration were significantly increased in CAD patients, while erythrocytes presented no difference between groups (Table I).

Table I: Patient clinical and anthropometric data.

	Control group (n=29)	CAD group (n=27)	*P*
Gender (male)	12 (41%)	19 (70%)	Ns
Age (years)	51.62 ± 1.28	56.96 ± 1.11	p<0.05
BMI (Kg/m²)	27.30 ± 0.74	27.79 ± 0.74	Ns
AHI (events/h)	7 (1-48)	17 (1-56)	p<0.05
Lowest O_2 saturation (%)	87.5 (63 - 93)	85.0 (77 - 90)	p<0.05
Hemoglobin (g/dL)	13.04 ± 0.24	13.77 ± 0.25	p<0.05
Erythrocytes (million erythrocytes/mL)	4.47 ± 0.06	4.65 ± 0.07	Ns

Categorical data are presented as n (%). Variables with normal distribution are presented as mean ± SE. Variables without normal distribution are presented as median (minimum–maximum). ns: $p > 0.05$. BMI: Body mass index (weight divided by the square of height, Kg/m^2). AHI: apnea-hypopnea index (apnea-hypopnea/hour of sleep).

Biochemical data in CAD groups: Table II presents biochemical data from the analyzed patients. The inflammatory marker, high-sensitivity C-reactive protein (CRP), as well as the analyzed steroid hormones testosterone, estradiol and progesterone were not significantly different between groups (Table II). CAD patients presented significantly increased ferritin levels; however there was no difference in transferrin or free iron concentrations (Table II). GsT activity, total glutathione (GSH+GSSG), GSH, and GSSG levels did not differ between controls and CAD patients (Table II). Nitrites and nitrates were significantly lower in CAD patients than controls (Table II).

Table II: Patient biochemical data.

	Control group (n=29)	CAD group (n=27)	*P*
CRP (mg/L)	1.59 (0.19 - 21.50)	2.00 (0.16 - 18.40)	ns
Available Iron (µg/dL)	84.79 ± 5.14	81.00 ± 4.62	ns
Ferritin (ng/mL)	144.00 (8.3 - 720.3)	302.35 (45.50 - 685.80)	p<0.05
Transferrin (mg/mL)	255 (183 - 371)	236 (160 - 296)	ns
Total testosterone (ng/mL)	0.63 (0.11 - 8.06)	3.55 (0.11 - 6.93)	ns
Estradiol (pg/mL)	18.10 (5.00 - 411.60)	25.00 (5.00 - 624.70)	ns
Progesterone (ng/mL)	0.29 (0.15 - 6.11)	0.26 (0.15 - 25.01)	ns
Total Glutathione (µmol/g Hb)	438.44 (52.63 - 5564.45)	440.30 (57.85 - 3854.17)	ns
GSH (µmol/g Hb)	329.67 (34.44 - 5445.85)	403.04 (85.30 - 3654.09)	ns
GSSG (µmol/g Hb)	78.62 (8.86 - 558.50)	86.65 (19.37 - 880.63)	ns
GsT (U/mL)	0.054 ± 0.004	0.047 ± 0.003	ns
Nitrites/nitrates (nmol/mg Hb)	0.76 (0.39 - 1.11)	0.57 (0.26 - 1.22)	p<0.05

Variables with normal distribution are presented as mean ± SE. Variables without normal distribution are presented as median (minimum–maximum). ns: $p > 0.05$. Total Glutathione: GSH+GSSG erythrocyte content. GSH: reduced glutathione. GSSG: oxidized glutathione.

Correlation analysis: In Spearman's correlation analysis, ferritin correlated with AHI (rho=0.394, P=0.003), age (rho=0.407, P=0.002), CRP (rho=0.280, P=0.042), transferrin (rho= -0.279, P=0.039), lowest O_2 saturation (rho= -0.352, P=0.010) and nitrites/nitrates (rho= -0.273, P=0.044). In Person's correlation analysis, ferritin and erythrocyte carbonyl levels were transformed to normal distribution using square root (sr) and natural logarithm (ln), respectively; (sr) ferritin correlated with (ln) erythrocyte carbonyl levels (pmol/g Hb) (r=0.307,

P=0.024). Spearman's analysis of total testosterone levels showed correlation with ferritin (rho=0.596, $P=2x10^{-6}$) and transferrin (rho= -0.344, P=0.010). Only statistically significant correlations are presented.

Regression models: The Poisson regression model was employed to predict which variables among the analyzed parameters were more relevant for predicting CAD (Table III). Two comparative models were developed. The first model revealed that total testosterone and available iron presented an inverse correlation with CAD, when controlling for ferritin, gender, and age. In the first model, a decrease of 1 ng/mL of total testosterone increased the risk of developing CAD by 26.4%, and a decrease of 1 µg/dL of available iron increased the risk of developing CAD by 1%. In the second model, total testosterone continued to present a significant inverse correlation with CAD when controlling for AHI, ferritin, gender, and age (Table III). In the second model, a decrease of 1 ng/mL of total testosterone increased the risk of developing CAD by 21.7%, and 1 unit of AHI/h increased the risk of developing CAD by 2.1%.

Table III: Results from Poisson regression model to predict coronary artery disease

Dependent variable	Obstructive coronary artery disease	
Regressor	RR (CI)	*P*
Total testosterone	0.736 (0.59 - 0.90)	0.004
Available Iron	0.990 (0.98 - 0.99)	0.029
Ferritin	1.001 (0.99 - 1.00)	ns
Gender	0.177 (0.073 - 0.42)	0.0001
Age	1.075 (1.01 - 1.13)	0.013
Total testosterone	0.783 (0.62 - 0.98)	0.034
Ferritin	1.000 (0.99 – 1.00)	0.0001
AHI	1.021 (1.00 - 1.03)	0.015
Gender	0.271 (0.10 - 0.68)	0.006
Age	1.057 (0.98 - 1.13)	ns

ns: $p > 0.05$. AHI: apnea-hypopnea index (apnea-hypopnea/ hour of sleep). Gender: male categorized as 0 (zero) and female as 1 (one). Age is expressed in years.

Discussion

A previous study of 2 290 male patients found an inverse relationship between atherosclerotic plaque area and testosterone serum concentration [29]. According, regression analysis identified an inverse relationship between CAD and total testosterone levels in our study, despite the reduced sample size and

presence of both genders in the analysis (Table III). Despite the frequency with which coronary heart disease is associated with low levels of testosterone in men, this relationship is still controversial in woman [32]. However, some studies have reported CAD association with low levels of androgens in postmenopausal woman [39], and surprisingly, high endogenous androgen is associated with reduced carotid thickness in pre- and postmenopausal women [40]. This profile was observed even when the analysis included both genders and young and old patients [33]. Moreover, decreased androgen levels, including testosterone, are inversely related to age in both genders [40], reinforcing the importance of including age and gender in linear regression analysis in hormone studies.

It is still controversial whether testosterone reduction has an anti- or pro-atherosclerotic effect. Testosterone is frequently considered to be a pro-oxidant hormone in oxidative stress studies [15], possibly causing deleterious vascular consequences. Manolakou (2009) defended the potential pro-atherosclerotic effect of testosterone, and considered testosterone to be deleterious in other vascular diseases [33]. Traish et al (2009) argued that testosterone supplementation may restore endothelial function and vasoreactivity, potentially reducing cardiovascular disease in hypogonadal men [30]. Also, testosterone infusion causes coronary vasodilation and improves blood flow in male CAD patients [41].

In regression analysis (Table III), testosterone decrease was an important predictor of CAD, even when AHI was present as the regressor. Studies have pointed out a relationship between hypoxia and testosterone levels. *In vitro* studies have shown that HIF-alpha is related to testosterone production [8] and androgen treatment regulates HIF-alpha [9, 19]. Therefore, hypoxia may be related to

testosterone decrease in SDB patients, contributing to the decreased testosterone profile found in CAD patients. Exogenous testosterone increases erythropoiesis and hemoglobin levels, however no difference is generally reported in transferrin receptor, erythropoietin, or ferritin levels [25, 26]. We found no relationship between testosterone and erythrocyte numbers or hemoglobin, however, correlation analysis found a positive correlation with ferritin and a negative correlation with transferrin concentrations. No significant correlation was found between testosterone and available iron (data not shown). It remains to be determined why CAD patients present lower testosterone levels and how this is related to iron homeostasis in atherosclerosis and hypoxic conditions.

