Investigación de la osteopontina y la integrina αvβ3 como marcadores de receptividad endometrial para la implantación embrionaria

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TESIS DOCTORAL

Departament d'Obstetrícia i Ginecologia, Pediatria,

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Facultat de Medicina

INVESTIGACIÓN DE LA OSTEOPONTINA Y LA INTEGRINA αvβ3 COMO MARCADORES DE RECEPTIVIDAD ENDOMETRIAL PARA LA IMPLANTACIÓN EMBRIONARIA

Memoria presentada por Gemma Casals Soler para aspirar al grado de Doctor por la Universitat de Barcelona

Directores: Prof. Juan Balasch Cortina i Dr. Jaume Ordi Maja

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Memoria presentada por Gemma Casals Soler para aspirar al grado de Doctor en Medicina por la Universitat de Barcelona, bajo la dirección del Profesor Juan Balasch Cortina, Catedrático de Obstetricia y Ginecología de la Universitat de Barcelona y Director del Institut Clínic de Ginecologia, Obstetrícia i Neonatología del Hospital Clínic de Barcelona, y el Doctor Jaume Ordi, Profesor Titular d'Anatomía Patológica de la Universitat de Barcelona, Consultor Senior del Hospital Clínic de Barcelona y Research professor del Centro de Investigación en Salud Internacional de Barcelona (CRESIB).

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HACEN CONSTAR: Que Dª Gemma Casals Soler ha realizado bajo nuestra dirección el trabajo titulado "Investigación de la osteopontina y la integrina αvβ3 como marcadores de receptividad endometrial para la implantación embrionaria" para aspirar al grado de Doctor en Medicina y que dicho trabajo está en condiciones de ser defendido por la aspirante a partir del día de la fecha.

Lo que hacemos constar a los efectos oportunos en Barcelona a 16 de julio de 2014.

Fdo.: Prof. J. Balasch.

Dr. J. Ordi.

Als meus pares

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PRESENTACIÓN

La presente Tesis Doctoral se estructura siguiendo las directrices de la normativa para la presentación de tesis doctorales como un compendio de publicaciones, aprobada por la Divisió de Ciències de la Salut de la Universitat de Barcelona en octubre del 1999 y asumida con modificaciones mínimas por la Comissió de Doctorat de la Facultat de Medicina el 19 de abril de 2006.

Los estudios que conforman esta Tesis Doctoral pertenecen a una misma línea de investigación. Los resultados obtenidos gracias a la realización de estos estudios, han aportado información relevante y novedosa sobre el tema y han sido recogidos en 3 artículos originales, publicados en diversas revistas de amplia difusión internacional.

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ABREVIATURAS UTILIZADAS EN ESTA TESIS DOCTORAL

ARNm: ácido ribonucleico mensajero

ER: receptor para estrógenos

FIV: Fecundación in vitro

FSH (follicle-stimulating hormone): hormona foliculoestimulante

HB-EGF (heparin binding-epidermal growth factor): factor de crecimiento epidérmico unido a heparina

hCG (human chorionic gonadotropin): gonadotropina coriónica humana

HOXA10: homeobox A10

ICSI (intracytoplasmic sperm injection): inyección intracitoplasmática de espermatozoides

IGF-II (insulin-like growth factor-II): factor de crecimiento tipo insulina II

IGFBP-1 (insulin-like growth factor binding protein 1: proteína de unión del factor de crecimiento tipo insulina-1

IL: interleucina

LDL (low density lipoproteins): lipoproteínas de baja densidad

LH (luteinizing hormone): hormona luteinizante

LIF (leukaemia inhibitory factor): factor inhibidor de leucemia

MMP (matrix metalloproteinases): metaloproteinasas de la matriz extracelular

MUC-1: mucina 1

PR: receptor para progesterona

TGF- β (transforming growth factor β): factor de crecimiento transformador beta

TIMP (tissue inhibitor of metalloproteinases): inhibidor tisular de las metaloproteinasas

VEGF (vascular endothelial growth factor) : factor de crecimiento del endotelio vascular

1. INTRODUCCIÓN: PLANTEAMIENTO GENERAL DEL PROBLEMA Y JUSTIFICACIÓN DE LA TESIS

El ciclo ovárico posee una doble función: la producción periódica de óvulos para ser fecundados y la secreción hormonal adecuada para la implantación del blastocisto en caso de que hubiese fecundación. Ambos procesos dependen de una serie de cambios funcionales propios del ciclo ovárico que tienen una clara traducción morfológica y estructural. Dichos cambios se inician con la selección y desarrollo folicular, la ovulación y la posterior formación y evolución el cuerpo lúteo. La valoración del ciclo ovárico, por tanto, constituye uno de los objetivos básicos en el estudio de la esterilidad (Balasch y Creus, 1999).

En concreto, la función del cuerpo lúteo, cuya misión fundamental es la secreción de progesterona, es de crucial importancia en el proceso reproductivo. La alteración de su función puede conducir a una insuficiencia del cuerpo lúteo. Uno de los requerimientos esenciales para una función luteínica normal es el desarrollo folicular adecuado, ya que el grado de luteinización depende de la acumulación de receptores para la LH en las células de la granulosa durante la fase folicular (Fritz y Fritz, 1991; Sasano y Suzuki, 1997). La función luteínica también depende de la secreción tónica de LH que, junto con la gonadotropina coriónica humana (HCG), son los principales factores luteotrópicos durante el ciclo menstrual y el inicio de la gestación (Patton y Stouffer, 1991; Nakano, 1997). La LH se une a sus receptores y estimula la síntesis de progesterona. El tercer requerimiento es la disponibilidad del colesterol como precursor para la biosíntesis de progesterona. El colesterol se origina a través de síntesis *de novo*, que es insuficiente para los requerimientos del cuerpo lúteo, o por captación a través de las lipoproteínas de baja densidad (LDL), por lo que es fundamental una adecuada vascularización de la granulosa tras la ovulación. Finalmente, la regulación de la función luteínica, que también depende de mecanismos autocrinos y paracrinos (Vega y Devoto, 1997) y de factores inmunológicos (Brännstroöm y Fridén, 1997), es aún en gran parte desconocida.

La secreción de progesterona inhibe el desarrollo folicular mediante una acción directa sobre el ovario, inhibiendo la aromatización, y un mecanismo central, actuando sobre los centros hipotálamo-hipofisarios, alterando la relación de FSH y LH liberadas e instaurando un ambiente gonadotrófico desfavorable para el desarrollo folicular.

El cuerpo lúteo también sintetiza y secreta estrógenos (básicamente 17β-estradiol), andrógenos (androstendiona) e inhibina A, pero esta producción hormonal disminuye de forma rápida a partir de los días 22-23 del ciclo. Esto se asocia a una involución morfológica progresiva del cuerpo lúteo. De hecho, la vida media del cuerpo lúteo no es posible prolongarla indefinidamente ni con estimulación gonadotrófica (Balasch y Creus, 1999). El cuerpo lúteo progresivamente parece ser menos sensible al estímulo de la LH y su capacidad esteroidogénica disminuye. La disminución de la secreción esteroidea ovárica supone el cese de su acción inhibitoria sobre los pulsos de liberación de FSH y LH, que aumentan su frecuencia y, de esta forma, se reactiva el sistema hipotálamo-hipófiso-gonadal y tiene lugar la finalización del ciclo menstrual.

En caso de existir una gestación, la HCG trofoblástica mantiene la función luteínica y el cuerpo lúteo sigue produciendo progesterona para mantener la gestación. La función del cuerpo lúteo es indispensable hasta la séptima semana de gestación, ya que a partir de la octava semana su función se reemplaza por la esteroidogénesis placentaria y tiene lugar la luteolisis.

