VALÉRIA AGUIAR GOMES

“EFEITO DO ANTICONCEPCIONAL ORAL SOBRE AS ALTERAÇÕES DE METALOPROTEINASES DA MATRIZ EXTRACELULAR EM PACIENTES COM SÍNDROME DO OVÁRIO POLICÍSTICO”

"EFFECT OF ORAL CONTRACEPTIVES ON CHANGES OF EXTRACELLULAR MATRIX METALLOPROTEINASES IN PATIENTS WITH POLYCYSTIC OVARY SYNDROME"

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Orientador/ Supervisor: Prof. Dr. José Eduardo Tanus dos Santos

"EFFECT OF ORAL CONTRACEPTIVES ON CHANGES OF EXTRACELLULAR MATRIX METALLOPROTEINASES IN PATIENTS WITH POLYCYSTIC OVARY SYNDROME"

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Farmacologia da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção de título de Doutora em Farmacologia.

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RESUMO

A síndrome do ovário policístico (SOP) é a endocrinopatia mais comum em mulheres na idade reprodutiva e está frequentemente associada a alguns fatores de risco cardiovascular. A grande maioria das doenças cardiovasculares (DCV) ocorre inicialmente com o remodelamento vascular, em que as metaloproteinases de matriz (MMPs) são os principais mediadores. Sendo assim, o objetivo do presente estudo foi comparar os níveis plasmáticos da MMP-2 e da MMP-9 e dos inibidores teciduais de MMPs (TIMPs) das pacientes com SOP com as controles saudáveis e examinar se os níveis desses biomarcadores estão associados com às características clínicas e bioquímicas da SOP. Além disso, avaliar o efeito do anticoncepcional oral sobre os níveis plasmáticos de MMPs e respectivos inibidores endógenos nas mulheres com SOP. Para isso, na primeira parte do estudo, avaliamos 65 controles ovulatórias e 80 pacientes com SOP. As concentrações plasmáticas de MMP-8, MMP-9, TIMP-1, TIMP-2 foram medidas por Elisa e, as de MMP-2, por zimografia. Os níveis de MMP-2, MMP-8, MMP-9 e TIMP-1 não foram significativamente diferentes entre os grupos ($p \geq 0,05$). Pacientes com SOP apresentaram menores níveis plasmáticos de TIMP-2 do que as controles saudáveis (182,30 ± 5,60 vs. 204,20 ± 7,28 ng/ml; $p \leq 0,05$). Além disso, a testosterona foi preditor independente dos níveis de TIMP-2 (estimativa = -0,35, p = 0,04) e da razão MMP-9/TIMP-1 (estimativa = 0,01, p = 0,04). Para avaliar se a redução do hiperandrogenismo iria promover alguma alteração no perfil das MMPs, foram analisadas 20 mulheres com SOP que queriam contracepção hormonal (grupo SOP- ACO), 20 mulheres ovulatórias que desejavam contracepção hormonal (grupo controle- ACO) e 15 mulheres ovulatórias que desejavam contracepção não-hormonal (grupo controle). O

tratamento com ACO contendo 30 mcg de etinilestradiol/2mg de acetato de clormadinona durante 6 meses reduziu significativamente as concentrações plasmáticas de MMP-2 no grupo controle (de 1,44 ± 0,11 unidades arbitrárias no tempo basal para 1,22 ± 0,07 unidades arbitrárias após 6 meses; p = 0,01), e no grupo SOP (de 1,43 ± 0,08 unidades arbitrárias no tempo basal para 1,25 ± 0,09 unidades arbitrárias após 6 meses; p = 0,007). O ACO reduziu as concentrações de TIMP-2 e TIMP-1 no grupo controle (todos p ≤0,05), mas não teve efeitos na MMP-9 plasmática e nas razões MMP-2/TIMP-2 e MMP-9/TIMP-1 (todos p≥ 0,05) nos grupos avaliados. Os achados do presente estudo indicam que as mulheres com SOP possuem um desequilíbrio nas razões MMP-2/TIMP-2 e MMP-9/TIMP-1, bem como níveis reduzidos de TIMP-2. Parte desses achados estão relacionados ao hiperandrogenismo presente nessas mulheres. Na segunda parte do estudo, observamos que a redução do hiperandrogenismo, promovido pelo tratamento em longo prazo com o ACO, reduziu as concentrações plasmáticas de MMP-2. Considerando o desequilíbrio no perfil das MMPs apresentado pelas mulheres com SOP e, as possíveis consequências decorrentes desse cenário, o tratamento com ACO se mostra benéfico nessas pacientes, podendo reduzir os riscos de futuras complicações cardiovasculares.

Palavras-chave: Metaloproteinase da matriz extracelular (MMP), Inibidor tecidual da matriz extracelular (TIMP), Síndrome do ovário policístico (SOP), Hiperandrogenismo, Anticoncepcional Oral (ACO), Risco cardiovascular.

ABSTRACT

The polycystic ovary syndrome (PCOS) is the most common endocrinopathy in women of reproductive age and it is often associated with some cardiovascular risk factors. The majority of cardiovascular disease (CVD) occurs initially with vascular remodeling in which matrix metalloproteinases (MMPs) are key mediators. Therefore, the aim of this study was to compare plasma levels of MMP-2 and MMP-9 and tissue inhibitors of MMPs (TIMPs) of PCOS patients with healthy controls and to examine whether the levels of these biomarkers are associated with clinical and biochemical characteristics of PCOS. In addition to it, our goal was to evaluate the effect of oral contraceptives on plasma levels of MMPs and their endogenous inhibitors in women with PCOS. In order to prove it, in the first part of the study we evaluated 65 controls and 80 patients with ovulatory PCOS. The plasma concentration of MMP-8, MMP-9, TIMP-1 and TIMP-2 were measured by Elisa, and MMP-2 by zymography. The levels of MMP-2, MMP-8, MMP-9 and TIMP-1 were not significantly different between groups ($p \geq 0.05$). PCOS patients had lower their plasma levels of TIMP-2 than healthy controls ones (182,30 ± 5,60 vs. 204,20 ± 7,28 ng/ml; p = 0,02). Furthermore, testosterone was an independent predictor of the levels of TIMP-2 (estimate = -0.35, p = 0.04) and the MMP-9/TIMP-1 ratio (estimate = 0.01, p = 0.04). To assess whether the reduction of hyperandrogenism would promote a change in the profile of MMPs, we analyzed 20 women with PCOS who wanted to hormonal contraception (OC-PCOS group), 20 ovulatory women who required hormonal contraception (OC-control group) and 15 ovulatory women who wanted non-hormonal contraception wanted a non-hormonal contraception (non-OC control group). Treatment with OC containing 2 mg chlormadinone acetate/30 μg ethinylestradiol for 6 months significantly reduced

plasma MMP-2 concentrations in the OC-control (from 1.44 ± 0.11 arbitrary units at baseline to 1.22 ± 0.07 arbitrary units after 6 months; p = 0.01) and the PCOS groups (from 1.43 ± 0.08 arbitrary units at baseline to 1.25 ± 0.09 arbitrary units after 6 months; p = 0.007) and TIMP-2 and TIMP-1 levels (448.0 ± 66.3 ng/mL versus 349.0 ± 40.9 ng/mL; p = 0.009) in the OC-control group (all p ≤0.05) but had no effects on MMP-9 concentrations or on MMP-2/TIMP-2 and MMP-9/TIMP- 1 ratios (all p≥ 0.05) in any group. The results of this study indicate that women with PCOS have an imbalance in the MMP-2/TIMP-2 and MMP-9/TIMP-1 ratios and reduced levels of TIMP-2. Parts of these findings are also related to hyperandrogenism presence in these women. In the second part of the study, we observed that the reduction of hyperandrogenism promoted by long-term treatment with the OC reduced plasma concentrations of MMP-2. Given the imbalance in the profile of MMPs presented by women with PCOS and the possible consequences of this scenario, treatment with OC shows beneficial in these patients may reduce the risk of future cardiovascular complications.

Keywords: Extracellular matrix metalloproteinase (MMP), Tissue inhibitor of extracellular matrix (TIMP), Polycystic ovary syndrome (PCOS), Hyperandrogenism, Oral Contraceptive (OC), Cardiovascular Risk.

LISTA DE SIGLAS E ABREVIATURAS

ACTH - adrenocorticotropina

ELISA - Enzyme-linked immunosorbent assay

DHEA - dehidroepiandrosterona

HDL - Lipoproteína de Alta Densidade

HOMA IR - Homeostasis model assessment insulin index- índice de resistência a insulina

IMC - Índice de massa corporal

LDL - Lipoproteína de Baixa Densidade

LH - Hormônio luteinizante

MEC - Membrana extracelular

mmHg - Milímetro de mercúrio

MMP - Metaloproteinases da Matriz extracelular

MT-MMP - Metaloproteinase da matriz extracelular do tipo membrana

PA - Pressão Arterial

SHBG - Hormônio sexual ligado a globulina

TIMP - Inibidor tecidual de metaloproteinase

SUMÁRIO

INTRODUÇÃO GERAL

Síndrome do Ovário Policístico

A síndrome do ovário policístico (SOP) foi descrita pela primeira vez em 1935 por Stein e Leventhal, que observaram uma associação entre a amenorréia, o hirsutismo, ovários com aspecto policístico e obesidade. A SOP é uma das endocrinopatias mais frequentes em mulheres na idade reprodutiva, acometendo cerca de 6-10% delas em todo o mundo [1]. Atualmente, a SOP é caracterizada pela presença de duas dentre as seguintes características: hiperandrogenismo clínico e/ou bioquímico, anovulação crônica e/ou a presença de ovários policísticos no ultrassom [2]. O emprego de tais critérios permite o diagnóstico da SOP em mulheres com quatro fenótipos diferentes: 1) hiperandrogenismo, anovulação crônica e presença de ovários policísticos; 2) hiperandrogenismo, anovulação crônica e ovários normais; 3) hiperandrogenismo e ovários policísticos, porém com ciclos ovulatórios; e 4) anovulação crônica, ovários policísticos e leve aumento na concentração de androgênio plasmático.

Independente da heterogeneidade clínica observada entre as mulheres com SOP, as características mais frequentes nelas são a presença de ovários com padrão policístico, hirsutismo, acne, *acantose nigricans*, irregularidades menstruais e resistência à insulina.

Devido a sua diversidade e complexidade, a fisiopatologia da SOP ainda não está totalmente elucidada. No entanto, sugere-se que o ovário das mulheres com SOP sejam predispostos a hipersecretarem andrógenos, provavelmente desde a vida intra-uterina, e durante a ativação do eixo hipotalo-hipófise-ovário que ocorre fisiologicamente no final da infância e início da puberdade.

Os níveis elevados de testosterona circulante estimulam a pituitária a uma hiperprodução de hormônio luteinizante (LH) e também amplifica a resistência à insulina. O aumento das concentrações de LH e insulina ampliam ainda mais a produção de androgênio pelos ovários e, portanto, podem contribuir para o mecanismo de anovulação.

Alguns distúrbios metabólicos são comumente associados à SOP, entre eles: a resistência à insulina (prevalência de 50 a 70% nas pacientes com SOP [3]), diabetes mellitus do tipo 2 de início precoce, dislipidemia, hipertensão e obesidade, que além de serem componentes da síndrome metabólica são conhecidamente fatores de risco para as doenças cardiovasculares (DCV) [4-7].

Um estudo recente observou que a prevalência de síndrome metabólica em mulheres com SOP é cerca de oito vezes maior do que em mulheres não-SOP na mesma faixa etária [4]. Devido ao aumento da prevalência dessas comorbidades que, por sua vez, estão intimamente relacionadas ao risco cardiovascular nas mulheres com SOP, acredita-se que esse grupo de mulheres estaria mais predisposto ao desenvolvimento precoce de DCV.

SOP e potenciais marcadores de doenças cardiovasculares

Diversos estudos observaram a frequência pronunciada de fatores de risco para DCV em mulheres com SOP quando comparados com mulheres na mesma faixa etária. Entretanto, ainda é incerto o aumento da mortalidade por DCV nessas mulheres, já que até o presente momento não há estudos clínicos prospectivos com esta finalidade.