We observed a positive correlation between ferritin and AHI and a negative correlation between ferritin and lowest O_2 saturation, which corroborated a previous report of ferritin upregulation by hypoxia [12]. Also, we found an important increase in ferritin levels in CAD patients (Table II). However, ferritin was not considered a predictor of CAD in either regression model (Table III). The importance of ferritin in atherosclerosis is still under discussion. The majority of CAD researchers consider ferritin to be an antioxidant, and consequently, anti-atherosclerotic by virtue of its role in iron sequestration [10]. However, as discussed by Halliwell & Gutteridge (2007), ferritin is more likely to cause oxidative stress than transferrin [15], possibly participating in LDL oxidation in atherosclerotic lesions [11]. The importance of oxidative stress, indicated by protein carbonylation, a marker of oxidative damage in proteins, has been shown in both CAD and SDB [5]. Interestingly, ferritin levels are positively correlated with erythrocyte carbonyl levels. Moreover, the positive correlation between AHI and

ferritin confirms the possible role of ferritin in the association between SDB and CAD. The increase in ferritin in CAD patients, concomitant with the strong correlation between ferritin and erythrocyte carbonyl and the inverse correlation found between ferritin and nitrites/nitrates, indicates that ferritin may cause oxidative stress and reduce $NO^{\bullet}$ vasodilator levels, thereby participating in the progression of atherosclerosis.

Concomitant with the testosterone decrease, an inverse relationship between available iron concentration and CAD were observed in a regression model (Table III). The hypoxia-inducible factors, HIF-1alpha and HIF-2alpha, contain IRE, and affect free iron levels [13]. In anemia occurring in the context of inflammation, ferritin levels increase and serum iron concentration decreases [42]. Despite the well known influence of inflammation on iron homeostasis, hypoxia may also be an important factor in the ferritin increase and available iron decrease observed in the current study, a conclusion supported by the lack of a CRP difference between groups (Table II). Also, the majority of patients presented higher CRP levels compared to clinical reference levels, and no correlation was found between CRP and AHI (data not shown).

The steroid hormones estradiol and progesterone are classically studied as antioxidant molecules, possibly mediating an anti-atherosclerotic effect [15]. Supporting this theory, postmenopausal hormone treatment is considered beneficial in SDB [20]. In our study, estradiol and progesterone were not significantly associated with AHI or CAD (Table II).

Reduced glutathione (GSH), as well as its oxidized form GSSG were not different between CAD patients and controls (Table II). Glutathione is considered

the most important intracellular non-enzymatic antioxidant [15]. A previous study reported reduced serum GSH levels in CAD patients, however diabetic and smoking patients were not excluded from the analysis [43]. The antioxidant enzyme GsT, as well as GSH and GSSG, were not associated with CAD (Table II) or AHI (data not shown). GsT uses GSH molecules to detoxify lipid peroxides and to solubilize aggregated proteins formed by oxidative damage [15, 44].

$NO^{\bullet}$ inhibits plaque formation, arterial constriction, platelet aggregation, and macrophage adhesion and penetration [45]. Nitrites and nitrates, an indirect $NO^{\bullet}$ measurement, was significantly lower in CAD patients (Table II), indicating that $NO^{\bullet}$ reduction may be related to atherosclerosis progression in our patients. Oxidative stress generated by hypoxia in SDB alters $NO^{\bullet}$-dependent vasodilation [46]. Thus, oxidative stress induced by hypoxia and ferritin increase may explain the inverse correlation found between nitrites/nitrates and ferritin. $NO^{\bullet}$ regulates HIF-1alpha in a hypoxia model *in vitro* [47], showing that this signaling free radical could participate both in CAD pathophysiological pathways and in SDB. However, nitrite and nitrate levels did not correlate with AHI in our study (data not shown).

Taken together, these findings provide evidence of the relevance of iron homeostasis, through ferritin increase and consequent available iron decrease, in the association of SDB and CAD. Decreased testosterone levels were confirmed as predictors of CAD, and may be involved in CAD pathophysiology and iron homeostasis. Ferritin may cause oxidative stress and reduced $NO^{\bullet}$ vasodilator in CAD patients, thereby participating in progression of atherosclerosis.

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CAPÍTULO 2

Artigo Científico 2

Artigo que submetido à revista *European Journal of Clinical Investigation.*

Oxidized-LDL and Paraoxonase-1 in the Association between Coronary Artery Disease and Sleep Disordered Breathing

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Abstract:

Sleep Disordered Breathing (SDB) and Coronary Artery Disease (CAD) are both considered to be oxidative stress-associated disorders. Intermittent hypoxia, consequent to SDB, induces inflammatory pathways and oxidative stress and reduces $NO^{\bullet}$ availability causing endothelial dysfunction and atherosclerosis progression. Low-density lipoprotein (LDL) peroxidation is directly involved in atherosclerotic plaque formation and has been reported in SDB. Oxidized LDL (ox-LDL) and malondialdehyde (MDA) are both lipid peroxidation markers. High-

density lipoprotein (HDL) mediates well known antiatherosclerotic properties related to paraoxonase-1 (PON-1). PON-1 hydrolyzes lipid peroxides, reducing ox-LDL accumulation. Obstructive sleep apnea (OSA) is associated with HDL dysfunction which may be related to decreased PON-1 activity. This study reports results from 29 controls and 27 cases with CAD (defined as > 30% coronary narrowing). AHI was increased in CAD patients, and PON-1 activity and HDL levels were decreased. Regression analyses showed that lower PON-1 activity and higher ox-LDL levels were important CAD predictors compared to HDL or MDA levels. Nitrite/nitrate level, an indirect $NO^{\bullet}$ marker, was positively correlated with PON-1. SDB was not correlated with decreased PON-1 activity or with increased ox-LDL, however AHI was inversely correlated with HDL levels. These results indicate that PON-1 and ox-LDL are important predictors of CAD; however, SDB may not be directly related to increased ox-LDL and decreased PON-1 activity found in CAD patients.

Key words: LDL, HDL, ox-LDL, PON-1, obstructive sleep apnea, atherosclerosis.

Abbreviations: AHI, apnea-hypopnea index; BMI, body mass index; CAD, coronary artery disease; CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MDA, malondialdehyde; Ox-LDL, oxidized low-density lipoprotein; $NO^{\bullet}$, nitric oxide; OSA, obstructive sleep apnea; RBC, red blood cell; ROS, reactive oxygen species; SDB, Sleep disordered breathing; TBARS, thiobarbituric acid.

Introduction

Coronary artery disease (CAD) is the main cause of mortality in western countries and is highly associated with sleep disordered breathing (SDB) (Mooe et al., 1996, Bradley and Floras, 2009, Luthje and Andreas, 2008). OSA is an important form of SDB and, as along with CAD, is considered to be an oxidative stress and inflammation-associated disorder (Bradley and Floras, 2009, Celen and Peker, 2010, Klein et al., 2010). SDB patients suffer hypoxia/reoxygenation processes as a consequence of intermittent hypoxia, which resemble ischemia–reperfusion events. These events induce inflammatory pathways and oxidative stress causing deleterious vascular endothelial consequences (Khayat et al., 2009, Bradley and Floras, 2009). Oxidative stress promotes fatty acid and cholesterol oxidation, recruitment of monocytes and vascular smooth muscle cells, macrophage uptake of oxidized cholesterols and the consequent formation and accumulation of foam cells (Singh and Jialal, 2006, Tavori et al., 2009), all of which contribute to atherosclerotic plaque development.

