Universidade Federal do Rio Grande Do Sul

Faculdade de Medicina

Programa de Pós-Graduação em Ciências Médicas: Endocrinologia

NÍVEIS SÉRICOS E NO FLUIDO PERITONEAL DE LEPTINA E INTERLEUCINA-6 E EXPRESSÃO GÊNICA E PROTÉICA DA LEPTINA E DO SEU RECEPTOR– ISOFORMA LONGA NO ENDOMÉTRIO TÓPICO E ECTÓPICO DE MULHERES COM ENDOMETRIOSE

Andrea Prestes Nácul

Porto Alegre, dezembro de 2010

Universidade Federal do Rio Grande Do Sul
Faculdade de Medicina
Programa de Pós-Graduação em Ciências Médicas: Endocrinologia

Andrea Prestes Nácul

Tese apresentada ao Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, como requisito parcial para obtenção do título de Doutor

Orientadora: Prof[a]. Dr[a]. Poli Mara Spritzer

Porto Alegre, dezembro de 2010

Agradecimentos

À minha orientadora, Prof[a]. Dr[a]. Poli Mara Spritzer pela amizade, confiança, oportunidade de aprendizado e crescimento profissional.

À colega Débora Martinho Morsch pela amizade, participação no estudo e disponibilidade.

À colega Sheila Lecke pela amizade, participação no estudo e incentivo constante.

A todos colegas da Unidade de Endocrinologia Ginecológica por tornar o ambiente de trabalho mais prazeroso e agradável.

À Miriam Sant'Helena e Natália Goulart, pelo auxílio, disponibilidade, amizade e carinho.

Ao colega Sérgio Galbinski pelo auxílio nas videolaparoscopias das pacientes do estudo.

À Dra. Maria Isabel Edelweiss pela participação no estudo e realização da imuno-histoquímica.

Aos meus pais, Leda e Augusto, e meus irmãos Miguel e Eduardo pelo amor, incentivo e ótima convivência.

Ao meu marido Telmo pelo amor, companheirismo, incentivo e compreensão.

À minha filha Isadora por proporcionar as horas de maior felicidade.

Às pacientes que participaram do estudo, pois são elas que nos incentivam na busca das respostas.

Ao Hospital Fêmina que disponibilizou as condições para a realização do estudo e é uma Instituição incentivadora da pesquisa científica.

Esta Tese de Doutorado segue o formato proposto pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, Metabolismo e Nutrição, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, sendo apresentada na forma de três manuscritos sobre o tema da Tese:

- Artigo de revisão: Aspectos atuais do diagnóstico e tratamento da endometriose (publicado na Revista Brasileira de Ginecologia e Obstetrícia – 2010 Jun; 32(6): 298-307)

- Artigo original 1: Gene expression of leptin and leptin long receptor isoform is increased and correlated in endometriosis

- Artigo original 2: Interleukin-6 levels in serum and peritoneal fluid of patients with endometriosis

SUMÁRIO

RESUMO

A endometriose é caracterizada pela presença de tecido endometrial localizado fora da cavidade uterina como peritônio, ovários e septo reto-vaginal e sua prevalência gira em torno de 6% a 10%. Mulheres com endometriose podem ser assintomáticas ou apresentar queixas de dismenorréia, dispareunia, dor pélvica crônica e/ou infertilidade. Em relação à patogênese, a teoria da menstruação retrógrada é bem aceita, embora alterações na biologia molecular do endométrio pareçam ser fundamentais para o desenvolvimento dos implantes endometrióticos. Evidências indicam que a endometriose está associada com aumento das concentrações de citoquinas pró-inflamatórias, fatores de crescimento e de angiogênese no fluido peritoneal (FP). Entre as citoquinas, a leptina é uma proteína derivada do gene da obesidade (Ob) que, além de ações no balanço energético, ingestão alimentar e controle do peso corporal também apresenta atividades imunorregulatórias e angiogênicas. A interleucina-6 (IL-6) é uma glicoproteína secretada por diversos tipos de células, incluindo macrófagos peritoneais e células estromais endometriais. A IL-6 é um marcador da resposta inflamatória de fase aguda e tem diversas atividades biológicas como indução da expressão do fator de crescimento do endotélio vascular (VEGF), crescimento e diferenciação de linfócitos B e ativação de linfócitos T. Estas citoquinas podem ter um papel na endometriose através das suas propriedades inflamatórias e angiogênicas.

No presente estudo, avaliamos a razão leptina/IMC e os níveis de IL-6 no soro e FP, bem como a expressão gênica da leptina e da forma longa do seu receptor (OB-R_L) no endométrio tópico e ectópico de 30 mulheres com endometriose e 19 controles com pelve normal à laparoscopia. A razão leptina/IMC no soro foi significativamente maior no grupo com endometriose em relação às controles normais 0.61 (0.41 – 0.95) e 0.41 (0.22 – 0.71) $P<0.05$, respectivamente. A expressão gênica da leptina e do OB-R_L foi significativamente maior no endométrio ectópico do que no endométrio tópico de pacientes com diferentes estágios da endometriose e controles. Uma correlação positiva entre níveis de RNAm da leptina e do OB-R_L foi observada no endométrio ectópico e tópico das pacientes com endometriose e no endométrio tópico das controles. A imuno-histoquímica do OB-R_L foi mais intensa nas células estromais e

epiteliais do endométrio tópico das pacientes e controles com maiores níveis de leptina no FP. Os níveis de IL-6 no FP foram significativamente maiores no grupo com endometriose do que no controle e também nas pacientes com endometriose III e IV em comparação a I e II e controles. Houve uma correlação positiva e significativa entre os níveis de IL-6 no FP e o escore de gravidade da endometriose da American Society of Reproductive Medicine-revised (ASRM-r).

Concluindo, nossos resultados sugerem que a razão leptina/IMC está associada com a presença da endometriose, o uso desta razão na prática clínica para predizer a presença de endometriose necessita ainda confirmação. Concentrações mais elevadas de leptina e OB-R_L no endométrio ectópico sugerem uma modulação positiva entre a leptina e seu receptor ativo com um papel da leptina no desenvolvimento dos implantes endometrióticos. Finalmente, nosso estudo sugere que a IL-6 esteja associada com a presença da endometriose pélvica e sua gravidade. Estudos avaliando a expressão gênica e protéica da IL-6 no endométrio tópico e ectópico são necessários para melhor elucidar o papel desta citoquina na patogênese da endometriose.

ABSTRACT

Endometriosis is characterized by the presence of endometrial tissue, localized out of the uterine cavity, like peritoneum, ovaries, and rectum-vaginal septum, with a prevalence of about 6% to 10%. Women with endometriosis may be asymptomatic or present dysmenorrhea, dyspareunia, chronic pelvic pain and/or infertility. Concerning its pathogenesis, while the retrograde menses theory is well accepted, disruption on endometrial molecular mechanisms seems to be critical to development of endometrial ectopic implants. Evidences support that endometriosis is associated with abnormal levels of proinflammatory cytokines, growth and angiogenic factors on peritoneal fluid (PF).

Leptin is an adipocyte derived protein and Ob-gene product. Besides the well established functions in energetic balance, food intake and body weight controls, leptin also presents imunoregulatory and angiogenic activities. Interleukin-6 (IL-6) is a glycoprotein secreted by several cell types, including peritoneal macrophages and endometrial stromal cells. IL-6 is a marker of the acute-phase inflammatory response and has several biologic activities including the induction of vascular endothelial growth factor (VEGF) expression, growth and differentiation of B lymphocytes and activation of T lymphocytes. These cytokines may play a role in endometriosis through its inflammatory and angiogenic proprieties.

In the present study, we assessed leptin/BMI ratio and IL-6 levels in serum and peritoneal fluid (PF) and evaluated the gene expression of leptin and its long form receptor (OB-R_L) in eutopic and ectopic endometrium of 30 women with endometriosis and 19 controls with normal pelvis at laparoscopy. Serum leptin/BMI ratio was significantly increased in endometriosis in comparison with controls [0.61 (0.41 – 0.95); 0.41 (0.22 – 0.71) $P<0.05$]. Leptin and OB-R_L gene expression was significantly higher in ectopic endometrium than in eutopic endometrium of patients with different stages of endometriosis and controls. A positive correlation between leptin mRNA and OB-R_L mRNA expression was observed in ectopic and eutopic endometrium in patients and in eutopic endometrium in controls. OB-R_L immunostaining was more intense in stromal and epithelial cells of eutopic endometrium in patients and controls with higher PF leptin levels. IL-6 levels in the PF were found to be

significantly higher in endometriosis group than in controls. In addition, IL6 levels in PF were significantly higher in patients with endometriosis III and IV in comparison to I and II and normal pelvis controls. There was a positive and significant correlation between IL-6 levels in PF and endometriosis ASRM-r score of severity.

In conclusion, our data suggest that serum leptin/BMI ratio is associated with the presence of endometriosis, although the clinical use of leptin/BMI ratio to predict endometriosis presence still needs confirmation. Moreover, the increased expression of leptin and OB-R_L in ectopic endometrium suggests a modulatory interaction between leptin and its active receptor and a role of leptin in the development of endometrial implants. In addition, our study suggests that IL-6 may be associated with the presence of pelvic endometriosis and its gravity. Further studies evaluating IL-6 gene and protein expression in topic and ectopic endometrium are needed to better elucidate the role of this cytokine on the pathogenesis of endometriosis.

Parte I

ASPECTOS ATUAIS DO DIAGNÓSTICO E TRATAMENTO DA ENDOMETRIOSE

ASPECTOS ATUAIS DO DIAGNÓSTICO E TRATAMENTO DA ENDOMETRIOSE

Este artigo foi publicado na Revista Brasileira de Ginecologia e Obstetrícia

(Rev Bras Ginecol Obstet. 2010 Jun; 32(6): 298-307)

Aspectos atuais do diagnóstico e tratamento da endometriose

Current aspects on diagnosis and treatment of endometriosis

Andrea Prestes Nácul[1]

Poli Mara Spritzer[2]

[1]Doutoranda do PPG: Endocrinologia, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul e médica da Unidade de Reprodução Humana do Hospital Fêmina- GHC.

[2]Coordenadora da Unidade de Endocrinologia Ginecológica, Serviço de Endocrinologia, Hospital de Clínicas de Porto Alegre; Professora Titular, Departamento de Fisiologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul; Coordenadora do Instituto Nacional de Ciência e Tecnologia de Hormônios e Saúde da Mulher/ CNPq.

Financiamento: INCT de Hormônios e Saúde da Mulher/CNPq (Conselho Nacional de Ciência e Tecnologia), FIPE (Fundo de Incentivo à Pesquisa do Hospital de Clínicas de Porto Alegre) e Gerência de Ensino e Pesquisa do Grupo Hospitalar Conceição/Fundo de Fomento à Pesquisa do Governo Federal

Resumo

A endometriose é caracterizada pela presença de tecido endometrial, localizado fora da cavidade uterina como superfície peritoneal, ovários e septo reto-vaginal. A prevalência gira em torno de 6% a 10%. Em relação à etiopatogenia, a teoria da menstruação retrógrada é aceita, porém alterações na biologia molecular do endométrio parecem ser fundamentais para o desenvolvimento dos focos ectópicos de endometriose. Mulheres com endometriose podem ser assintomáticas ou apresentar queixas de dismenorréia, dispareunia, dor pélvica crônica e/ou infertilidade. Embora o diagnóstico definitivo da endometriose necessite de uma intervenção cirúrgica, preferencialmente por videolaparoscopia, diversos achados no exame físico, de imagem e laboratoriais já podem predizer com alto grau de confiabilidade que a paciente apresenta endometriose. Os tratamentos mais difundidos atualmente são a cirurgia, a terapia de supressão ovariana ou a associação de ambas. Tratamentos farmacológicos que não inibem a função ovariana estão em investigação.

Palavras-chave: endometriose; laparoscopia; estrogênios; progestógenos; infertilidade; dor pélvica

Abstract

Endometriosis is characterized by the presence of endometrial tissue, localized out of the uterine cavity, like peritoneum, ovaries, and rectum-vaginal septum. The prevalence is about 6% to 10%. Concerning the etiopathogenesis the retrograde menses theory is well accepted, although disruption in endometrial molecular biology seems to be fundamental to development of endometriotic ectopic implants. Women with endometriosis may be asymptomatic or present complaints like dysmenorrhea, dispareunia, pelvic pain and/or infertility. Although the definitive diagnosis of endometriosis needs a surgical intervention, mainly by laparoscopy, many findings in the physical examination, image and laboratorial tests can predict with high degree of certainty that a patient has endometriosis. Current treatments include surgery, ovarian suppression therapy or both. Pharmacological treatments that don't inhibit ovarian function are being under investigation.

Key-words: endometriosis; laparoscopy; estrogen; progestins, infertility; pelvic pain

Introdução

A endometriose é caracterizada pela presença de tecido funcional semelhante ao endométrio localizado fora da cavidade uterina, mais comumente no peritônio pélvico, nos ovários e septo reto-vaginal e, mais raramente, no pericárdio, pleura e sistema nervoso central.

Os estudos apontam uma prevalência de até 20% das mulheres em idade reprodutiva[1] e 30 a 50% das mulheres inférteis apresentam endometriose[2].

A etiopatogenia ainda não está bem estabelecida, porém as evidências indicam que a combinação de fatores genéticos, hormonais e imunológicos poderia contribuir para a formação e o desenvolvimento dos focos ectópicos de endometriose[3]. A teoria mais aceita para explicar o desenvolvimento da endometriose é a teoria da implantação, descrita por Sampson em 1927. De acordo com este autor, ocorreria o refluxo de tecido endometrial através das trompas de falópio durante a menstruação, com subseqüente implantação e crescimento no peritônio e ovário[4]. Um estudo recente[1], confirmando a teoria de Sampson, verificou que a distribuição dos implantes endometrióticos é assimétrica e relacionada tanto com a anatomia abdomino-pélvica quanto com o fluxo do líquido peritoneal. Um dos aspectos discutidos a respeito desta teoria é que, embora 70-90% das mulheres apresentem menstruação retrógrada, apenas uma minoria irá desenvolver a doença[1]. Isto sugere que outros fatores - genéticos, hormonais ou ambientais, poderiam determinar uma maior suscetibilidade para desenvolver a doença. A expressão aumentada de genes envolvidos com o mecanismo de apoptose celular como o c-fos, por exemplo, pode aumentar a sobrevida destas células dentro da cavidade peritoneal que, interagindo com moléculas de adesão, irão se aderir à superfície peritoneal[5]. A presença de quantidades elevadas de macrófagos no líquido peritoneal pode também estar associada à secreção de diversas citocinas, fatores de crescimento e de angiogênese que culminarão na implantação e invasão deste tecido endometrial ectópico.

Por outro lado, a manutenção e o crescimento dos implantes ectópicos são estimulados pelos estrogênios. As células estromais do tecido endometriótico apresentam a capacidade de sintetizar estrogênios a partir do colesterol, pois expressam as enzimas esteroidogênicas. Algumas evidências sugerem que a endometriose é caracterizada por resistência à ação de

progesterona cuja ação, antagônica aos estrogênios, leva à atrofia do endométrio. Enquanto o tecido endometrial tópico expressa proteínas transcritas por ambos os receptores de progesterona A e B (PRA e PRB)[6], o tecido endometriótico apresenta apenas proteínas transcritas pelo PRA - a forma truncada do receptor que tem ação repressora sobre o PRB. Alterações na razão PRA/PRB em certos tecidos alvos podem modificar a ação final da progesterona através da regulação diferencial da resposta gênica à progesterona[7,8].

Quadro clínico

O quadro clínico da paciente com endometriose é bastante variável. A paciente pode ser assintomática, referir apenas infertilidade ou ter sintomas como dismenorréia severa, dispareunia profunda, dor pélvica crônica, dor ovulatória, sintomas urinários ou evacuatórios perimenstruais e fadiga crônica. O exame ginecológico pode ser normal, mas a presença de dor à mobilização uterina, retroversão uterina ou aumento do volume ovariano é sugestiva de endometriose, embora não seja específica. Outras condições como síndrome do cólon irritável, doença inflamatória pélvica e cistite intersticial podem apresentar sintomatologia semelhante e devem entrar no diagnóstico diferencial. Os sinais sugestivos de endometriose profunda infiltrativa são nodulações palpáveis no fórnice vaginal posterior ou septo reto-vaginal, espessamento dos ligamentos útero-sacros ou lesões violáceas na vagina[3].

Avaliação diagnóstica da endometriose

Embora o diagnóstico definitivo da endometriose necessite de uma intervenção cirúrgica, preferencialmente por videolaparoscopia, diversos achados no exame físico, de imagem e laboratoriais já podem predizer com alto grau de confiabilidade que a paciente apresenta endometriose.