Contudo, diferentes estudos demonstraram alterações na função endotelial

e aumento de alguns marcadores bioquímicos de DCV, assim como PAI-I, proteína C-reativa, adiponectina, endotelina-1 e marcadores de estresse oxidativo nas pacientes com SOP [4,8-11].

Achados como disfunção endotelial, que é um preditor para o desenvolvimento de DCV, aumento na espessura da camada íntima-média [12-14] e uma maior rigidez na artéria carótida [15] já foram relatados em mulheres com SOP. Os três fatores também são considerados marcadores precoces de mudanças estruturais na artéria que, posteriormente, podem resultar em eventos cardiovasculares como hipertrofia do ventrículo esquerdo e infarto do miocárdio. É importante ressaltar que a maior parte dos estudos inclui pacientes obesas e/ou hipertensas e/ou resistentes à insulina, fatores que poderiam, por si só, explicar parte desses resultados. Entretanto, um estudo recente mostrou aumento no índice da rigidez e redução na distensibilidade da artéria carótida em pacientes jovens e não obesas com SOP sem fatores de risco clássicos para doença cardiovascular [16].

A detecção precoce de mudanças estruturais em mulheres jovens com SOP é extremamente relevante, pois essas alterações estão associadas ao aumento da morbidade e mortalidade por DCV. Além disso, mulheres na pós-menopausa com características clínicas de SOP têm probabilidade de apresentar doença arterial coronariana 2,5 vezes maior do que as controles da mesma idade [17].

Em um estudo prospectivo foram avaliadas 61 mulheres com SOP e 85 mulheres controles durante nove anos. Observou-se que as mulheres com SOP apresentaram maior prevalência de calcificação na artéria coronária (45,9% *vs*

30,6%) e na aorta (68,9% *vs* 55,3%) do que as controles. Esses resultados estão associados à presença de aterosclerose subclínica [18].

Sabe-se que o processo aterosclerótico é caracterizado por um remodelamento vascular da matriz extracelular e que as metaloproteinases de matriz (MMPs) têm sido implicadas como mediadores principais no estágio inicial de remodelamento vascular. Estudos também têm demonstrado o aumento da expressão de algumas MMPs na placa aterosclerótica, uma vez que a ativação das MMPs parece facilitar a instalação da aterosclerose, a desestabilização da placa e a agregação plaquetária [19-21]. Além de participar de processos patológicos como a formação de placa aterosclerótica, as MMPs também participam de processos fisiológicos como a ovulação [22] e o crescimento folicular [23].

Metaloproteinases da Matriz Extracelular

As metaloproteinases da matriz extracelular (MMPs) são uma família de mais de 20 subtipos de proteases zinco e cálcio-dependentes, estruturalmente relacionadas. Elas são caracterizadas pela habilidade de degradarem componentes da matriz extracelular, como o colágeno, fibronectina e várias proteoglicanas [24].

As MMPs participam das etapas de proliferação celular, diferenciação, remodelamento da matriz extracelular, vascularização e migração celular [25]. Elas exercem papeis importantes durante o remodelamento tecidual fisiológico como: o desenvolvimento embrionário, a morfogênese, a reprodução e reabsorção tecidual e, ainda, em processos patológicos que incluem reações

inflamatórias, destruição da cartilagem na artrite, ruptura de placas ateroscleróticas, reestenose miocárdica, aneurismas, invasão neoplásica, entre outros.

As MMPs são classificadas de acordo com os substratos que degradam [26]. Desse modo, elas podem ser agrupadas em:

1. Colagenases (MMP-1, MMP-8, MMP-13 e MMP-18). Colagenases são responsáveis por clivar o colágeno fibrilar (colágenos do tipo I, II e III);
2. Gelatinases (MMP-2 e MMP-9). Degradam principalmente o colágeno desnaturado (gelatina);
3. Estromelisinas (MMP-3 e MMP-10). Apesar de possuírem similaridade em relação ao substrato, a MMP-3 possui uma maior eficiência proteolítica quando comparada a MMP-10;
4. Matrilisinas (MMP-7 e MMP-26). Não possuem o domínio hemopexina;
5. MMPs do tipo membrana (MT1-MMP a MT8-MMP). A MT1-MMP pode degradar o colágeno do tipo I, II e II e outros componentes da matriz extracelular;
6. Metaloelastases (MMP-12).

Independentemente do substrato que degradam, as MMPs apresentam algumas similaridades estruturais. De um modo geral, elas constituem-se de um peptídeo sinal, um pró-domínio autoinibitório (domínio pró-peptídico), um domínio catalítico e um domínio hemopexina. O pró-domínio possui um domínio N-terminal, permitindo que a enzima seja transportada para o meio extracelular.

O pró-domínio também contém uma cisteína que protege o domínio catalítico da enzima. A presença do íon zinco no domínio catalítico e o resíduo de cisteína são características comuns a todas MMPs. O domínio catalítico das gelatinases MMP-2 e MMP-9 é exclusivo, já que é o único que contém três fibronectinas do tipo 2, que formam um domínio de ligação com o colágeno, permitindo a junção e subsequente clivagem do mesmo. Com exceção das matrilisinas (MMP-7 e MMP-27), as MMPs contêm uma região flexível (de dobradura) que é conhecida como domínio hemopexina, o qual está ligado à cauda C-terminal.

As MMPs são secretadas na forma de precursores inativos (zimogênios) cuja latência é mantida através da interação entre o resíduo de cisteína, que está presente no pró-domínio, e o zinco presente no domínio catalítico, o que impede o acesso ao sítio ativo pelo substrato. Elas também podem ser ativadas por outras MMPs (ex: MT-MMPs) e por outras classes de proteases, como, por exemplo, a plasmina, que promove a clivagem do domínio pró-peptídico, deixando o sítio catalítico da enzima livre para interação com o respectivo substrato [24], ou por meio da ação não-proteolítica como o estresse oxidativo e detergentes [27].

A regulação da atividade proteolítica dessas enzimas pode ocorrer em vários níveis: 1) através da transcrição gênica; 2) tradução e síntese de zimogênios; 3) secreção dos zimogênios; 4) ativação dos zimogênios nos tecidos; 5) interação com inibidores teciduais de metaloproteinases (TIMPs) [24,28]. Esses inibidores teciduais são pequenas proteínas que agem formando um complexo na proporção de 1:1 com o zinco do domínio catalítico das MMPs promovendo, assim, um impedimento estérico dessas com os seus substratos.

O equilíbrio tecidual entre MMPs e TIMPs é primordial para a dinâmica da degradação da matriz extracelular, sendo fundamental para a manutenção da homeostase tecidual [26]. Vários hormônios, assim como citocinas, angiotensina II, fatores de crescimento, estresse de cisalhamento e estresse oxidativo também podem interferir nessa regulação [29,26].

Envolvimento das MMP-2 e MMP-9 em doenças cardiovasculares

As ações proteolíticas das MMPs, entre outras funções, desempenham um importante papel no remodelamento vascular e na migração celular.

Dentre as diversas MMPs conhecidas, duas delas merecem destaque: a MMP-2 e a MMP-9. Essas duas MMPs degradam gelatina e colágeno do tipo IV e V e participam de alterações estruturais e funcionais observadas no remodelamento vascular presente em diversas doenças cardiovasculares [19,20].

Estudos com animais knock-out para a MMP-2 e MMP-9 têm observado que essas MMPs estão envolvidas na disfunção cardíaca, na ruptura após infarto do miocárdio e no desenvolvimento de aneurisma abdominal da aorta [30-32]. Além disso, diversos grupos têm demonstrado, tanto em estudos clínicos quanto experimentais um aumento significativo na atividade e expressão das MMP-2 e MMP-9 em várias doenças cardiovasculares, tais como aterosclerose, hipertensão e insuficiência cardíaca [33-35].

A MMP-2 é expressa constitutivamente e é amplamente distribuída pela maioria das células do tecido conjuntivo (fibroblastos), células endoteliais e epiteliais.

Alguns trabalhos têm demonstrado o aumento da MMP-2 em amostras do miocárdio de pacientes com cardiomiopatia [35] e em modelos experimentais de hipertensão [36-38]. Estudos clínicos observaram, ainda, um aumento da MMP-2 plasmática em pacientes com insuficiência cardíaca [39-41] e na cardiomiopatia hipertrófica com disfunção sistólica [41]. Além disso, um aumento na expressão de MMP-2 foi evidenciado em aneurisma de aorta [42,19,43], e na restenose coronariana [42,19]. Além disso, a participação da MMP-2 também foi demonstrada durante a formação de lesões arteriais no processo aterosclerótico [44,45].

A MMP-9 também tem participação em algumas doenças cardiovasculares, podendo até ser considerada como um potencial marcador, sobretudo nas patologias com componente inflamatório, uma vez que ela é sintetizada e secretada por diversas células inflamatórias, como macrófagos e neutrófilos. Essa enzima também participa dos processos de migração e proliferação de células musculares lisas vasculares, pois permite que essas células rompam a barreira de tecido conjuntivo ao redor [42,46].

Os trabalhos que avaliaram as regiões vulneráveis de placas ateroscleróticas observaram que essa MMP estava altamente expressa, Dessa maneira, acredita-se que ela possua uma participação importante no remodelamento associado à aterosclerose e à ruptura dessas placas, o que pode resultar em eventos cardiovasculares fatais [21,20,19].

Níveis elevados da enzima também foram relatados em pacientes com angina instável [40], aneurisma de aorta [47], e naqueles que posteriormente apresentaram um evento cardiovascular fatal [48]. A elevação dos níveis foi

relacionada à presença e severidade de DCV e à rigidez arterial em pacientes com doença arterial coronariana [39,40,49].

Estudos recentes têm revelado a participação da MMP-8, além da MMP-9 e da MMP-2, em placas instáveis na carótida [50,51]. O aumento dos níveis séricos e da expressão da MMP-8 também foi observados em lesões ateroscleróticas. O aumento da lesão foi proporcional com o aumento da expressão dessa enzima [52,53]. Trabalhos recentes encontraram uma associação entre a presença e severidade de doenças cardiovasculares com o aumento plasmático de MMP-8.

A fisiopatologia da SOP ainda não está totalmente esclarecida. Apesar disso, sabe-se que a SOP está intrinsecamente associada à resistência insulina, obesidade, hipertensão e dislipidemia que são fatores de risco para DC. Entretanto ainda não foram elucidados os mecanismos responsáveis pelo desenvolvimento de hipertensão e de outras comorbidades associadas com DC em mulheres com SOP. Sendo assim, o entendimento da fisiopatologia da SOP é essencial para a redução dos riscos cardiovasculares, aos quais as mulheres com SOP estão frequentemente expostas.

Como exposto, a MMP-2 e a MMP-9 são enzimas conhecidas por estarem envolvidas no desenvolvimento de hipertrofia ventricular esquerda [54,55], hipertensão [55,37,38], e no processo de ruptura de placa aterosclerótica [40,56]. Além disso, por estarem relacionadas a doenças cardiovasculares, torna-se pertinente avaliar as concentrações circulantes dessas enzimas, bem como investigar se o tratamento com o anticoncepcional oral pode alterar as concentrações dessas MMPs nas mulheres com SOP. Pois,

o anticoncepcional oral é a droga de primeira escolha no tratamento das mulheres com SOP que desejam contracepção, uma vez q ele regula o ciclo menstrual, promove proteção endometrial e reduz o hiperandrogenismo nessas pacientes.

OBJETIVOS

OBJETIVOS

Os objetivos do primeiro artigo foram:

1. Determinar se existe alterações significativas nas concentrações plasmáticas de MMP-2 e MMP-9 e do TIMP-1 e TIMP-2 em pacientes com SOP quando comparadas com as do grupo controle.
2. Avaliar se os níveis desses biomarcadores estão associados com às características clínicas e bioquímicas da SOP.

O objetivo do segundo artigo foi:

1. Avaliar o efeito do anticoncepcional oral sobre os níveis plasmáticos de MMPs e respectivos inibidores endógenos nas mulheres com SOP.