Low-density lipoprotein (LDL) oxidation increases inflammatory pathways and directly participates in atherosclerotic plaque formation (Levitan et al., 2010). Serum oxidized LDL (ox-LDL) is generated during the endothelium atherosclerotic process in coronary artery disease (CAD) patients (Levitan et al., 2010); however, the source of the main reactive oxygen species (ROS) in this process has not been elucidated. Malondialdehyde (MDA), an aldehyde lipid peroxidation product, is present in ox-LDL complexes and was detected in myocardial infarction patients (Levitan et al., 2010). Specific oxidative modifications in LDL structure cause

cholesterol accumulation as a result of ineffective ox-LDL degradation by macrophages, resulting in foam cell formation (Levitan et al., 2010).

Lipid peroxidation in CAD patients may be related to SDB. Previous studies have demonstrated increased ox-LDL levels in OSA patients (Tan et al., 2006, Gozal and Kheirandish-Gozal, 2008); and thiobarbituric acid (TBARS), a lipid peroxidation marker, has been associated with OSA (Tan et al., 2006). However, in at least one study, OSA patients did not exhibit a significant difference in lipid peroxidation levels using TBARS (Gozal and Kheirandish-Gozal, 2008).

High-density lipoprotein (HDL) is responsible for cellular cholesterol efflux; and reverse cholesterol transport has been proposed as an important antiatherosclerotic process (Tan et al., 2006, Jayakumari and Thejaseebai, 2009). HDL has well established antioxidant properties of lipid oxidation inhibition and reduces endothelial cell inflammation (Tan et al., 2006). The HDL antioxidant property is mainly related to an esterase, paraoxonase-1 (PON-1), present in this lipoprotein complex in blood (Lavie et al., 2004, Jayakumari and Thejaseebai, 2009). PON-1 hydrolyzes lipid peroxides reducing ox-LDL accumulation (Durrington et al., 2001). This is confirmed by knockout PON-1 models (Tward et al., 2002, Ng et al., 2008) and the fact that avians do not possess PON-1 and avian HDL is unable to metabolize lipid peroxides (Durrington et al., 2001). Beyond the important protective effect of PON-1 upon LDL oxidation, it is important to point out that its activity also reduces HDL and fatty acid lipid peroxidation (Lavie et al., 2004). OSA is associated with HDL dysfunction; and in atherosclerosis the reduced ability of HDL to hydrolyze ox-LDL may be related to decreased PON-1 activity (Tan et al., 2006).

The potent vasodilator nitric oxide ($NO^{\bullet}$) is a product of nitric oxide synthase (NOS). Endothelial NOS (eNOS) transiently produces superoxide radical ($O_2^{\bullet -}$) in hypoxic conditions, reducing $NO^{\bullet}$ availability and increasing oxidative damage (Singh and Jialal, 2006). It is known that OSA patients exhibit reduced $NO^{\bullet}$ availability (Khayat et al., 2009) which reduces the $NO^{\bullet}$ antiatherosclerotic effect.

8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OHdG) is an important oxidative DNA damage marker. Increased 8-OHdG levels are frequently related to CAD (Gackowski et al., 2001, Satoh et al., 2008, Collins et al., 1998) and SDB (Khayat et al., 2009, Atanasiu et al., 2007).

In the present work, which is a logical extension of our previous study (Klein et al., 2010), we assessed SBD by AHI along with laboratory markers HDL, PON-1, ox-LDL, MDA, and 8-OHdG among a cohort of CAD patients. We found that reduced PON-1 activity and elevated ox-LDL levels, concomitantly with higher AHI, were important CAD predictors.

Materials and methods

Patients and CAD Study

The project was approved by the Hospital de Clínicas de Porto Alegre ethics committee, and all participants signed an informed consent form. A cross-sectional study was conducted by screening patients referred for diagnostic or therapeutic coronary angiography. The exclusion criteria were: age less than 35 or greater than 65 years; smoking in the previous 6 months; clinical diagnosis, dietary, or pharmacological treatment for diabetes mellitus; anginal pain in the

previous week; use of anxiolytic medication; treatment for chronic pulmonary disease; use of vitamin supplement; body mass index (BMI) ≥ 40 kg/ m^2; any physical, psychological, or social issue that would interfere with conducting the home polysomnographic test; previous coronary intervention (myocardial revascularization or angioplasty). Hypertension, past history of smoking, and medication use were not criteria for exclusion, but the numbers of these conditions was similar between controls and CAD patients. All patients were assessed by quantitative angiography using the same equipment and projection, with the table and image intensifier kept at constant height. A seven inch magnification was used for all images. Image quantification was carried out by the same investigator, who was blinded with respect to clinical and biochemical variables. Patients with luminal narrowing of 30% or higher were considered to have CAD. Patients without narrowing or narrowing less than 30% were included in the control group.

Sleep Study

In the polysomnographic study, a Level III portable monitor (SomnoCheck, Weinmann, Germany) was used at home by the patients. A nasal cannula connected to a pressure transducer was employed to quantify air flow and snoring. Inspiratory effort, pulse oximetry, heart rate, and sleep position were also verified. The polysomnography respiratory analyses were made by a sleep specialist who was blinded to clinical and biochemical variables. Apnea was defined as airflow reduction to 10% or less of the baseline value persisting for 10 seconds or more; hypopnea was defined as airflow reduction of 50% or more, associated with a reduction of 3% or more in oxygen saturation. The AHI was defined as

apnea/hypopnea episodes per hour, calculated by dividing the sum of total apneas and hypopneas by the hours of recorded polysomnography.

Laboratory measurements

Approximately 20 mL arterial blood was collected from each patient from femoral artery puncture during catheterization. Blood was collected in vials containing coagulation inhibitors, EDTA and citrate. Immediately after collection, the samples were refrigerated to 0°C, centrifuged for 10 min at 0°C, aliquoted, and stored at -80°C. Hemolysates were prepared by lysing red blood cells with 2% ethanol (ratio 1:10) followed by centrifugation to obtain crude extracts. High-sensitivity C-reactive protein, total protein, triglycerides, total cholesterols, HDL, and LDL were quantified in routine clinical laboratory analysis, additional tests were performed at the research laboratory.

Nitrites/nitrates assay

The Griess reaction, which measures nitrites and nitrates in the sample, was employed to determine circulating $NO^{\bullet}$ concentration (Bryan and Grisham, 2007). Hemolysates were centrifuged and maintained on ice. A standard curve was made using 1 mM sodium nitrite ($NaNO_2$) diluted in 100 mM phosphate buffer to concentrations of 2.5 nmol/mL, 5 nmol/mL, 15 nmol/mL and 30 nmol/mL. In the microplate (all wells in triplicate) 100 µL sample or standards were added, followed by 50 µL of Griess reagent (0.02 g naphthylenediamine dichloride, 0.2 g sulfanilamide, 500 µL orthophosphoric acid and 10 mL MilliQ water). The microplate was protected from light during a 10 min incubation. Absorbance was

read at A_{543} (Grisham et al., 1996).

PON-1, Ox-LDL, and MDA assays

Serum paraoxonase-1 activity was measured in spectrophotometer and monitored on a microplate for 5 min at 25°C at 412nm. The assay is based on the p-nitrophenol formation by paraoxon hydrolysis. The p-nitrophenol molar extinction coefficient is ε_{412}^{M} 169,000 $M^{-1}cm^{-1}$. The assay was employed using 1.0 mM paraoxon, 1.0 mM $CaCl_2$ in 50 mM glycine buffer, pH 10.5. One U of paraoxonase activity produces 1 nmol of p-nitrophenol per min, and activity was expressed as units-per mL of serum (Eckerson et al., 1983, van Himbergen et al., 2005). Serum ox-LDL levels were quantified by commercial kit (OxLDL-β2GPI human ELISA Kit-Cayman-USA). MDA levels were measured by HPLC as previously reported (Alabarse et al, 2010).

8-OHdG assay

The 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OHdG) assay was performed using a commercial kit (8-Hydroxy-2-Deoxyguanosine, EIA Kit-Cayman-USA). DNA was extracted from leucocytes using a commercial kit (Genomic DNA Extraction Kit - Real Biotech Corp) and the purified DNA was quantified spectrophotometrically at A_{260} (Abs=1.0 corresponds to 50 µg of DNA).