No obstante, la progesterona no sólo es indispensable para el mantenimiento de la gestación, sino que tiene un papel fundamental para la preparación del endometrio para la implantación embrionaria. De hecho, el endometrio es el principal órgano diana de la progesterona durante el ciclo menstrual. Previamente, durante la fase folicular del ciclo ovárico, los estrógenos deben preparar el endometrio para la acción posterior de la progesterona, estimulando su proliferación y los cambios característicos en las glándulas, estroma y epitelio, así como induciendo la aparición de los receptores de progesterona, que deben estar presentes en una concentración

adecuada para que la progesterona pueda ejercer su función de forma correcta después de la ovulación (Strowitzki y cols., 2006). Posteriormente, la progesterona transforma el endometrio en secretor e inhibe la síntesis de sus propios receptores y de los del estradiol (Young y Lessey, 2010). El endometrio, por tanto, constituye un órgano diana sensible al influjo hormonal ovárico y su análisis se ha considerado un excelente método de estudio de los cambios hormonales del ovario.

La progesterona es un elemento crítico para el desarrollo de una serie de cambios profundos y complejos en el endometrio que le conducirán a un estado receptivo para la implantación del embrión (Lessey, 2011). Se conoce como receptividad endometrial a la capacidad del endometrio de aceptar y acomodar al embrión en desarrollo (Lessey, 2011). Cuando la implantación embrionaria no tiene lugar, la destrucción de la estructura endometrial conduce a la menstruación. Cuando existe implantación, el endometrio continua desarrollando cambios morfológicos y moleculares para las subsiguientes etapas de implantación embrionaria (Cakmak y Taylor, 2011). El endometrio es receptivo a la implantación del blastocisto durante una "ventana" restringida espacial y temporalmente llamada "ventana de implantación". Este periodo, en la fase secretora media, tiene lugar entre los días 20 a 24 del ciclo (Makker y Singh, 2006) y se caracteriza por la expresión de diferentes factores de crecimiento endometriales, citoquinas y moléculas de adhesión (Aghajanova y cols., 2008). De esta forma, el endometrio se convierte en uno de los tejidos más fascinantes del cuerpo humano, cuyo propósito es la implantación de un embrión, aunque solo permite que éste se implante en determinadas condiciones óptimas (Lessey, 2011).

A parte de un endometrio receptivo, la implantación embrionaria exitosa requiere el desarrollo sincrónico de un blastocisto competente para implantar, de forma que se establece un diálogo sincronizado entre el tejido materno y el embrión (Guzeloglu-Kayisli y cols., 2007; Revel, 2012). El diálogo molecular entre el embrión y el endometrio implica interacciones célula-célula y célula-matriz extracelular, mediante

diferentes moléculas de adhesión, factores de crecimiento, citoquinas, proteínas moduladoras, enzimas de degradación de la matriz, hormonas, prostaglandinas, radicales libres, etc. Parece ser que cada uno de ellos, cuando se expresa o inhibe de forma apropiada, contribuye a la receptividad o no receptividad endometrial para la implantación (Makker y Singh, 2006). Por tanto, la consecución del estado de receptividad endometrial para la implantación depende del desarrollo de una red compleja en la que participan numerosos factores. Aunque muchos de ellos ya se han descrito, su función individual y su papel en el complejo desarrollo endometrial no son claramente conocidos (Strowitzki y cols., 2006). Como consecuencia de ello, uno de los principales obstáculos para el éxito reproductivo es nuestra incapacidad para diagnosticar o tratar con eficacia el endometrio no receptivo (Sharkey y Smith, 2003).

Por tanto, el ciclo periódico de formación y desaparición del cuerpo lúteo constituye un evento esencial en la reproducción humana. Su principal producto de secreción, la progesterona, es indispensable para conseguir un estado de receptividad endometrial para la implantación embrionaria. El endometrio es un órgano diana de la secreción hormonal ovárica y, por tanto, el estudio de los cambios que presenta en respuesta a este influjo hormonal es de gran interés para determinar alteraciones que puedan conducir a esterilidad o infertilidad. Un endometrio no receptivo o inadecuado puede considerarse como uno de los principales factores determinantes de fertilidad, pero el establecimiento del estado de receptividad endometrial depende de una red compleja que incluye múltiples factores. De todo lo anterior se deduce la necesidad y a la vez la dificultad de disponer de marcadores endometriales fiables y asequibles en la clínica práctica para el estudio de la receptividad endometrial en la paciente estéril o infértil.

1.1. Valoración de la función luteínica y de la receptividad endometrial

La progesterona, principal producto hormonal secretado por el cuerpo lúteo, es un elemento básico para la adecuada preparación endometrial para la implantación del concepto y el mantenimiento de la gestación en sus fases iniciales. Bajo el término de defecto luteínico se engloba un espectro de trastornos que incluye alteraciones tanto del ciclo ovárico como endometrial. En condiciones normales ambos ciclos suelen coincidir, pero en condiciones patológicas y en los ciclos inducidos existe con frecuencia una disociación entre el funcionalismo ovárico y el endometrial. Así, se establece que la verdadera insuficiencia luteínica correspondería a una situación de déficit de producción de progesterona por parte del cuerpo lúteo, que se traduce en una deficiente transformación secretora del endometrio. Por otro lado, la insuficiencia progestacional de endometrio se caracterizaría por una transformación secretora deficiente en presencia de niveles circulantes de progesterona normales. Por este motivo, los dos métodos tradicionalmente empleados en la valoración de la función luteínica en esterilidad han sido las determinaciones de progesterona plasmática y la biopsia endometrial.

1.1.1. Progesterona plasmática

El método ideal para el diagnóstico de la insuficiencia luteínica sería la determinación diaria de la progesterona plasmática durante la fase lútea. No obstante, a parte de no ser aplicable en la práctica rutinaria, este método presenta otros inconvenientes. Por un lado, el patrón de secreción de la progesterona es pulsátil e incluso su análisis durante tres días a lo largo de la fase lútea no permite predecir el estado funcional del endometrio (Balasch y cols., 1982; Young y Lessey, 2010). Por otro lado, la determinación aislada de progesterona plasmática no permite la valoración del endometrio como elemento esencial en el estudio de la función luteínica. De hecho, la mayoría de defectos progestacionales del endometrio (85-100%) se asocian a niveles normales de progesterona y, por tanto, su determinación

no es predictiva de las características funcionales del endometrio (Balasch y Vanrell, 1987; Soules y cols., 1989; Hecht y cols., 1990).

Esta discrepancia entre niveles de progesterona plasmática normales y transformación secretora endometrial deficiente podría explicarse por un déficit de receptores hormonales que impide que el endometrio responda de forma adecuada al estímulo de la progesterona. Esta entidad, denominada "pseudoinsuficiencia luteínica" (Keller y cols., 1979), está hoy en discusión, ya que los diferentes estudios sobre receptores hormonales y defectos luteínicos aportan resultados contradictorios: mientras algunos autores han detectado concentraciones de receptores similares entre biopsias endometriales normales y deficientes, otros reportan una disminución de estos receptores en los endometrios con déficit luteínico (Young y Lessey, 2010). De todas formas, el estudio de los receptores para el estradiol y la progesterona en el endometrio con déficit luteínico puede tener interés etiopatogénico y científico, pero carece de aplicación en la clínica diaria.

Por tanto, la determinación plasmática de progesterona es un mal predictor del grado de transformación secretora endometrial y carece de aplicación práctica en la clínica diaria para valorar la receptividad del endometrio.

1.1.2. Biopsia de endometrio

Tal como ya se ha comentado, una implantación exitosa requiere un embrión viable en estadio de blastocisto y un endometrio receptivo, de forma que debe existir una buena sincronía entre el desarrollo de ambos elementos. El endometrio humano experimenta una serie de cambios de gran complejidad con el objetivo de presentar las características necesarias que le permitan ser receptivo para la implantación del embrión. El periodo de receptividad endometrial o ventana de implantación es limitado en el tiempo y se sigue de una fase de no receptividad en la que el ambiente uterino resulta hostil para el blastocisto (Revel, 2012).