CAPÍTULO

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Imbalanced circulating matrix metalloproteinases in polycystic ovary syndrome

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Abstract Altered levels of matrix metalloproteinases (MMPs) may reflect relevant pathogenetic mechanisms of disease conditions. The objective of this study was to compare the plasma levels of MMPs and tissue inhibitors of MMPs (TIMPs) in polycystic ovary syndrome (PCOS) patients with those found in healthy ovulatory controls and to examine whether the levels of these biomarkers are associated with clinical and biochemical features of this syndrome. Sixty-five healthy ovulatory subjects (controls) and 80 patients with PCOS were include in this study. MMP-2, MMP-8, MMP-9, TIMP-1, TIMP-2 concentrations were measured in plasma samples by gelatin zymography or enzyme-linked immunoassays. MMP-2, MMP-8, MMP-9, and TIMP-1 levels were similar in PCOS patients and in healthy controls ($P > 0.05$). PCOS patients had lower plasma TIMP-2 levels than healthy controls ($P < 0.05$). We found higher MMP-2/TIMP-2 and MMP-9/TIMP-1 ratios in PCOS patients than in healthy controls (all $P < 0.05$). Testosterone levels correlated positively with the MMP-9/TIMP-1 ratio and negatively with TIMP-2 levels ($r = 0.26$, $P < 0.01$ and $r = -0.21$, $P = 0.02$, respectively). In addition, only testosterone was an independent predictor of TIMP-2 levels (estimate $= -0.35$, $P = 0.04$) and the MMP-9/TIMP-1 ratio (estimate $= 0.01$, $P = 0.04$). We found evidence indicating that the balance between MMPs and TIMPs in women with PCOS is altered, probably due to androgen excess found in these women.

Keywords Polycystic ovary syndrome · Hyperandrogenism · Metalloproteinases · Tissue inhibitors of metalloproteinases

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Introduction

Polycystic ovary syndrome (PCOS) is the most common female endocrinopathy, affecting about 6–10% [1] of women of reproductive age. PCOS is characterized by hyperandrogenism, chronic anovulation, and/or polycystic ovaries on ultrasound [2]. The syndrome is also a multifaceted metabolic disease that is associated with insulin resistance, obesity, and metabolic syndrome [3–6], and this may contribute to increased risk of developing cardiovascular disease.

Although PCOS has been associated with a wide variety of cardiovascular risk factors, there is no definitive evidence for increased cardiovascular morbidity and mortality in women with PCOS. Nevertheless, endothelial dysfunction and elevated biochemical markers of cardiovascular and inflammatory disease have been demonstrated in women with PCOS [7–9]. In addition, studies found an increased intima-media thickness [10, 11] and increased stiffness in the carotid artery [12] in PCOS patients, both of which are early signs of arterial structural changes. However, the

majority of studies included obese and/or hypertensive and/or insulin resistant patients, and these factors per se could explain part of these findings. In this respect, a recent study demonstrated an increased common carotid artery stiffness index and reduced distensibility of the carotid artery in young, non-obese PCOS patients without classical risk factors for cardiovascular disease [13]. These findings are associated with the presence of subclinical atherosclerosis.

It is known that the atherosclerotic process is characterized by vascular remodeling of the extracellular matrix. Matrix metalloproteinases (MMPs) have been implicated as primary mediators of this remodeling [14–16]. MMPs and tissue inhibitors of metalloproteinases (TIMPs) have been shown to play significant roles in many physiological conditions, including embryo implantation, angiogenesis, bone remodeling, ovarian follicular growth, and ovulation. They also contribute to pathological states, such as atherosclerosis, inflammation, and arthritis. MMP-2 and MMP-9 have been associated with cardiovascular disease [17], including atherosclerosis, coronary artery disease, and stroke [18–20], thus giving further support to the suggestion that MMP-2 and MMP-9 could play an important role in the development of cardiovascular diseases. Recently, it was suggested that circulating MMP-8 may be a marker of atherosclerosis [21–23]. The extent of remodeling of the extracellular matrix depends upon the critical equilibrium between MMPs and TIMPs. Studies have shown that alterations of circulating MMPs/TIMPs concentrations are implicated in the pathophysiology of a variety of cardiovascular diseases. The imbalance between MMPs and TIMPs has been observed in different contexts, including metabolic syndrome [24], obese children [25], and hypertensive disorders of pregnancy [26]. At present, only a limited number of studies have investigated possible alterations of circulating MMPs/TIMPs concentrations in women with PCOS [27–29]. In addition, there has been no evaluation of the circulating levels of MMP-8 in PCOS.

Due to the important association of the MMPs/TIMPs ratio with many cardiovascular diseases [23, 30], in the present study we assessed (1) whether there are significant alterations in the plasma concentrations of MMPs, TIMPs, and MMPs/TIMPs in PCOS young and no-obese patients compared with those found in healthy ovulatory controls and (2) the association between these markers and clinical and biochemical characteristics present in women with PCOS.

Materials and methods

Subjects and study protocol

A cross-sectional study was conducted at the University Hospital of the Faculty of Medicine of Ribeirao Preto, University of São Paulo (HC-FMRP-USP), Brazil. The study protocol was approved by the local institutional review board, and all volunteers gave written informed consent. Eighty women with PCOS were included in the study immediately after diagnosis, and 65 healthy ovulatory women were recruited at a basic health unit before the prescription of a contraceptive method. Inclusion criteria were age between 18 and 35 years and BMI <30 kg/m^2. The diagnosis of PCOS was confirmed by the presence of at least two of the three criteria of the Rotterdam Consensus [31]: chronic anovulation, clinical, and/or biochemical signs of hyperandrogenism, and polycystic ovaries. Exclusion criteria for all subjects were smoking; alcoholism; drug addiction; current pregnancy; current or previous use (up to 2 months before the study) of oral, vaginal, monthly injectable, or transdermal hormonal contraceptives; current or previous use (up to 6 months before the study) of a long-lasting hormonal contraceptive method (injectable, implant, or intrauterine device); use of antiandrogenic or hypoglycemic drugs, anti-inflammatory drugs, or statins; presence of systemic diseases (diabetes mellitus type 2, cardiovascular diseases, autoimmune diseases, liver disease, thyroid disease, or congenital renal hyperplasia); personal history of arterial or venous thrombosis; chronic or acute inflammatory processes; and puerperium of 12 weeks or less. Inclusion criteria for ovulatory women were regular menstrual cycles, absence of clinical and laboratorial hyperandrogenism, and ultrasonography performed during the early follicular phase to confirm normal ovarian morphology.

Anthropometric measurements and laboratory tests

The following anthropometric variables were determined: weight, height, body mass index (BMI), and waist circumference (the lowest measurement found between the iliac crest and the inferior margin of the last rib). Blood samples were collected in the Gynecology Laboratory of HC-FMRP-USP between 8:00 and 9:00 a.m. after at least 10 h of fasting and always during the follicular phase (third to seventh day of the cycle) for control women and for women with PCOS and oligomenorrhea. PCOS patients with amenorrhea were evaluated after a pelvic ultrasonography showing no evidence of either a follicle equal to or greater than 10 mm or a corpus luteum. Samples of whole blood (20 ml) were collected and divided into tubes without anticoagulant (for serum separation) and in plastic conical tubes (BD-Becton-Dickinson, Plymouth, UK) with no vacuum and containing sodium citrate anticoagulant at 3.2% (in a fixed proportion of 9 parts whole blood to 1 part anticoagulant).

The blood samples were processed within a maximum of 2 h after collection. Serum was stored at -80°C for

simultaneous determination of the following serum variables: fasting serum glucose determined by the oxidase method using a Konelab 60i analyzer (Wiener Lab®, Rosario, Argentina); total cholesterol, HDL-cholesterol, and triglycerides (TG) determined by an enzymatic method using the BT 3000 plus analyzer (Wiener lab®); LDL-cholesterol as calculated according to the Friedewald formula [LDL-cholesterol = total cholesterol−(HDL-C + TG/5)] because none of the samples contained triglyceride levels exceeding 400 mg/dl [32]; ultrasensitive C reactive protein (CRP), sex hormone binding globulin (SHBG), and insulin were measured by chemoluminescence with the DPC Immulite® 2000 analyzer (Diagnostic Products Corporation, Los Angeles, CA, USA®); interleukin-6 (IL-6) was determined by chemoluminescence using the DPC Immulite® 1000 analyzer and the respective kits for Immulite® 1000 (Siemens®, CA, USA); and total testosterone was determined by radioimmunoassay using the Tri Carb 2100 TR scintillator (Packard® Instrument Company, Illinois, USA). The free androgen index (FAI) was calculated using the following formula: total testosterone (nmol/l)/SHBG (nmol/l) × 100 [33]. Insulin resistance was determined according to the homeostasis model assessment-insulin resistance (HOMA-IR) index, i.e., HOMA-IR = fasting serum glucose (mg/dl) × 0.05551 × fasting insulin (μU/ml)/22.5 [34].

Measurement of plasma MMP-9, MMP-8, TIMP-1, and TIMP-2 concentrations

Whole blood was centrifuged at 120×*g* (700 rpm) in a Sorvall RC 3 centrifuge (Sorvall Kendro Laboratory Products GmbH, Langenselbold, Germany) at room temperature (mean 22°C; range 18–24°C) for 15 min. Plasma was obtained by centrifuging the samples at 1600×*g* (2500 rpm) for 30 min using a Universal 32 R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) at 4°C. Plasma aliquots were stored at −70°C until they were analyzed. Concentrations of MMP-8, MMP-9, TIMP-1, and TIMP-2 were measured using a commercially available enzyme-linked immunosorbent (ELISA) assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

SDS-polyacrylamide gel electrophoresis (PAGE) gelatin zymography of MMP-2

Gelatin zymography of MMP-2 from plasma samples was performed as previously described [35–37]. Briefly, plasma samples were subjected to electrophoresis on 7% SDS-PAGE co-polymerized with gelatin (1%) as the substrate. After electrophoresis was complete, the gel was incubated for 1 h at room temperature in a 2% Triton X-100 solution and incubated at 37°C for 16 h in Tris–HCl buffer, pH 7.4, containing 10 mmol/l $CaCl_2$. The gels were stained with 0.05% Coomassie Brilliant Blue G-250 and then destained with 30% methanol and 10% acetic acid. Gelatinolytic activities were detected as unstained bands against the background of Coomassie blue-stained gelatin. Enzyme activity was assayed by densitometry using ImageJ version 1.42q (Wayne Rasband National Institutes of Health, USA). MMP-2 was identified as a band at 72 kDa by the relation of log Mr to the relative mobility of Sigma SDS-PAGE LMW marker proteins.

Statistical analysis

All the results were expressed as mean + SEM. An unpaired Student's *t* test was used to compare normally distributed variables. The Mann–Whitney *U* test was used to compare non-normally distributed variables. Spearman's correlation was applied to calculate the correlation between the variables. Data were analyzed using SPSS 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

Univariate analyses were performed to assess the relationship between MMPs or TIMPs concentrations and clinical variables that could influence their levels. HOMA, BMI, PCOS, and testosterone levels were included as independent variables in a multiple linear regression model to explain changes in MMPs, TIMPs, and the MMPs/TIMPs ratio using SAS 9.0 software (SAS Institute Inc., Cary, NC, USA). The results were defined as statistically significant when $P < 0.05$.

Results

The clinical characteristics of all participants are summarized in Table 1. We found no significant differences in age, diastolic arterial pressure (DAP), lipid profile, fasting serum glucose, IL-6, and CRP when controls were compared with patients with PCOS (all $P > 0.05$). However, patients with PCOS had higher BMIs and HOMA (all $P < 0.05$) than in controls. The mean systolic arterial pressure and waist circumference were higher in PCOS patients than in controls but was within normal limits (all $P < 0.01$). In addition, PCOS patients had a greater ovarian volume. Similarly, total testosterone and FAI were higher in PCOS patients than in controls, and SHBG levels were lower in women with PCOS than in controls (all $P < 0.01$).