Statistical analysis

Categorical variables are presented as absolute values and were analyzed by chi-square test. The Kolmogorov-Smirnov test of normality was employed to

verify distribution of variables. Variables with normal distribution are presented as mean ± standard error (SE); means were compared by student's t-test. Variables without normal distribution are presented as median (minimum–maximum); medians were compared by Mann-Whitney U test. Spearman coefficient was employed to test correlation between variables without normal distribution, and Pearson coefficient was used to test correlation between variables with normal distribution. Poisson Regression analysis was employed to predict CAD, using five variables, according to the number of analyzed patients. $P \leq 0.05$ was considered statistically significant.

Results

Clinical and anthropometric data in CAD groups: Table I presents anthropometric and clinical data of analyzed patients. Gender and BMI did not differ between groups (Table I). CAD patients were significantly older than controls (Table I). In the CAD group AHI was significantly higher and the lowest O_2 saturation was decreased (Table I).

Table I: clinical and anthropometric characteristics of 56 included patients.

	Controls (n=29)	CAD (n=27)	*P*
Male Gender (%)	12 (41%)	19 (70%)	ns
Age	51.62 ± 1.28	56.96 ± 1.11	P≤0.05
BMI (Kg/m²)	27.30 ± 0.74	27.79 ± 0.74	ns
AHI	7 (1-48)	17 (1-56)	P≤0.05
Lowest O_2 saturation (%)	87.5 (63-93)	85.0 (77-90)	P≤0.05

Categorical data are presented as n (%). Variables with normal distribution are presented as mean ± SE. Variables without normal distribution are presented as median (minimum–maximum). ns: $p > 0.05$. BMI: Body mass index (weight divided by the square of height, Kg/m^2). AHI: apnea-hypopnea index (apnea-hypopnea/hour of sleep).

Biochemical data: Table II presents biochemical data from the analyzed patients. Total cholesterol levels, HDL levels, and PON-1 activity were significantly decreased in CAD patients compared to controls (Table II). High-sensitivity C-reactive protein (CRP), triglycerides, LDL, ox-LDL, MDA, and 8-OHdG levels did not differ between controls and CAD patients (Table II).

Table II: biochemical data of 56 included patients.

	Controls (n=29)	CAD (n=27)	*P*
C-reactive protein (mg/L)	1.59 (0.19- 21.50)	2.00 (0.16-18.40)	ns
Triglycerides (mg/dL)	93 (20-640)	134 (60-295)	ns
Total Cholesterol (mg/dL)	185.68 ± 8.54	158.52 ± 7.45	P≤0.05
HDL (mg/dL)	51.00 ± 2.40	40.44 ± 2.21	P≤0.05
LDL (mg/dL)	109.70 ± 6.95	91.27 ± 7.04	ns
Ox-LDL (U/mL)	0.06 (0.02-0.35)	0.07 (0.03-0.38)	ns
MDA (μM)	6.879 (2.90-12.78)	7.17 (3.04-12.62)	ns
8OHdG (pg/mL)	1.63 (1.00-4.73)	1.47 (0.58-4.34)	ns
PON-1 (U/mL)	5.73 (1.10-10.50)	2.44 (0.40-10.10)	P≤0.05

Variables with normal distribution are presented as mean ± SE. Variables without normal distribution are presented as median (minimum–maximum). ns: $p > 0.05$. HDL: high-density lipoprotein. LDL: low-density lipoprotein. Ox-LDL: oxidized low-density lipoprotein. MDA: malondialdehyde. 8-OHdG: 8-oxo-7,8-dihydro-2'-deoxyguanosine. PON-1: paraoxonase-1.

Correlation analysis: in Spearman's correlation analysis, PON-1 correlated with BMI (rho=-0.288, P=0.037), and nitrites/nitrates (rho= 0.383, P=0.004). Spearman's analysis of ox-LDL levels showed correlation with age (rho= 0.276, P= 0.039), nitrites/nitrates (rho= -0.322, P = 0.015), and HDL (rho= -0.276, P=0.039). Spearman's analysis revealed a correlation of HDL with AHI (rho= -0.277, P =

0.037). Only statistically significant correlations are presented.

Regression models: Regarding the analyzed lipid oxidation parameters ox-LDL and MDA, Poisson regression model was employed to predict which variables were more relevant for predicting CAD (Table III). In the regression analysis, ox-LDL concentration was normalized relative to LDL levels. A comparative model was developed. The comparative model showed that increased ox-LDL levels and higher AHI were important predictors of CAD, but MDA was not, even when controlling for BMI, gender, and age (Table III).

Table III: Poisson stepwise regression model to predict coronary artery disease

Dependent variable	Obstructive coronary artery disease	
Regressor	RR (CI)	*P*
Ox-LDL	24.12 (1.79-324.00)	0.016
Age	1.05 (0.99-1.09)	ns
Gender	0.67 (0.35-1.26)	ns
BMI	1.03 (0.94-1.12)	ns
AHI	1.02 (1.00-1.03)	0.005
MDA	1.06 (0.95-1.18)	ns
Age	1.04 (0.99-1.09)	ns
Gender	0.65 (0.33-1.24)	ns
BMI	1.03 (0.94-1.12)	ns
AHI	1.02 (1.00-1.03)	0.025

ns: $p > 0.05$. Ox-LDL: oxidized low-density lipoprotein. Gender: male categorized as 0 (zero) and female as 1 (one). Age is expressed in years. BMI: body mass index (weight divided by the square of the height; Kg/m^2). AHI: apnea-hypopnea index (apnea-hypopnea/hour of sleep). MDA: malondialdehyde. RR>1: positive correlation with dependent variable. RR<1: negative correlation with dependent variable.

Poisson regression models were employed to predict which antioxidant system variables, PON-1 or HDL, were relevant for predicting CAD (Table IV). A comparative model was developed. The model showed that a decrease in PON-1 activity, but not decreased HDL levels, was an important CAD predictor (Table IV). An increase in AHI was an important predictor in both analyses (Table III and IV).

Table IV: Poisson stepwise regression model to predict coronary artery disease

Dependent variable	Obstructive coronary artery disease	
Regressor	RR (CI)	*P*
PON-1	0.87 (0.76-1.00)	0.049
AHI	1.02 (1.01-1.03)	0.008
Gender	0.69 (0.37-1.27)	Ns
Age	1.04 (0.99-1.08)	Ns
BMI	1.00 (0.92-1.09)	Ns
HDL	0.97 (0.94-1.00)	Ns
AHI	1.01 (1.00-1.02)	0.046
Gender	0.76 (0.40-1.41)	Ns
Age	1.05 (1.00-1.09)	0.033
BMI	1.02 (0.93-1.09)	Ns

ns: $p > 0.05$. PON-1: paraoxonase-1. Gender: male categorized as 0 (zero) and female as 1 (one). Age is expressed in years. BMI: body mass index (weight divided by the square of the height; Kg/m^2). AHI: apnea-hypopnea index (apnea-hypopnea/hour of sleep). HDL: high-density lipoprotein. RR>1: positive correlation with dependent variable. RR<1: negative correlation with dependent variable.

Discussion

As previously reported by our group (Klein et al., 2010), oxidative stress is

intrinsically involved in SDB and CAD pathophysiology. In all analyses performed in the current study, higher AHI was related to CAD (Table I, III, and IV), reaffirming the relevance of SDB in development of atherosclerosis.