Por todo ello, la valoración de la función luteínica mediante la biopsia de endometrio se ha considerado tradicionalmente como un elemento básico en el estudio de la paciente estéril o infértil (Jones, 1975; Wentz, 1988). Existen varios factores que han otorgado a la biopsia endometrial un papel principal en la valoración de la función luteínica (Jones, 1975): a) es simple de practicar; b) el endometrio es el órgano diana más directo de la secreción hormonal ovárica; c) el estudio morfológico de las glándulas, el epitelio y el estroma, los tres elementos fundamentales del endometrio, permite el diagnóstico de la ovulación y el pronóstico de la menstruación subsiguiente con un error de ± 1 día; d) su estudio permite la investigación directa de la preparación del endometrio para la implantación embrionaria.

La valoración de la biopsia endometrial se ha realizado tradicionalmente siguiendo los criterios de Noyes y cols. (1950), a partir de los cuales se puede establecer el datado del endometrio con un error de ± 1 día mediante la valoración de parámetros morfológicos con microscopía óptica. El datado histológico resultante de la aplicación de estos criterios se compara con el datado cronológico del ciclo de la mujer, según el primer día de la siguiente regla (que se considera como día 28 del ciclo) o bien según la ovulación (día 14 del ciclo) determinada mediante temperatura basal, detección del pico de LH o ecografía. Cuando el datado histológico presenta un retraso mayor de 2 ó 3 días (según los diferentes autores) con respecto al datado cronológico, se considera que el endometrio está "fuera de fase", sinónimo de fase lútea deficiente. El defecto luteínico objetivado con este método debe evidenciarse al menos en dos ciclos menstruales diferentes para otorgarle valor clínico (Jones, 1975; Wentz, 1888).

No obstante, Noyes y cols, en su artículo de 1950 únicamente establecieron que el datado histológico y cronológico coinciden en ± 1 día en aproximadamente el 80% de los ciclos, pero el objetivo de estos autores no era discriminar entre fase lútea adecuada y deficiente. Sin embargo, sus criterios han sido ampliamente aplicados al

estudio de la fase lútea, especialmente en fase premenstrual inmediata, cuando el endometrio ha recibido todo el influjo hormonal procedente del ovario y, por tanto, debería reflejar al máximo su función hormonal. Por este motivo, la biopsia endometrial se ha practicado de forma rutinaria entre los días 25 a 27 del ciclo tipo, es decir, pasada la fase de receptividad endometrial (Balasch y Vanrell, 1987; Balasch y cols., 1985, 1992).

1.1.3. Limitaciones de la biopsia de endometrio en el estudio de la función luteínica en esterilidad

En la dos últimas décadas se ha puesto en duda la utilidad del datado histológico endometrial mediante los criterios de Noyes y cols., que se había considerado el gold standard para la evaluación endometrial y el diagnóstico de los defectos luteínicos (Balasch y cols. 1992; Castelbaum y cols., 1994; Coutifaris y cols., 2004; Makker y Singh, 2006; Strowitzki y cols., 2006; Cakmak y Taylor, 2011). A continuación se resumen sus principales limitaciones:

a) De acuerdo con resultados de nuestro grupo y de otros autores, el 85-100% de las mujeres con biopsia de endometrio deficiente presentan niveles plasmáticos de progesterona normales (Balasch, 1987; Balasch y Vanrell, 1987).

b) Los resultados de la biopsia de endometrio no guardan relación con la fertilidad de la mujer. Ello queda bien demostrado en dos artículos clave sobre el tema de nuestro grupo (Balasch y cols, 1986, 1992) en los que se demuestra que:

    i. la incidencia de defectos luteínicos diagnosticados por biopsia endometrial es la misma en mujeres estériles que en mujeres fértiles,

    ii. el diagnóstico de un defecto luteínico mediante la biopsia de endometrio es un fenómeno aleatorio,

iii. la evidencia de un defecto progestacional de endometrio o de un funcionalismo normal en el mismo ciclo del embarazo o en ciclos previos de forma repetida, no guarda relación con la evolución del embarazo en la mujer estéril, y

iv. el tratamiento del defecto luteínico endometrial en pacientes estériles no influye sobre la consecución del embarazo ni sobre su evolución (a término o aborto), comparado con las pacientes control no tratadas.

c) La biopsia de endometrio en fase lútea tardía es un mal predictor de la calidad de la fase lútea. Así, se ha demostrado que muchas pacientes estériles con biopsia premenstrual normal presentan un retardo madurativo evidente en la fase lútea media, cuando debería producirse la implantación del blastocisto (Batista y cols., 1993; Castelbaum y cols., 1994).

Estudios posteriores han confirmado que el datado histológico endometrial no tiene la precisión necesaria para el diagnóstico de los defectos luteínicos ni tampoco es útil en el manejo clínico de las pacientes con fallo reproductivo (Coutifaris y cols., 2004; Murray y cols., 2004). No obstante, a pesar de estas notables limitaciones de la biopsia de endometrio, no hay duda del importante papel de una adecuada preparación de la mucosa endometrial para la implantación del embrión y de la relación de la maduración del endometrio con los estímulos hormonales recibidos. Por tanto, se continua considerando que el estudio del endometrio es de crucial importancia en la valoración de la fertilidad de la mujer, pero se ha señalado que el análisis de la biopsia endometrial mediante otras técnicas y a nivel molecular podría ser más predictivo del estado de receptividad endometrial para la implantación (Cakmak y Taylor, 2011).

Por todo ello, se insiste repetidamente en la literatura acerca de la necesidad de buscar nuevos marcadores que permitan establecer la potencial

receptividad del endometrio para la implantación del blastocisto (Makker y Singh, 2006; Strowitzki y cols., 2006; Aghajanova y cols., 2008; Lessey, 2011).

1.2. Receptividad endometrial y endometriosis

La endometriosis es una patología crónica caracterizada por la presencia de tejido endometrial viable fuera de la cavidad uterina (Cakmak y Taylor, 2011). Afecta al 6-10% de las mujeres en edad reproductiva y al 25-50% de las pacientes estériles (Giudice, 2010; Cakmak y Taylor, 2011).

El origen de la endometriosis continua siendo una cuestión sin resolver, ya que no existe ninguna teoría que clarifique los diferentes aspectos de la enfermedad de forma global. Los mecanismos concretos que condicionan la supervivencia y posterior implantación del tejido endometrial ectópico permanecen desconocidos (Burney y Giudice, 2012).

Uno de los factores que pueden influenciar la capacidad de las células endometriales para proliferar, adherirse al mesotelio y/o evadir los procesos inmunológicos que las destruirían son las alteraciones hormonales. La endometriosis se ha considerado tradicionalmente una patología estrógeno-dependiente. De hecho, se ha demostrado que las lesiones endometriósicas presentan un incremento de expresión de la aromatasa y una disminución de 17β-hidroxi-esteroide-dehidrogenasa tipo 2, lo que comporta un gran incremento local de la concentración de estradiol, que a su vez estimula la producción de prostaglandina E2 y ésta estimula aún más la actividad aromatasa (Zeitoun y cols., 1998; Noble y cols., 1997). Además de su condición estrógeno-dependiente, la endometriosis también se caracteriza por presentar resistencia a la progesterona. Las lesiones endometriósicas muestran una reducción de la expresión del receptor de la progesterona y ausencia del receptor PR-B (Attia y cols., 2000). También se ha detectado una regulación anómala de los genes

relacionados con la respuesta al estímulo de la progesterona en la fase lútea endometrial (Burney y cols., 2007).