Patients with PCOS had significant lower plasma TIMP-2 concentrations compared with those found in controls (182.30 ± 5.60 vs. 204.20 ± 7.28 ng/ml; $P = 0.02$; Table 2), while MMP-2, MMP-9, MMP-8, and TIMP-1 levels did not differ significantly between groups (all $P > 0.05$; Table 2). However, we found higher MMP-2/

Table 1 Clinical and laboratory characteristics of the study groups

	Controls ($n = 65$)	PCOS ($n = 80$)	P value
Age (years)	23.54 ± 0.56	24.40 ± 0.44	0.09[a]
BMI (kg/m^2)	22.86 ± 0.39	26.43 ± 0.73	<0.01[a]
WC (cm)	72.81 ± 0.91	86.06 ± 1.79	<0.01[a]
SAP (mmHg)	112.10 ± 1.07	117.60 ± 1.24	<0.01[a]
DAP (mmHg)	76.26 ± 0.89	78.69 ± 0.92	0.06[b]
Ovarian volume (cm^3)	6.60 ± 0.21	11.70 ± 0.40	<0.01[a]
TChol (mg/dl)	168.60 ± 2.57	173.10 ± 3.69	0.53[a]
TG (mg/dl)	76.56 ± 5.49	92.99 ± 5.94	0.22[a]
HDL (mg/dl)	50.80 ± 0.90	49.84 ± 1.03	0.37[a]
LDL (mg/dl)	101.60 ± 2.14	104.70 ± 3.11	0.62[a]
Glycemia (mg/dl)	88.67 ± 0.56	87.55 ± 1.05	0.34[a]
Insulin (μU/ml)	5.50 ± 0.31	11.07 ± 1.44	0.01[a]
HOMA-IR	1.22 ± 0.07	2.45 ± 0.34	0.03[a]
Testosterone (ng/dl)	52.18 ± 1.66	77.67 ± 3.58	<0.01[a]
SHBG (nmol/l)	46.93 ± 1.40	35.51 ± 2.12	<0.01[a]
FAI (%)	4.44 ± 0.20	9.64 ± 0.89	<0.01[a]
Interleukin-6 (pg/ml)	1.71 ± 0.10	2.02 ± 0.15	0.58[a]
CRP (mg/l)	3.83 ± 0.62	2.46 ± 0.36	0.36[a]

Values are the mean ± S.E.M

BMI body mass index, *WC* waist circumference, *SAP* systolic arterial pressure, *DAP* diastolic arterial pressure, *TChol* total cholesterol, *TG* Triglycerides, *HOMA-IR* homeostasis model assessment-insulin resistance, *SHBG* sex hormone binding globulin, *FAI* free androgen index, *CRP* C reactive protein

[a] P value obtained by the Mann–Whitney test

[b] P value obtained by the unpaired t test

TIMP-2 and MMP-9/TIMP-1 ratios in women with PCOS than in controls (all $P < 0.05$; Table 2).

We examined the correlation between MMPs and TIMPs with clinical characteristics of all participants in both groups. MMP-9 was positively correlated with MMP-8 ($r = 0.20$, $P = 0.01$), and MMP-8 levels were positively correlated with TIMP-1 ($r = 0.43$, $P < 0.01$) and negatively with triglycerides levels ($r = -0.24$, $P < 0.01$). Waist circumference ($r = 0.20$, $P = 0.02$), glucose ($r = 0.21$, $P = 0.02$), and testosterone ($r = 0.26$, $P < 0.01$) were positively correlated with the MMP-9/TIMP-1 ratio.

To determine the influence of PCOS and certain variables (HOMA, BMI, and testosterone) on MMPs, TIMPs, and MMPs/TIMPs ratios, we performed a multiple linear regression analysis (Table 3). HOMA-IR was significantly related to the MMP-9/TIMP-1 ratio when not considering BMI in the multiple regression model (data not shown). However, testosterone was an independent predictor of TIMP-2 levels (estimate = −0.35, $P = 0.04$) and of the MMP-9/TIMP-1 ratio (estimate = 0.01, $P = 0.04$).

Discussion

The main findings of the present study in women with PCOS are that: (i) compared with healthy subjects, patients with PCOS have an imbalance between MMPs and TIMPs, including lower concentrations of TIMP-2 and increased MMP-2/TIMP-2 and MMP-9/TIMP-1 ratios and (ii) TIMP-

Table 2 MMP and TIMP profile in PCOS patients and controls

	Controls ($n = 65$)	PCOS ($n = 80$)	P value
MMP-2 (arbitrary units)	0.99 ± 0.03	1.01 ± 0.03	0.66
MMP-9 (ng/ml)	146.80 ± 10.52	175.90 ± 12.45	0.07
MMP-8 (ng/ml)	496.10 ± 49.54	598.50 ± 68.13	0.78
TIMP-2 (ng/ml)	204.20 ± 7.28	182.30 ± 5.60	0.02
TIMP-1 (ng/ml)	479.10 ± 29.11	427.30 ± 21.54	0.30
MMP-2/TIMP-2 ratio (×1000)	5.19 ± 0.25	5.91 ± 0.25	0.02
MMP-9/TIMP-1 ratio	0.36 ± 0.03	0.48 ± 0.04	0.01

Values are the mean ± S.E.M

P value obtained by the Mann–Whitney test

Table 3 Results from multiple linear regression analyses for MMP, TIMPs, and MMPs/TIMPs ratios using PCOS as an independent variable

	MMP-2 ($R^2 = 0.05$)		MMP-9 ($R^2 = 0.03$)		MMP-8 ($R^2 = 0.03$)		TIMP-2 ($R^2 = 0.07$)		TIMP-1 ($R^2 = 0.06$)		MMP-2/TIMP-2 ($R^2 = 0.03$)		MMP-9/TIMP-1 ($R^2 = 0.09$)	
	E	P	E	P	E	P	E	P	E	P	E	P	E	P
PCOS	0.05	0.45	0.03	0.78	0.26	0.16	1.37	0.90	0.02	0.83	0.001	0.70	0.01	0.94
HOMA	0.01	0.66	0.02	0.28	−0.02	0.61	−2.41	0.27	−0.01	0.64	0.001	0.17	0.03	0.21
Testosterone	−0.01	0.11	0.01	0.45	0.01	0.67	−0.35	0.04	−0.01	0.06	0.001	0.54	0.01	0.04
BMI	−0.01	0.07	−0.01	0.99	−0.01	0.96	−0.31	0.74	−0.01	0.33	−0.001	0.17	0.01	0.45

E estimate

2 was negatively related to testosterone levels, while the MMP-9/TIMP-1 ratio was positively related to testosterone levels. To our knowledge, this is the first study showing evidence for a negative association between testosterone and TIMP-2 and a positive association between testosterone and the MMP-9/TIMP-1 ratio. Our results suggest that the hyperandrogenism present in most women with PCOS can be a contributor to cardiovascular risk because there is a link between the imbalance in MMPs/TIMPs ratios and cardiovascular disease.

The MMPs constitute a large family of proteolytic enzymes that degrade the extracellular matrix and facilitate remodeling under normal and pathological conditions [30]. MMPs can be strictly regulated at multiple levels through the control of gene transcription, posttranslational activation of zymogens, and the interactions of secreted MMPs with TIMPs, which are small proteins that inhibit MMPs by noncovalently binding them with a 1:1 stoichiometry. TIMP-1 and TIMP-2 are the major inhibitors of MMP-9 and MMP-2, respectively [30]. In the present study, we found reduced levels of TIMP-2 and an elevated MMP-2/TIMP-2 ratio in women with PCOS when compared with healthy controls. In addition to inhibiting MMPs, TIMP-2 may inhibit the migration and apoptosis of macrophages and foam cells. Moreover, studies have demonstrated that TIMP-2 is also involved in the activation of MMP-2. At low concentrations, TIMP-2 serves as a receptor for MMP-2, forming the membrane-type matrix metalloproteinase 1-TIMP-2 (MT1-MMP-TIMP-2) complex and consequently leading to MMP-2 activation [38, 39]. However, at high concentrations, TIMP-2 neutralizes membrane-type matrix metalloproteinase 1 (MT-MMP-1) and prevents MMP-2 activation [38, 39]. Therefore, changes in TIMP-2 can contribute to an imbalance in the MMP-2/TIMP-2 ratio, favoring extracellular degradation. In spite of higher MMP-9 levels and lower TIMP-1 levels in the group of PCOS women, these differences were not statistically significant when compared with control group. However, theses alterations contributed to higher MMP-9/TIMP-1 ratio observed in the PCOS group.

Our findings showing that women with PCOS have higher MMP-2/TIMP-2 and MMP-9/TIMP-1 ratios than ovulatory controls are in agreement with previous studies [27, 29]. These results are important because a critical equilibrium between MMPs and TIMPs determines extracellular matrix degradation, and alterations in the MMPs/TIMPs ratios may lead to disease conditions. Therefore, the assessment of MMP-9/TIMP-1 and MMP-2/TIMP-2 ratios may be better markers of net MMPs activities.

The MMP-9 and MMP-2 levels in the present study were not significantly different between PCOS patients and healthy controls. These findings are in contrast with previous studies [27, 29] that reported higher serum concentrations of MMP-9 [27, 29] and MMP-2 [29] in women with PCOS than in controls. It is possible that the small sample size and the use of serum samples instead of plasma, could explain the difference observed between the studies. Indeed, previous studies used serum samples to assess circulating levels of MMPs, and serum samples can artificially increase MMP-9 levels [35–37].

Most women with PCOS exhibit a clustering of cardiovascular risk factors, including obesity and insulin resistance. Indeed, BMI and HOMA-IR were significantly higher in PCOS women compared with control women enrolled in the present study, even though BMI <30 kg/m^2 was used as an inclusion criteria. We evaluated the potential impact of BMI and insulin resistance on MMPs and TIMPs levels. However, theses markers of cardiovascular risk were not relevant predictors of MMPs or TIMPs levels. These findings are not enough to completely rule out the possibility that insulin resistance and obesity contribute to imbalanced MMPs/TIMPs under other conditions.

We found a negative correlation between plasma TIMP-2 concentrations and testosterone levels. We also found a positive correlation between the MMP-9/TIMP-1 ratio and testosterone. These results are consistent with reduced levels of TIMP-2 and an increased MMP-9/TIMP-1 ratio, which confirms the findings obtained when we compared PCOS patients with ovulatory women. Hyperandrogenism is a major component of PCOS, and the association between testosterone, TIMP-2, and MMP-9/TIMP-1 ratio suggests a role for testosterone in the augmentation of cardiovascular risk factors. In a multivariate model, testosterone was also an independent predictor of TIMP-2 levels and the MMP-9/TIMP-1 ratio. The Rotterdam criteria for PCOS include four phenotypes of polycystic ovary syndrome, and only one phenotype does not require clinical hyperandrogenism for the diagnosis [40]. Women with the three phenotypes that include hyperandrogenism have higher levels of cardiovascular risk markers [41, 42]. Previously, Luque-Ramírez [42] compared control women with PCOS patients, and the carotid intima-media thickness was increased in PCOS women independently of obesity and was directly related to hyperandrogenism, suggesting that androgen excess is associated with cardiovascular risk. Our data suggest that hyperandrogenism, and not the PCOS diagnosis, is the major factor in the imbalance in the MMPs/TIMPs ratios found in women with PCOS. However, future studies are necessary to confirm these findings and to determine the mechanisms explaining the interaction between MMPs/TIMPs ratios and testosterone.

In conclusion, we found evidence that the plasma MMPs/TIMPs profile is altered in PCOS. Patients with PCOS have lower TIMP-2 levels and higher MMP-2/TIMP-2 and MMP-9/TIMP-1 ratios when compared with

controls. Furthermore, total testosterone is an independent predictor of TIMP-2 levels and MMP-9/TIMP-1 ratio. Together, these results suggest that hyperandrogenism is a key characteristic in women with PCOS that may contribute to an imbalance between MMPs and TIMPs, therefore this scenario can favor an increased risk of developing cardiovascular diseases in this group of women with PCOS. Pharmacological interventions focusing on MMPs may be justified in patients with PCOS.