Na Figura 1 apresentamos um fluxograma de investigação da endometriose. Até o momento nenhum marcador bioquímico pode ser considerado como de eleição para diagnóstico de endometriose, porém o Ca-125, quando coletado no primeiro ou segundo dia do ciclo menstrual,

pode ser útil para o diagnóstico da endometriose em estádio avançado, principalmente quando os valores são superiores a 100 UI/ml[9]. Embora concentrações normais não excluam a doença, casos com níveis elevados no pré-operatório podem auxiliar no acompanhamento da paciente e na suspeita clínica de recidiva da endometriose. Mais recentemente, algumas citocinas vêm sendo estudadas como novos marcadores não cirúrgicos da endometriose. A interleucina-6 (IL-6) parece ter um desempenho melhor do que outras citocinas em discriminar pacientes com endometriose[10]. O primeiro exame de imagem a ser solicitado na paciente com história e exame físico sugestivo de endometriose é a ultrassonografia pélvica transvaginal, preferencialmente com preparo intestinal. Um estudo de Abrao et al., 2007[11], avaliando a acurácia deste exame, demostrou uma sensibilidade de 94% e uma especificidade de 98% na identificação de focos de endometriose profunda. Se o exame é normal, a paciente pode não ter endometriose ou ter doença inicial não infiltrativa. Por outro lado, se o exame for conclusivo para endometriose ovariana, do septo reto vaginal ou reto-sigmóide ou do trato urinário, o tratamento pode ser indicado sem exames de imagem adicionais. Para avaliação de endometriomas maiores do que 2 cm, a ultrassonografia transvaginal é um método eficiente, segundo Moore et al., 2002[12]. A presença de massas ovarianas com hipótese diagnóstica duvidosa pode ser melhor avaliada com a ressonância magnética (RM). Alterações sugestivas de doença do septo reto-vaginal, ligamentos útero-sacros ou do reto-sigmóide podem ser confirmadas por ecoendoscopia retal ou RM. A ecoendoscopia retal permite identificar a distância entre a lesão e a luz retal, assim como compressões extrínsecas e lesões da submucosa do reto[13.] A RM também permite identificar doença profunda com invasão do trato intestinal, porém não possibilita precisar a camada intestinal acometida pela lesão[14]. A ultrassonografia transvaginal para o diagnóstico de endometriose de bexiga tem sido relatada como método eficaz, com sensibilidade de 71,4% e especificidade de 100%[15]. Ultrassonografia sugestiva de endometriose vesical ou ureteral pode ser complementada com a urografia excretora, que poderá evidenciar estreitamentos ureterais. A urorressonância pode ser utilizada como método alternativo à urografia excretora para avaliação de dilatações do sistema coletor renal. Apesar dos exames de imagem disponíveis apresentarem

boa acurácia no diagnóstico da endometriose, a videolaparoscopia com biópsia das lesões para análise anátomo-patológica ainda é o padrão-ouro no diagnóstico da endometriose.

Classificação da endometriose

Após a realização da videolaparoscopia, a endometriose pode ser classificada de acordo com o tipo histológico dos implantes, a localização anatômica da doença – peritônio, ovário ou septo reto-vaginal ou através da extensão da doença sobre os órgãos pélvicos. A classificação mais utilizada atualmente é a da American Society of Reproductive Medicine - revisada em 1996[16]. Esta classificação gradua a endometriose em mínima, leve, moderada ou grave pela extensão da doença no peritônio e ovários, bem como pela presença de aderências tubo-ovarianas e bloqueio do fundo-de-saco de Douglas. (tabela 1) Esta classificação, embora com algumas limitações, é bastante útil na orientação do tratamento pós-cirúrgico, especialmente quando a queixa da paciente é infertilidade.

Análise crítica dos tratamentos para a endometriose

A abordagem terapêutica da endometriose varia, dependendo se a queixa da paciente é dor pélvica ou infertilidade, embora muitas vezes estas queixas estejam associadas. Os tratamentos mais difundidos atualmente são a cirurgia, a terapia de supressão ovariana ou a associação de ambas. Nas pacientes em que a queixa é dor pélvica, podemos iniciar um tratamento empírico com anticoncepcionais orais sem o diagnóstico definitivo quando a avaliação clínica for sugestiva de endometriose mínima ou leve.[3] Se a paciente não melhorar em três meses ou houver a suspeita de endometriose profunda infiltrativa, podemos usar análogos do GnRH (GnRHa) por três meses e após manter com anticoncepcionais orais. Se a paciente apresentar recidiva da dor, exame de imagem sugestivo de endometrioma maior que 3 cm ou suspeita de aderências a cirurgia deve ser indicada (Quadro 1).

Tratamentos de reprodução assistida para endometriose

Os tratamentos de reprodução assistida – Inseminação intra-uterina e Fertilização *in vitro* podem ser indicados para pacientes com endometriose e infertilidade, levando em conta o grau da doença, o envolvimento das trompas, a idade, o tempo de infertilidade e a presença de outros fatores associados[3].

A inseminação intra-uterina com indução da ovulação é um tratamento eficaz para os casos de endometriose mínima ou leve. A anatomia da pelve deve estar preservada, pelo menos uma trompa deve estar pérvia e em boas condições e o exame de capacitação espermática deve mostrar valores de espermatozóides acima de 5 milhões/ml. O tratamento deve ser oferecido por até seis ciclos. Pacientes com mais de 35 anos podem partir diretamente para FIV.

A FIV é o tratamento apropriado para os casos de endometriose grau 3 ou 4 com comprometimento tubário, se houver fator masculino associado ou se os tratamentos prévios falharam. Não parece haver correlação significativa do número de ciclos de FIV com recorrência da endometriose[17]. (Figuras 3 e 4)

Tratamento cirúrgico da endometriose

O tratamento cirúrgico da endometriose compreende desde procedimentos de baixa complexidade como cauterização de focos superficiais e liberação de aderências velamentosas até intervenções complexas nos ovários, fundo de saco de Douglas, intestino, bexiga e ureteres, exigindo, em alguns casos, uma equipe multidisciplinar.

Por vários anos, o tratamento cirúrgico da endometriose se baseou nos princípios oncológicos de remoção radical das lesões. Este princípio ainda é utilizado quando se trata de casos de estenose intestinal ou ureteral ou massas ovarianas de característica duvidosa. No entanto, atualmente, sabemos que não há correlação entre a extensão da doença com a gravidade dos

sintomas, bem como com o prognóstico reprodutivo e de recorrência de dor a longo-prazo[18]. Além disso, muitas pacientes apresentam infertilidade associada à dor, exigindo que o procedimento cirúrgico seja conservador. Baseado nestas considerações, alguns autores preconizam tratamento cirúrgico apenas para pacientes que não respondam ao tratamento medicamentoso bem como para aquelas pacientes que desejam engravidar espontaneamente[19]. Existem poucos ensaios clínicos randomizados publicados, avaliando o resultado do tratamento cirúrgico da endometriose sintomática. Uma revisão de Vercellinni[19] descreve melhora sintomática após o tratamento conservador em torno de 60-80%, com recorrência dos sintomas e índice de re-operação variando entre os estudos de 12 a 58%.

Para a paciente com infertilidade, a ablação dos focos e a adesiólise parece melhorar a fertilidade nos graus mínimo e leve da doença[2]. Já nos casos de graus moderado ou severo não há ensaios clínicos randomizados ou meta-análises disponíveis para responder se a ressecção dos focos aumentaria os índices de gestação.

Endometriomas ovarianos

Os endometriomas ovarianos não respondem adequadamente ao tratamento medicamento, sendo a cirurgia indicada nos casos de endometriomas sintomáticos ou grandes[20].A ooforectomia deve ser reservada para os casos de recidiva da dor, especialmente em mulheres na peri-menopausa. A cirurgia conservadora deve ser realizada em mulheres jovens ou com desejo de gestar. As opções de cirurgia conservadora incluem a exérese da pseudocápsula, a drenagem e ablação do cisto ou punção e esvaziamento. Nestes casos, recomenda-se enviar parte da pseudo-cápsula para análise histopatológica para confirmação do diagnóstico clínico e exclusão de malignidade que é em torno de 0,7%[21.] A cirurgia excisional está associada com menor recorrência dos sintomas de dismenorréia, dispareunia e dor não-menstrual em relação à drenagem e ablação da cápsula. A cirurgia excisional também diminui a recorrência do endometrioma e da necessidade de re-intervenção, bem como aumenta os índices de gestação espontânea, nas pacientes com sub-fertilidade[21]. Parece haver uma melhor resposta folicular ovariana à estimulação com citrato

de clomifeno e gonadotrofinas nas pacientes que realizaram cirurgia excisional[22]. Entretanto, não há evidências de qual a melhor abordagem cirúrgica dos endometriomas em relação aos índices de gestação após tratamento de reprodução assistida.

Endometriose profunda infiltrativa

Os resultados da cirurgia nas pacientes que apresentam endometriose infiltrativa das paredes posterior da vagina e anterior do reto foram avaliados na maioria dos casos através de estudos observacionais ou retrospectivos, não comparados e com um número limitado de pacientes. Existe apenas um ensaio clínico não-randomizado, comparando cirurgia laparotômica com manejo expectante. Após um seguimento de mais de dois anos, o tempo para recorrência da dor moderada à severa foi significativamente mais longo no grupo das pacientes operadas. Além disso, houve uma melhora significativa de dispareunia profunda, tenesmo e dismenorréia no grupo operado. Já em relação a taxas de gravidez, não houve diferença entre os grupos[23]. Uma coorte retrospectiva demonstrou que a presença de endometriose intestinal piora ainda mais o prognóstico reprodutivo de pacientes inférteis com endometriose moderada e grave. Além disso, as pacientes que fizeram cirurgia completa com ressecção intestinal segmentar tiveram melhores taxas de gestação tanto espontânea quanto por fertilização in vitro (FIV) em relação às pacientes que fizeram cirurgia incompleta[24.] Os índices de complicações maiores e menores com a cirurgia cólon-retal na endometriose profunda variam amplamente, de 0 a 13%[19]. A maioria das complicações graves deste tipo de cirurgia está associada especificamente com perfuração inadvertida ou ressecção incidental do reto. A segunda complicação mais freqüente é a formação de fístula reto-vaginal, com um risco relatado de até 10%, mesmo em mãos experientes[25]. Um balanço entre os possíveis benefícios e os riscos potenciais deste tipo de procedimento deve ser realizado quando considerar cirurgia para alívio da dor em mulheres com endometriose profunda. Os resultados obtidos com a cirurgia da endometriose infiltrativa são dependentes da habilidade do cirurgião. A cirurgia da endometriose do septo reto-vaginal deve ser considerada em pacientes altamente motivadas, após consentimento detalhado e esclarecido, sempre tendo em mente que a tendência à progressão da doença do septo é limitada.

Assim, o tratamento cirúrgico da endometriose é uma alternativa para pacientes que não respondem ou não toleram o tratamento com associações estro-progestogênicas ou que apresentem estreitamento da luz intestinal. Pacientes com dispareunia profunda ou tenesmo devem ser consideradas boas candidatas, pois a remoção dos nódulos nestes casos é mais efetiva do que a terapia medicamentosa em aliviar a dor do tipo orgânica. A cirurgia conservadora deve ser indicada para pacientes com dor intolerável desejando gestar. Entretanto, é importante que tanto o médico quanto a paciente estejam cientes de que a cirurgia conservadora implica em índices elevados de recorrência da dor a médio e longo-prazo. A histerectomia com salpingo-ooforectomia também pode ser considerada para pacientes com prole completa e falha dos tratamentos prévios, tendo a certeza de que todos os focos visíveis tenham sido ressecados juntamente[26].

Tratamento farmacológico da endometriose

Dentre os tratamentos farmacológicos mais difundidos para a dor associada à endometriose estão as combinações estro-progestogênicas, progestogênios isolados, e análogos do GnRH. (Quadro 1). Basicamente, estes agentes inibem o crescimento dos implantes por decidualização e atrofia do endométrio ou através da supressão dos hormônios esteróides ovarianos e indução de um estado de hipoestrogenismo. Os estudos que avaliaram estes tratamentos hormonais mostraram que eles são igualmente efetivos, porém seus efeitos adversos e custos diferem de forma significativa[27,28.] É importante salientar que todos os tratamentos disponíveis para a dor associada com a endometriose têm efeito contraceptivo. Por outro lado, não é rara a associação de dor e infertilidade, principalmente nos graus mais severos da doença, o que impossibilita o uso destes tratamentos. Não há evidências de que a supressão ovariana isolada, com a terapia hormonal, seja efetiva para o tratamento da infertilidade em pacientes com endometriose de qualquer grau, além de retardar a possibilidade de gravidez pelo efeito anticoncepcional[29]. Além disso, o tratamento pré ou pós-operatório com drogas supressoras da função ovariana não parecem melhorar a fertilidade nestas pacientes[30].

A única indicação de terapia de supressão ovariana em pacientes inférteis com endometriose é previamente à FIV. Uma revisão recente da Cochrane demonstrou que o uso de análogos do GnRH por 3-6 meses antes da FIV aumentaria em quatro vezes as chances de gravidez[31]. Porém, os resultados dessa revisão foram baseados em apenas um ensaio clínico com uma amostra pequena de pacientes e problemas metodológicos. Assim, a indicação do uso prévio de análogos do GnRH deve ser individualizada, observando a presença de fatores que possam piorar a resposta da paciente à estimulação ovariana, como por exemplo uma reserva ovariana comprometida.

Combinações estro-progestogênicas

Os anticoncepcionais combinados (AC) são considerados primeira linha no tratamento da dor associada à endometriose peritoneal, com presença ou não de endometriomas menores que quatro centímetros.

As vantagens destes fármacos são a possibilidade de uso por períodos prolongados, a boa tolerabilidade e a fácil administração. Como a endometriose é uma doença crônica e progressiva, com recorrência dos sintomas no caso do retorno da ovulação, devemos planejar para que o tratamento possa ser usado por tempo prolongado (anos) sem que haja efeitos adversos graves, pouca tolerabilidade ou custo elevado.

Os ACO podem ser administrados de forma cíclica ou contínua. A recomendação é que quando o uso cíclico dos ACO não melhora a dor associada com o sangramento mensal, a administração contínua pode ser uma alternativa efetiva e segura para estas pacientes. Os ACO combinados contendo progestogênios mais androgênicos (derivados da 19-nortestosterona) são tradicionalmente prescritos para a endometriose, porém os derivados da 17-α-hidroxiprogesterona também tem se mostrado efetivos[32.]

Progestogênios isolados

Os progestogênios isolados são largamente utilizados para o tratamento da dor associada à endometriose pelos mesmos motivos das associações estro-progestogênicas – possibilidade de uso por tempo prolongado e boa tolerabilidade. As apresentações orais são o acetato de noretisterona, o dienogest (não disponível no Brasil), o acetato de ciproterona e o levonorgestrel. Todas as apresentações são para uso contínuo, e apresentam eficácia semelhante aos AC na melhora da dismenorréia, dispareunia, e dor pélvica. O pior controle do ciclo menstrual é uma desvantagem em relação aos anticoncepcionais combinados com estrogênios[32,33].

Alternativamente à via oral, os progestogênios podem ser utilizados pela via intra-muscular, sub-cutânea, sub-dérmica e intra-uterina. O acetato de medroxiprogesterona na forma de depósito (DMPA), intra-muscular e sub-cutâneo, tem eficácia é semelhante aos análogos do GnRH na melhora da dor, porém a perda mineral óssea é menos significativa e reversível apenas com o progestogênio[34]. As vantagens do DMPA de depósito são a baixa incidência de sintomas de hipoestrinismo, o baixo custo e a administração a cada três meses. Porém, o controle do sangramento é errático, o tratamento não pode ser interrompido na vigência de para-efeitos (ganho de peso, diminuição da libido, acne, queda de cabelo, mastalgia, sintomas depressivos) e o retorno dos ciclos menstruais regulares pode demorar entre sete meses até um ano para se restabelecerem. Assim, a melhor indicação para o DMPA de depósito seria pacientes histerectomizadas com doença residual.

Existe pouca experiência ainda em relação ao uso do implante sub-dérmico de etonogestrel, um metabólito ativo do desogestrel, para o tratamento dos sintomas dolorosos da endometriose. A eficácia do implante para redução da dor, controle do sangramento e a incidência de efeitos adversos parece ser semelhante às relatadas com o DMPA[35].

Outra opção que tem sido utilizada para reduzir sintomas dolorosos da endometriose é o sistema intra-uterino com levonorgestrel (LNG-IUS) que tem duração de cinco anos. O levonorgestrel

vai exercer atividade androgênica e anti-estrogênica diretamente no endométrio, podendo levar à amenorréia e melhora da dismenorréia, com menor impacto no metabolismo da paciente.

A eficácia é semelhante ao GnRHa no tratamento da dor crônica, embora o controle do sangramento seja pior com o LNG-IUD[36]. Após um ano de uso, poucas pacientes relatam sangramento inter-menstrual e entre 20 e 30% estão em amenorréia.

Análogos do GnRH (GnRHa)

O GnRHa pode ser administrado diariamente através de spray nasal (acetato de nafarelina) ou por injeção sub-cutânea, esta última com formulações para uso diário, mensal ou trimestral (acetato de leuprolida, acetato de goserelina). Os efeitos colaterais são decorrentes do estado de "pseudo-menopausa" e incluem fogachos, vagina seca, diminuição da libido, depressão, irritabilidade, fadiga e perda mineral óssea. O GnRHa pode ser usado por períodos de três a seis meses com alívio significativo da dor, embora o tratamento por três meses seja tão efetivo quanto o de seis meses[37]. O uso do GnRHa, associado com um estrogênio e progestogênio, denominado terapia de "add-back", reduz a severidade dos sintomas de hipoestrinismo e a perda mineral óssea, principalmente quando se planeja manter o tratamento por mais de seis meses[38]. Várias opções de associações de estrogênios e progestogênios tem se mostrado eficazes na terapia de "add back". Não há diferença entre diferentes doses de estrogênios (1,25 ou 0,625 mg EC) em relação à perda óssea[39]. A tibolona também se mostrou eficaz em impedir a perda de massa óssea, e parece ser uma boa alternativa já que associa ações estrogênicas, progestogênicas e androgênicas[40]. Ainda faltam estudos avaliando esquemas com doses mais baixas de TH e com agentes reguladores do cálcio.