Lipid peroxidation has been associated with CAD and SDB. LDL peroxidation and plaque formation may be related to SDB hypoxic events. In comparative regression analyses, MDA was found not to be a predicator of CAD; however, increased ox-LDL was found to be a predictor, even when controlled for AHI, gender, age, and BMI (Table III). Increased TBARS levels were reported in CAD patients (Jayakumari and Thejaseebai, 2009, Lavie et al., 2004); however TBARS is an indirect and nonspecific lipid peroxidation marker compared to MDA measurement by HPLC, as employed in this study. Interestingly, ox-LDL is constantly removed by macrophages from circulation and degraded. however, an *in vitro* study showed that the presence of MDA in the LDL complex inhibits this process (Lavie et al., 2004). In the current work, MDA did not correlate with CAD (Table II and III), but the importance of ox-LDL in CAD pathophysiology was reaffirmed. Evidence for increased levels of both TBARS (Lavie et al., 2004) and ox-LDL (Tan et al., 2006) in OSA patients were previously reported; however, we found no relationship between MDA or ox-LDL and AHI (data not shown). These differences may be due to the rigid exclusion criteria employed in the present work, where even diabetes mellitus and smoking patients were criteria for exclusion.

Regression analyses comparing HDL levels and PON-1 activity as CAD predictors showed that decreased PON-1, but not HDL, was an important predictor, even when controlling for AHI, gender, age, and BMI (Table IV). PON-1 activity was

decreased in CAD patients (Table II and Table IV), as has been reported (Lavie et al., 2004, Jayakumari and Thejaseebai, 2009). Surprisingly, PON-1 activity was not correlated with HDL levels, showing that its decreased activity was not directly related to lower HDL found in CAD patients (Table II). The mechanisms involved in PON-1 activity decrease have yet to be evaluated, however, inflammation is a possible explanation (Durrington et al., 2001, Novak et al., 2010) which has to be investigated. Also, no significant correlation between PON-1 and LDL or ox-LDL was found (data not shown). Reduced levels of HDL and PON-1 have been reported in CAD patients (Durrington et al., 2001, Jayakumari and Thejaseebai, 2009); and pharmacologic treatment may be employed to increase HDL levels (Barish and Evans, 2004, Kurban and Mehmetoglu, 2010). In addition, a correct diet may be an important means to increase HDL levels and PON-1 activity (Durrington et al., 2001). Corroborating this approach, correlation analysis demonstrated a negative association between BMI and PON-1 activity, reaffirming that diet and life-style are important for preventing atherosclerotic plaque formation and progression.

PON-1 was not associated with AHI or Lowest O_2 saturation in the present study (data not shown), leading to the conclusion that SDB is not directly involved in the PON-1 profile found in CAD subjects. Interestingly, HDL levels were inversely related to AHI in our correlation analysis. Decreased HDL levels were previously described in elderly OSA patients (Roche et al., 2009); however, other studies failed to find this association (Tamaki et al., 2009, Drager et al., 2005, Szaboova et al., 2007).

Reduced $NO^{\bullet}$ availability is associated to OSA and CAD (Jelic et al., 2008),

and $NO^{\bullet}$ is increased by HDL (Tan et al., 2006). In the present study, nitrites and nitrates were positively correlated with PON-1, but not with HDL (data not shown), showing that hydrolysis of lipid peroxides by PON-1 may be important in $NO^{\bullet}$ endothelial availability. Corroborating this idea, nitrites and nitrates levels were negatively correlated with ox-LDL levels.

A previous study reported higher urinary 8-OHdG excretion in OSA patients compared to healthy subjects; however, diabetic and smoking patients were not excluded from the analysis (Yamauchi et al., 2005). In previous work, increased 8-OHdG was found in endothelial progenitor cells from CAD patients (Satoh et al., 2008). Higher lymphocyte 8-OHdG was found in CAD patients in the absence of strict exclusion criteria (Gackowski et al., 2001). In our study, 8-OHdG was not significantly associated either with AHI or CAD (Table II).

We found no differences in levels of triglycerides or LDL between CAD patients and controls (Table II). However in another study, LDL levels were found to be lower and triglycerides higher in CAD patients (Jayakumari and Thejaseebai, 2009). Interestingly, despite a narrow range of age (12 – 14 years old), a correlation was observed between ox-LDL and age in our work.

Taken together, these findings provide evidence that the decrease in PON-1 activity in CAD patients is associated with reduced HDL levels. PON-1 may be involved, not only in preventing lipid peroxidation in CAD patients, but also in $NO^{\bullet}$ endothelial availability. Increased ox-LDL levels, but not MDA concentration, was associated with CAD. Despite the fact that SDB was not directly associated with decreased PON-1 activity or increased ox-LDL in the analyzed subjects, PON-1 may be related to decreased HDL levels found in CAD patients.

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DISCUSSÃO

A participação do estresse oxidativo na associação entre a DAC e os DRS foi anteriormente evidenciada nestes mesmos pacientes por nosso grupo de pesquisa (KLEIN *et al*., 2010). Os grupamentos carbonil, marcadores de dano oxidativo em proteínas, são maiores em eritrócitos de pacientes com DAC e apresentam correlação positiva com o IAH, mostrando-se também preditores da DAC pela análise de regressão (KLEIN *et al*., 2010). No presente estudo (Artigo científico 1) a concentração sérica de ferritina é maior em pacientes com DAC e apresentou correlação positiva com a carbonilação em eritrócitos e com o IAH, sugerindo participação da homeostase do ferro na associação da DAC e DRS. Ainda, a redução da concentração do ferro disponível no soro foi evidenciada nos pacientes com DAC. Nosso trabalho sugere que a redução da concentração de ferro disponível é decorrente da alta concentração de ferritina (Artigo científico 1), e as proteínas de homeostase do ferro podem estar sofrendo influência das alterações na concentração de testosterona.

A ferritina é um complexo que armazena ferro sob a forma de Fe(III). Como discutido por You e Wang (2005), sua capacidade de estocar ferro, evitaria o estresse oxidativo gerado pela clássica reação de Fenton, $Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + OH^{\bullet}$ (HALLIWELL & GUTTERIDGE, 2007). Porém, durante a estocagem do ferro pela ferritina, ocorre formação de H_2O_2, sendo a transferrina a forma mais segura de armazenamento de ferro sob o ponto de vista oxidativo (HALLIWELL & GUTTERIDGE, 2007). Também, é mais provável o Fe(III) passe a Fe(II) pelo $O_2^{\bullet-}$ na ferritina (mostrado apenas in vitro) que na transferrina (HALLIWELL & GUTTERIDGE, 2007, YOU & WANG, 2005), tornando possível que a reação de

Fenton ocorra. Corroborando com este ponto de vista, estudos epidemiológicos têm demonstrado relação direta entre ferritina e fatores de risco para doenças cardiovasculares (SALONEN *et al*., 1994, SALONEN *et al*., 1992, TUOMAINEN *et al*., 1998). Além disso, a correlação negativa entre ferritina e nitritos e nitratos encontrada em nosso estudo (Artigo científico 1) sugere que esta reduz o $NO^{\bullet}$ circulante, podendo participar na progressão da placa de ateroma.

A relação entre níveis de testosterona e aterosclerose ainda é controversa. Um importante estudo demonstrou que baixos níveis de testosterona endógena estão associados à maior mortalidade em pacientes homens com DAC (MALKIN *et al*., 2010), apesar destes níveis serem considerados anormalmente baixos, podendo ser classificados como hipogonadismo. Infelizmente, marcadores de homeostase do ferro não são comumente avaliados nos estudos epidemiológicos envolvendo testosterona e DAC. É importante ressaltar que não está estabelecido se a redução da testosterona é consequência do processo fisiopatológico da CAD ou se pacientes com baixos níveis de testosterona tendem a desenvolver DAC. Porém, um estudo em modelo animal mostrou evidências da segunda opção. Nettleship (2007) demonstrou, em estudo realizado em murinos, que a redução artificialmente induzida da produção de testosterona leva ao desenvolvimento da aterosclerose. Ainda, este processo é revertido pela administração de testosterona em concentrações fisiológicas (NETTLESHIP *et al*., 2007)

A administração do hormônio em indivíduos com baixas concentrações de testosterona ainda é controversa e precisa ser melhor estudada. A maior parte dos estudos apresenta conclusões negativas em relação à administração em pacientes (HADDAD *et al*., 2007, WU & VON ECKARDSTEIN, 2003, FADINI *et al*.,

2009, MANOLAKOU *et al*., 2009). O mecanismo pelo qual a testosterona influencia o desenvolvimento da placa de ateroma ainda não é claro.