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Oral Contraceptive Containing Chlormadinone Acetate and Ethinylestradiol Reduces Plasma Concentrations of Matrix Metalloproteinase-2 in Women with Polycystic Ovary Syndrome

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Abstract: Biochemical markers of cardiovascular disease, including matrix metalloproteinases (MMPs), are altered in women with polycystic ovary syndrome (PCOS), with many of these alterations thought to be due to excess androgen concentrations. Despite oral contraceptives (OCs) being the first-line pharmacological treatment in women with PCOS and the importance of MMPs in many physiological conditions and pathological states, including cardiovascular diseases, no study has yet evaluated whether OCs alter plasma concentrations of MMPs. We therefore assessed whether treatment with an OC containing the anti-androgenic progestogen alters MMP profiles in women with PCOS. We analysed 20 women with PCOS who wanted hormonal contraception (OC-PCOS group), 20 ovulatory women who required hormonal contraception (OC-control group) and 20 ovulatory women who wanted non-hormonal contraception (non-OC-control group). OC consisted of cyclic use of 2 mg chlormadinone acetate/30 μg ethinylestradiol for 6 months. Plasma concentrations of MMP-2, MMP-9, TIMP-1 and TIMP-2 were measured by gelatin zymography or enzyme-linked immunoassays. OC treatment for 6 months significantly reduced plasma MMP-2 concentrations in the OC-control and OC-PCOS groups and TIMP-2 and TIMP-1 concentrations levels in the OC-control group (all $p < 0.05$), but had no effects on MMP-9 concentrations or on MMP-2/TIMP-2 and MMP-9/TIMP-1 ratios in any group (all $p > 0.05$). These findings indicated that long-term treatment with an OC containing chlormadinone acetate plus ethinylestradiol reduced plasma MMP-2 concentrations in both healthy and PCOS women. As the latter have imbalances in circulating matrix MMPs, treatment of these women with an OC may be beneficial.

Matrix metalloproteinases (MMPs) are a large endogenous family of proteolytic enzymes that degrade extracellular collagen and participate in vascular remodelling. In addition, the equilibrium between MMPs and their endogenous tissue inhibitors (TIMPs) is crucial for regulating the degradation of extracellular matrix, and its remodelling. MMPs play significant roles in many physiological conditions and are involved in several pathological states, including atherosclerosis.

Endothelial dysfunction and increased concentrations of biochemical markers of cardiovascular diseases have been observed in women with polycystic ovary syndrome (PCOS) [1–8]. Several MMPs [9–11], especially MMP-2 and MMP-9 [12–15], are involved in cardiovascular diseases including atherosclerosis, coronary artery disease and stroke [16–18]. Few studies to date, however, have investigated whether the levels of circulating MMPs and TIMPs are altered in women with PCOS [19–22], although we recently reported that women with PCOS have imbalances in circulating MMPs and that these alterations were associated with excess circulating androgen in these women [22].

The first-line pharmacological therapy used in PCOS patients who do not want to conceive is an oral contraceptive (OC) because it effectively reduces androgen excess. To our knowledge, no study has evaluated whether the use of OC can alter plasma concentrations of MMPs in women with PCOS. We therefore investigated whether the administration to women with PCOS of an OC containing the anti-androgenic agent progestogen, thus reducing hyperandrogenism, could alter their MMP profiles.

Materials and Methods

Subjects and study protocol. This study was approved by the Institutional Review Board at the Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brazil, and each subject provided written informed consent. The present work was carried out in accordance with the ethics standards of the Helsinki Declaration.

We evaluated 20 women with PCOS who wanted hormonal contraception (OC-PCOS group), 20 ovulatory women who required hormonal contraception (OC-control group) and 20 ovulatory women who wanted a method of non-hormonal contraception (condoms or a copper intrauterine device) (non-OC-control group). All of these women had visited a basic health unit of the Faculty of Medicine of

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Ribeirão Preto, University of São Paulo (FMRP-USP), Brazil, between January 2008 and January 2010. Each woman with PCOS was matched by age and body mass index (BMI) to two ovulatory controls, 1 OC and 1 non-OC. Women were included if they were 18–35 years old, sexually active, wanted a method of contraception, had a BMI $\geq$ 18 and <30 kg/m^2 and had normal menstrual cycles (duration of between 24 and 32 days with individual variation of ±3 days). The ovulatory women had pre-ovulatory symptoms, and those with PCOS were diagnosed using the Rotterdam criteria (2004) [23].

Women were excluded at screening if they had any clinical condition corresponding to category 3 or 4 of the World Health Organization medical eligibility criteria for OC use [24]. Other exclusion criteria included smoking, alcoholism, recreational drug use, any systemic disease (systemic arterial hypertension, diabetes mellitus, immune system diseases or thyroid diseases) except PCOS, current or previous (up to 2 months before the study) use of an oral, vaginal, monthly injectable or transdermal combined hormonal contraceptive or current or previous use (up to 6 months before the study) of a long-lasting hormonal contraceptive method (injectable, implant or intrauterine device). Women who had given birth within 12 weeks of study entry, those currently breastfeeding or who had stopped breastfeeding within 2 months of the screening visit and those with chronic and/or acute inflammatory processes were also excluded. All participants provided written informed consent, and the study was approved by the institutional review board of the FMRP-USP.

The OC used contained 2 mg of chlormadinone acetate (CMA) and 30 µg of ethinylestradiol (EE) (Belara®; Janssen Cilag GmBH, Grünenthal, Germany). No participant in the OC-PCOS and OC-control groups was excluded from the study. Of the 20 participants in the non-OC-control group, 5 were excluded, including 3 who changed to a hormonal method and 2 who abandoned the protocol; thus, after 6 months, 15 women were evaluated (fig. 1).

Anthropometric measurements and laboratory tests. Weight, height, BMI and waist circumference (the lowest measurement between the iliac crest and the inferior margin of the last rib) were measured at screening. Blood samples were collected in the Gynecology Laboratory of the University Hospital of the FMRP-USP between 8:00 and 9:00 a.m. after at least 10 hr of fasting. Samples were collected from control women and women with PCOS and oligomenorrhoea during the follicular phase (third to seventh day of the cycle). PCOS patients with amenorrhoea were evaluated after pelvic ultrasonography showed no evidence of either a follicle $\geq$ 10 mm or a corpus luteum. Whole blood (20 mL) samples were divided into tubes without anticoagulant (for serum separation) and into plastic conical tubes (BD-Becton Dickinson, Plymouth, UK) with no vacuum and containing 3.2% sodium citrate, in a fixed proportion of nine parts whole blood to one part anticoagulant.

All blood samples were processed within 2 hr after collection, with serum samples stored at -80°C. Fasting serum glucose concentration was determined by the oxidase method using a Konelab 60i analyser (Wiener Lab®, Rosario, Argentina). The concentrations of total cholesterol, HDL-cholesterol and triglycerides (TG) were measured enzymatically using a BT 3000 plus analyser (Wiener lab®); LDL-cholesterol concentrations were calculated according to the Friedewald formula [LDL-cholesterol = total cholesterol − (HDL-C + TG/5)] because none of the samples contained triglyceride concentrations >400 mg/dL [25]. Sex hormone-binding globulin (SHBG) and insulin were measured by chemoluminescence with the DPC Immulite® 2000 analyser (Diagnostic Products Corporation®, Los Angeles, CA, USA), and total testosterone was determined by radioimmunoassay using the Tri Carb 2100 TR scintillator (Packard® Instrument Company, IL, USA). The free androgen index (FAI) was calculated using the formula: total testosterone (nM)/SHBG (nM) × 100 [26]. Insulin resistance was determined according to the homeostasis model assessment-insulin resistance (HOMA-IR) index, that is HOMA-IR = fasting serum glucose (mg/dL) × 0.05551 × fasting insulin (µU/mL)/22.5 [27].

Measurement of plasma MMP-9, TIMP-1 and TIMP-2 concentrations. Whole blood samples collected into anticoagulant were centrifuged at 120 × *g* (700 rpm) in a Sorvall RC 3 centrifuge (Sorvall Kendro Laboratory Products GmbH, Langenselbold, Germany) at room temperature (mean 22°C; range 18–24°C) for 15 min. Plasma was obtained by centrifuging the samples at 1600 × *g* (2500 rpm) for 30 min. in a Universal 32 R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) at 4°C, and plasma aliquots were stored at −70°C until analysed. MMP-9, TIMP-1 and TIMP-2 concentrations were measured using commercially available enzyme-linked immunosorbent (ELISA) assay kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

SDS-polyacrylamide gel electrophoresis (PAGE) gelatin zymography of MMP-2. Gelatin zymography of MMP-2 from plasma samples was performed as described [28–30]. Briefly, plasma samples were subjected to electrophoresis on 7% SDS-PAGE co-polymerized with gelatin (1%). The gel was incubated for 1 hr at room temperature in 2% Triton X-100 solution and subsequently at 37°C for 16 hr in Tris–HCl buffer, pH 7.4, containing 10 mM $CaCl_2$. The gels were stained with 0.05% Coomassie Brilliant Blue G-250 and destained with 30% methanol/10% acetic acid. Gelatinolytic activities were detected as unstained bands against the background of Coomassie blue-stained gelatin. Enzyme activity was assayed densitometrically using ImageJ version 1.42q (Wayne Rasband National Institutes of Health, USA). MMP-2 was identified as a 72 kDa band.

Statistical analysis. The mean TIMP-2 concentration in women with PCOS [22] indicated that the inclusion of at least 16 of these women would be necessary to observe a difference of one standard deviation between the pre- and post-treatment measurements, with a test power of 80% and an alpha of 5%.

All results were expressed as mean ± standard error of the mean (S.E.M.). Non-normally distributed variables were compared using the Kruskal–Wallis test with post hoc Dunn's multiple comparison test, and normally distributed variables were compared by one-way ANOVA with post hoc Tukey's test. Comparisons within each group were assessed using Student's paired *t*-tests. Results were considered statistically significant when $p < 0.05$.

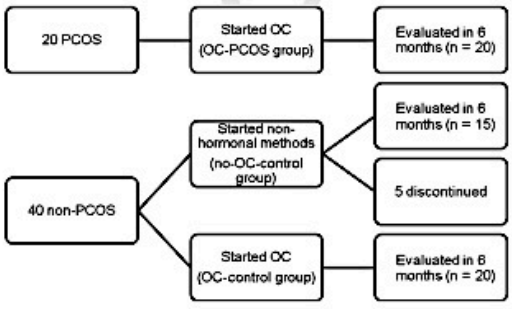

Fig. 1. Study flow chart.

Results

The clinical characteristics of all participants are summarized in table 1. The mean ages of the OC-POS (25.1 ± 0.9 years),

Table 1.

Clinical and laboratorial characteristics of study subjects.