Danazol e Gestrinona

Apesar de terem eficácia comprovada em diversos estudos, atualmente estas duas opções de tratamento para endometriose têm ficado como terceira opção por apresentarem pouca tolerabilidade devido aos efeitos colaterais.

O danazol induz amenorréia por inibição do pico de LH, inibição das enzimas esteroidogênicas e aumento da testosterona livre. Assim, associa tanto para-efeitos devido ao hipoestrogenismo (vaginite atrófica, fogachos) quanto ao hiperandrogenismo (acne, seborréia, hirsutismo, alteração da voz, dislipidemia).

A gestrinona é um derivado da 19-noresteróide e tem ações antiestrogênicas, anti-progestogênicas e androgênicas. Tem eficácia semelhante ao danazol e aos análogos do GnRH[41]. A baixa tolerabilidade devido aos efeitos androgênicos tem limitado o uso.

Inibidores da aromatase

Há uma parcela significativa de pacientes que permanecem sintomáticas ou apresentam recorrência da dor após o tratamento com GnRHa ou após a pan-histerectomia[42].

Os inibidores da aromatase impedem a conversão de androgênios em estrogênios não só no ovário, mas também no tecido adiposo, na pele e no próprio tecido endometriótico que possui esta enzima. O único ensaio clínico randomizado disponível na literatura[43], demonstrou que a associação de inibidor da aromatase com GnRHa é mais eficaz do que GnRHa isolado na melhora dos sintomas dolorosos no pós-operatório de pacientes com endometriose severa. Nas pacientes pré-menopáusicas, deve ser feita alguma forma de supressão ovariana associada com os inibidores da aromatase. Se o ovário não for suprimido concomitantemente, a depleção de estrogênio estimula a secreção de FSH pelo hipotálamo que irá, por sua vez, estimular a secreção ovariana de estradiol[44]. Nestes casos, os inibidores da aromatase podem ser associados com GnRHa, com progestogênio isolado ou associado com estrogênio. Nos casos, relativamente raros de endometriose pós-menopáusica, os inibidores da aromatase parecem ser a primeira escolha já que, nestes casos, o estrogênio circulante provém de outros sítios que não o ovário. Entretanto, este tratamento é considerado ainda investigativo, não estando aprovado para uso clínico.

Referências

1. Bricou, A., Batt, R.E., and Chapron, C. 2008. Peritoneal fluid flow influences anatomical distribution of endometriotic lesions: why Sampson seems to be right. *Eur J Obstet Gynecol Reprod Biol* 138:127-134.
2. Jacobson, T.Z., Barlow, D.H., Koninckx, P.R., Olive, D., and Farquhar, C. 2002. Laparoscopic surgery for subfertility associated with endometriosis. *Cochrane Database Syst Rev*:CD001398.
3. Kennedy, S., Bergqvist, A., Chapron, C., D'Hooghe, T., Dunselman, G., Greb, R., Hummelshoj, L., Prentice, A., and Saridogan, E. 2005. ESHRE guideline for the diagnosis and treatment of endometriosis. *Hum Reprod* 20:2698-2704.
4. Sampson, J.A. 1927. Metastatic or Embolic Endometriosis, due to the Menstrual Dissemination of Endometrial Tissue into the Venous Circulation. *Am J Pathol* 3:93-110 143.
5. Morsch, D.M., Carneiro, M.M., Lecke, S.B., Araujo, F.C., Camargos, A.F., Reis, F.M., and Spritzer, P.M. 2009. c-fos gene and protein expression in pelvic endometriosis: a local marker of estrogen action. *J Mol Histol* 40:53-58.
6. Conneely, O.M., Mulac-Jericevic, B., and Lydon, J.P. 2003. Progesterone-dependent regulation of female reproductive activity by two distinct progesterone receptor isoforms. *Steroids* 68:771-778.
7. Attia, G.R., Zeitoun, K., Edwards, D., Johns, A., Carr, B.R., and Bulun, S.E. 2000. Progesterone receptor isoform A but not B is expressed in endometriosis. *J Clin Endocrinol Metab* 85:2897-2902.
8. Reis FM, Lhullier C, Edelweiss MI, Spritzer PM. In vivo assessment to the regulation of transforming growth factor alpha, epidermal growth factor (EGF) and EGF receptor in the human endometrium by medroxyprogesterone acetate. Journal of Assisted Reproduction and Genetics. 22(1): 19-24, 2005.
9. Abrao, M.S., Podgaec, S., Pinotti, J.A., and de Oliveira, R.M. 1999. Tumor markers in endometriosis. *Int J Gynaecol Obstet* 66:19-22.

10. Othman Eel, D., Hornung, D., Salem, H.T., Khalifa, E.A., El-Metwally, T.H., and Al-Hendy, A. 2008. Serum cytokines as biomarkers for nonsurgical prediction of endometriosis. *Eur J Obstet Gynecol Reprod Biol* 137:240-246.
11. Abrao, M.S., Goncalves, M.O., Dias, J.A., Jr., Podgaec, S., Chamie, L.P., and Blasbalg, R. 2007. Comparison between clinical examination, transvaginal sonography and magnetic resonance imaging for the diagnosis of deep endometriosis. *Hum Reprod* 22:3092-3097.
12. Moore, J., Copley, S., Morris, J., Lindsell, D., Golding, S., and Kennedy, S. 2002. A systematic review of the accuracy of ultrasound in the diagnosis of endometriosis. *Ultrasound Obstet Gynecol* 20:630-634.
13. Roseau, G., Dumontier, I., Palazzo, L., Chapron, C., Dousset, B., Chaussade, S., Dubuisson, J.B., and Couturier, D. 2000. Rectosigmoid endometriosis: endoscopic ultrasound features and clinical implications. *Endoscopy* 32:525-530.
14. Bazot, M., and Darai, E. 2005. Sonography and MR imaging for the assessment of deep pelvic endometriosis. *J Minim Invasive Gynecol* 12:178-185; quiz 177, 186.
15. Bazot, M., Thomassin, I., Hourani, R., Cortez, A., and Darai, E. 2004. Diagnostic accuracy of transvaginal sonography for deep pelvic endometriosis. *Ultrasound Obstet Gynecol* 24:180-185.
16. American Society for Reproductive Medicine. 1997. Revised American Society for Reproductive Medicine classification of endometriosis:1996. Fertil Steril 67: 817-821.
17. Benaglia, L., Somigliana, E., Vercellini, P., Benedetti, F., Iemmello, R., Vighi, V., Santi, G., and Ragni, G. 2009. The impact of IVF procedures on endometriosis recurrence. *Eur J Obstet Gynecol Reprod Biol* 148:49-52.
18. Vercellini, P., Fedele, L., Aimi, G., Pietropaolo, G., Consonni, D., and Crosignani, P.G. 2007. Association between endometriosis stage, lesion type, patient characteristics and severity of pelvic pain symptoms: a multivariate analysis of over 1000 patients. *Hum Reprod* 22:266-271.

19. Vercellini, P., Crosignani, P.G., Abbiati, A., Somigliana, E., Vigano, P., and Fedele, L. 2009. The effect of surgery for symptomatic endometriosis: the other side of the story. *Hum Reprod Update* 15:177-188.
20. Chapron, C., Vercellini, P., Barakat, H., Vieira, M., and Dubuisson, J.B. 2002. Management of ovarian endometriomas. *Hum Reprod Update* 8:591-597.
21. Hart, R., Hickey, M., Maouris, P., Buckett, W., and Garry, R. 2005. Excisional surgery versus ablative surgery for ovarian endometriomata: a Cochrane Review. *Hum Reprod* 20:3000-3007.
22. Alborzi, S., Ravanbakhsh, R., Parsanezhad, M.E., Alborzi, M., Alborzi, S., and Dehbashi, S. 2007. A comparison of follicular response of ovaries to ovulation induction after laparoscopic ovarian cystectomy or fenestration and coagulation versus normal ovaries in patients with endometrioma. *Fertil Steril* 88:507-509.
23. Vercellini, P., Pietropaolo, G., De Giorgi, O., Daguati, R., Pasin, R., and Crosignani, P.G. 2006. Reproductive performance in infertile women with rectovaginal endometriosis: is surgery worthwhile? *Am J Obstet Gynecol* 195:1303-1310.
24. Stepniewska, A., Pomini, P., Bruni, F., Mereu, L., Ruffo, G., Ceccaroni, M., Scioscia, M., Guerriero, M., and Minelli, L. 2009. Laparoscopic treatment of bowel endometriosis in infertile women. *Hum Reprod* 24:1619-1625.
25. Darai, E., Thomassin, I., Barranger, E., Detchev, R., Cortez, A., Houry, S., and Bazot, M. 2005. Feasibility and clinical outcome of laparoscopic colorectal resection for endometriosis. *Am J Obstet Gynecol* 192:394-400.
26. Lefebvre, G., Allaire, C., Jeffrey, J., Vilos, G., Arneja, J., Birch, C., and Fortier, M. 2002. SOGC clinical guidelines. Hysterectomy. *J Obstet Gynaecol Can* 24:37-61; quiz 74-36.
27. Davis, L., Kennedy, S.S., Moore, J., and Prentice, A. 2007. Modern combined oral contraceptives for pain associated with endometriosis. *Cochrane Database Syst Rev*:CD001019.
28. Selak, V., Farquhar, C., Prentice, A., and Singla, A. 2007. Danazol for pelvic pain associated with endometriosis. *Cochrane Database Syst Rev*:CD000068.

29. Hughes, E., Brown, J., Collins, J.J., Farquhar, C., Fedorkow, D.M., and Vandekerckhove, P. 2007. Ovulation suppression for endometriosis. *Cochrane Database Syst Rev*:CD000155.
30. Yap, C., Furness, S., and Farquhar, C. 2004. Pre and post operative medical therapy for endometriosis surgery. *Cochrane Database Syst Rev*:CD003678.
31. Sallam, H.N., Garcia-Velasco, J.A., Dias, S., and Arici, A. 2006. Long-term pituitary down-regulation before in vitro fertilization (IVF) for women with endometriosis. *Cochrane Database Syst Rev*:CD004635.
32. Vercellini, P., De Giorgi, O., Mosconi, P., Stellato, G., Vicentini, S., and Crosignani, P.G. 2002. Cyproterone acetate versus a continuous monophasic oral contraceptive in the treatment of recurrent pelvic pain after conservative surgery for symptomatic endometriosis. *Fertil Steril* 77:52-61.
33. Muneyyirci-Delale, O., and Karacan, M. 1998. Effect of norethindrone acetate in the treatment of symptomatic endometriosis. *Int J Fertil Womens Med* 43:24-27.
34. Crosignani, P.G., Luciano, A., Ray, A., and Bergqvist, A. 2006. Subcutaneous depot medroxyprogesterone acetate versus leuprolide acetate in the treatment of endometriosis-associated pain. *Hum Reprod* 21:248-256.
35. Walch, K., Unfried, G., Huber, J., Kurz, C., van Trotsenburg, M., Pernicka, E., and Wenzl, R. 2009. Implanon versus medroxyprogesterone acetate: effects on pain scores in patients with symptomatic endometriosis--a pilot study. *Contraception* 79:29-34.
36. Petta, C.A., Ferriani, R.A., Abrao, M.S., Hassan, D., Rosa e Silva, J.C., Podgaec, S., and Bahamondes, L. 2009. A 3-year follow-up of women with endometriosis and pelvic pain users of the levonorgestrel-releasing intrauterine system. *Eur J Obstet Gynecol Reprod Biol* 143:128-129.
37. Hornstein, M.D., Yuzpe, A.A., Burry, K.A., Heinrichs, L.R., Buttram, V.L., Jr., and Orwoll, E.S. 1995. Prospective randomized double-blind trial of 3 versus 6 months of nafarelin therapy for endometriosis associated pelvic pain. *Fertil Steril* 63:955-962.
38. Surrey, E.S., and Hornstein, M.D. 2002. Prolonged GnRH agonist and add-back therapy for symptomatic endometriosis: long-term follow-up. *Obstet Gynecol* 99:709-719.

39. Sagsveen, M., Farmer, J.E., Prentice, A., and Breeze, A. 2003. Gonadotrophin-releasing hormone analogues for endometriosis: bone mineral density. *Cochrane Database Syst Rev*:CD001297.

40. Wender MC, Edelweiss MI, Campos LS. Castro JA, Spritzer PM. Endometrial assessment in patients using tibolone or placebo: one-year randomized trial and tow-year observational study. Menopause. 11(4): 423-429, 2004.

41. 1996. Gestrinone versus a gonadotropin-releasing hormone agonist for the treatment of pelvic pain associated with endometriosis: a multicenter, randomized, double-blind study. Gestrinone Italian Study Group. *Fertil Steril* 66:911-919.

42. Waller, K.G., and Shaw, R.W. 1993. Gonadotropin-releasing hormone analogues for the treatment of endometriosis: long-term follow-up. *Fertil Steril* 59:511-515.

43. Soysal, S., Soysal, M.E., Ozer, S., Gul, N., and Gezgin, T. 2004. The effects of post-surgical administration of goserelin plus anastrozole compared to goserelin alone in patients with severe endometriosis: a prospective randomized trial. *Hum Reprod* 19:160-167.

44. Attar, E., and Bulun, S.E. 2006. Aromatase and other steroidogenic genes in endometriosis: translational aspects. *Hum Reprod Update* 12:49-56.

Tabela 1 - Classificação da American Society for Reproductive Medicine – revisada em 1996.

Estágio I (mínima)		1-5		
Estágio II (leve)		6-15		
Estágio III (moderada)		16-40		
Estágio IV (severa)		>40		
Peritônio	Endometriose	< 1 cm	1-3 cm	> 3 cm
	Superficial	1	2	4
	Profunda	2	4	6
Ovário	D superficial	1	2	4
	Profunda	4	16	20
	E superficial	1	2	4
	Profunda	4	16	20
Obliteração do fundo de saco posterior		Parcial 4		Completa 40
Ovário	Aderências	< 1/3 Envolvido	1/3-2/3 Envolvido	> 2/3 Envolvidos
	D velamentosa	1	2	4
	Densa	4	8	16
	E velamentosa	1	2	4
	Densa	4	8	16
Trompa	D velamentosa	1	2	4
	Densa	4*	8*	16
	E velamentosa	1	2	4
	Densa	4*	8*	16

*Se as fímbrias tubárias estiverem totalmente envolvidas por aderências, mude o escore para 16.
De acordo com American Society for Reproductive Medicine, 1997(15).
O escore final da endometriose é a soma dos escores parciais da extensão da doença no peritônio, no ovário direito e esquerdo, da obliteração do fundo de saco posterior e das aderências ovarianas e tubárias à direita e à esquerda.

Quadro 1 - Opções terapêuticas na mulher com endometriose sintomática sem desejo de gestação.

Terapias de primeira-linha
Doença peritoneal e cistos endometrióticos < 4 cm
Combinações estro-progestogênicas usadas cíclica ou continuamente* (oral, intra-vaginal, transdérmico)
Progestogênio isolado via oral contínuo (desogestrel)
Doença do septo reto-vaginal
Acetato de noretisterona, 2,5 mg/dia contínuo*
Terapias de segunda-linha
Análogos do GnRH de depósito + terapia "add back" (ex: tibolona 2,5 mg/dia)
Progestogênios alternativos (ex: acetato de medroxiprogesterona, acetato de ciproterona)
Terapias de terceira-linha
Danazol em baixa-dose (ex: 200 mg/dia, oral ou intra-vaginal)
Gestrinona (2,5 mg 2x/semana)
Situações especiais
Mulheres multíparas com dismenorréia como sintoma principal
LNG-IUD
Mulheres histerectomizadas com doença residual
Acetato de medroxiprogesterona de depósito (150 mg IM trimestral)
*Pausa de 7 dias é sugerido em casos de sangramento de escape durante o uso contínuo.

Legenda das Figuras

Figura 1 - Fluxograma de avaliação da paciente com suspeita de endometriose pélvica.

Figura 2 - Conduta clínica na paciente com dor pélvica crônica e suspeita de endometriose.

Figura 3: - Conduta clínica na paciente com endometriose grau I e GII e infertilidade.

Figura 4 - Conduta clínica na paciente com endometriose grau III ou IV e infertilidade.