Aunque no se ha podido establecer una relación causa-efecto entre esterilidad y endometriosis, existen muchos datos que avalan la asociación entre ambas entidades (Practice Commitee of the American Society for Reproductive Medicine, 2012). No obstante, mientras que los estadios mas avanzados de la enfermedad producen una distorsión anatómica que claramente implica un problema de fertilidad para la mujer, los mecanismos responsables de la esterilidad en los estadios iniciales son menos obvios. Se han propuesto múltiples mecanismos para explicar la esterilidad de estas pacientes: foliculogénesis alterada, mala calidad ovocitaria, capacidad de fecundación reducida, embriogénesis anómala y la disminución de la capacidad de implantación embrionaria (Gupta y cols., 2008; Cakmak y Taylor, 2011).

El análisis de los resultados de la Fecundación *in vitro* en estas pacientes nos debería permitir valorar individualmente cada una de las etapas del ciclo reproductivo y, por tanto, ayudar a esclarecer cual de los anteriores mecanismos es el responsable de la disminución de la fertilidad en la endometriosis. Sin embargo, no existe consenso entre los diferentes autores y, mientras algunos no detectan diferencias entre la tasa de implantación de mujeres con o sin endometriosis, otros publican tasas de implantación y de embarazo significativamente menores en pacientes con esta enfermedad (Pal y cols., 1998; Bukulmez y cols., 2001; Barnhart y cols., 2002; Kuivasaari y cols., 2005).

Debido a que la implantación depende tanto de un endometrio adecuado como de un embrión funcional, existe controversia sobre si la posible disminución de la capacidad de implantación de las pacientes con endometriosis se debe a uno u otro de estos factores (Garrido y cols., 2002). Con el objetivo de determinar la posible presencia de cambios en el endometrio de estas pacientes que pudieran afectar su

estado de receptividad, el estudio de marcadores de implantación en las pacientes con endometriosis constituye una área de intensa investigación.

Por tanto, el estudio de los marcadores de receptividad endometrial en las pacientes con endometriosis constituye una área de gran interés para intentar conocer mejor los mecanismos responsables de la esterilidad en estas pacientes, principalmente en los estadios iniciales de la enfermedad, donde no existe una causa anatómica evidente de esterilidad.

1.3. Nuevos marcadores de receptividad endometrial

Tal como se ha comentado, el desarrollo de un endometrio receptivo requiere de la estrecha colaboración de un gran número de factores. Dado que la valoración histológica añade escasa información clínicamente relevante, la búsqueda de nuevos marcadores de función luteínica y receptividad endometrial debe encaminarse a la evaluación funcional del endometrio (Revel, 2012). Por tanto, los esfuerzos deben centrarse en el estudio de la función individual de los diferentes elementos involucrados en la preparación del endometrio receptivo y de su papel dentro de la compleja trama de cambios que tienen lugar. Las relaciones complejas e íntimamente ligadas entre la endocrinología y la inmunología en el proceso de implantación embrionaria nos otorgan el potencial para descubrir nuevos marcadores de receptividad endometrial (Lessey, 2011).

El objetivo, por tanto, es identificar marcadores válidos y reproducibles, que puedan ayudar a la identificación clínica de la ventana de implantación (Revel, 2012). Además, el marcador perfecto tendría que ser funcionalmente relevante dentro del proceso de implantación (Lessey, 2011). Por último, la técnica ideal para valorar la función endometrial y predecir la receptividad del endometrio debería ser de fácil aplicación en la clínica diaria (Strowitzki y cols., 2006; Cakmak y Taylor, 2011).

1.3.1. Marcadores endometriales

I. Marcadores bioquímicos

a) Receptores hormonales

Tal como se ha comentado anteriormente, el influjo hormonal ovárico es crucial para el desarrollo de un endometrio adecuadamente preparado para la implantación embrionaria. Para responder a este estímulo hormonal, las células endometriales presentan receptores para estrógenos (ERα y ERβ) y progesterona (PR-A y PR-B) (Guzeloglu-Kayisli y cols., 2007). Su expresión varia a lo largo del ciclo menstrual: mientras en la fase proliferativa el estradiol induce la expresión de ER y PR, en la fase secretora la progesterona la inhibe (Makker y Singh, 2006). La pérdida selectiva de PR en el epitelio endometrial parece ser crítica para el establecimiento de la receptividad endometrial y su persistencia anómala podría estar asociada con esterilidad (Makker y Singh, 2006). No obstante, los receptores PR-B disminuyen en el epitelio glandular pero persisten en el estroma (Strowitzki y cols., 2006). Por tanto, la acción de la progesterona durante la ventana de implantación probablemente tiene lugar a través de los receptores PR-B del compartimento estromal (Strowitzki y cols., 2006).

No obstante, tal como ya se mencionó antes, los estudios sobre déficit luteínico y receptores esteroideos han aportado resultados contradictorios y, a la espera de nuevas investigaciones, su valoración carece de aplicación clínico-práctica.

b) Factores de crecimiento y citoquinas

Un gran número de factores de crecimiento y citoquinas se expresan de forma cíclica en el endometrio durante diferentes fases del ciclo menstrual y algunas de ellas lo hacen durante la fase secretora media, por lo que se han estudiado como posibles factores asociados con el proceso de implantación.

El *heparin binding-epidermal growth factor* (HB-EGF) presenta dos formas activas: la forma transmembrana presente en el epitelio luminal media la adhesión del embrión en el endometrio a través de su receptor presente en el blastocisto, mientras

que la forma soluble favorece el desarrollo embrionario. Estudios *in vitro* han demostrado que HB-EGF estimula la expresión epitelial de proteínas clave en el endometrio como LIF, HOXA-10 y la subunidad β3 de integrina (Lessey y cols., 2002a). El *insulin-like growth factor binding protein 1* (IGFBP-1) parece interaccionar con el IGF-II producido por el citotrofoblasto y, además, posee una secuencia peptídica RGD que le permite unirse a la integrina αvβ1 en el citotrofoblasto inhibiendo su capacidad de invasión (Strowitzki y cols., 2006). El *transforming growth factor β* (TGF-β) también podría tener un papel inhibitorio sobre la invasión del trofoblasto (Strowitzki y cols., 2006). La alteración de la expresión de un gen perteneciente a su familia, el *endometrial bleeding associated factor* (ebaf), parece estar asociada a varias formas de esterilidad y podría tratarse de un defecto molecular oculto de receptividad endometrial (Tabibzadeh y cols., 2000). El *vascular endothelial growth factor* (VEGF) parece participar en el incremento de la angiogénesis y de la permeabilidad vascular necesarias para la correcta implantación embrionaria (Makker y Singh, 2006).