Apesar de evidências sugerirem aumento da GSH na aterosclerose (ASHFAQ *et al*., 2006, HOEKSTRA *et al*., 2003) não foi encontrada relação entre a atividade da enzima GsT ou as concentrações de GSH totais, GSH ou GSSG com DAC no presente estudo, assim como não houve relação com o IAH (Artigo científico 1). A atividade da enzima GsT também não apresentou correlação com a DAC ou IAH.

O marcador de dano oxidativo ao DNA, 8-OHdG, não apresentou relação com a DAC ou IAH em nosso estudo (dados não apresentados). Desta forma, é possível que o estresse oxidativo, já evidenciado nestes pacientes pelos marcadores de carbonilação de proteínas e ox-LDL, não estejam levando à mutagênese. Porém, é importante ressaltar que o 8-OHdG foi mensurado a partir do DNA de leucócitos, não excluindo a possibilidade de que diferentes processos possam estar ocorrendo no endotélio destes pacientes.

Poucos estudos relacionam as alterações dos hormônios esteroides aos DRS (SHAHAR *et al*., 2003, YOUNG *et al*., 2003, KIRBAS *et al*., 2007). O presente estudo não encontrou relação entre a concentração sérica de estradiol ou progesterona com a DAC ou IAH (dados não apresentados). Grande parte dos estudos envolvendo progesterona e estradiol estão relacionados a DAC, devido à alta incidência da doença no período pós-menopausa (ARNAL *et al*., 2006, GOURDY *et al*., 2008) em que a concentração destes hormônios é reduzida. A administração do estradiol é amplamente estudada na DAC, devido ao uso do hormônio como contraceptivo e na reposição hormonal pós-menopausa. O estudo

dos hormônios esteroides tem se mostrado de grande importância, devido ao fato de participarem na fisiopatologia de muitas doenças, tornando necessário grande cuidado e estudo para sua administração.

Um importante estudo na érea da associação entre DRS e DAC, desenvolvido por Tan (2006), mostrou que pacientes com AOS possuem disfunção das HDL, tendo estas reduzida capacidade de hidrolisar ox-LDL *ex vivo.* O estudo sugere ainda que esta disfunção possa estar relacionada à menor expressão ou baixa atividade da PON-1 (TAN *et al*., 2006). A atividade da enzima PON-1 já havia sido previamente discutida na associação entre DAC e DRS. Um estudo prévio falhou em mostrar relação entre pacientes com AOS e a atividade de PON-1, mas mostrou que está reduzida em pacientes com DAC e AOS (LAVIE *et al*., 2004). Como discutido neste estudo (Artigo científico 2), apesar da relação entre AOS e PON-1 ainda parecer inconsistente, não existem dúvidas da sua relação com a DAC, tanto em estudos epidemiológicos como em modelo animal (LAVIE *et al*., 2004, JAYAKUMARI & THEJASEEBAI, 2009, FU & WU, 2010). Porém, os processos que levam à redução da atividade da enzima na aterosclerose ainda são discutidos. Evidências apontam que o processo inflamatório pode ser uma possível explicação (DURRINGTON *et al*., 2001, NOVAK *et al*., 2010). Não foi encontrada relação entre o marcador inflamatório proteína-C-reativa e a atividade da PON-1 em nosso estudo (dado não apresentado), mas não descartamos a possibilidade, que precisa ser melhor investigada. É preciso levar em consideração que os valores de referência para o uso PCR como um marcador inflamatório em doenças cardiovasculares são: <1,0 mg/L de baixo risco, de 1,0 a 3,0 mg/L de médio risco e >3,0 mg/L de alto risco

(PEARSON *et al*., 2003), e em nosso estudo a maior parte dos pacientes apresenta valores superiores a 2,0mg/L. Este é um importante viés do presente estudo, por que todos os pacientes foram indicados para coronarioangiografia, e mesmo não apresentando DAC evidente ou IAH considerável, não podem ser considerados indivíduos saudáveis.

O presente trabalho mostra que a associação entre os DRS e a DAC está relacionada a uma complexa rede de alterações fisiopatológicas, e mais dúvidas do que respostas formam levantadas. A grande variabilidade existente, tanto genética quanto de estilo de vida e alimentação torna esta rede ainda maior. Estudos em modelo animal serão necessários para confirmar as hipóteses levantadas, mas fica evidente a necessidade de diagnóstico precoce e adequado para ambos as doenças, principalmente para os distúrbios do sono, que ainda não são encarados com a devida importância.

CONCLUSÃO

Este trabalho mostra a hipótese da participação da testosterona na homeostase do ferro, alterando a concentração da ferritina e ferro disponível em pacientes com DAC e DRS. O dano oxidativo, já evidenciado nestes pacientes por nosso grupo, pode ser conseqüência do aumento dos níveis de ferritina. Os níveis do vasodilatador $NO^{\bullet}$ são mais baixos em pacientes com DAC e estão correlacionados negativamente com a concentração de ferritina, mostrando que esta pode estar relacionada à diminuição do $NO^{\bullet}$. A ferritina está correlacionada positivamente com o IAH, indicando que os DRS podem estar participando destas

alterações. Os pacientes com DAC apresentaram menores níveis de testosterona e ferro disponível, como já havia sido evidenciado anteriormente. Além disso, a concentração de ox-LDL mostrou-se um melhor marcador de peroxidação lipídica em paciente com DAC em comparação ao MDA. A atividade da enzima PON-1 foi considerada melhor preditora de DAC comparada à concentração de HDL na circulação. Porém, não houve relação entre ox-LDL e PON-1 com o IAH, mostrando que os DRS não participaram nestas alterações fisiopatológicas. Também, a concentração de $NO^{\bullet}$ na circulação destes pacientes é diretamente proporcional à atividade da PON-1 e inversamente proporcional as ox-LDL, indicando que a atividade da PON-1 sobre as ox-LDL pode influenciar na concentração deste vasodilatador na circulação. A atividade da PON-1 foi inversamente proporcional ao IMC destes pacientes, mostrando que a alimentação e estilo de vida dos paciente com DAC não alteram apenas a concentração dos colesteróis, mas também a atividade de uma importante enzima antioxidante. Os principais dados apresentados estão esquematizados abaixo:

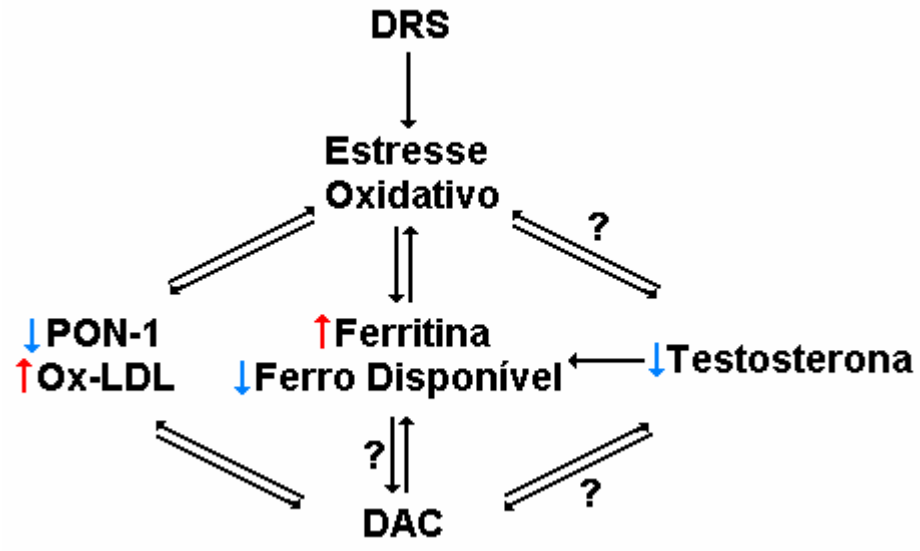

Figura 2: Esquema representativo dos resultados obtidos através das análises de regressão e correlação. DRS: distúrbios respiratórios do sono; PON-1: paraoxonase-1; Ox-LDL: lipoproteínas de baixa densidade oxidadas; DAC: doença arterial coronariana.