Figura 1:

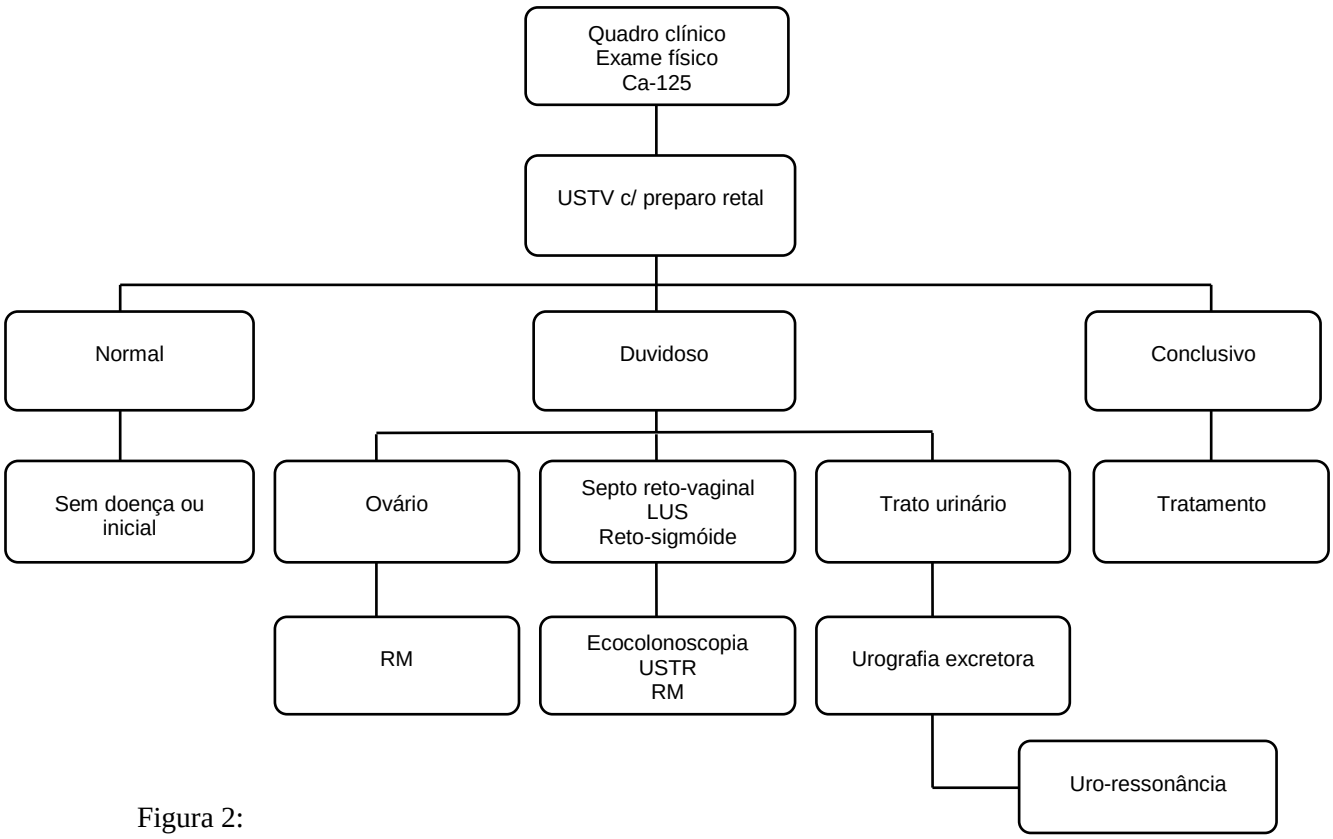


Figura 2:

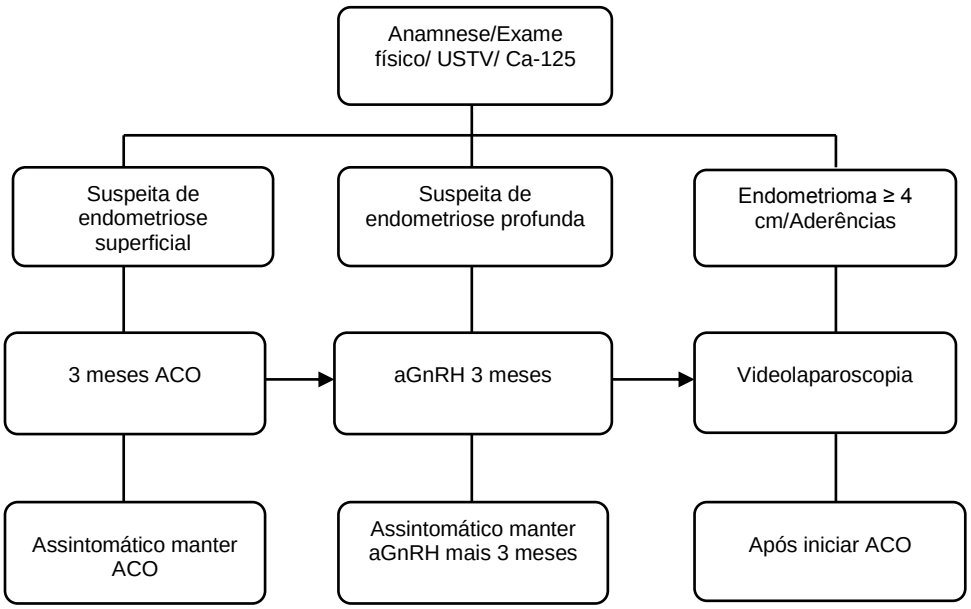
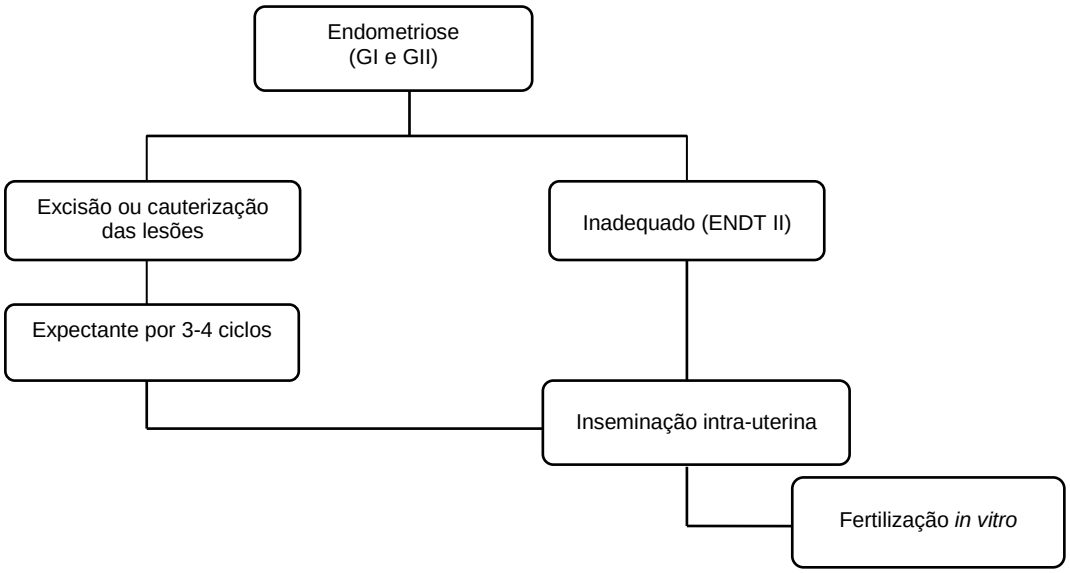
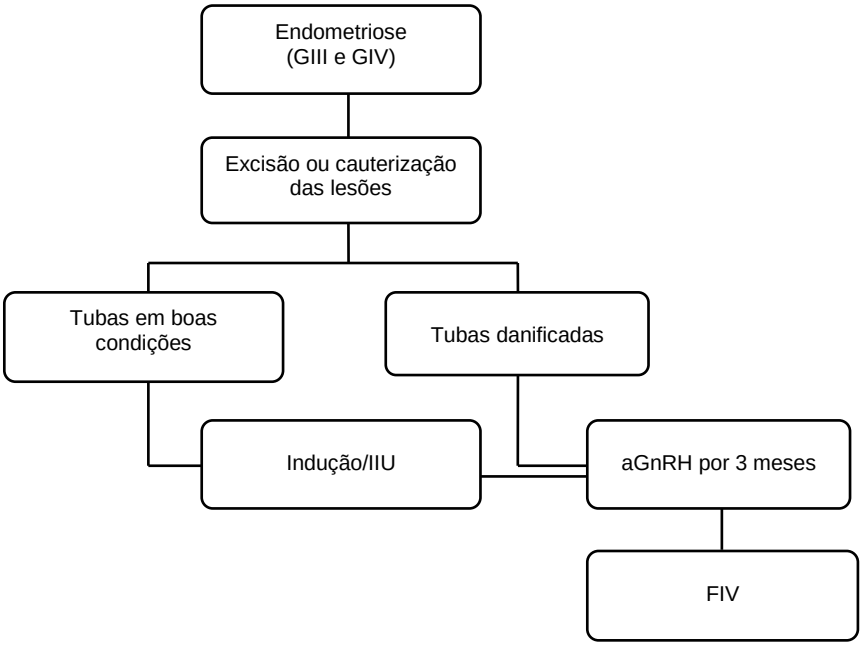


Figura 3:



Obs: mulheres > 35 anos, pular etapas

Figura 4:



Parte II

Artigo Original 1:

Gene expression of leptin and leptin long receptor isoform is increased and correlated in endometriosis

Gene expression of leptin and leptin long receptor isoform is increased and correlated in endometriosis

Running title: Leptin and OB-R_L in endometriosis

Gene expression of leptin and leptin long receptor isoform is increased and correlated in endometriosis

A. P. Nácul[1,2], S. B. Lecke[1], M. I. Edelweiss[3], D. M. Morsch[1], P. M. Spritzer[1,4]

[1]Gynecological Endocrinology Unit, Division of Endocrinology, Hospital de Clínicas de Porto Alegre and National Institute of Hormones and Women's Health- CNPq, Porto Alegre, 90035-003, Brazil; [2]Human Reproduction Unit, Hospital Fêmina- Grupo Hospitalar Conceição, Porto Alegre, 90430-001, Brazil; [3]Division of Pathology, Hospital de Clínicas de Porto Alegre, Porto Alegre, 90035-003, Brazil; [4]Laboratory of Molecular Endocrinology, Department of Physiology, Universidade Federal do Rio Grande do Sul, Porto Alegre, 90050-170, Brazil.

Corresponding author

Poli Mara Spritzer, MD, PhD

Division of Endocrinology, Hospital de Clínicas de Porto Alegre

Rua Ramiro Barcelos, 2350

CEP 90035-003 – Porto Alegre, RS, Brazil

Tel/Fax: +55 51-3359-8027 Fax: +55 51-3359-8777

E-mail: spritzer@ufrgs.br

Running title: Leptin and OB-R_L in endometriosis

Gene expression of leptin and leptin long receptor isoform is increased and correlated in endometriosis

A. P. Nácul[1,2], S. B. Lecke[1], M. I. Edelweiss[3], D. M. Morsch[1], P. M. Spritzer[1,4]

[1]Gynecological Endocrinology Unit, Division of Endocrinology, Hospital de Clínicas de Porto Alegre and National Institute of Hormones and Women's Health- CNPq, Porto Alegre, 90035-003, Brazil; [2]Human Reproduction Unit, Hospital Fêmina- Grupo Hospitalar Conceição, Porto Alegre, 90430-001, Brazil; [3]Division of Pathology, Hospital de Clínicas de Porto Alegre, Porto Alegre, 90035-003, Brazil; [4]Laboratory of Molecular Endocrinology, Department of Physiology, Universidade Federal do Rio Grande do Sul, Porto Alegre, 90050-170, Brazil.

Corresponding author

Poli Mara Spritzer, MD, PhD

Division of Endocrinology, Hospital de Clínicas de Porto Alegre

Rua Ramiro Barcelos, 2350

CEP 90035-003 – Porto Alegre, RS, Brazil

Tel/Fax: +55 51-3359-8027 Fax: +55 51-3359-8777

E-mail: spritzer@ufrgs.br

ABSTRACT

Endometriosis is a chronic inflammatory condition characterized by implantation and growth of endometrial tissue outside the uterus. Leptin presents immunoregulatory and angiogenic properties and might play a role in the pathogenesis of endometriosis. We assessed leptin/BMI ratio in serum and peritoneal fluid (PF) and evaluated the gene expression of leptin and of the long form leptin receptor (OB-R_L) in eutopic and ectopic endometria of 29 women with endometriosis and 19 controls with laparoscopically normal pelvis. Age and BMI were similar between the groups and between different endometriosis stages. Serum leptin/BMI ratio was significantly increased in endometriosis in comparison with controls [0.61 (0.41 – 0.95); 0.41 (0.22 – 0.71) $P<0.05$]. Leptin and OB-R_L gene expression was significantly higher in ectopic endometrium vs. eutopic endometrium of patients with different stages of endometriosis and controls. A positive correlation between leptin mRNA and OB-R_L mRNA expression was observed in ectopic and eutopic endometria in patients and in eutopic endometrium in controls. OB-R_L immunostaining was more intense in stromal and epithelial cells of eutopic endometrium in patients and controls with higher PF leptin levels. The increased expression of leptin and OB-R_L in ectopic endometrium suggests a modulatory interaction between leptin and its active receptor and a role of leptin in the development of endometrial implants.

Key-words: endometriosis / immunohistochemistry / leptin / leptin receptor / Real-time RT-PCR.

INTRODUCTION

Endometriosis has been defined as a chronic inflammatory disease. The eutopic endometrium of women with endometriosis has specific characteristics that favor tissue survival, adhesion and growth outside uterine cavity. Several studies have demonstrated that endometriosis is associated with abnormal peritoneal and endometrial production of proinflammatory cytokines and growth and angiogenic factors (Matarese *et al*., 2000; Gazvani and Templeton, 2002).

Expression of leptin, a cytokine produced mainly by adipocytes and implicated in the regulation of sex hormone production, ovulation, endometrial cell physiology, and early embryo development and implantation (Mitchell *et al.* 2005), has been demonstrated in the endometrium (Kitawaki *et al.*, 2000). Leptin may play a role in endometriosis through its inflammatory and angiogenic proprieties. Nevertheless, studies evaluating serum and peritoneal fluid (PF) levels of leptin in patients with endometriosis report conflicting results: some have observed increased levels (Matarese *et al*. 2000; De Placido *et al.* 2001; Mahutte *et al.* 2003; Bedaiwy *et al.*, 2006; Gungor *et al.*, 2009), while others found no significant differences between patients with endometriosis and controls (Viganò *et al.*, 2002; Wertel *et al.*, 2005; Gungor *et al.*, 2009; Wertel *et al.*, 2005; Barcz *et al.*, 2008). Moreover, the possibility of an association between PF leptin levels and severity of endometriosis is also controversial, with some studies reporting a negative correlation (Matarese *et al.*, 2000; De Placido *et al.*, 2001; Mahutte *et al.*, 2003), and others suggesting a positive correlation with more severe forms of peritoneal endometriosis (Wertel *et al.*, 2005; Bedaiwy *et al.*, 2006; Milewski *et al.*, 2008; Gungor *et al.*, 2009).

Although leptin and its receptors are expressed in human endometrium (Gonzalez *et al.*, 2000), only a few studies so far have evaluated leptin receptor gene and/or protein expression in endometrial tissue of women with endometriosis (Lima-Couy *et al.*, 2004; Wu *et al.*, 2002). Lima-Coy *et al.* (2004) evaluated the three isoforms of leptin receptor – total (OB-R_T), long (OB-R_L) and short (HuB219.3) – in the topic endometrium of patients with moderate and severe endometriosis and observed increased receptor expression in the period corresponding to

embryo implantation, without difference between patients and controls. Wu *et al.* (2002) reported expression of leptin receptor in both topic and ectopic endometrium.

Therefore, the aims of the present study were a) to assess leptin and OB-R_L gene expression in ectopic and eutopic endometrium of women with endometriosis and in eutopic endometrium of normal controls, b) to determine leptin/BMI ratio in serum and PF in both groups, c) to assess the immunoreactive presence of OB-R_L in endometrium and endometriotic implants and d) to investigate the relationship among these variables.

MATERIALS AND METHODS

Subjects

Twenty-nine women with pelvic endometriosis and 19 women with laparoscopically normal pelvis were consecutively selected among patients undergoing gynecological laparoscopy for infertility, pelvic pain, ovarian pathology or tubal ligation (TL) between September 2007 and March 2009. Infertility was defined as absence of pregnancy after one year of sexual intercourse without contraception. Chronic pelvic pain was defined as non-cyclical pelvic pain during at least six months, severe enough to cause functional limitation or require medical attention (American College of Obstetricians and Gynecologysts). Endometriosis, defined as ectopic presence of endometrial glands and/or stroma, was confirmed by histology in all patients with suspected lesions at laparoscopy. Endometriosis was classified according to the revised classification of the American Society of Reproductive Medicine (ASRM, 1997). Peritoneal endometriotic lesions were observed in 26 patients, and ovarian superficial endometrioma in 3.

Inclusion criteria were (i) a premenopausal status, (ii) the need for laparoscopy, and (iii) no use of hormonal medication in the previous three months. Exclusion criteria were body mass index above 35. The study protocol was approved by the local Ethics Committee (IRB-equivalent), and written informed consent was obtained from all subjects.

Study protocol

All participants underwent physical examination, including measurement of height and weight and estimation of body mass index (BMI). Laparoscopy with biopsy of endometriotic implants and concomitant biopsy of eutopic endometrium were performed preferentially on the second half of the menstrual cycle. However, in around 20% of participants laparoscopy and biopsy were performed in the proliferative phase. A single sample of superficial peritoneal endometriotic tissue was obtained from the largest lesion. Topic endometrial samples were collected with curettage. The same surgeon was in charge of all laparoscopic evaluations (AN).