En el apartado de las citoquinas, el *leukaemia inhibitory factor* (LIF), miembro de la familia de interleucina 6 (IL-6), es un claro ejemplo de marcador de la ventana de implantación (Aghajanova y cols., 2008). Los estudios de delección de su gen en el ratón sugirieron que LIF era esencial para la implantación, pero los resultados obtenidos en el ratón no son extrapolables a nuestra especie y los datos en humanos son aún insuficientes (Strowitzki y cols., 2006). LIF estimula la expresión de genes regulados por la progesterona en el epitelio luminal del ratón y favorece el crecimiento y diferenciación de las células trofoblásticas. Otros estudios sugieren su papel como mediador en las interacciones entre leucocitos deciduales y el citotrofoblasto durante la fase de invasión (Guzeloglu-Kayisli y cols., 2007). Por otro lado, el patrón espaciotemporal de expresión endometrial de IL-6 y principalmente IL-11 nos permite postular sobre su papel en la implantación embrionaria (Guzeloglu-Kayisli y cols.,

2007), mientras que IL-1, IL-11 y IL-15 se consideran importantes en el proceso de invasión (Strowitzki y cols., 2006).

c) Metaloproteinasas de la matriz extracelular (MMP)

Durante la fase secretora del ciclo, la composición de la matriz extracelular endometrial experimenta una serie de cambios para favorecer la implantación. El endometrio humano produce diferentes tipos de MMPs, enzimas que juegan un papel importante en la disolución tisular y cuya expresión está regulada por estradiol y progesterona, así como por los inhibidores tisulares de las MMPs (TIMPs), cuya producción viene determinada por factores endocrinos y paracrinos (Makker y Singh, 2006). Las principales MMPs expresadas durante el proceso de implantación son MMP-2 y MMP-9 (Strowitzki y cols., 2006). El adecuado balance entre MMPS y TIMPs parece ser crucial para la remodelación tisular durante el ciclo menstrual y estas moléculas podrían ser mediadores clave para la degradación de la matriz extracelular durante los procesos de implantación y decidualización (Makker y Singh, 2006; Guzeloglu-Kayisli y cols., 2007).

d) Genes homeobox (HOX)

Los genes Hox son factores de transcripción que actúan como reguladores de la morfogénesis y diferenciación embrionaria (Guzeloglu-Kayisli y cols., 2007). Los genes HoxA10 y HoxA11 se expresan en las glándulas y el estroma endometrial a lo largo del ciclo menstrual, con un gran incremento durante la ventana de implantación que se mantiene durante el resto de fase secretora y, si existe gestación, su expresión permanece elevada en la decidua (Guzeloglu-Kayisli y cols., 2007). Aunque se desconocen los mecanismos exactos a través de los cuales estos genes están implicados en el proceso de implantación embrionaria, se ha observado una alteración en su patrón de expresión en pacientes con endometriosis, hidrosálpinx y síndrome de ovarios poliquísticos (Makker y Singh, 2006; Strowitzki y cols., 2006). Además,

estudios con ratones con HoxA10 mutado han mostrado una alteración en la expresión de genes que responden al estímulo de la progesterona en las células estromales y, por tanto, se ha sugerido que HoxA10 podría tener un papel importante en este sentido (Guzeloglu-Kayisli y cols., 2007).

e) Hormonas peptídicas

Estudios inmunohistoquímicos han mostrado expresión de prolactina en células del epitelio glandular y en estroma endometriales durante la fase secretora media y avanzada (Makker y Singh, 2006). Se ha detectado un fallo de expresión endometrial de esta hormona durante la ventana de implantación en algunas pacientes con esterilidad idiopática y en pacientes con abortos de repetición (Garzia y cols., 2004).

f) Moléculas antiadhesión

En la superficie apical del epitelio endometrial, una barrera de glicoproteínas con propiedades antiadherentes llamadas mucinas evitan la interacción entre embrión y endometrio (Makker y Singh, 2006). La mucina más estudiada en el ámbito de la implantación es la MUC-1, que se expresa durante la ventana de implantación y cuyos cambios en el patrón de glicosilación podrían permitir o evitar la adhesión del embrión en la superficie endometrial y seleccionar el lugar más apto para la implantación (Strowitzki y cols., 2006; Lessey, 2011).

g) Componentes de la matriz extracelular y moléculas de adhesión

El proceso de implantación embrionaria se inicia con la aposición del blastocisto al epitelio luminal del endometrio y, posteriormente, el embrión se adhiere firmemente a la pared uterina y las células trofoblásticas penetran a través del espesor endometrial. Esta secuencia de eventos permitió plantear que los componentes de la matriz extracelular y especialmente las moléculas de adhesión podrían tener un papel crucial en las primeras etapas de la implantación y, por tanto, ser marcadores clave para definir el estado de receptividad endometrial.

La glicodelina A es una de las glicoproteínas más abundantemente secretadas por el endometrio secretor y la decidua durante las primeras etapas gestacionales (Strowitzki y cols., 2006). Se cree que su función estaría relacionada con la tolerancia inmune mediante la inhibición de la actividad de las células Natural Killer (Makker y Singh, 2006; Strowitzki y cols., 2006). La L-selectina, por otra parte, se encuentra expresada en la superficie del embrión preimplantatorio. Sus características funcionales, junto con el incremento de la expresión de ligandos para L-selectina en el endometrio durante la ventana de implantación, han permitido plantear la siguiente hipótesis: el embrión seria transportado a través de la cavidad uterina por mucina hasta que la L-selectina se une a sus receptores en la superficie endometrial y esto permite establecer una posterior interacción más firme endometrio-embrión mediante otras moléculas de adhesión llamadas integrinas (Strowitzki y cols., 2006).

Las integrinas son el grupo de moléculas de adhesión más estudiadas en la investigación de marcadores de receptividad endometrial (Strowitzki y cols., 2006). Tienen una distribución transmembrana y presentan una estructura heterodimérica, conformada por dos subunidades, α y β, de cada una de las cuales existen varios subtipos y, combinándose entre sí, forman las diferentes moléculas completas (Lessey, 2011). Las integrinas se encuentran ampliamente distribuidas en los diferentes tipos celulares y funcionan principalmente como moléculas de adhesión. Su dominio extracelular actúa como receptor de diferentes ligandos de la matriz extracelular como la fibronectina, el colágeno o la laminina (Strowitzki y cols., 2006).

La mayoría de integrinas se expresan de forma constitutiva en el endometrio y se localizan tanto en el epitelio como en el estroma y las células endoteliales. No obstante, la expresión endometrial de algunas integrinas presenta variaciones a lo largo del ciclo menstrual (Lessey, 2002a). En concreto, algunas de ellas se expresan exclusivamente durante la ventana de implantación: la aparición de β3 y el cese de expresión de α4 coincide con la apertura de la ventana de implantación y esto apoya la

hipótesis de que las integrinas podrían ser marcadores clave de receptividad endometrial (Guzeloglu-Kayisli, 2007).

En concreto, la integrina αvβ3 aparece súbitamente en la superficie apical de las células luminales y glandulares coincidiendo con el inicio de la ventana de implantación, continua expresándose hasta el final del ciclo menstrual y persiste en las primeras etapas de la gestación (Lessey y cols., 1992; Lessey y cols., 1994a). Debido a su aparición en el inicio de la fase receptiva endometrial y a sus características funcionales, esta integrina ha sido extensamente estudiada como marcador de receptividad endometrial y, de hecho, es uno de los marcadores aún hoy mejor caracterizados (Lessey, 2011). Además, se ha publicado que existe una disminución de la expresión de la integrina αvβ3 en el endometrio de pacientes con endometriosis, hidrosálpinx, esterilidad idiopática y síndrome de ovarios poliquísticos (Lessey y cols., 1994b, 1995; Meyer y cols., 1997; Apparao y cols., 2002).

No obstante, aunque se sigue considerando que las integrinas presentan un importante papel en el proceso de implantación embrionaria, su uso como marcador individual de receptividad endometrial ha sido cuestionado por estudios de nuestro grupo y otros debido a que: a) la expresión de la integrina αvβ3 en el endometrio está íntimamente relacionada con el grado de maduración endometrial, independientemente de que el endometrio esté o no "fuera de fase"; b) no se observan diferencias en la expresión de este marcador en el endometrio de pacientes estériles y mujeres de fertilidad probada; c) su expresión endometrial no guarda relación con la consecución de gestación espontánea o no por parte de la paciente estéril y; d) presenta baja reproducibilidad y alta variabilidad ciclo a ciclo (Creus y cols., 1998; Creus y cols., 2002; Ordi y cols., 2002; Ordi y cols., 2003b).