	Non-OC-control group		OC-control group		OC-PCOS group	
	BL	6M	BL	6M	BL	6M
BMI (kg/m$^{2)}$	21.76 ± 0.68	22.17 ± 0.88	23.53 ± 0.75T	24.03 ± 0.83	22.52 ± 0.96	22.70 ± 0.95
WC (cm)	69.53 ± 1.93	70.80 ± 2.11	73.58 ± 1.78	75.80 ± 2.21*	78.05 ± 2.24**	77.05 ± 2.17
SAP (mmHg)	109.20 ± 2.21	112.10 ± 2.05	113.30 ± 1.75	114.00 ± 1.52	113.90 ± 1.38	112.4 ± 1.97
DAP (mmHg)	75.07 ± 1.72	77.33 ± 1.58	77.95 ± 1.34	74.10 ± 1.53*	76.65 ± 1.47	74.50 ± 1.06
Ovarian volume (cm^3)	5.97 ± 0.20	5.87 ± 0.32	6.28 ± 0.32	5.25 ± 0.32	10.72 ± 0.59***	7.35 ± 0.50
TChol (mg/dL)	146.20 ± 6.73	148.30 ± 6.02	161.30 ± 6.93	161.00 ± 4.48	168.50 ± 5.63	188.90 ± 7.98*
TG (mg/dL)	56.93 ± 6.85	59.13 ± 7.72	75.55 ± 6.70	92.90 ± 6.24	75.55 ± 13.52	127.10 ± 16.07*
HDL (mg/dL)	46.53 ± 1.58	45.80 ± 1.63	49.50 ± 1.92	53.95 ± 1.99*	51.10 ± 1.80	61.40 ± 1.86*
LDL (mg/dL)	109.90 ± 6.43	84.47 ± 4.78*	97.85 ± 4.61	85.90 ± 2.75*	102.60 ± 4.45	101.10 ± 6.80
Glycaemia (mg/dL)	85.73 ± 1.91	82.53 ± 3.57	85.30 ± 1.39	75.45 ± 3.08*	87.00 ± 1.43	80.80 ± 1.63*
Insulin (μU/mL)	3.02 ± 0.43	2.28 ± 0.21	5.94 ± 0.88**	4.84 ± 0.75	5.24 ± 0.77**	6.02 ± 1.09
HOMA-IR	0.72 ± 0.14	0.62 ± 0.15	1.30 ± 0.19**	0.94 ± 0.15*	1.15 ± 0.17	1.26 ± 0.25
SHBG (nM)	59.87 ± 3.43	56.98 ± 5.19	58.99 ± 4.69	154.30 ± 8.83*	42.00 ± 3.83**	217.90 ± 25.28*
FAI (%)	2.90 ± 0.39	3.45 ± 0.37	3.57 ± 0.43	1.57 ± 0.28*	9.69 ± 2.61**	0.92 ± 1.66*

BMI, body mass index; WC, waist circumference; SAP, systolic arterial pressure; DAP, diastolic arterial pressure; TChol, total cholesterol; TG, triglycerides; HOMA-IR, homoeostasis model assessment-insulin resistance; SHBG, sex hormone-binding globulin; FAI, free androgen index; CRP, C reactive protein; PCOS, polycystic ovary syndrome.

Values are the mean ± S.E.M.

[1]Significantly different by the Mann–Whitney *U*-test.

[2]Significantly different by the unpaired *t*-test.

*$p < 0.05$ *versus* baseline by two-tailed (baseline *versus* 6 months) paired *t*-test.

**$p < 0.05$ *versus* baseline by two-tailed (baseline *versus* control-baseline) Kruskal–Wallis test with post hoc Dunn's multiple comparison test for normally distributed variables or by one-way ANOVA with post hoc Tukey's test for non-normally distributed variables.

***Xxxxxxxx.

OC-control (24.0 ± 1.3 years) and non-OC-Control (27.5 ± 0.9 years) groups were similar ($p = 0.09$). Similarly BMI, systolic arterial pressure (SAP), diastolic arterial pressure (DAP) and concentrations of total cholesterol (TChol), triglycerides, HDL, LDL and fasting serum glucose were similar in the three groups ($p > 0.05$ each). However, patients with PCOS had higher ovarian volume, waist circumference and insulin ($p < 0.05$ each) than controls. Insulin concentrations (5.9 ± 0.8 IU/mL *versus* 3.0 ± 0.4 IU/mL, $p < 0.05$) and HOMA-IR score (1.3 ± 0.1 *versus* 0.7 ± 0.1, $p < 0.05$) were significantly higher in the OC-Control than in the non-OC-Control group, but remained within normal limits. In addition, FAI was significantly higher, and SHBG concentrations were significantly lower in the OC-PCOS than in the control groups ($p < 0.01$ each).

At baseline, the MMP-2 concentrations were similar among the three groups ($p > 0.05$), whereas TIMP-2 concentrations were significantly lower in the OC-PCOS than in the OC-Control group (161.0 ± 6.5 ng/mL *versus* 206.2 ± 11.2 ng/mL; $p = 0.01$; fig. 2B). As expected, the baseline MMP-2/TIMP-2 ratio was significantly higher in the OC-PCOS group than in the OC-Control group (0.009 ± 0.0005 ng/mL *versus* 0.007 ± 0.0004 ng/mL; $p = 0.005$; fig. 2C). However, MMP-9 and TIMP-1 concentrations and the MMP-9/TIMP-1 ratio were similar in the three groups ($p > 0.05$ each) (fig. 3).

Treatment with OC increased waist circumference and HDL concentration, and reduced DAP, LDL and serum glucose concentrations in the OC-Control group. In the OC-PCOS group, 6 months of OC increased TChol, triglyceride and HDL concentrations, while reducing serum glucose concentrations and FAI.

Treatment with OC for 6 months significantly reduced MMP-2 concentrations in the OC-control group, from 1.44 ± 0.11 arbitrary units at baseline to 1.22 ± 0.07 arbitrary units after 6 months ($p = 0.01$), and in the OC-PCOS group, from 1.43 ± 0.08 arbitrary units at baseline to 1.25 ± 0.09 arbitrary units after 6 months ($p = 0.007$) (fig. 2A). Moreover, OC treatment of women in the OC-Control group significantly reduced TIMP-1 (448.0 ± 66.3 ng/mL *versus* 349.0 ± 40.9 ng/mL; $p = 0.009$; fig. 3B) and TIMP-2 (206.2 ± 11.3 ng/mL *versus* 181.7 ± 10.4 ng/mL; $p = 0.03$; fig. 2B) concentrations after 6 months. However, OC treatment did not alter MMP-9 concentrations or the MMP-9/TIMP-1 and MMP-2/TIMP-2 ratios after 6 months ($p > 0.05$ each).

Discussion

We have shown here for the first time that the long-term treatment with an OC containing chlormadinone acetate plus combined with ethinylestradiol reduced plasma MMP-2 concentrations levels in both PCOS and healthy women.

Hyperandrogenism, a key component of PCOS, is often associated with increased metabolic and cardiovascular risks in women with PCOS. According to the Rotterdam criteria, only one of the four phenotypes of PCOS does not require clinical hyperandrogenism for diagnosis [31]. Women with the three phenotypes that include hyperandrogenism have higher levels of cardiovascular risk markers [32], such as increased carotid intima-media thickness [33]. Reducing circulating androgen concentrations may therefore decrease parameters associated with cardiovascular risk.

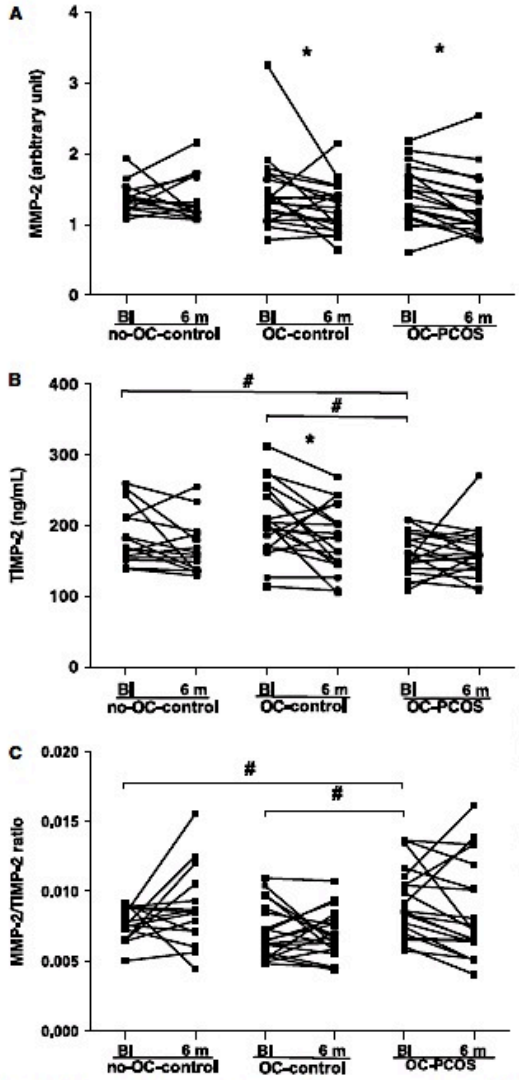

Fig. 2. Effect of oral contraceptive on plasma concentrations of (A) MMP-2 and (B) TIMP-2, and on (C) the MMP-2/TIMP-2 ratio in non-OC-Controls (N = 15), OC-Controls (N = 20) and OC-PCOS (N = 20) women after 6 months. $^{*}p < 0.05$ *versus* baseline by two-tailed (baseline *versus* baseline) unpaired *t*-test. $^{*}p < 0.05$ *versus* baseline by two-tailed (baseline *versus* 6 months) paired *t*-test.

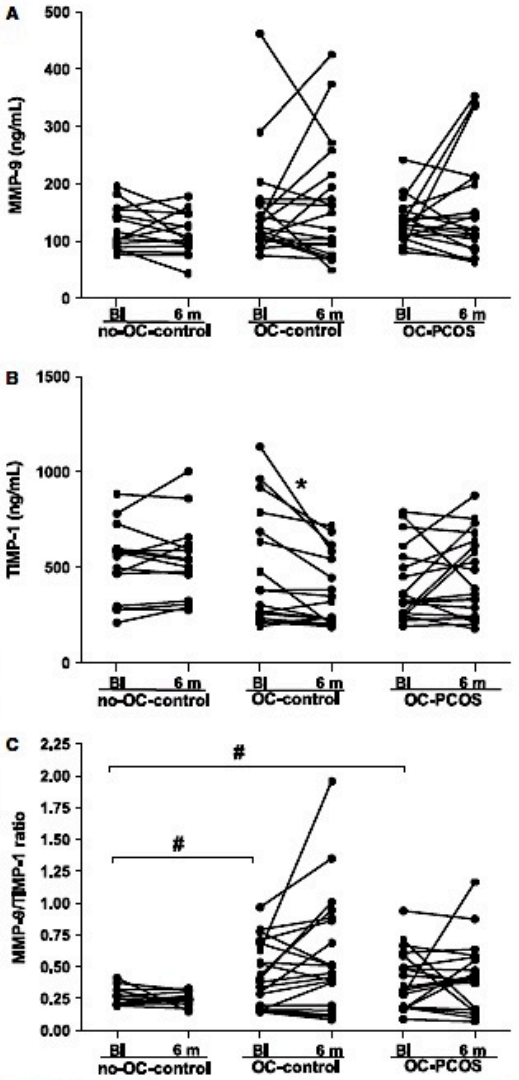

Fig. 3. Effect of oral contraceptive on plasma concentrations of (A) MMP-9 and (B) TIMP-1, and on (C) the MMP-9/TIMP-1 ratio in non-OC-Controls (N = 15), OC-Controls (N = 20) and OC-PCOS (N = 20) women after 6 months. $^{*}p < 0.05$ *versus* baseline by two-tailed (baseline *versus* baseline) unpaired *t*-test. $^{*}p < 0.05$ *versus* baseline by two-tailed (baseline *versus* 6 months) paired *t*-test.

Oral contraceptives is the medical treatment most widely used to reduce hyperandrogenism in women with PCOS. As expected, we found that an anti-androgenic OC containing ethinylestradiol and chlormadinone acetate increased total cholesterol, triglyceride and HDL concentrations in women with PCOS, but not in women without PCOS. OC use reduced serum glucose concentrations in both groups. All the alterations in biochemical metabolism we observed were within normal limits and were in agreement with results of previous studies [34, 35].

We found that use of an anti-androgenic OC containing ethinylestradiol and chlormadinone acetate reduced plasma MMP-2 concentrations in women with and without PCOS. These findings suggest that the reduction of free testosterone induced by this anti-androgenic contraceptive may be involved in the reduction in MMP-2 concentrations we observed. While this is the first study showing such effects for OC, there is growing experimental [36–39] and clinical [40] evidence that some cardiovascular drugs may affect MMP levels, we have not included

patients taking any other medications in the present study and therefore, our findings reflect the effects exerted by OC only.

Few studies have evaluated the relationship between MMP-2 and testosterone. Androgen has been shown to stimulate MMP-2 expression via androgen receptor transactivation in human prostate cancer LNCaP cells [41], and two androgen response elements involved in androgen-induced MMP-2 expression have been identified [42]. Although testosterone reduced MMP-2 activity in the ovaries of a rat model of PCOS but not in control rats [43], testosterone had no effect on the MMP-2 gene and protein expression in human aortic smooth muscle cells [44], but in this study, the testosterone concentrations were based on the average level of free testosterone in an adult male. Additional studies are necessary to confirm these findings and to determine the mechanisms linking MMP-2 and testosterone interaction.