Peripheral venous blood samples were collected immediately before anesthetic induction for laparoscopy. PF samples were collected immediately after the start of the procedure from Douglas cull de sac. All samples were kept on ice for transport to the laboratory and stored in aliquots at –80°C until assayed. Endometriotic and endometrial samples were fractioned and one portion was immediately frozen in liquid nitrogen and stored at –80°C until mRNA extraction, while the other was fixed in 10% buffered formalin and embedded in paraffin for subsequent histological diagnosis and immunohistochemistry, as described (Morsch *et al.*, 2009).

Based on histological and laparoscopic findings, three types of tissue were studied: 1) eutopic endometrium from disease-free patients, 2) eutopic endometrium from patients with endometriosis, and 3) ectopic endometrium from patients with endometriosis.

Serum and peritoneal fluid measurements

Serum estradiol and progesterone concentrations were assayed by eletrochemiluminescence (Roche Diagnostic, Mannhein, Germany). Serum and peritoneal leptin levels were determined using a Human Leptin ELISA (LINCO Research, Missouri, USA).

RNA isolation

Endometrial tissue total RNA extraction was carried out in phenol-guanidine isothiocyanate (Trizol®, Invitrogen™ Life Technologies, Foster City, USA) as previously described

(Oliveira *et al.*, 2003; Morsch *et al.*, 2009). Concentration and quality of total RNA were assessed using GeneQuant spectrophotometer (Pharmacia Biotech, Cambridge, England).

Real time RT-PCR protocol

Reverse transcription of 1 µg of total RNA into cDNA was carried out using the Superscript II First-Strand Synthesis System for RT-PCR (Invitrogen™ Life Technologies, Foster City, USA), according to the manufacturer's instructions in a PCT-100™ Programmable Thermal Controller (MJ Research Inc., Watertown, USA).

Real-time PCR was performed in triplicate in a 7500 Fast Real-Time PCR System thermal cycler with 7500 Fast System Sequence Detection 1.4 Software (Applied Biosystems, Foster City, USA). Experiments were performed by monitoring in real time the increase in fluorescence of the SYBR® Green dye as previously described (Higuchi R. 1992, Higuchi R. 1993, Zipper 2004). Primers were designed by Primer Express 3.0 Software for Real-Time PCR (Applied Biosystems, Foster City, USA) and acquired from Invitrogen™ (Life Technologies, Foster City, USA). Primer sequences were projected to target two exons of an mRNA with respect to known splice variants and single-nucleotide polymorphism positions. The forward and reverse primer sequences designed for leptin (NM_000230.2) were (5' to 3') TCCCCTCTTGACCCATCTC and GGGAACCTTGTTCTGGTCAT, respectively. These primers anneal between residues 858 to 876 (forward) and 967 to 948 (reverse), producing a PCR product of 110 bp. The forward and reverse primer sequences for leptin receptor (NM_001003679.2) were (5' to 3') AGGAAGCCCGAAGTTGTGTT and TCTGGTCCCGTCAATCTGA, respectively. These primers anneal between residues 3,617 to 3,636 (forward) and 3,716 to 3,698 (reverse), resulting in an amplicon of 100 bp. Beta-2-microglobulin (NM_004048.2) was used to normalize mRNA quantitation. CTATCCAGCGTACTCCAAAG and ACAAGTCTGAATGCTCCACT (5' to 3') forward and reverse B2M primer sequences, anneal between residues 119 to 138 (forward) and 283 to 264 (reverse), resulting in an amplicon of 165 bp. cDNA samples (1.0 ng/µL) were mixed with a predetermined forward and reverse primer volume (0.9 and 0.7 µL for leptin, 0.9 and 0.3 µL for leptin receptor, 0.7 and 0.9 µL for B2M) and 12.5 µL of 2X Fast

SYBR® Green Master Mix (Applied Biosystems, Foster City, USA) in a total of 25 µL. Protocol conditions consisted of denaturation at 94°C for 2 min followed by 50 cycles (30 sec at 94°C and 30 sec at 60°C). Primers generated amplicons that produced a single sharp peak during melting curve analysis. Data were analyzed by relative quantitation using the comparative C_T method (Applied Biosystems 2004). Validation assays were performed by amplification of the target and reference genes, separately, using serial dilutions of an mRNA sample. Both target and reference mRNAs presented equal efficiencies of amplification. The $\Delta\Delta C_T$ method calculates changes in gene expression as relative fold difference between an experimental and the calibrator sample, correcting non-ideal amplification efficiencies (Livak and Schmittgen, 2001).

Immunohistochemistry

Formalin-fixed, paraffin-embedded endometrial samples were cut into 5 µm slices, which were stained by immunohistochemistry using the avidin-biotin-peroxidase method (Hsu and Raine, 1981), as previously described (Spritzer *et al.*, 1996; Reis *et al.*, 1999). Following deparaffinization and rehydration with a graded series of ethanol, immunohistochemistry was performed using peroxidase reaction. Sections were incubated with 3% H_2O_2 in room temperature for 5 minutes in order to suppress endogenous peroxidase activity. The samples were then incubated in a humidity chamber at room temperature for 30 minutes with a 0.5 µg/mL goat antihuman leptin receptor antibody C20 (Santa Cruz Biotechnology, CA, USA). Phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (W/V) replaced the primary antibody in negative controls. After 20-minute incubation with the linker, streptavidin-peroxidase was used for 5 min to stain the slices. Subsequent to each incubation step, the tissues were washed three times with PBS 50 mM Tris – HCl buffer. Counterstaining was carried out with Mayer's hematoxylin and the slices were mounted. A positive reaction was characterized by the presence of granular brown staining in the cytoplasm. The intensity of immunostaining in epithelium and stroma was evaluated by two independent observers and classified as negative,

weak, moderate or intense, and converted by a semiquantitative scale into 0–3 arbitrary units (Reis, *et al.*, 2002).

Statistical analysis

Data are presented as means ± SD or median and interquartile range. Comparisons between group means were analyzed by Student's test. Median values were compared using the Mann-Whitney U test. Comparisons of median values involving three groups were analyzed using the Kruskal-Wallis test. The chi-square test was used to compare qualitative variables. Pearson's rank or Spearman's correlation coefficients were calculated using a two-tailed significance test for variables with a Gaussian or non-Gaussian distribution, respectively. Wilcoxon's non-parametrical, paired test was used if required based on the number of subjects and the non-homogeneous features of each group. The gamma test was used for qualitative comparison of more than two groups.

All analyses were performed using the Statistical Package for the Social Sciences 16 (SPSS, Chicago, IL, USA). Data were considered to the significant at $P < 0.05$.

RESULTS

The age of participants ranged from 21 to 50 years. Endometrial biopsies were unsatisfactory in two patients with endometriosis and two normal pelvis controls. Therefore, the results of gene expression and immunohistochemistry refer to data from 28 patients with endometriosis and 16 control participants.

Endometriosis was classified as stage I (minimal) in 13 patients, stage III (moderate) in 6, and stage IV (severe) in 10. Laparoscopy was performed for infertility investigation in 15 patients (31.3%), tubal ligation in 14 (29.2%), chronic pelvic pain in 10 (20.8%), adnexal pathology in 6 (12.5%), association of infertility and chronic pelvic pain in 2 (4.2%), and association of tubal ligation and chronic pelvic pain in 1 (2.1%) patient. Table I presents clinical profile of the endometriosis and control groups. Circulating levels of estradiol and progesterone in the proliferative and secretory phases of the menstrual cycle in each group also appear in

Table I. While progesterone levels were higher in the secretory than in the proliferative phase, no differences were observed in estradiol and progesterone levels between the endometriosis and control groups. Serum leptin/BMI ratio was similar in both proliferative and secretory phases of the cycle, respectively, in endometriosis [0.56 (0.27 – 1.24) vs. 0.61 (0.41 – 0.91) *P*=0.97] and control [0.33 (0.21 – 0.53) vs. 0.37 (0.18 – 0.68), *P*=0.77] groups. PF leptin/BMI ratio was found to also be similar in proliferative and secretory phases, respectively, in endometriosis [1.15 (0.35 – 1.97) vs. 0.62 (0.39 – 1.04), *P*=0.64] and control [0.46 (0.12 – 0.72) vs. 0.39 (0.26 – 0.74), *P*=0.73] groups. Therefore, posterior analyses were performed including all patients, without referring the cycle phase.

PLEASE INSERT TABLE I HERE

As shown in Table II, serum leptin/BMI ratio was significantly higher in endometriosis than in control groups. PF leptin/BMI ratio was similar between the groups and different between different endometriosis stages. There were no significant differences in serum and PF leptin/BMI ratio when controls were compared with patients with minimal/mild or moderate/severe endometriosis.

PLEASE INSERT TABLE II

mRNA encoding for leptin and OB-R_L were detectable in 28 samples (96.5%) of ectopic endometrium. In eutopic endometria of patients and controls, leptin mRNA and OB-R_L were detectable in 25 out of 28 and 16 out of 17 tested samples (89% and 94%, respectively). Figure 1A shows that leptin mRNA expression was significantly higher in ectopic lesions compared with eutopic endometrium of patients with endometriosis ($P < 0.001$). OB-R_L mRNA expression was also significantly higher in ectopic lesions as compared to eutopic endometrium of endometriosis group ($P < 0.05$).

Leptin (Figure 1B) and OB-R_L mRNA (Figure 1C) expression were also significantly higher in ectopic endometrium when endometriosis patients were stratified into minimal/mild and moderate/severe endometriosis groups as compared to eutopic endometrium of normal pelvis controls. Conversely, no differences were found between the different stages of endometriosis. Leptin and OB-R_L transcripts were also similar in eutopic endometria of patients with endometriosis and controls (data not shown).

PLEASE INSERT FIGURES 1 HERE

A positive and significant correlation was observed between leptin and OB-R_L transcripts in ectopic endometria of patients with endometriosis (Figure 2A), and also in eutopic endometria of both endometriosis and control participants (Figure 2B).

PLEASE INSERT FIGURE 2 HERE

Figure 3 shows immunostaining of OB-R_L in representative biopsies of eutopic endometrium from control and endometriosis groups and ectopic endometrium. Cytoplasmic staining was observed at both stromal and epithelial compartments from women with different stages of endometriosis and controls.

PLEASE INSERT FIGURE 3 HERE

OB-R_L immunostaining was also analyzed considering median PF leptin levels for the overall group (< 13.6 ng/mL or ≥ 13.6 ng/mL). Figure 4 shows that in women with PF leptin ≥ 13.6 ng/mL, immunostaining of OB-R_L was significantly more intense in epithelial cells (Figure 4A) and stromal cells (Figure 4B) in eutopic endometria from both endometriosis and control participants than in those with lower PF leptin levels.

PLEASE INSERT FIGURE 4 HERE

DISCUSSION

In the present study, we found a significant higher serum leptin/BMI ratio in endometriosis group as well as an also significantly higher expression of leptin and OB-R_L transcripts in ectopic compared to eutopic endometrium of patients with endometriosis and normal pelvis controls. These results suggest a putative role of leptin in the development of endometrial implants.

Only few studies have previously analyzed the leptin/BMI ratio in steady of leptin concentrations in women with endometriosis (Wertel et al, 2005), despite the fact that circulating leptin is strongly related to BMI. In this sense, using leptin/BMI ratio allows to control the influence of individual body weight in leptin secretion and obtaining more accurate results.

We did not find differences in PF leptin/BMI ratio between endometriosis and control groups, similar to results obtained by others (Viganò *et al.*, 2002). In contrast, some reports have shown higher PF leptin in endometriosis than in controls (Matarese *et al*, 2000; De Placido *et al.*, 2001). Conversely, while in our study, serum and PF leptin/BMI ratio did not correlated with the severity of endometriosis, Mahutte *et al.* (2003) found an inverse correlation between PF leptin levels and the severity of the endometriosis, Wertel *et al*. (2005), Bedaiwy *et al.* (2006) and Gungor *et al.* (2008) found a positive correlation between these aspects and Barcz *et al.* (2008) did not observe any correlation. Such discrepancies are not surprising given the fact that studies differ widely concerning patient characteristics, endpoints, endometriosis severity, stratification (or not) by anatomical location, age and BMI. Nevertheless, despite the specificities of each study, all seem to indicate (at least with the current commercially available kits) that PF leptin levels are not good markers to screen the presence, location or severity of endometriosis.

In the present study, laparoscopies were scheduled preferentially in the secretory phase in order to evaluate the association between leptin and endometrial differentiation, rather than proliferation. However, because some participants had their samples collected in the proliferative phase, we observed that serum and PF leptin/BMI ratios were comparable in the two phases in both endometriosis and control participants. In addition, the estradiol and progesterone levels recorded in each cycle phase (proliferative or secretory) were similar in endometriosis patients and control participants. Thus, our control group may be regarded as adequate for the purpose of this study.

In the present study, expression of leptin and OB-R_L transcripts was significantly higher in ectopic vs. eutopic endometrium of patients with endometriosis and normal pelvis controls. Moreover, the positive correlation between leptin mRNA and OB-R_L mRNA in both groups suggests a positive modulation between leptin and its receptor not only in endometriosis, but also in eutopic endometrium. This idea is supported by the observation that OB-R_L immunoreactivity was more intense in epithelial and stromal cells from eutopic endometrium of patients with greater levels of PF leptin.

Our results differ from those of Wu *et al.* (2002). While in both studies expression of leptin mRNA was higher in ectopic endometrium in comparison with eutopic endometrium of controls, we detected leptin mRNA in almost all eutopic and ectopic endometrium samples. This finding might be attributable, at least in part, to the methods used, since real-time RT-PCR used in our study is more sensitive to smaller amounts of mRNA.

Other authors have identified leptin and OB-R transcripts in eutopic endometrium. Kitawaki *et al.* (2000) identified the long form of the receptor in 84% of endometrial samples, vs. 91% in our study, including eutopic endometria of patients and controls. In an *in vitro* study, González *et al.* (2000) demonstrated that the presence of leptin and leptin receptor mRNA in endometrial epithelial cells and embryos could be related to the embryo implantation process. Kitawaki *et al.* (2000) also showed fluctuations in the expression of OB-R in endometrium, with a peak in the early secretory phase.

Our choice to analyze only the long form of the leptin receptor was based on the evidence that this isoform has the highest transcriptional activity (Cioffi *et al.*, 1996). In addition, Lima-Coy *et al.* (2004) assessed the total, long, and short isoforms of leptin receptor in eutopic endometrium of patients with moderate and severe endometriosis and observed an increase in receptor expression during the period of embryo implantation, without differences between eutopic endometrium from patients with endometriosis and controls. Taken together our results and those from Lima-Coy corroborate the idea that the functioning of eutopic endometrium is normal in endometriosis.

One limitation of our study concerns to the purity of cell populations of ectopic samples. Tissues obtained from lesions contain a mixture of cells types including leukocytes and peritoneal fibroblasts in addiction to ectopic endometrium. (Bulmer *et al*., 1998) Thus, there is a small risk of involvement of peritoneal cells in determining the results shown.

In conclusion, our data suggest that serum leptin/BMI ratio is associated with the presence of endometriosis, although the clinical use of leptin/BMI ratio to predict endometriosis presence still needs confirmation. Moreover, the increased expression of leptin and OB-R_L in ectopic endometrium suggests a modulatory interaction between leptin and its active receptor, and a role of leptin, an inflammatory and angiogenic cytokine, in the initiation or development of endometrial implants.

FUNDING

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico [CNPq INCT 573747/2008-3] and Fundo de Apoio à Pesquisa do Hospital de Clínicas de Porto Alegre [FIPE-HCPA 07-339], Brazil.

REFERENCES

Barcz E, Milewski L, Radomski D, Dziunycz P, Kamiński P, Roszkowski PI and Malejczyk J (2008) A relationship between increased peritoneal leptin levels and infertility in endometriosis." Gynecol Endocrinol 24, 526-530.

Bedaiwy MA, Falcone T, Goldberg JM, Sharma RK, Nelson DR and Agarwal A (2006) Peritoneal fluid leptin is associated with chronic pelvic pain but not infertility in endometriosis patients. Hum Reprod 21, 788-791.

Bulmer JN, Jones RK, Searle RF. (1998) Intraepithelial leukocytes in endometriosis and adenomyosis: comparison of eutopic and ectopic endometrium with normal endometrium. Human Reprod 13, 2910-2915.

Cioffi JA, Shafer AW, Zupancic TJ, Smith-Gbur J, Mikhail A, Platika D and Snodgrass HR (1996) Novel B219/OB receptor isoforms: possible role of leptin in hematopoiesis and reproduction. Nat Med 2, 585-589.

De Placido G, Alviggi C, Carravetta C, Pisaturo ML, Sanna V, Wilding M, Lord GM and Matarese G (2001) The peritoneal fluid concentration of leptin is increased in women with peritoneal but not ovarian endometriosis. Hum Reprod 16, 1251-1254.

Gazvani R and Templeton A (2002) Peritoneal environment, cytokines and angiogenesis in the pathophysiology of endometriosis. Reproduction 123, 217-226.

Gonzalez RR, Caballero-Campo P, Jasper M, Mercader A, Devoto L, Pellicer A and Simon C (2000) Leptin and leptin receptor are expressed in the human endometrium and endometrial leptin secretion is regulated by the human blastocyst. J Clin Endocrinol Metab 85, 4883-4888.