PERSPECTIVAS

As principais perspectivas para este estudo são: a utilização de um modelo murino de deficiência (NETTLESHIP *et al*., 2007) e suplementação de testosterona, estudando sua participação na regulação da ferritina e transferrina, assim como sua participação na formação das placas de ateroma, frequente neste modelo (NETTLESHIP *et al*., 2007); estudar a possibilidade de utilização da PON-1 como preditor clínico de risco para CAD, assim como a influência das estatinas (grupo de fármacos utilizados no controle da hipercolesterolemia) em sua transcrição e tradução (HARANGI *et al*., 2009).

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CURRICULUM VITAE

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Formação Acadêmica/Titulação:

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2004 - 2008	Graduação em Ciências Biológicas. Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil.

Formação complementar:

2010 - 2010	Extensão universitária em Criminalística e Locais de Crime. 8h. Renova Cursos, Brasil.
2007 - 2007	Extensão universitária em Introdução ao Dreamweaver. 20h. Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil.
2004 - 2004	Extensão universitária em XI Curso de Técnicas Histológicas. 45h. Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil.
2004 - 2004	Extensão universitária em Terapia Celular e Manipulação Genética. 8h. Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil.

Participação em Projetos:

2009 - Atual	Estresse Oxidativo e Hormônios Esteróides na Associação entre Apnéias-Hipopnéias Obstrutivas do Sono e Doença Aterosclerótica Coronariana.
2007 - Atual	Defesas Antioxidantes E Dano oxidativo em espécies patogênicas de candida não-albicans.
2005 - Atual	Determinação da expressão e atividade enzimática das defesas antioxidantes em ratos ao longo do envelhecimento.

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26. Atividades Das Enzimas Catalase (Cat), Superóxido Dismutase (Sod), Glutationa Peroxidase (Gpx) E A Carbonilação De Proteínas No Coração De Ratos Machos Com E Sem Atividade Reprodutiva In: XIX Salão de Iniciação Científica, Porto Alegre, 2007, v.2. p.468 – 468.

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27. Catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) activities and the protein carbonylation among naive and experienced male rat heart In: V Meeting of SFRBM-South American Group, Montevideo, 2007, p.120 – 120.

HACKENHAAR, F.S., SALOMON, T.B., ALABARSE, P.V.G., BENFATO, M.S.
28. Comparação Da Atividade Pulmonar De Enzimas Antioxidantes E Dano Em Proteína Em Ratos Machos Reprodutores E Não Reprodutores In: XIX Salão de Iniciação Científica, Porto Alegre, 2007, v.2. p.467 – 468.

ALABARSE, P.V.G., SALOMON, T.B, HACKENHAAR, F.S., BENFATO, M.S.
29. Glutathione peroxidase and superoxide dismutase activities and protein damage in brains of naive and experienced male rats In: V Meeting of SFRBM-South American Group, Montevideo, 2007. p.111 – 111.

HACKENHAAR, F. S., ALABARSE, P. V. G., SALOMON, T.B, BENFATO, M.S.
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BENFATO, M.S., HACKENHAAR, F.S., SALOMON, T.B, ALABARSE, P.V. G.
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ALABARSE, P.V.G., BINS, S.C., HACKENHAAR, F.S., SALOMON, T.B, EHRENBRINK, G., DORNELLES, P., BENFATO, M.S.
33. Atividade Enzimática Da Catalase E Superóxido Dismutase Em Ratos Machos De 12 Meses Reprodutores E Não-Reprodutores. In: VIII Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular do Centro de

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Produção Técnica:

Websites:

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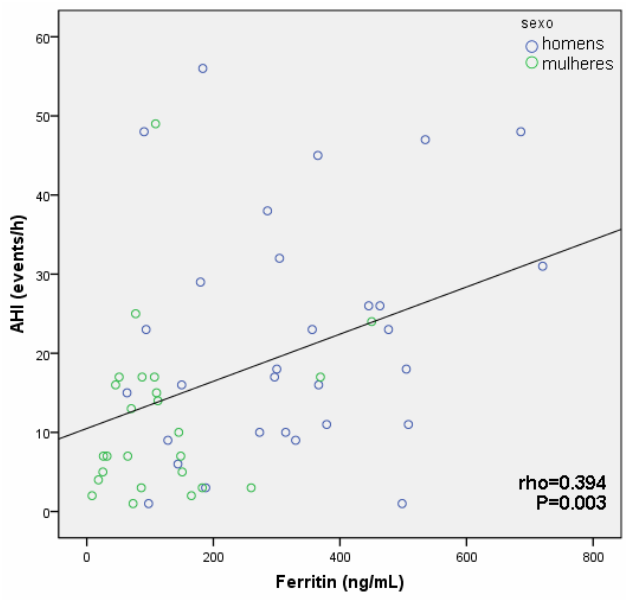
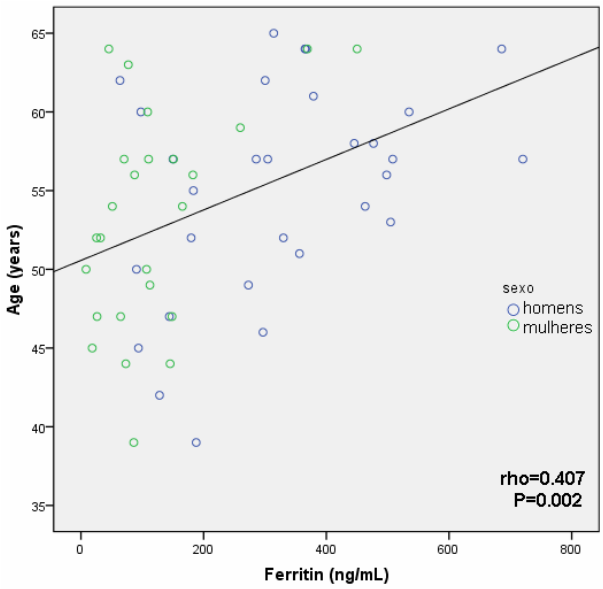
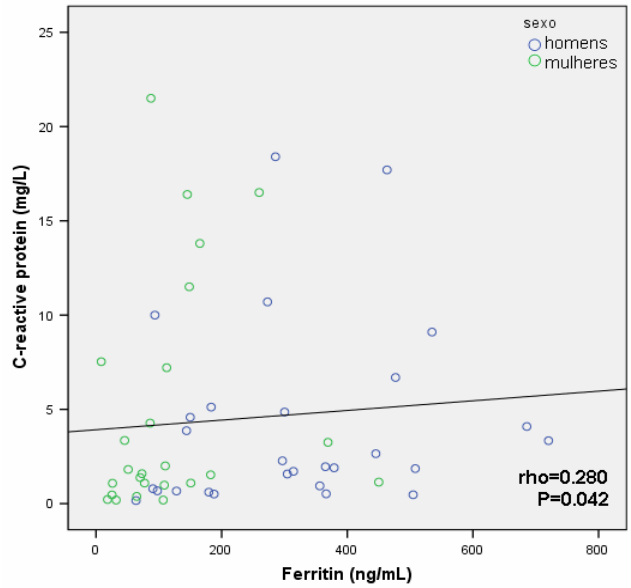
ANEXOS