Otra molécula que suscita gran interés en el contexto de la implantación embrionaria es la osteopontina, el principal ligando de la integrina αvβ3 (Apparao y cols., 2001; DuQuesnay y cols., 2009). Es una proteína glicosilada y fosforilada,

originariamente identificada como constituyente de la matriz ósea y que contiene una secuencia tripeptídica (RGD) capaz de unirse a la integrina αvβ3 (Apparao y cols., 2001). La osteopontina se encuentra expresada en múltiples tejidos humanos. En el endometrio, se detecta en las células del epitelio luminal y glandular durante la fase secretora media y avanzada del ciclo (Apparao y cols., 2001; von Wolff y cols., 2001), en las secreciones uterinas durante la fase secretora (von Wolff y cols., 2001) y en la decidua en caso de existir gestación (Apparao y cols., 2001; von Wolff y cols., 2004). Se postula que su papel no se limitaría a la adhesión del embrión al epitelio endometrial, sino que podría participar en estadios posteriores de la implantación embrionaria, concretamente en la invasión del trofoblasto, y que también podría estar implicada en la modulación de la inmunidad celular mediante la regulación del conjunto de citoquinas liberadas por las células inmunológicas (von Wolff y cols., 2001).

La osteopontina y su receptor, la integrina αvβ3, se expresan de forma coordinada en el endometrio humano durante la fase secretora del ciclo (Apparao y cols., 2001; von Wolff y cols., 2001). Se ha propuesto que, durante la fase de receptividad endometrial, la osteopontina procedente de las secreciones del epitelio endometrial entraría en el lumen y se uniría a su receptor, la integrina αvβ3, en la superficie apical del epitelio (Apparao y cols., 2001). Por tanto, su aparición conjunta en la superficie endometrial durante la ventana de implantación podría constituir un elemento crucial para promover las primeras fases de la implantación embrionaria y, de este modo, la expresión endometrial del binomio integrina αvβ3 / osteopontina podría ser clave para determinar el estado receptividad endometrial para la implantación.

II. Marcadores morfológicos: pinópodos

Los pinópodos son estructuras microscópicas que están presentes en la superficie apical del epitelio luminal durante el periodo de receptividad, en forma de protrusiones esféricas sin microvellosidades que corresponden a proyecciones

citoplasmáticas sobre la superficie celular (Psychoyos y Martel, 1990; Nikas, 2000; Strowitzki y cols., 2006). También presentan un patrón de expresión temporo-espacial característico a lo largo del ciclo menstrual: en el ciclo menstrual normal se observarían entre los días 19 y 21, es decir, durante la primera mitad de la fase de receptividad uterina (Hoozemans y cols., 2004; Lessey, 2011). Su formación está estimulada por la progesterona (Strowitzki y cols., 2006). La función de los pinópodos no está clara, pero se cree que podrían estar implicados en la concentración de fluido endometrial cerca del lugar de implantación, facilitando el proceso de adhesión del embrión (Stavreus-Evers y cols., 2001).

A pesar de que los pinópodos han sido motivo de múltiples investigaciones en las dos últimas décadas, algunas de ellas por parte de nuestro grupo, su validez como marcadores de receptividad endometrial ha sido puesta en duda (Creus y cols., 2002; Ordi y cols., 2003a; Casper, 2011; Lessey, 2011).

III. Marcadores inmunológicos: células Natural Killer uterinas

Las células Natural Killer uterinas son diferentes a las células Natural Killer circulantes, tanto fenotípicamente como funcionalmente, ya que se caracterizan por presentar una baja citotoxicidad directa (Guzeloglu-Kayisli y cols., 2007). En humanos, su presencia en el útero varía a lo largo del ciclo menstrual, de forma que son escasas en la fase proliferativa e incrementan gradualmente durante la fase lútea media hasta llegar al máximo número en la fase secretora tardía. Si no existe gestación, unos 2 días antes de la menstruación, las células Natural Killer uterinas empiezan a experimentar cambios nucleares similares a la muerte por apoptosis. Si existe gestación, siguen presentes en el útero gestante, principalmente en la zona de contacto con las células trofoblásticas durante el proceso de invasión. Su presencia llega a ser máxima durante el primer trimestre, cuando representan el 70% de los linfocitos (Guzeloglu-Kayisli y cols., 2007). El estímulo que desencadena este reclutamiento masivo de células no es bien conocido y en la actualidad se está

investigando sobre las citoquinas responsables de este fenómeno (Wold y Arici, 2005). Por otra parte, estudios con ratones han permitido determinar la contribución de las células Natural Killer uterinas a la implantación embrionaria y a la formación de la placenta (Guimond y cols., 1998).

En resumen, el estudio de los marcadores bioquímicos, morfológicos e inmunológicos hasta ahora expuestos ha contribuido a un mejor conocimiento sobre su expresión endometrial y ha permitido establecer hipótesis sobre su función individual durante el proceso de implantación embrionaria. No obstante, ninguno de ellos ha demostrado ser útil de forma individual como indicador de un endometrio receptivo funcional.

1.3.2. Limitaciones del estudio individual de marcadores de receptividad endometrial

Tal como se ha comentado anteriormente, el desarrollo de un endometrio receptivo requiere de la colaboración de un gran número de factores. Existen varias limitaciones para la identificación de un elemento que, de forma individual, constituya un marcador de receptividad endometrial para la implantación con aplicabilidad en la práctica clínica (Sharkey y Smith, 2003; Aghajanova y cols., 2008; Cakmak y Taylor, 2011):

a) Existe variabilidad entre los diferentes sujetos estudiados, debido al gran número de factores implicados y de cambios que deben tener lugar para llegar al estado de receptividad. El momento exacto en el que se producen estos cambios y la magnitud de cada uno de ellos puede ser variable, incluso en mujeres con ciclos regulares y sometidas a un riguroso control del mismo.

b) La heterogeneidad dentro de los grupos de estudio puede condicionar que se incluyan en un mismo grupo a mujeres con defectos luteínicos, con defectos ocultos en la expresión de determinados marcadores a pesar de presentar

una histología endometrial normal, o pacientes subfértiles en las que también pueden existir cambios en la expresión de los marcadores.

c) El endometrio presenta plasticidad en su diferenciación. Así, los estudios que han utilizado más de una biopsia en el mismo ciclo menstrual han mostrado que estas muestras seriadas pueden presentar un resultado anómalo en la fase secretora precoz y una total normalidad en una fase más avanzada.

d) La falta de correlación entre la expresión de un determinado marcador y el pronóstico reproductivo de los pacientes estudiados nos indica que no es útil para el diagnóstico y manejo clínico.

e) Existe una cantidad enorme de factores implicados en el proceso de implantación embrionaria. Por tanto, la identificación de un único marcador como responsable del establecimiento de la receptividad endometrial es extremadamente compleja. Además, existe redundancia funcional, es decir, que un miembro de una familia de genes puede compensar funcionalmente a otro.

f) Los resultados de los estudios con animales no siempre son superponibles al humano, ya que el proceso de implantación varía entre las diferentes especies. Por tanto, estas investigaciones nos proporcionan solo una evidencia indirecta de la función de estos marcadores.

g) Los estudios *in vivo* se han realizado en ciclos no concepcionales y, por tanto, no sujetos a las señales embrionarias que influencian de forma directa o indirecta al endometrio desencadenando cambios morfológicos, bioquímicos e inmunológicos.

Todas estas limitaciones han dificultado la valoración del papel exacto de los diferentes marcadores de receptividad endometrial mediante su estudio individualizado. El desarrollo de las técnicas de genómica y proteómica ha

permitido el estudio simultáneo de gran número de genes y proteínas endometriales y, de esta forma, ha permitido arrojar luz sobre algunas de estas limitaciones, lo que a su vez ha llevado a un interés renovado por los diferentes potenciales marcadores de receptividad endometrial.