Gungor T, Kanat-Pektas M, Karayalcin R and Mollamahmutoglu L (2009) Peritoneal fluid and serum leptin concentrations in women with primary infertility. Arch Gynecol Obstet 279, 361-364.

Hsu SM and Raine L (1981) Protein A, avidin, and biotin in immunohistochemistry. J Histochem Cytochem 29, 1349-1353.

Kitawaki J, Koshiba H, Ishihara H, Kusuki I, Tsukamoto K and Honjo H (2000) Expression of leptin receptor in human endometrium and fluctuation during the menstrual cycle. J Clin Endocrinol Metab 85, 1946-1950.

Lima-Couy I, Cervero A, Bonilla-Musoles F, Pellicer A and Simón C (2004) Endometrial leptin and leptin receptor expression in women with severe/moderate endometriosis. Mol Hum Reprod 10, 777-782.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.

Mahutte NG, Matalliotakis IM, Goumenou AG, Vassiliadis S, Koumantakis GE and Arici A (2003) Inverse correlation between peritoneal fluid leptin concentrations and the extent of endometriosis. Hum Reprod 18, 1205-1209.

Matarese G, Alviggi C, Sanna V, Howard JK, Lord GM, Carravetta C, Fontana S, Lechler RI, Bloom SR and De Placido G (2000) Increased leptin levels in serum and peritoneal fluid of patients with pelvic endometriosis. J Clin Endocrinol Metab 85, 2483-2487.

Milewski L., Barcz E, Dziunycz P, Radomski D, Kamiński P, Roszkowski PI, Korczak-Kowalska G and Malejczyk J (2008) Association of leptin with inflammatory cytokines and lymphocyte subpopulations in peritoneal fluid of patients with endometriosis. J Reprod Immunol 79, 111-117.

Mitchell M, Armstrong DT, Robker RL and Norman RJ (2005) Adipokines: implications for female fertility and obesity. Reproduction 130, 583-597.

Morsch DM, Carneiro MM, Lecke SB, Araújo FC, Camargos AF, Reis FM and Spritzer PM (2009) c-fos gene and protein expression in pelvic endometriosis: a local marker of estrogen action. J Mol Histol 40, 53-58.

Oliveira IO, Lhullier C, Brum IS and Spritzer PM (2003) Gene expression of type 2 17 beta hydroxysteroid dehydrogenase in scalp hairs of hirsute women. Steroids 68: 641-649.

Reis FM, Maia AL, Ribeiro MF and Spritzer PM (1999) Progestin modulation of c-fos and prolactin gene expression in the human endometrium. Fertil Steril 71, 1125-1132.

Reis FM, Ribeiro MF, Maia AL and Spritzer PM (2002) Regulation of human endometrial transforming growth factor beta1 and beta3 isoforms through menstrual cycle and medroxyprogesterone acetate treatment. Histol Histopathol 17, 739-745

Revised American Society for Reproductive Medicine classification of endometriosis: 1996 (1997) Fertil Steril 67, 817-21.

Spritzer, PM, Ribeiro MF, Oliveira MC, Barbosa-Coutinho LM, Silva IS, Dahlem N, Cericatto R and Pavanato MA (1996) Effects of tamoxifen on serum prolactin levels, pituitary immunoreactive prolactin cells and uterine growth in estradiol-treated ovariectomized rats. Horm Metab Res 28, 171-176.

Viganò P, Somigliana E, Matrone R, Dubini A, Barron C, Vignali M and di Blasio AM (2002) Serum leptin concentrations in endometriosis. J Clin Endocrinol Metab 87, 1085-1087.

Wertel I, Gogacz M, Polak G, Jakowicki J and Kotarski J (2005) Leptin is not involved in the pathophysiology of endometriosis-related infertility. Eur J Obstet Gynecol Reprod Biol 119, 206-209.

Wu MH, Chuang PC, Chen HM, Lin CC and Tsai SJ (2002) Increased leptin expression in endometriosis cells is associated with endometrial stromal cell proliferation and leptin gene up-regulation. Mol Hum Reprod 8, 456-464.

FIGURE LEGENDS

Figure 1: Leptin and long leptin receptor isoform (OB-R_L) gene expression (A) in ectopic endometrium and eutopic endometrium of patients with endometriosis. Values are expressed as *n* fold change differences in relation to the calibrator sample (ΔΔCt method). **: $P< 0.001$ (leptin mRNA ectopic vs. eutopic endometrium); *: $P<0.05$ (OB-R_L mRNA ectopic vs. eutopic endometrium) (Wilcoxon signed ranks test). Leptin gene expression (B) and long leptin receptor isoform (OB-R_L) (C) in eutopic endometrium of normal pelvis controls, ectopic endometrium of minimal/mild and moderate/severe endometriosis. Values are expressed as *n* fold change differences in relation to the calibrator sample (ΔΔCt method). Asterisks indicate significant difference in comparison to controls. *: $P<0.05$; **: $P<0.01$ and ***: $P<0.001$(Mann-Whitney-U test)

Figure 2: Relationship between leptin mRNA and long leptin receptor isoform (OB-R_L) mRNA expression (A) ectopic endometrium of patients with endometriosis (R=0.57, $P<0.01$) and (B) eutopic endometrium of patients with endometriosis and normal pelvis controls (Endometriosis R=0.52 $P<0.01$; Normal pelvis R=0.57 $P<0.02$). The slope and intercepts of the regression lines in figure (B) were similar between groups, $P = 0.464$ and $P = 0.871$ respectively.

Figure 3: Immunostaining of long leptin receptor isoform (OB-R_L) in eutopic endometrium of normal pelvis controls (A), eutopic (B), and ectopic endometrium (C) of patients with endometriosis. Original magnification: x 200

Figure 4: Immunostaining intensity of long leptin receptor isoform (OB-R_L) in topic epithelial cells ($P<0.05$ Gamma Test) (A) and stromal cells ($P< 0.05$ Gamma test) (B) according to peritoneal fluid leptin levels in patients with endometriosis and normal pelvis controls.

Table I

Characteristics of women with endometriosis and normal pelvis controls

	Endometriosis	**Controls**
N	29	19
Age (years)[a]	32 ± 7	33 ± 5
BMI (kg/m²)[a]	25.6 ± 4.5	25.2 ± 3.5
Estradiol (pg/ml)		
Proliferative	122 (37-160)	64 (18-144)
	(n=5)	(n=4)
Secretory	104 (56-226)	91 (55-132)
	(n=24)	(n=12)
Progesterone (ng/ml)		
Proliferative	0.2 (0.18-0.79)	0.42 (0.21-0.86)
	(n=5)	(n=4)
Secretory	3.6 (0.56-11.25)	4.15 (0.59-9.1)
	(n=24)	(n=13)
Main indication for laparoscopy *n* (%)		
Infertility	11 (36.7)	4 (21.1)
Pelvic pain	11 (36.7)	0 (0)
Adnexal mass	6 (20)	0 (0)
Tubal ligation	1 (3.3)	13 (68.4)
Infertility and pelvic pain	1 (3.3)	1 (5.3)
Tubal ligation and pelvic pain	0 (0)	1 (5.3)

[a]: Age and BMI are expressed as mean ± SD

No significant difference in age, BMI, estradiol and progesterone between endometriosis and control groups (Student's t test)

BMI, body mass index.

Table II

Serum and peritoneal fluid leptin/BMI ratio in normal pelvis controls and endometriosis patients stratified according to stage of endometriosis

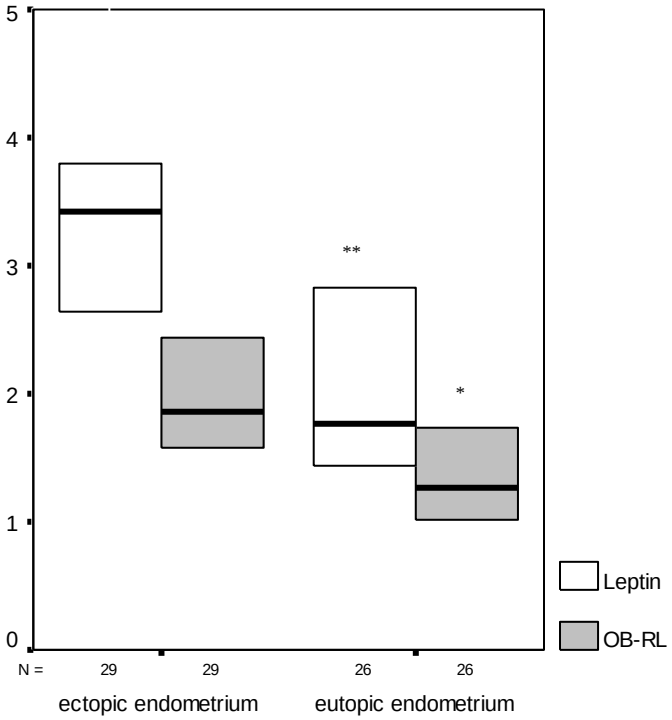
				rASRM		
	Controls	**Endometriosis**	***P***	**Stage I/II**	**Stage III/IV**	***P*[a]**
Serum leptin/BMI	0.41 (0.22–0.71) (*n* = 18)	0.61 (0.41–0.95) (*n* = 28)	0.04	0.56 (0.28–0.99) (*n* = 13)	0.78 (0.43–0.96) *n* = 15	0.08
Peritoneal fluid leptin/BMI	0.44 (0.28–0.73) (*n* = 17)	0.7 (0.45–1.18) (*n* = 23)	0.07	0.54 (0.28–1.36) *n* = 12	0.71 (0.59–1.15) *n* = 11	0.12

Data are presented as median and interquartile range (Mann-Whitney)

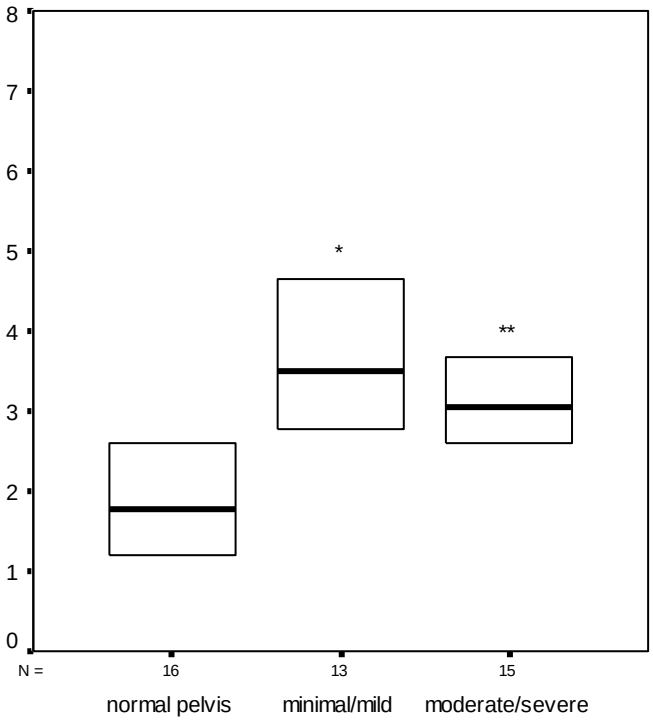
[a] Controls *vs.* rASRM stage I/II *vs* rASRM stage III/IV (Kruskal-Wallis)

rASRM stages, revised American Society for Reproductive Medicine classification (1997).

Figure 1:
A


5
4
3
2
1
0
**
*
Leptin
OB-RL
N =
29
29
26
26
ectopic endometrium
eutopic endometrium

B


8
7
6
5
4
3
2
1
0
*
**
N =
16
13
15
normal pelvis
minimal/mild
moderate/severe

C

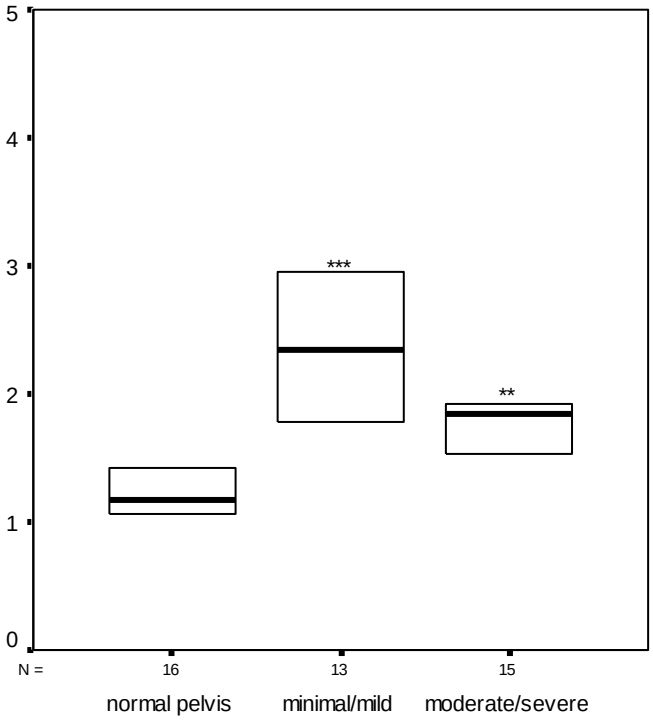
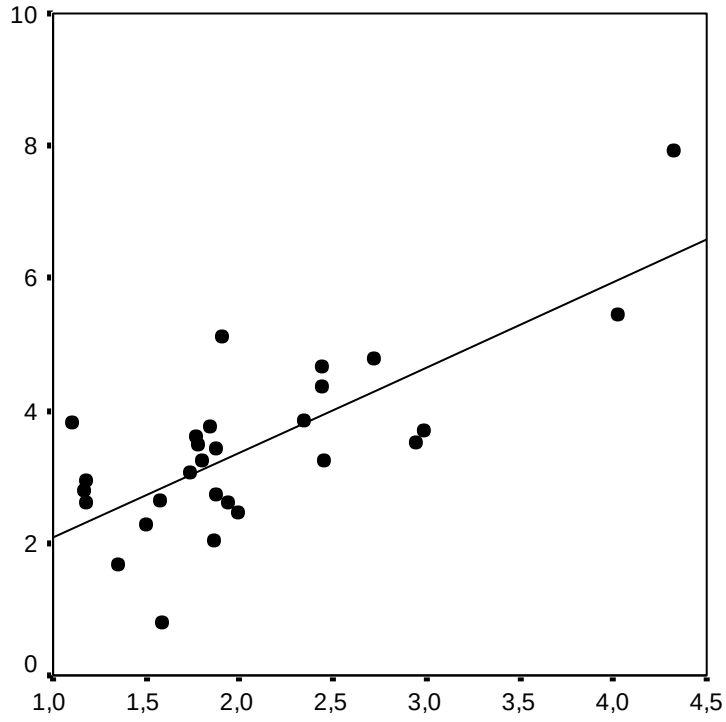

5
4
3
2
1
0
***
**
N =
16
13
15
normal pelvis
minimal/mild
moderate/severe

Figure 2:
A


10
8
6
4
2
0
1,0
1,5
2,0
2,5
3,0
3,5
4,0
4,5
OB-RL mRNA expression in ectopic endometrium (n fold change)

B

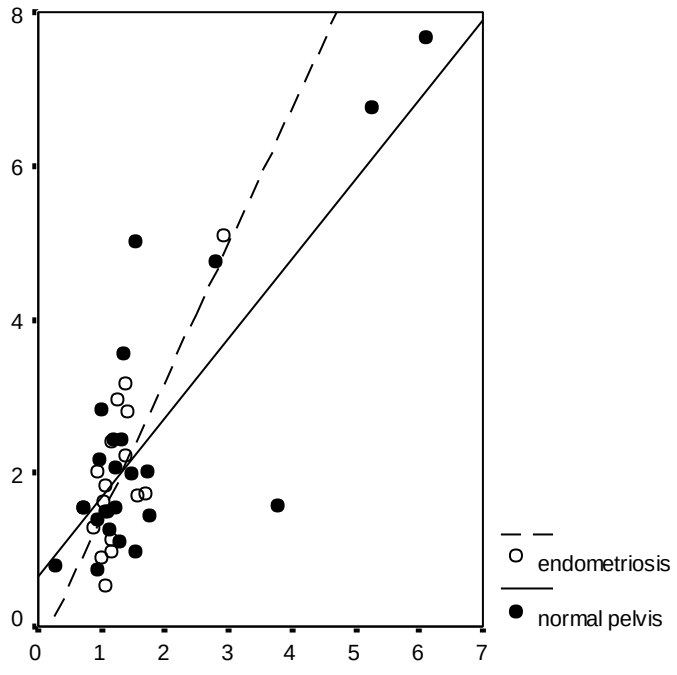

8
6
4
2
0
0
1
2
3
4
5
6
7
endometriosis
normal pelvis
OB-RL mRNA expression in topic endometrium (n fold change)

Figure 3:

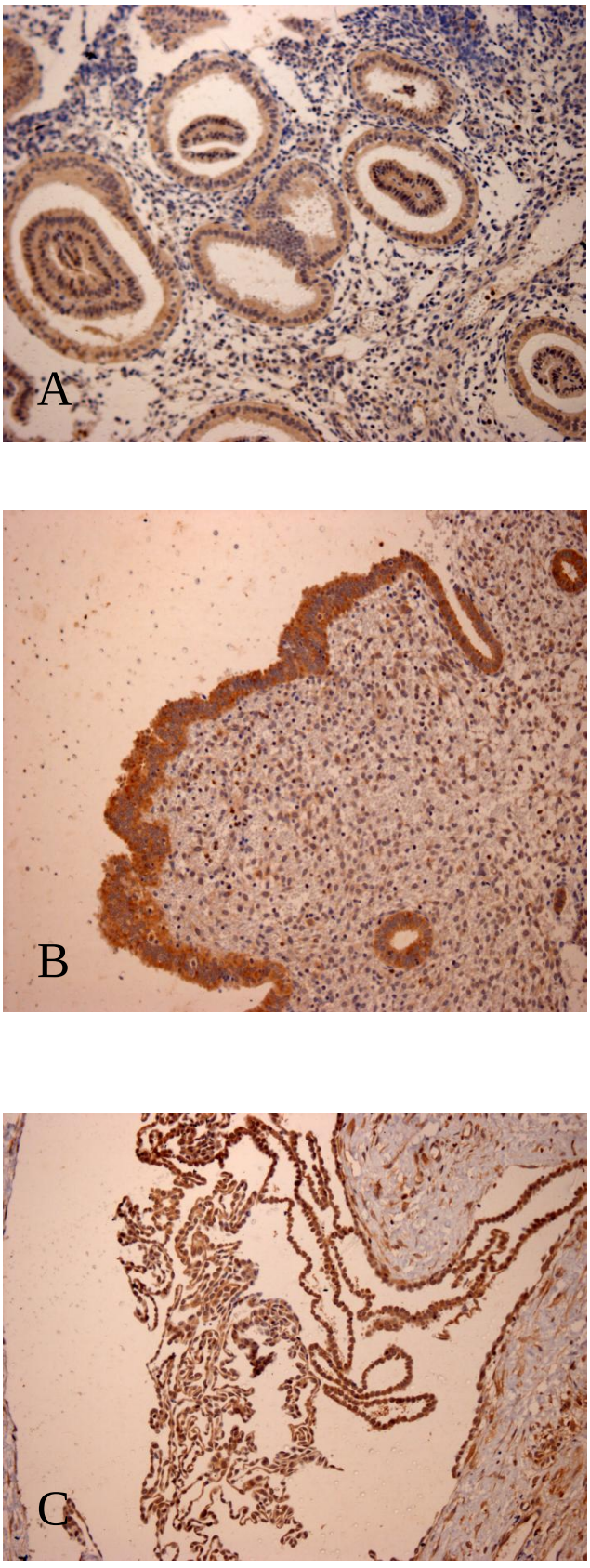
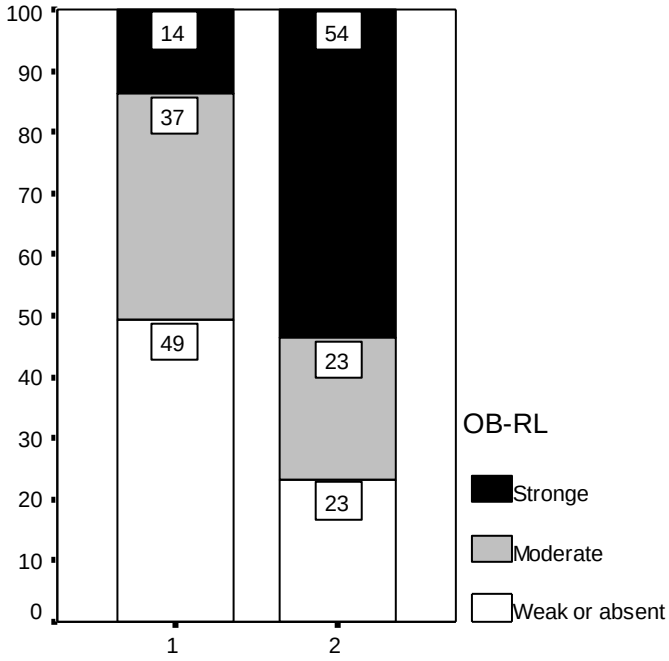
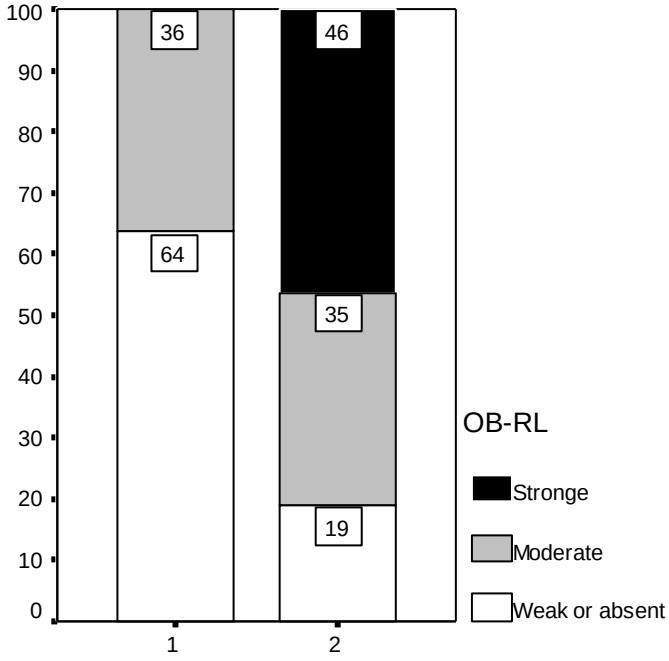


Figure 4:
A



Peritoneal fluid leptin (1:< 13.6; 2:>= 13.6)

B



Peritoneal fluid leptin (1: <13.6; 2>=13.6)

Part III

Artigo original 2:

Interleukin-6 levels in serum and peritoneal fluid of patients with endometriosis

Interleukin-6 levels in serum and peritoneal fluid of patients with endometriosis

A.P. Nácul[1,2], S. B. Lecke[1], D. M. Morsch[1], P. M. Spritzer[1,3]

[1]Gynecological Endocrinology Unit, Division of Endocrinology, Hospital de Clínicas de Porto Alegre and National Institute of Hormones and Women's Health - CNPq, Porto Alegre, 90035-003, Brazil; [2]Human Reproduction Unit, Hospital Fêmina- Grupo Hospitalar Conceição, Porto Alegre, 90430-001, Brazil; [3]Laboratory of Molecular Endocrinology, Department of Physiology, Universidade Federal do Rio Grande do Sul, Porto Alegre, 90050-170, Brazil.

Corresponding author

Poli Mara Spritzer, MD, PhD

Division of Endocrinology, Hospital de Clínicas de Porto Alegre

Rua Ramiro Barcelos, 2350

CEP 90035-003 – Porto Alegre, RS, Brazil

Tel/Fax: +55 51-3359-8027 Fax: +55 51-3359-8777

E-mail: spritzer@ufrgs.br

ABSTRACT

Endometriosis is a chronic inflammatory condition characterized by implantation and growth of endometrial tissue outside the uterine cavity, which seem to depend on an abnormal peritoneal microenvironment with increased levels of pro-inflammatory cytokines and growth factors. Interleukin-6 (IL-6) is a glycoprotein secreted by peritoneal macrophages and endometrial stromal cells. IL-6 has immunoregulatory and angiogenic properties and may have a role in the pathogenesis of endometriosis. This study assessed IL-6 levels in serum and peritoneal fluid (PF) of patients with pelvic endometriosis and evaluated the associations of this cytokine with menstrual phases and severity of endometriosis. Forty-eight women submitted to laparoscopy because of infertility, chronic pelvic pain or tubal ligation agreed to participate and were included in the study. Laparoscopy revealed that 30 had endometriosis; 18 had a normal pelvis and were included in the control group. The severity of endometriosis was graded according to the revised 4-stage American Society for Reproductive Medicine classification (1997). Blood was collected immediately before laparoscopy; PF was collected immediately after starting the procedure and stored in aliquots at -80° C. IL-6 levels were measured using a human IL-6 enzyme immunometric assay. Age and BMI were similar between groups and between the different stages of endometriosis. Serum IL-6 levels were above the test detection limit in only eleven patients, eight in the endometriosis group and three in the control group. In contrast, IL-6 levels in the PF were detectable in 42 participants and were significantly higher in the endometriosis group than in the control group [49.1 (34.2 – 96.2) and 21 (13.2 – 36.2), p=0.04 MWU]. In addition, IL-6 levels in PF were significantly higher in patients with stage III/IV endometriosis [76.9 (48.8 – 134.7)] than in those with stage I/II disease [36 (12.4 – 42.5)] and in control patients with a normal pelvis ([21.3 (12.2 – 36.1] p=0.0001 and p=0.001) (Mann-Whitney U). There was a positive and significant correlation between IL-6 levels in PF and the r-ASRM score of endometriosis severity (RS= 0.77 p<0.001). Our findings suggest that IL-6 may be associated with pelvic endometriosis and its severity. Studies evaluating IL-6 gene and protein expression in topic and ectopic endometrium should be conducted to elucidate the role of this cytokine in the pathogenesis of endometriosis.

Key words: Endometriosis; cytokines; IL-6; peritoneal fluid; infertility; chronic pelvic pain; endometrioma

INTRODUCTION

Endometriosis is a chronic inflammatory condition characterized by implantation and growth of endometrial tissue outside the uterine cavity, which seem to depend on an abnormal peritoneal microenvironment with increased levels of pro-inflammatory cytokines and growth factors. Interleukin-6 (IL-6) is a glycoprotein secreted by several cell types, such as peritoneal macrophages and endometrial stromal cells. IL-6 is a marker of inflammatory response in the acute phase and has several biological activities, such as induction of vascular endothelial growth factor (VEGF) expression (Motro, Itin et al. 1990), growth and differentiation of B lymphocytes and activation of T lymphocytes (Nothnick 2001). IL-6 also has important functions in reproductive physiology, as it regulates ovarian steroid production, folliculogenesis and early events associated with implantation (Akoum, Lemay et al. 1996). Some studies found elevated IL-6 concentrations in peritoneal fluid (PF) of patients with endometriosis in comparison with controls without the disease (Velasco, Acien et al.; Punnonen, Teisala et al. 1996; Harada, Yoshioka et al. 1997), whereas others failed to show this difference (Khan, Masuzaki et al. 2002; Bedaiwy, El-Nashar et al. 2007). Initially, it was believed that PF IL-6 was produced by macrophages, which are found at high concentrations in the PF of patients with endometriosis (Harada, Yoshioka et al. 1997). Later, Tsudo et al. (2000) found that IL-6 was similarly produced by cultured macrophages and endometriotic stromal cells of patients with endometriosis. Moreover, IL-6 mRNA expression and protein production were higher in

endometrioid cells than in eutopic endometrial cells of in vitro controls. TNF-α induced the expression of IL-6 mRNA and the production of IL-6 in both endometrioid and endometrial stromal cells, although endometrioid cells were more sensitive to TNF-α stimulation in a dose-dependent manner (Tsudo, Harada et al. 2000). Bergqvist (2001) examined homogenized eutopic and ectopic endometrial samples from normal controls and patients with endometriosis and found significantly higher levels of IL1-β and IL-6 in ectopic endometrium than in the eutopic endometrium of controls. In contrast, TNF-α levels were higher in the eutopic endometrium of controls. The limitation of their study was the impossibility to determine whether interleukin levels were elevated because of macrophages, endometrial tissue, or both (Bergqvist, Bruse et al. 2001). Several authors have studied serum markers of endometriosis to define parameters to detect patients with initial stages of endometriosis, when ablation or excision of lesions improves reproductive outcomes. (Mihalyi, Gevaert et al.; Gagne, Rivard et al. 2003; Somigliana, Vigano et al. 2004; Bedaiwy, El-Nashar et al. 2007; Martinez, Garrido et al. 2007; Agic, Djalali et al. 2008; Othman Eel, Hornung et al. 2008; Seeber, Sammel et al. 2008). IL-6 seems to be the serum marker with the best discriminatory power, as its sensitivity ranges from 71% to 90%, and specificity, from 51% to 83% using different cut-off values (Bedaiwy, El-Nashar et al. 2007; Martinez, Garrido et al. 2007; Othman Eel, Hornung et al. 2008). Other studies demonstrated that combined analyses of different markers might improve the accuracy of endometriosis diagnosis (Mihalyi, Gevaert et al.; Gagne, Rivard et al. 2003; Somigliana, Vigano et al. 2004; Agic, Djalali et al. 2008; Seeber, Sammel et al. 2008), but results revealed that no highly accurate marker for endometriosis has been defined. Therefore, this study assessed IL-6 levels in serum and peritoneal fluid of patients with pelvic endometriosis and evaluated the associations of this cytokine with menstrual phases and severity of endometriosis.

MATERIAL AND METHODS

Patients

This study enrolled patients who underwent gynecological laparoscopy in the Human Reproductive Unit of Hospital Fêmina and had a diagnosis of endometriosis confirmed during the procedure. Patients without evidence of pelvic disease according to laparoscopic and histopathological examinations were included in the control group (normal pelvis). For 18 months from September 2007 to March 2009, 30 women with endometriosis and 18 women with a normal pelvis were included in the study. Infertility was defined as absence of pregnancy after one year of sexual intercourse without contraception. Chronic pelvic pain was defined as non-cyclical pelvic pain during at least six months and severe enough to cause limitations or request medical treatment (American College of Obstetricians and Gynecologysts). Endometriosis was diagnosed if atypical implants were seen during laparoscopy or confirmed by histological examination of sites with signs suggestive of active endometriosis. The criteria for inclusion were: (1) patients were pre-menopausal (range 21-50 yr) and (2) had not received hormones for at least 3 months before surgery. The exclusion criterion was body mass index (BMI) above 35. The study protocol was approved by the local Ethics Committee (IRB-equivalent), and written informed consent was obtained from all participants.

Study protocol

All the women underwent clinical examination, including measurement of height and weight. Mean BMI was calculated. All laparoscopies were performed by the same surgeon, who diagnosed and staged endometriosis according to the revised American Society for Reproductive Medicine classification (1997).

Procedures were scheduled preferably for the secretory phase of the cycle (74%), but some patients were included in the proliferative phase (20%). Peripheral venous blood samples were collected immediately before laparoscopy, and peritoneal fluid (PF), immediately after the beginning of the procedure. Samples were stored in ice until processing in the laboratory. Cell components from blood and PF were removed by centrifugation at 300 rpm for 20 minutes. The resulting sera were collected and stored in aliquots at -80° C until assayed.

Dates of the menstrual cycle phase were confirmed by histological examination and defined according to the criteria described by Noyes et al. (Noyes et al, 1950). In two patients the endometrial biopsies were unsatisfactory, and one patient had a hysterectomy before the study was completed.

Serum hormone measurements

Serum estradiol and progesterone concentrations were assayed using electrochemiluminescence (Roche Diagnostic, Mannheim, Germany) at a 5.0 pg/ml detection limit and intra-and interassay coefficients of variation below 5%.

IL-6 in serum and peritoneal fluid measurements

IL6 levels were determined using a human IL-6 enzyme immunometric assay (Assay Designs, Ann Arbor, MI) at a detection limit of 3.91 pg/ml. The intra- and interassay coefficients of variation were 5.3 and 12.4.

Statistical analysis

Results are presented as mean ± SD or median and interquartile range. Comparisons between the two group means were analyzed using the Student *t* test; median values were compared using the Mann-Whitney U test. The chi-square test was used to compare qualitative variables. The Pearson rank or the Spearman correlation coefficient was calculated for variables with a Gaussian or non-Gaussian distribution using a two-tailed significance test.

All analyses were performed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA). The level of significance was set at $p<0.05$.

RESULTS

Thirty patients with a diagnosis of endometriosis and 18 patients with a normal pelvis according to laparoscopic results were included in the study. The two groups had a similar mean age (32.6 ± 7.4 versus 33 ± 5.8 p= 0.98, t-test) and BMI (25.5 ± 4.5 versus 25.2 ± 3.5 p=0.86, t-

test). Endometriosis was classified as minimal (stage I) in 13 patients, moderate (stage III) in 7 patients and severe (stage IV) in 10 patients. Active endometriosis, characterized by red lesions, was predominant in seventeen (56.7%) patients; black and white lesions were found in nine (30%) patients; and endometrioma as the main finding of laparoscopy was seen in four (13.3%) patients. In the endometriosis group, laparoscopy was performed for infertility in 11 (36.7%), pelvic pain in 10 (33.3%), adnexal pathology in 7 (23.3%) and tubal ligation in 1 (3.3%). In the control group, laparoscopy was performed for tubal ligation in 13 (72.2%), infertility in 4 (22.2%) and for both tubal ligation and pelvic pain in 1 (5.6%).

Estradiol and progesterone concentrations

Table 1 shows the distribution of patients with endometriosis and control patients according to serum estradiol and progesterone levels and the menstrual cycle phase. Estradiol and progesterone levels were similar in the comparison of grade I/II, grade III/IV and control groups in proliferative as well in secretory phases of the cycle.