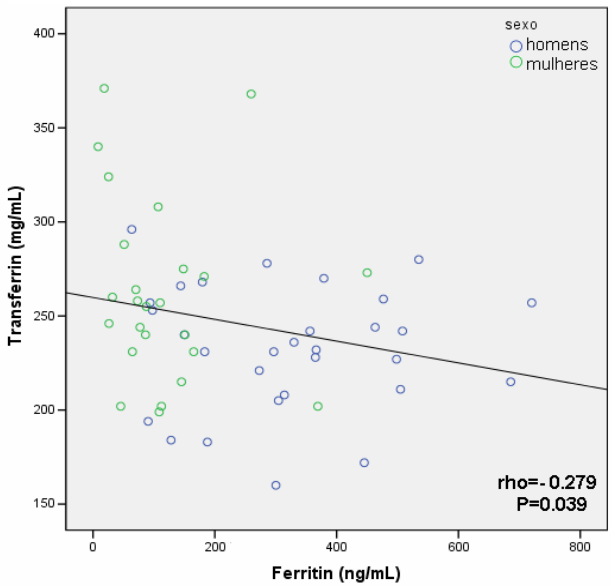
Anexo 1: gráficos de dispersão das análises de correlação dos artigos

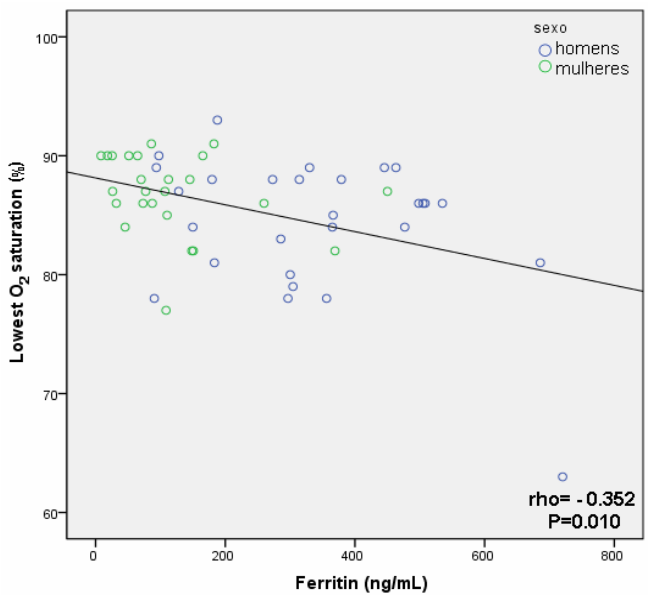
1.1: Artigo Científico 1

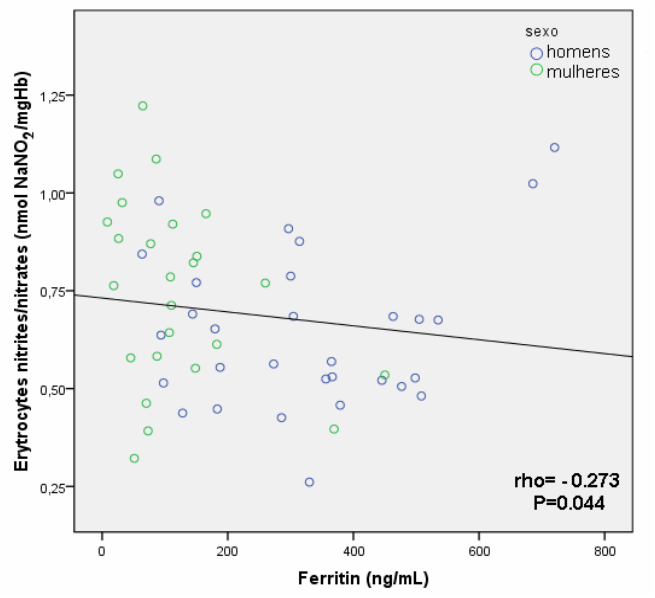


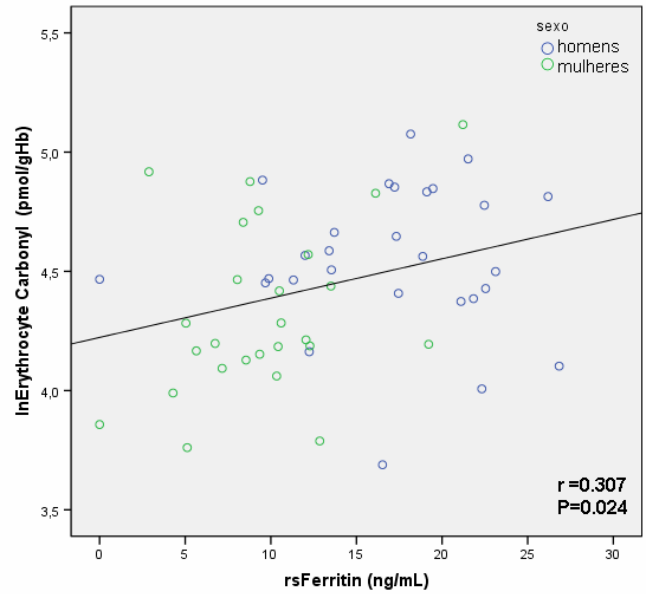


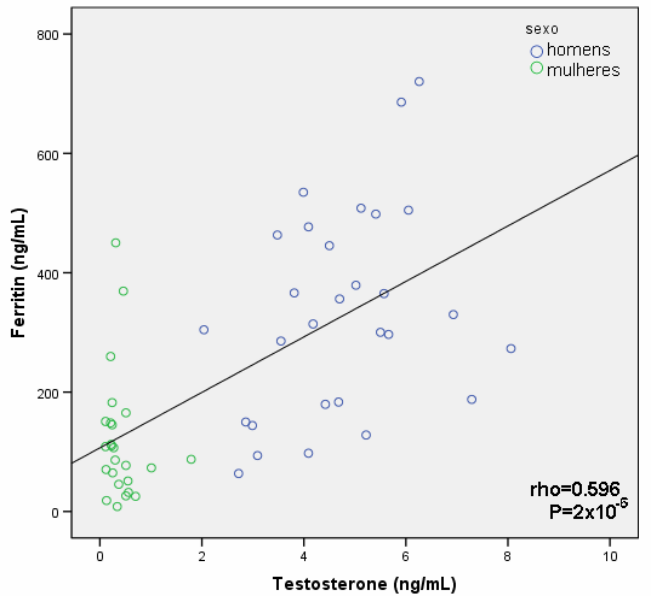


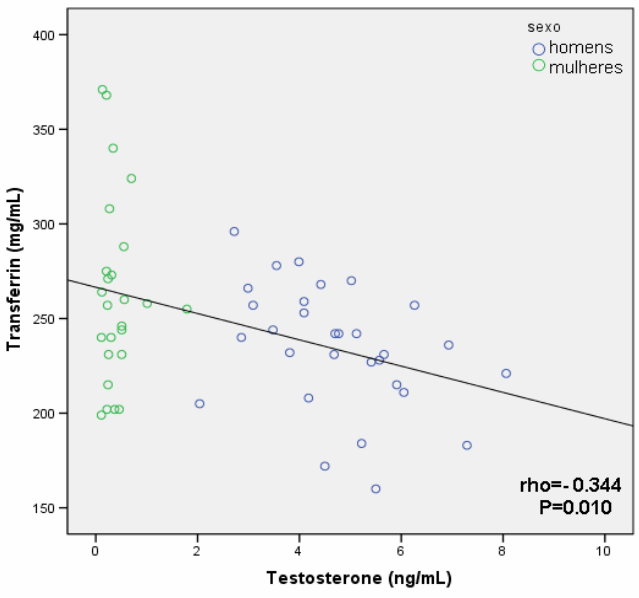



sexo
homens
mulheres
Lowest O_2 saturation (%)
Ferritin (ng/mL)
rho= - 0.352
P=0.010


sexo
homens
mulheres
Erytrocytes nitrites/nitrates (nmol $NaNO_2$/mgHb)
Ferritin (ng/mL)
rho= - 0.273
P=0.044


sexo
homens
mulheres
lnErythrocyte Carbonyl (pmol/gHb)
rsFerritin (ng/mL)
r =0.307
P=0.024


sexo
homens
mulheres
Ferritin (ng/mL)
Testosterone (ng/mL)
rho=0.596
P=2x10-6


sexo
homens
mulheres
Transferrin (mg/mL)
Testosterone (ng/mL)
rho=- 0.344
P=0.010

1.2 : Artigo Científico 2