1.3.3. Nuevas tecnologías aplicadas a la búsqueda de marcadores de receptividad endometrial: las "ómicas"

La aparición de las llamadas "ómicas" ha permitido realizar comparaciones a gran escala de células y tejidos en diferentes condiciones. En los últimos años han aparecido las primeras publicaciones sobre el uso de la proteómica y la secretómica para la investigación de la receptividad endometrial, pero la técnica más utilizada hasta hoy ha sido la genómica.

El desarrollo de las técnicas de genómica, principalmente a través del uso de *microarrays*, ha constituido una nueva y poderosa herramienta para identificar marcadores de receptividad mediante un abordaje global. Estas técnicas permiten el análisis simultáneo de miles de mRNAs y deben asociarse a sofisticadas herramientas de bioinformática para analizar los resultados obtenidos. El objetivo principal es captar un perfil molecular característico del endometrio receptivo (Hoozemans y cols., 2004).

Mediante estas técnicas se ha observado que los genes que aumentaron su expresión durante la fase secretora media corresponden mayoritariamente a proteínas de la superficie celular, componentes de la matriz extracelular, citoquinas, factores de crecimiento, genes que codifican para la señalización intracelular, proteínas del ciclo celular y moléculas relacionadas con el sistema inmunitario (Aghajanova y cols., 2008). De esta forma, la genómica ha permitido comprender procesos biológicos y rutas bioquímicas y de señalización que tienen lugar durante la ventana de implantación en el endometrio humano.

No obstante, existen discrepancias notables entre los resultados de los estudios publicados hasta hoy, que pueden atribuirse a diferencias en el diseño utilizado: el número de pacientes y muestras incluidas en el estudio, la edad de los pacientes, el tipo de *array* utilizado, las fases del ciclo comparadas, los criterios para determinar el momento de realizar la biopsia de endometrio, el utilizar o no *pools* de muestras y los criterios utilizados en el análisis estadístico de los resultados (Aghajanova y cols., 2008; Koot y cols., 2012; Ruiz-Alonso y cols., 2012). De hecho, entre los genes que aumentan su expresión durante la fase de receptividad, el único en común en los primeros estudios publicados fue el de la osteopontina (Aghajanova y cols., 2008). Aunque estas discrepancias no han hecho posible establecer un consenso sobre los genes involucrados en la fase receptiva, los diferentes estudios muestran la existencia de un perfil transcriptómico típico de la ventana de implantación.

Los estudios de genómica, por tanto, han identificado centenares de genes diferencialmente expresados y que podrían ser potenciales marcadores. No obstante, mientras el número de marcadores potenciales crece exponencialmente, la validación de los mismos está siendo limitada y la translación de esta investigación básica a la clínica diaria está siendo compleja (Strowitzki y cols., 2006; Lessey, 2011). El reto es identificar marcadores válidos y reproducibles que puedan ayudar a la valoración clínica de la receptividad endometrial (Casper, 2011; Revel, 2012). Sin embargo, el gran incremento de potenciales candidatos dificulta centrarse en un determinado biomarcador. Los datos obtenidos mediante esta tecnología dan lugar a un problema de interpretación de la relevancia funcional de estos genes que podría solucionarse mediante estudios funcionales posteriores (Makker y Singh, 2006).

En resumen, las técnicas de genómica han permitido identificar un gran número de genes sobreexpresados durante la ventana de implantación. No obstante, las limitaciones técnicas expuestas anteriormente dificultan la identificación de marcadores de receptividad endometrial mediante esta

metodología y, por tanto, hoy por hoy, la aplicabilidad de estas técnicas a la clínica diaria. Por otro lado, la forma más simple y eficiente de estudiar la expresión génica es la identificación de proteínas específicas mediante anticuerpos en estudios de citometría de flujo, inmunocitoquímica e inmunohistoquímica (Uhlen y Ponten, 2005; Serafini y cols., 2009), ya que la recomposición del ARNm, su modificación y procesamiento altera la producción de las proteínas funcionales (Azad y cols., 2006). La inmunohistoquímica, técnica utilizada en los estudios que conforman la presente Tesis Doctoral, permite el análisis directo de la expresión proteica y proporciona información sobre la localización histológica y la distribución de la proteína a nivel subcelular y, por tanto, es particularmente apropiada para estudios clínicos. Tal como se señala en la literatura, la inmunohistoquímica posee gran número de aplicaciones prácticas en patología ginecológica (Yaziji y Gown, 2001). Por último, esta técnica se considera fundamental para determinar las características clinicopatológicas de las proteínas identificadas mediante estudios de proteómica y genómica (Pei y cols., 2007).

Por otro lado, resulta de gran interés el hecho de que, a diferencia de muchas de las moléculas anteriormente estudiadas por su implicación en el proceso de implantación y, a pesar de las discrepancias existentes entre los diferentes estudios de genómica, la osteopontina, motivo de investigación en esta tesis, esté presente en la mayoría de estos estudios como molécula que experimenta un incremento de su expresión durante la fase receptiva para la implantación embrionaria. Estos datos apoyan el posible papel crucial en el establecimiento de la receptividad endometrial de este marcador y de su receptor, la integrina αvβ3, uno de los biomarcadores más estudiados y con el que comparte el mismo patrón de expresión en el endometrio. El estudio del binomio osteopontina / integrina αvβ3 podría por tanto en teoría, contribuir a la identificación de marcadores clínicamente útiles para definir la ventana de

implantación y el funcionalismo uterino, tanto en condiciones fisiológicas, como la respuesta a cambios de los niveles hormonales, como patológicas. En este último ámbito, el estudio de estos marcadores en las pacientes con endometriosis podría contribuir a conocer mejor los mecanismos implicados en la esterilidad de estas pacientes, principalmente en los estadios iniciales de la enfermedad, donde la causa responsable de la no consecución del embarazo no está bien establecida.

Y es en este sentido que se comprenden la hipótesis de trabajo y los objetivos de esta Tesis Doctoral tal como se exponen a continuación.

2.HIPÓTESIS DE TRABAJO

1. Estudios previos de nuestro grupo han demostrado que el estudio histológico del endometrio carece de utilidad clínica práctica para valorar la receptividad endometrial para la implantación. Sin embargo, dicha evaluación de la receptividad es un requisito indispensable en el estudio de la esterilidad.

2. El endometrio constituye un elemento esencial en el proceso de implantación embrionaria, en el que tienen lugar profundos y complejos cambios y reestructuraciones programadas en los períodos pre- y post-implantación en sus diferentes tipos celulares.

3. Se ha descrito que tanto la integrina αvβ3 como la osteopontina presentan unos patrones de expresión temporales y espaciales específicos en los diferentes tipos celulares del endometrio, patrones de expresión que definirían la fase de receptividad endometrial para la implantación embrionaria o "ventana de implantación".

En base a lo anterior, nuestra **hipótesis de trabajo** es que la identificación y estudio de la osteopontina y la integrina αvβ3 como marcadores endometriales de implantación embrionaria en diferentes situaciones clínicas, debería permitir ir más allá de los simples criterios clásicos morfo-histológicos valorados por microscopia óptica y de esta forma poder definir de una manera más precisa y objetiva el grado de receptividad endometrial para la implantación embrionaria.