IL-6 in serum and peritoneal fluid

Table 2 shows serum IL-6 concentrations in the endometriosis and control groups. Serum IL-6 levels were above the test limit of detection in only eleven patients, eight in the endometriosis group and three in the control group. However, IL-6 levels were detected in the peritoneal fluid of all patients for whom this cytokine was measured (25 patients with endometriosis and 17 control patients). Table 3 shows similar PF IL-6 levels in the proliferative and secretory phases of the cycle. PF IL-6 levels were significantly higher in the endometriosis than in the control group, 21.3 (12.2 – 36.1) and 43.2 (43.2 -76.9), respectively (Figure 1 - A). In addition, PF IL-6 levels were significantly higher in the group of patients with stage III/IV disease [76.9 (48.8 – 134.7)] than in the stage I/II group [36 (12.4 – 42.5)] or control patients [21.3 (12.2 – 36.1] ($p=0.0001$ and $p= 0.001$) (Mann-Whitney U) (Figure 1 – B). However, there were no significant differences in IL-6 levels between patients with stage I/II endometriosis and

the control group (normal pelvis). Figure 2 shows a positive and significant correlation between IL-6 levels in PF and r-ASRM scores of endometriosis severity. (RS= 0.77; p=0.0001)

DISCUSSION

This study found an increase in IL-6 levels in PF of patients with endometriosis in comparison with patients with a normal pelvis. In addition, patients with moderate and severe disease had significant higher levels of PF IL-6 than patients with minimal and mild endometriosis and control patients. Moreover, a strong positive correlation was observed between PF IL-6 and endometriosis severity.

Our results agree in part with the findings reported by Velasco et al (2010), who described increased PF IL-6 levels in patients with moderate or severe endometriosis, but no correlation of IL-6 with endometriosis severity. This might be explained by the fact that only patients with more advanced stages of the disease were included in their study. Punnonen et al. (1996) also demonstrated increased levels of IL-6 and IL-10 in PF of patients with endometriosis, although also without any correlation with disease severity (Punnonen, Teisala et al. 1996). Khan et al (2002) did not find any differences in IL-6 in PF of patients with endometriosis and in controls without the disease, although patients with stage I/II disease had higher levels of IL-6 than those in the stage III/IV group. Laparoscopy revealed that patients with red lesions had higher levels of IL-6, hepatocyte growth factor (HGF), estradiol and progesterone than patients with black and white lesions or controls (Khan, Masuzaki et al. 2002). It is widely believed that atypical lesions (red) are more biologically active than typical ones (black) (Khan, Masuzaki et al. 2004). Patients with early stages of endometriosis but with more active lesions might have an increased level of cytokine secretion that might explain the symptoms of pain and infertility. Active endometriosis lesions, rather than lesion extension, seem to be a more accurate parameter of disease severity. In a previous study, Harada et al. (1997) correlated PF IL-6 levels, its soluble receptor and the tumor necrosis factor-α (TNF-α) with the number and size of endometrial implants in infertile patients. In addition to increased levels of IL-6 and TNF-α in patients with endometriosis, they found a positive correlation

between red lesions and cytokine levels. They suggested that increased levels of cytokines in PF might be associated with infertility and the pathogenesis of endometriosis. Also, the production of IL-6 might be the result of stimulation by increased levels of TNF-α (Harada, Yoshioka et al. 1997). Our endometriosis sample was characterized by active peritoneal and ovarian lesions in 70% of patients. This may be, at least partially, the reason why we found a positive and significant correlation of PF IL-6 levels and severity of the disease.

Harada et al (1997) found significant levels of IL-6 in PF obtained in the secretory phase of the cycle (Harada, Yoshioka et al. 1997). Other authors did not find significant differences in cytokine levels in PF or serum in proliferative or secretory phases (Khan, Masuzaki et al. 2002; Othman Eel, Hornung et al. 2008). In our study, laparoscopies were scheduled preferentially for the secretory phase to evaluate the association between IL-6 and endometrial differentiation, rather than proliferation. However, the material from some participants was collected in the proliferative phase, and serum and PF IL-6 levels could be compared in the two phases for both patients with endometriosis and control participants. Our findings suggest that IL-6 production is not modulated by steroid hormone fluctuations during the menstrual cycle.

According to some studies, different cut-off values for serum levels of IL-6 seen to be good predictors of endometriosis (Bedaiwy, Falcone et al. 2002; Martinez, Garrido et al. 2007; Othman Eel, Hornung et al. 2008). Others have suggested that an association of serum markers might increase diagnostic accuracy (Mihalyi, Gevaert et al.; Somigliana, Vigano et al. 2004) Our study findings are in agreement with those reported by Kalu et al (2007), who did not find any differences in serum IL-6 of patients with endometriosis in comparison with patients without endometriosis (Kalu, Sumar et al. 2007). One limitation of our study was that the levels of IL-6 were detectable in the samples of only a few patients because of the test detection limit. Variations in assay methods, patient selection, or both may also have accounted for differences in our results.

In conclusion, our study suggests that IL-6 may be associated with pelvic endometriosis and its severity. Further studies should evaluate IL-6 gene and protein expression in topic and ectopic endometrium to elucidate the role of this cytokine in the pathogenesis of endometriosis.

FUNDING

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico [CNPq INCT 573747/2008-3] and Fundo de Apoio à Pesquisa of Hospital de Clínicas de Porto Alegre [FIPE-HCPA 07-339], Brazil.

REFERENCES:

(1997). "Revised American Society for Reproductive Medicine classification of endometriosis: 1996." Fertil Steril **67**(5): 817-21.

Agic, A., S. Djalali, et al. (2008). "Combination of CCR1 mRNA, MCP1, and CA125 measurements in peripheral blood as a diagnostic test for endometriosis." Reprod Sci **15**(9): 906-11.

Akoum, A., A. Lemay, et al. (1996). "Secretion of interleukin-6 by human endometriotic cells and regulation by proinflammatory cytokines and sex steroids." Hum Reprod **11**(10): 2269-75.

Bedaiwy, M. A., S. A. El-Nashar, et al. (2007). "Effect of ovarian involvement on peritoneal fluid cytokine concentrations in endometriosis patients." Reprod Biomed Online **14**(5): 620-5.

Bedaiwy, M. A., T. Falcone, et al. (2002). "Prediction of endometriosis with serum and peritoneal fluid markers: a prospective controlled trial." Hum Reprod **17**(2): 426-31.

Bergqvist, A., C. Bruse, et al. (2001). "Interleukin 1beta, interleukin-6, and tumor necrosis factor-alpha in endometriotic tissue and in endometrium." Fertil Steril **75**(3): 489-95.

Gagne, D., M. Rivard, et al. (2003). "Development of a nonsurgical diagnostic tool for endometriosis based on the detection of endometrial leukocyte subsets and serum CA-125 levels." Fertil Steril **80**(4): 876-85.

Harada, T., H. Yoshioka, et al. (1997). "Increased interleukin-6 levels in peritoneal fluid of infertile patients with active endometriosis." Am J Obstet Gynecol **176**(3): 593-7.

Kalu, E., N. Sumar, et al. (2007). "Cytokine profiles in serum and peritoneal fluid from infertile women with and without endometriosis." J Obstet Gynaecol Res **33**(4): 490-5.

Khan, K. N., H. Masuzaki, et al. (2002). "Association of interleukin-6 and estradiol with hepatocyte growth factor in peritoneal fluid of women with endometriosis." Acta Obstet Gynecol Scand **81**(8): 764-71.

Khan, K. N., H. Masuzaki, et al. (2004). "Higher activity by opaque endometriotic lesions than nonopaque lesions." Acta Obstet Gynecol Scand **83**(4): 375-82.

Martinez, S., N. Garrido, et al. (2007). "Serum interleukin-6 levels are elevated in women with minimal-mild endometriosis." Hum Reprod **22**(3): 836-42.

Mihalyi, A., O. Gevaert, et al. "Non-invasive diagnosis of endometriosis based on a combined analysis of six plasma biomarkers." Hum Reprod **25**(3): 654-64.

Motro, B., A. Itin, et al. (1990). "Pattern of interleukin 6 gene expression in vivo suggests a role for this cytokine in angiogenesis." Proc Natl Acad Sci U S A **87**(8): 3092-6.

Nothnick, W. B. (2001). "Treating endometriosis as an autoimmune disease." Fertil Steril **76**(2): 223-31.

Othman Eel, D., D. Hornung, et al. (2008). "Serum cytokines as biomarkers for nonsurgical prediction of endometriosis." Eur J Obstet Gynecol Reprod Biol **137**(2): 240-6.

Punnonen, J., K. Teisala, et al. (1996). "Increased levels of interleukin-6 and interleukin-10 in the peritoneal fluid of patients with endometriosis." Am J Obstet Gynecol **174**(5): 1522-6.

Seeber, B., M. D. Sammel, et al. (2008). "Panel of markers can accurately predict endometriosis in a subset of patients." Fertil Steril **89**(5): 1073-81.

Somigliana, E., P. Vigano, et al. (2004). "Use of the concomitant serum dosage of CA 125, CA 19-9 and interleukin-6 to detect the presence of endometriosis. Results from a series of reproductive age women undergoing laparoscopic surgery for benign gynaecological conditions." Hum Reprod **19**(8): 1871-6.

Tsudo, T., T. Harada, et al. (2000). "Altered gene expression and secretion of interleukin-6 in stromal cells derived from endometriotic tissues." Fertil Steril **73**(2): 205-11.

Velasco, I., P. Acien, et al. "Interleukin-6 and other soluble factors in peritoneal fluid and endometriomas and their relation to pain and aromatase expression." J Reprod Immunol **84**(2): 199-205.

Table 1: Distribution of patients with endometriosis and patients with a normal pelvis (control group) according to serum estradiol and progesterone levels and phase of menstrual cycle.

	Endometriosis		**Controls**	
	I/II	**III/IV**		
N	13	17	18	P*
Phase of cycle n (%)				
Proliferative	3 (23.1)	3 (18.8)	4 (22.2)	
Secretory	10 (76.9)	13 (81.2)	12 (66.7)	
Estradiol (pg/ml)[b]				
Proliferative	122 (11 – 164)	157 (63 – 169)	64.9 (18 – 144)	0.55
Secretory	143 (65 – 231)	102 (42 – 214)	91 (55 – 132)	0.64
Progesterone (ng/ml)[b]				
Proliferative	0.20 (0.16 – 0.79)	0.73 (0.2 – 0.8)	0.42 (0.21 – 0.86)	0.71
Secretory	6.12 (0.92 – 13.22)	1.05 (0.37 – 9.5)	4.15 (0.59 – 9.16)	0.44

[b]: Data presented as median and interquartile range

*: Kruskall-Wallis

Table 2: Serum IL-6 concentrations in patients with a normal pelvis (control group) and patients stratified according to endometriosis stages using the revised American Society for Reproductive Medicine classification (1997). Note: IL-6 was detectable in serum samples of only 8 patients with endometriosis (2 minimal/mild and 6 moderate/severe) and 3 patients with a normal pelvis.

	Control group	Endometriosis	P*
Serum IL-6 (pg/ml)	9.5 (7.9 – 12.4) (n=3)	10.2 (8.5 – 12.6) (n=8)	0.77

*: normal pelvis (control group) x endometriosis (Mann-Whitney U)

Table III: IL-6 in peritoneal fluid in proliferative and secretory phases of the cycle in patients with endometriosis

	Proliferative phase (n=6)	Secretory phase (n=18)	P*
Peritoneal fluid IL-6 (ng/ml)	37.5 (13-238)	47 (31-72)	0.82

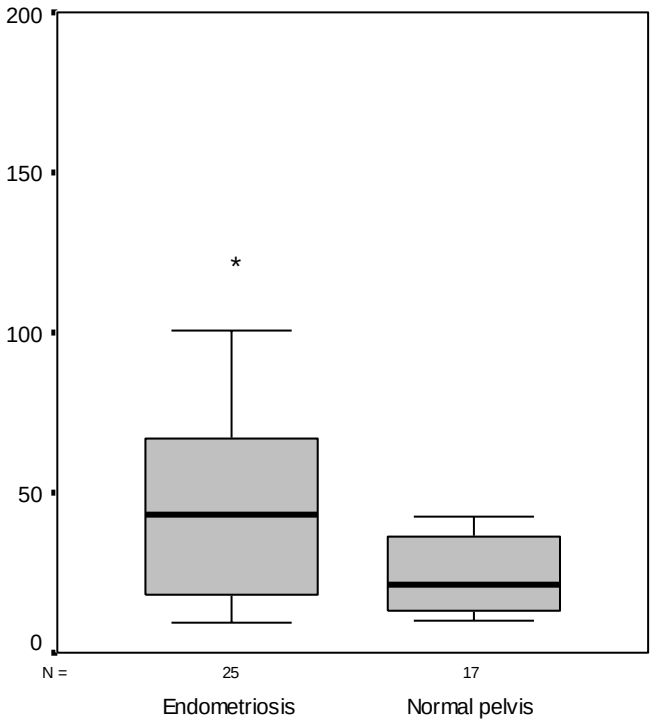
*: Mann-Whitney U

FIGURE LEGENDS:

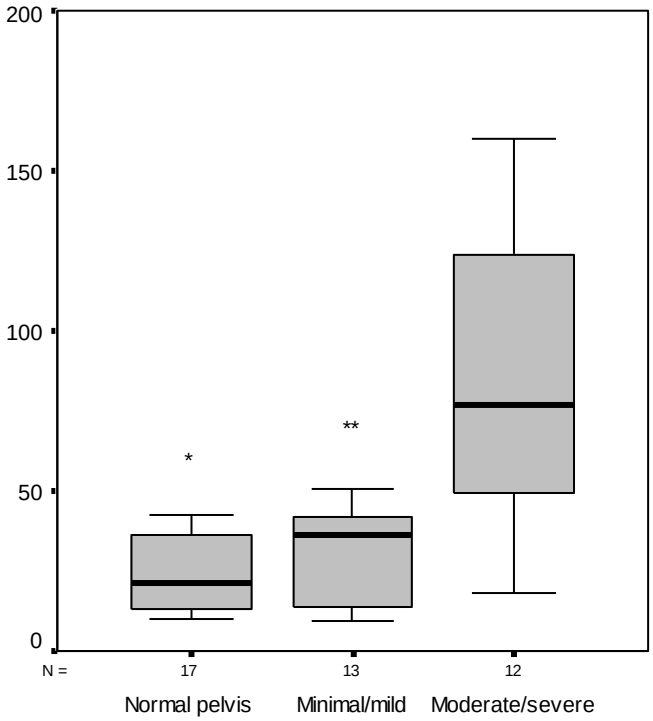
Figure 1: Peritoneal fluid IL6 concentrations in patients with endometriosis and patients with a normal pelvis (control group), *: p=0.047 (MWU) (A) and stratified according to stages of endometriosis using the revised American Society for Reproductive Medicine classification (1997), *: p=0.001 (MWU) (normal pelvis x moderate/severe) and **: p=0.0001 (MWU) (minimal/mild x moderate/severe) (B).

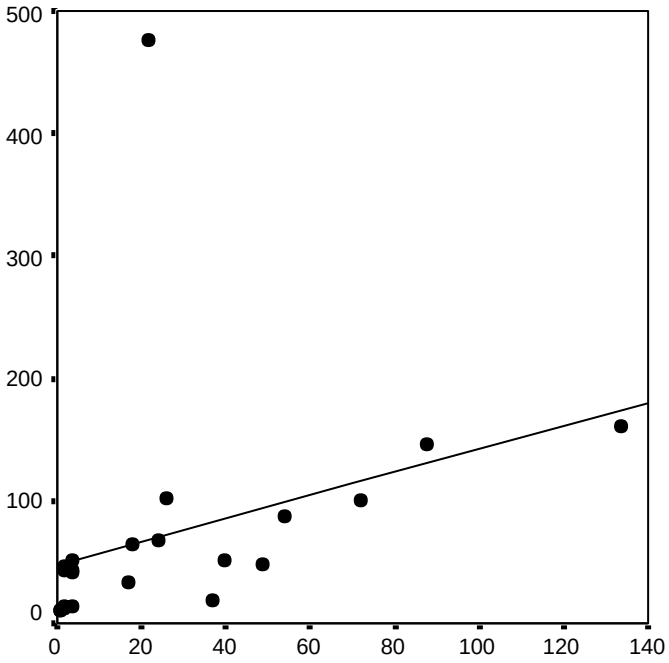
Figure 2: Association between peritoneal fluid IL-6 and r-ASRM score in patients with endometriosis, rs=0.77 p=0.0001(Spearman correlation coefficient).

A


200
150
100
50
0
*
N =
25
17
Endometriosis
Normal pelvis

B


200
150
100
50
0
*
**
N =
17
13
12
Normal pelvis
Minimal/mild
Moderate/severe


500
400
300
200
100
0
0
20
40
60
80
100
120
140
r-ASRM endometriosis score

N125n Nácul, Andrea Prestes

Níveis séricos e no fluido peritoneal de leptina e interleucina-6 e expressão gênica e protéica da leptina e do seu receptor- isoforma longa no endométrio tópico e ectópico de mulheres com endometriose / Andrea Prestes Nácul ; orient. Dra. Poli Mara Spritzer. – 2010.

83 f. : il. color.

Tese (doutorado) – Universidade Federal Rio Grande do Sul. Faculdade de Medicina. Programa de Pós-Graduação em Ciências Médicas: Endocrinologia. Porto Alegre, BR-RS, 2010.

1. Endometriose 2. Leptina 3. Interleucina-6 4. Expressão gênica 5. Endométrio I. Spritzer, Poli Mara II. Título.

NLM: WP 390

Catalogação Biblioteca FAMED/HCPA