Matrix metalloproteinases clearly play a role in cardiovascular remodelling, especially MMP-2 [45–48]. In fact, imbalanced MMPs have been shown in many conditions associated with increased cardiovascular risk [49–52], and it is possible that altered circulating MMP levels contribute to this increased risk. Consequently, the ability of an anti-androgenic OC to reduce MMP-2 concentrations in women with PCOS may reduce their cardiovascular risk.

Alterations in MMP and TIMP concentrations have been observed in women with PCOS [19, 21, 22]. Moreover, we reported that hyperandrogenism, one of the main characteristics of PCOS, was an independent predictor of reduced TIMP-2 concentrations and increased MMP-9/TIMP-1 ratios [22]. We have shown here that the reduction of hyperandrogenism, promoted by treatment with OC, reduced MMP-2 levels in women with PCOS.

This study had several limitations. Circulating MMPs and TIMPs may be released into the bloodstream by various tissue sources, including cardiac, ovarian and vascular tissues, as well as peripheral blood neutrophils. Therefore, our results should be interpreted with caution because plasma MMP and TIMP levels may not reflect local cardiovascular tissue concentrations. Although OC reduced MMP-2 concentrations in both women with PCOS and healthy controls, it is not known whether the cardiovascular risk of these women will be affected in the future. In addition, we have not studied the effects of other therapies for PCOS on MMPs.

In conclusion, we showed that an OC containing 2 mg chlormadinone acetate and 30 μg of ethinylestradiol with anti-androgenic effects reduced plasma concentrations of MMP-2 in women with PCOS, suggesting that this may be an important benefit of OCs in this group.

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DISCUSSÃO

DISCUSSÃO

A SOP além de ser uma endocrinopatia frequente entre as mulheres com idade reprodutiva está frequentemente associada às comorbidades que são sabidamente fatores de risco cardiovascular. Deste modo, acredita-se que mulheres com SOP estejam predispostas a desenvolver DCV precocemente. Assim, já que as MMPs estão implicadas a doenças cardiovasculares, torna-se pertinente avaliar as concentrações plasmáticas de MMPs e TIMPs em pacientes com SOP.

Nossos achados iniciais são que: (1) as pacientes com SOP têm um desequilíbrio entre MMPs e TIMPs, incluindo concentrações mais baixas de TIMP-2 e aumento nas razões MMP-2/TIMP-2 e MMP-9/TIMP-1 em comparação com indivíduos saudáveis e (2) a testosterona foi relacionada negativamente com os níveis plasmáticos de TIMP-2 e positivamente com a razão MMP-9/TIMP-1.

Esse é primeiro estudo que mostra uma associação entre a testosterona e os níveis de TIMP-2 e com a razão MMP-9/TIMP-1, sugerindo que o hiperandrogenismo, observado na maioria das mulheres com SOP, pode ser um fator contribuinte para o risco cardiovascular, devido à participação dessas enzimas e seus inibidores teciduais em DCV.

Inicialmente, o principal achado desse trabalho é que as mulheres com SOP possuem um desequilíbrio entre MMPs e TIMPs. Nossos resultados mostram um aumento das razões MMP-2/TIMP-2 e MMP-9/TIMP-1 em mulheres com SOP quando comparadas com mulheres saudáveis. Esses resultados são importantes porque é, justamente, o equilíbrio crítico entre MMPs e TIMPs que determina a degradação da matriz extracelular.

Atualmente, tem-se sugerido que a razões entre MMPs/TIMPs sejam melhores marcadores do que as MMPs e os TIMPs isoladamente. Pois, alterações no equilíbrio entre esses dois parâmetros, favorecendo o aumento da degradação da matriz, podem resultar em alterações estruturais e funcionais cardíacas associadas à DCV como, por exemplo, a hipertensão [26,57].

Além disso, quando comparamos a concentração plasmática de TIMP-2 entre as mulheres saudáveis e as mulheres com SOP, as últimas tiveram valores significativamente inferiores. A atividade das MMPs é regulada principalmente pela interação dessas enzimas com os TIMPs. Até o momento, existem quatro TIMPs conhecidos até o momento e eles são responsáveis por inibir mais de 25 MMPs. Contudo, embora os TIMPs possam inibir qualquer MMP, os TIMP-1 e TIMP-2 são os principais inibidores da MMP-9 e da MMP-2, respectivamente [58].

É importante salientar que as ações dos TIMPs não se limitam a inibir a ação proteolítica das MMPs. Os TIMPs participam na regulação da migração celular, proliferação e apoptose. Além disso, estudos também têm demonstrado que os TIMPs podem estimular a produção de colágeno por fibroblastos cardíacos [59,24,60-62].

Recentemente, Kandalam *et al* [59], demonstraram que o TIMP-2 exerce papel chave na regulação das respostas cardíacas ao infarto do miocárdio. Portanto, é possível considerar que o TIMP-2 possa contribuir para as alterações cardiovasculares em mulheres com PCOS. Além disso, o TIMP-2, quando está em altas concentrações inibe a MMP-2. Por outro lado, quando esse inibidor está em baixas concentrações participa da ativação da MMP-2 [63,64]. A ativação da pró-MMP-2 pela MT1-MMP requer a formação de um complexo trimolecular entre a

pró-MMP-2, MT1-MMP e o TIMP-2, em que o domínio C-terminal do TIMP-2 liga-se ao domínio hemopexina da pró-MMP-2 e, o domínio N-terminal desse inibidor se liga ao sítio ativo da MT1-MMP. Uma vez formado o complexo, a MT1-MMP adjacente ao complexo cliva o pró-domínio da MMP-2, tornando-a ativa [64,63].

Portanto, as baixas concentrações plasmáticas de TIMP-2 em mulheres com SOP é um achado importante, pois além de favorecer o desequilíbrio da razão entre MMP-2/TIMP-2, que pode induzir ao aumento da degradação da matriz extracelular como citado anteriormente, também pode favorecer a ativação da MMP-2.

Sabe-se, ainda, que essa MMP exerce importante papel no remodelamento cardiovascular envolvido no mecanismo patológico de diversas DCVs. Um trabalho recente relatou a presença de MMP-2 no interior do cardiomiócito, região em que essa enzima atua sobre os substratos intracelulares, entre eles a troponina I [65].

Além da troponina I, já se descobriram outros substratos não relacionadas à matriz extracelular para a MMP-2. Estes substratos incluem a big endotelina-1 [66], o peptídeo relacionado ao gene da calcitonina (CGRP) [67] e a adrenomedulina (AM) [68]. Ao clivar esses substratos, a MMP-2 gera metabólitos com ações vasoconstritoras potentes.

Recentes estudos sugeriram a participação da MMP-8 em algumas DCVs, entre elas a aterosclerose [53]. As concentrações plasmáticas da MMP-8 foram positivamente associadas à presença e severidade de doença arterial coronariana [53,69]. O Aumento nos níveis plasmáticos de MMP-8 já foram relatados em pacientes com síndrome metabólica. O presente estudo foi o primeiro a propor a

avaliação das concentrações plasmáticas dessa MMP em pacientes com ovário policístico. Os nossos resultados não indicam a participação dessa MMP na SOP, já que não encontramos diferença significativa nos níveis de MMP-8 nas mulheres com SOP quando comparadas com mulheres saudáveis.

O aumento das razões MMP-2/TIMP-2 e MMP-9/TIMP-1 observados nesse estudo corroboram com os demais estudos que avaliaram os mesmos parâmetros nas pacientes com SOP [70,71]. Embora as razões MMPs/TIMPs estejam aumentadas nas pacientes com SOP, a elevação dos níveis de MMP-9 e a redução dos níveis de TIMP-1 que foram observados nessas pacientes, não foram estatisticamente significativos quando comparados com indivíduos saudáveis. Apesar disso, essas alterações contribuíram para o aumento da razão MMP-9/TIMP-1 observado no grupo SOP. No entanto, esses achados estão em contraste com os estudos anteriores [70,72], que encontraram um aumento nas concentrações séricas de MMP-2 [72] e de MMP-9 [72,70] em pacientes com SOP quando comparadas com pacientes saudáveis. Por outro lado, no estudo de Lewandowski *et al* [72], o aumento dos níveis de MMP-9 e MMP-2 em mulheres com PCOS pode ser devido à obesidade presente no grupo SOP.

Os resultados contrastantes entre o presente trabalho e os anteriores podem ser parcialmente explicados pelas diferenças entre os estudos. Uma das diferenças diz respeito ao tipo de método empregado nos trabalhos para determinar os níveis plasmáticos de MMP-2. No presente estudo utilizamos zimografia já, no trabalho citado anteriormente o ensaio utilizado foi o Elisa. Outra diferença entre os estudos é o tipo de amostra empregada para determinar os níveis plasmáticos de MMPs e TIMPs. Nos trabalhos anteriores foram utilizados

soro ao invés de plasma. Diferenças a respeito do tipo de amostra utilizada para determinar os níveis plasmáticos de MMPs já foram evidenciadas em estudos anteriores. Nesses trabalhos foi observado que amostras de soro não são apropriadas para a determinação de MMPs [73,74], visto que no soro, durante a coagulação sanguínea, ocorre a liberação de diversas proteases que podem ativar a MMP-9. Além disso, também ocorre a liberação de MMPs por plaquetas e/ou leucócitos durante a ativação plaquetária, resultando em níveis artificialmente aumentados quando comparados com os níveis plasmáticos [73-75].

Além dessas diferenças, o tamanho amostral dos estudos anteriores é inferior ao do presente estudo, o que pode, em parte, explicar a divergência entre os estudos. Além disso, a presença e severidade das comorbidades presentes nas mulheres com SOP avaliadas podem ter impacto sobre os níveis circulantes das MMPs.

Sabe-se que é frequente a presença de fatores de risco cardiovascular, como resistência à insulina e obesidade em mulheres com SOP quando comparadas a mulheres saudáveis. Um dos critérios de inclusão no estudo foi o IMC <30 kg/m^2. Apesar disso, na primeira parte do estudo, as mulheres do grupo SOP apresentaram IMC e HOMA-IR significantemente maiores do que as controles. A medida da circunferência abdominal, a média da pressão sistólica e os níveis de insulina também foram estatisticamente diferentes, entretanto os valores estão no limiar normal.

Nós também avaliamos dois marcadores inflamatórios, a interleucina 6 e a proteína C reativa (CRP), e ambos não foram diferentes entre os dois grupos analisados. Vários trabalhos já se propuseram estudar esses dois marcadores e

os resultados obtidos foram conflitantes, pois alguns deles sugerem o aumento dos níveis em mulheres com SOP quando comparadas a indivíduos saudáveis, enquanto que outros não observaram essas diferenças. Uma meta-análise realizada recentemente sugere que as mulheres com SOP possuem níveis elevados de CRP quando comparadas a mulheres saudáveis. Porém, não foi constatado diferenças nas concentrações de interleucina 6 entre mulheres com SOP e mulheres saudáveis [76].

Para avaliar a influência de algumas variáveis comumente observadas na SOP (IMC, HOMA e testosterona), assim como a própria SOP sobre as concentrações plasmáticas de MMPs, TIMPs e das razões entre MMPs e TIMPs, realizamos uma análise por regressão linear múltipla. Apesar de alguns trabalhos mostrarem aumento dos níveis de MMPs na obesidade [77], inclusive em mulheres obesas [78,79], o IMC não foi preditor de nenhuma das MMPs e nem dos TIMPs estudados no presente estudo. Contudo, a diferença significativa do IMC entre as pacientes com SOP e as controles saudáveis deve-se ao sobrepeso apresentado por algumas pacientes do primeiro grupo e não a obesidade.

O índice HOMA também não foi preditor de nenhum dos parâmetros avaliados, mesmo tendo estudos que mostram que a hiperinsulinemia aumenta tanto a MMP-2 (em aproximadamente 6 vezes) quanto a MMP-9 (em aproximadamente em 13 vezes) [80,81]. Embora, a média do índice HOMA tenha sido maior nas SOPs, os valores desse parâmetro estão na faixa de normalidade. Todavia, esses achados não são suficientes para eliminar completamente a possibilidade de que a resistência à insulina e/ou à obesidade possam contribuir para o desequilíbrio das razões entre MMPs e TIMPs observado na SOP.

DISCUSSÃO

Um dos principais achados desse estudo é que a testosterona foi um preditor independente dos níveis de TIMP-2 e da razão MMP-9/TIMP-1, sendo um preditor negativo para o TIMP-2 e positivo para a razão MMP-9/TIMP-1. Este é o primeiro estudo mostrando a evidência dessas associações. Esse resultado corrobora com outro resultado do mesmo estudo, em que encontramos uma correlação negativa entre as concentrações plasmáticas de TIMP-2 e os níveis de testosterona e uma correlação positiva entre a razão MMP-9/TIMP-1, novamente com a testosterona.

Uma das principais características da SOP é a presença de hiperandrogenismo clínico e/ou laboratorial. Os nossos achados também sugerem a participação da testosterona no aumento dos fatores de risco cardiovascular nas mulheres com SOP, que é aqui representado pelo desequilíbrio da relação entre MMPs e TIMPs e com a redução plasmática de TIMP-2 observada nessas pacientes.

Os critérios de Rotterdam para o diagnóstico da SOP abrange quatro fenótipos, e somente um deles não necessita do hiperandrogenismo para o diagnóstico [82]. Logo, o hiperandrogenismo, que é observado em aproximadamente 60-80% das pacientes com SOP, é uma das características fundamentais dessa síndrome. Alguns estudos já se propuseram a avaliar quais fenótipos representam maiores e menores riscos cardiovasculares. Um deles observou que o único fenótipo que não possui o hiperandrogenismo foi o que apresentou características endócrinas e metabólicas mais leves [83]. Já, as mulheres com os três fenótipos que incluem hiperandrogenismo possuem níveis mais elevados de marcadores de risco cardiovascular (41, 42), como por exemplo,

o aumento da espessura da camada íntima - média da carótida observada nas mulheres com SOP está correlacionada com o hiperandrogenismo [84,13].

Apesar desses achados, sugerindo o envolvimento dos andrógenos endógenos no desenvolvimento de DCV, os poucos estudos com mulheres sem SOP, que examinaram a associação entre andrógenos endógenos e o desenvolvimento de DCV não observaram participação importante dos andrógenos nesse processo [85]. Entretanto, recentemente um grande estudo associou as altas concentrações de testosterona com os marcadores de aterosclerose [86].

A disfunção endotelial também tem sido relacionada com o aumento da testosterona livre [87]. Além disso, as concentrações de androgênios no soro foram relatadas como preditor da pressão sanguínea em mulheres jovens sem SOP [88]. A testosterona livre, por sua vez, foi caracterizada como preditor nas mulheres jovens com SOP [89]. Embora, nenhum estudo tenha provado a relação causa-efeito entre a testosterona e a DCV em um trabalho com modelo experimental, a administração de testosterona em primatas fêmeas foi associado ao aumento da aterogênese [90].

Contudo, apesar de várias evidências, tanto bioquímicas quanto clínicas, ainda não se sabe, até o presente momento, qual o papel preciso da testosterona endógena no desenvolvimento da aterosclerose e de outras DCVs. Nossos resultados sugerem uma possível participação do hiperandrogenismo no desequilíbrio entre MMPs e TIMPs observado nas mulheres com SOP. Este panorama de alteração do equilíbrio entre MMPs e TIMPs, consequentemente, pode favorecer um aumento do risco de desenvolvimento de doenças

cardiovasculares neste grupo de mulheres. Portanto, intervenções farmacológicas, focando a redução do hiperandrogenismo e/ou das MMPs podem ser benéficas nas pacientes com SOP.

Na primeira parte do estudo não foi observado um aumento nas concentrações plasmáticas de MMP-2 e MMP-9 em mulheres com SOP. Entretanto, as relações MMP-2/TIMP-2 e MMP-9/TIMP-1 estavam aumentadas, o que poderia predispô-las a um risco cardiovascular maior. De acordo com os nossos achados, a testosterona poderia ser um preditor do desequilíbrio entre MMPs e TIMPs observado na SOP.

A droga de primeira escolha para a redução do hiperandrogenismo em mulheres com SOP que não desejam a contracepção é o anticoncepcional oral (ACO). Dessa maneira, na segunda parte do estudo foi investigado se a redução do hiperandrogenismo com a administração de ACO com propriedades antiandrogênicas em mulheres com SOP é acompanhado da redução dos níveis de MMPs nessas mulheres.

Acredita-se que o hiperandrogenismo presente na SOP seja de origem multifatorial, onde o ovário possui a maior parcela de contribuição tendo portanto uma participação primordial. Em menor proporção temos a contribuição das adrenais e por último do tecido adiposo.

Diversas drogas que visam bloquear a produção ovariana de andrógenos ou sua ação periférica são utilizadas no tratamento do hiperandrogenismo. Uma das drogas mais utilizadas em mulheres que desejam a contracepção é o ACO. A redução do hiperandrogenismo pelo ACO é uma ação conjunta dos dois componentes presentes na sua formulação. No nosso caso, o ACO utilizado é

composto por etinilestradiol e acetato de clormadinona. O etinilestradiol aumenta os níveis circulantes de hormônio sexual ligado à globulina (SHBG) que, por sua vez, reduz a concentração de testosterona livre circulante, uma vez que o SHBG liga-se preferencialmente à testosterona. O componente progestágeno inibe a 5α-redutase e atua como antagonista no receptor de androgênios [91]. Além disso, o ACO também diminui a produção de androgênio pela adrenal, através de um mecanismo ainda pouco esclarecido. Mas, possivelmente, seja devido a uma diminuição na produção hormonal de adrenocorticotropina (ACTH).

O principal achado desse trabalho foi a redução dos níveis plasmáticos da MMP-2 depois de seis meses de tratamento com o ACO, contendo etinilestradiol e acetato de clormadinona em mulheres com SOP. Vale ressaltar, que nenhum trabalho prévio avaliou o perfil das MMPs nas mulheres com SOP após o tratamento com ACO. Estas descobertas sugerem que a redução da testosterona livre induzida por esse contraceptivo antiandrogénico pode estar envolvida na diminuição da MMP-2 plasmática observada no presente estudo.

O aumento da expressão e atividade da MMP-2 já foi demonstrado diversas vezes em DCV [34,39,45,20], assim como o aumento plasmático. Além disso, essa MMP está associada às alterações morfológicas arteriais em modelo experimental de hipertensão [37,38] e de aterosclerose [20]. Do mesmo modo, a MMP-2 pode induzir ações vasoconstritoras, por alterar as concentrações teciduais de alguns peptídeos vasoativos, como citado anteriormente. Portanto, é possível que o aumento da atividade da MMP-2 possa contribuir para uma disfunção endotelial e para o aumento da resistência vascular periférica. Estes

estudos também propõem a participação da MMP-2 no remodelamento vascular relacionado à DCV. Portanto, medidas que visam a redução de marcadores que tenham envolvimento com a instalação e progressão de DCV nas mulheres com SOP é relevante. Porém, poucos estudos avaliaram a relação entre a MMP-2 e a testosterona para sugerirmos um suposto mecanismo para a redução da MMP-2 por consequência da redução da testosterona disponível.

Em um estudo com células LNCaP demonstrou-se que o androgênio regula a expressão de MMP-2 via receptor de androgênios de maneira dependente da PI3K [92], sugerindo que o androgênio possa ser um possível modulador da MMP-2. Recentemente, identificou-se que dois elementos de resposta ao androgênio estão envolvidos na indução da MMP-2 pelo androgênio [93]. Contudo, em um segundo estudo com células musculares lisas de humanos, a testosterona não teve nenhum efeito sobre a expressão do gene da MMP-2 [94].

De modo contrário aos demais achados, Henmi *et al* [95] observaram redução da atividade da MMP-2 no ovário de ratas estimuladas com dehidroepiandrosterona (DHEA) durante sete e quinze dias. Portanto, esses resultados sugerem que a regulação da MMP-2 é dependente de estímulo específico e do tipo celular. Mesmo assim, são necessários estudos adicionais para determinar os mecanismos de interação entre a MMP-2 e a testosterona.

O tratamento com ACO não alterou significativamente os níveis de MMP-9, TIMP-1 e TIMP-2 nas mulheres com SOP. Também não foram observadas modificações relevantes nas razões MMP-2/TIMP-2 e MMP-9/TIMP-1. Uma limitação importante dos nossos estudos é que não conseguimos delimitar a principal fonte das MMPs e TIMPs circulantes, pois os dois marcadores podem ser

liberados na corrente sanguínea por diferentes fontes teciduais, visto que numerosos tipos celulares podem expressar essas enzimas, incluindo células vasculares, cardiomiócitos, células endoteliais, células musculares lisas, fibroblastos e neutrófilos. Desse modo, os resultados dos nossos estudos devem ser interpretados com prudência, visto que os níveis plasmáticos, tanto de MMPs quanto de TIMPs podem não refletir as concentrações plasmáticas do sistema cardiovascular.

Ademais, mesmo não sendo o foco principal do nosso trabalho, as concentrações plasmáticas de alguns marcadores de risco cardiovascular que refletem disfunção metabólica foram avaliados, uma vez que os ACOs possuem alguns efeitos conhecidos sobre o metabolismo lipídico. Como esperado, o uso do ACO durante 6 meses aumentou as concentrações plasmáticas do colesterol total, dos triglicérides e do HDL e reduziu os níveis de glicose. Esses achados, além de estarem de acordo com estudos anteriores, [91,96,97] também estão dentro dos limites desejáveis.

As principais conclusões do estudo mostram que: 1) em comparação com indivíduos saudáveis, as pacientes com SOP têm um desequilíbrio entre MMPs e TIMPs, incluindo menores concentrações de TIMP-2 e aumento das razões MMP-2/TIMP-2 e MMP-9/TIMP-1; 2) o TIMP-2 foi negativamente relacionado aos níveis de testosterona, enquanto que a razão MMP-9/TIMP-1 foi positivamente relacionada aos níveis de testosterona e 3) o uso do anticoncepcional oral, contendo etinilestradiol e acetato de clomardinona por 6 meses reduz os níveis plasmáticos de MMP-2, o que sugere que esse tratamento pode reduzir o risco de futuro evento cardiovascular neste grupo de mulheres.

CONCLUSÃO GERAL

CONCLUSÕES

Nossos dados demonstram que as razões MMP-2/TIMP-2 e MMP-9/TIMP-1 estão aumentadas nas mulheres com SOP e que os níveis plasmáticos de TIMP-2 estão reduzidos nessas pacientes, quando comparadas às controles. Além disso, a testosterona total foi um preditor independente dos níveis de TIMP-2 e da razão MMP-9/TIMP-1. Juntos, esses resultados revelam que o hiperandrogenismo, característica-chave em mulheres com SOP, pode contribuir para o desequilíbrio observado entre MMPs e TIMPs nas pacientes. Na segunda etapa do nosso estudo, observamos que o uso do anticoncepcional oral (2 mg de acetato de clormadinona e 0,03 mg de etinilestradiol) durante 6 meses reduziu os níveis de MMP-2, tanto nas controles saudáveis quanto nas portadoras de SOP, além de reduzir os níveis de TIMP-2 e TIMP-1 nas primeiras. Sendo assim, a diminuição do hiperandrogenismo com o uso do anticoncepcional oral nas pacientes com SOP pode possuir efeito benéfico, uma vez que diminui os níveis de MMP-2 plasmática, o que pode contribuir também para a redução do risco cardiovascular nessas mulheres. Entretanto, são necessários estudos maiores e com maior tempo de tratamento para confirmar os efeitos benéficos ou não do uso de anticoncepcional oral em pacientes com SOP.

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