3. OBJETIVOS

1. Estudiar si la expresión de osteopontina e integrina αvβ3 constituye o no una manifestación funcional independiente del datado histológico clásico del endometrio **(Estudio 1).**

2. Investigar si existe o no una buena correlación temporal entre la expresión de osteopontina e integrina αvβ3 y determinar si delimitan de forma adecuada la ventana de implantación **(Estudio 1).**

3. Establecer si existen o no diferencias en la expresión y coexpresión de osteopontina e integrina αvβ3 durante la ventana de implantación en el endometrio de pacientes estériles y mujeres de fertilidad probada **(Estudio 1).**

4. Investigar si la expresión y la coexpresión de osteopontina e integrina αvβ3 durante la ventana de implantación se relaciona con la causa de esterilidad y con la instauración ulterior de la gestación **(Estudio 1).**

5. Determinar los efectos sobre la expresión endometrial de osteopontina e integrina αvβ3 de diferentes terapéuticas hormonales empleadas en esterilidad o en el control de la fertilidad (inductores de la ovulación, estimulación ovárica para Fecundación *in vitro*, gestágenos, tratamiento hormonal sustitutivo para preparación endometrial, contraceptivos orales) **(Estudio 2).**

6. Estudiar si la expresión o coexpresión endometrial de osteopontina e integrina αvβ3 permite discriminar la receptividad endometrial en pacientes con estadios iniciales de endometriosis (grados I y II) **(Estudio 3).**

4.INVESTIGACIONES REALIZADAS,

MÉTODOS Y RESULTADOS

La descripción de las pacientes, así como la metodología utilizada en las investigaciones llevadas a cabo para el logro de los objetivos planteados antes, se encuentran detalladamente expuestas en las secciones de "Material y Métodos" de cada uno de los tres artículos que constituyen el cuerpo doctrinal de la presente Tesis Doctoral.

Dichos artículos se incluyen a continuación tal como han sido publicados en la literatura científica (páginas 41 a 77).

ESTUDIO 1

"Expresión de la osteopontina y la integrina αvβ3 en el endometrio de mujeres estériles y fértiles"

"Osteopontin and αvβ3 integrin expression in the endometrium of infertile and fertile women"

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Article

Osteopontin and $\alpha_v\beta_3$ integrin expression in the endometrium of infertile and fertile women

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Abstract

Osteopontin and its receptor $\alpha_v\beta_3$ integrin have recently been proposed as a major complex to promote embryo attachment, and thus they would be useful as markers of endometrial receptivity. In the current study $\alpha_v\beta_3$ integrin and osteopontin expression and co-expression in in-phase and out-of-phase endometrial biopsies from normal healthy women ($n = 12$) and infertile patients ($n = 107$) were investigated. Two endometrial biopsies (post-ovulatory day +6 to +8, and 4 days later) were performed during a single menstrual cycle in each subject. Oestradiol and progesterone serum concentrations were quantified on the same days as endometrial sampling. No statistically significant difference regarding $\alpha_v\beta_3$ integrin and osteopontin expression and their co-expression was found between fertile controls and infertile patients irrespective of endometria being in-phase or out-of-phase, infertility factors detected or whether patients became spontaneously pregnant or not. Although a co-ordinate high concentration of both glycoproteins on post-ovulatory day 8 onwards was observed, there was an evident lack of temporal co-expression of these markers during the implantation window. It is concluded that the functional significance of the osteopontin:$\alpha_v\beta_3$ integrin complex as a marker of endometrial receptivity and implantation potential in women seems to be untenable.

Keywords: *endometrium, implantation, infertility, integrins, osteopontin*

Introduction

Implantation of the human blastocyst is a complex process involving the ovary, endometrium and embryo. Successful implantation requires a receptive endometrium and the ovary provides the hormonal stimulus for establishment of a successful pregnancy (Lessey, 2002; Achache and Revel, 2006). In this process, the embryo must be able to interact with the endometrium, but it has been estimated that the embryo itself is responsible for only one-third of implantation failures, whereas inadequate endometrial receptivity would explain approximately two-thirds of these failures (Achache and Revel, 2006). Thus, the human endometrium as a fertility-determining factor is currently a matter of great interest (Sharkey and Smith, 2003; Hoozemans *et al.*, 2004; Bulletti *et al.*, 2005; Minas *et al.*, 2005; Achache and Revel, 2006; Aplin, 2006; Strowitzki *et al.*, 2006; Quenby *et al.*, 2007).

The receptive endometrium develops when an adequate secretory transformation of the oestrogen-primed endometrium occurs in response to progesterone. A number of architectural, cellular, biochemical and molecular events occur in the endometrium within the so-called window of implantation (i.e. 6–8 days after ovulation) (Castelbaum *et al.*, 1994; Santoro *et al.*, 2000). Investigation of endometrial function in infertility has traditionally been assessed by dating premenstrual endometrial biopsy specimens according to the morphological criteria reported more than half a century ago by Noyes *et al.* (1950). Until recently, premenstrual endometrial dating was considered as the 'gold standard' for endometrial function evaluation (Balasch, 2000), but over the past 10–15 years the relationship between histological changes and endometrial receptivity has been seriously questioned (Balasch *et al.*, 1992; Castelbaum *et al.*, 1994; Somkuti *et al.*, 1995; Coutifaris *et al.*, 2004).

In addition to morphological changes, a number of biomarkers of normal endometrial development and receptivity have been described recently (Sharkey and Smith, 2003; Hoozemans *et al.*, 2004; Bulletti *et al.*, 2005; Minas *et al.*, 2005; Achache and Revel, 2006; Makker and Singh, 2006; Quenby *et al.*, 2007). On the basis that initial attachment of the embryo on the endometrial epithelium would involve cell adhesion events, cell adhesion molecules have been proposed as the markers of choice to assess uterine receptivity in infertility, with $\alpha_v\beta_3$ integrin being the best characterized cell adhesion molecule on the luminal surface of the endometrium (Lessey, 2002; Sharkey and Smith, 2003; Hoozemans *et al.*, 2004; Achache and Revel, 2006; Aplin, 2006). The $\alpha_v\beta_3$ integrin ligand is osteopontin (OPN), and this has been shown to bind to several different integrin receptors, but $\alpha_v\beta_3$ integrin has been recognized as a primary receptor for OPN (Lessey, 2002). In fact, OPN and its receptor $\alpha_v\beta_3$ integrin have been found to be co-ordinately expressed in the human menstrual epithelium across the menstrual cycle in normally cycling fertile women, with both glycoproteins being maximally expressed at the time of the implantation window (Apparao *et al.*, 2001). Thus, a complementary role for these proteins in endometrial function and implantation has been suggested, and it has been proposed that an OPN:$\alpha_v\beta_3$ integrin complex may occur at the apical surface of human uterine luminal epithelium to promote embryo attachment, as has been suggested in other species (von Wolff *et al.*, 2001; Lessey, 2002; Johnson *et al.*, 2003; Makker and Singh, 2006).

Based on the above evidence, the current study was undertaken to investigate $\alpha_v\beta_3$ integrin and OPN expression and co-expression in normal and out-of-phase endometrial biopsies form both normal healthy women and infertile patients.

Materials and methods

Patients and study cycle

This study included 107 consecutive infertile patients undergoing a routine work-up, and 12 fertile controls who were undergoing tubal sterilization. All women involved gave informed consent to participate in the present study, which was approved by the Ethics Committee of the Hospital Clínic. The mean (±SEM) age of infertility patients was 31.8 ± 1.6 years (range 23–42 years) and all of them had a history of normal ovulatory menstrual cycles (25–33 days). The main causes of infertility in these women were: non-absolute male factor (sperm concentration >5 × 10^6/ml to <20 × 10^6/ml, and/or <50% motile sperm grades 'a' and 'b' according to World Health Organization criteria) (Rowe *et al.*, 2000) (n = 30); endometriosis (n = 24); unexplained (n = 22); tubal factor with no hydrosalpinges (n = 14); other (incomplete work-up, multiple causes, etc.) (n = 17). As male infertility in a couple may imply a normal female fecundity potential, and endometriosis and unexplained infertility have been reported to be associated with increased aberrant integrin expression (Lessey *et al.*, 1994, 1995), results were analysed considering these different aetiologies of infertility. The control group included 12 fertile (mean parity 1.4, range 1–4) healthy women aged 29–41 years (mean 33.8 ± 1.1 years). These control women had regular menstrual cycles (27–32 days) and were taking no medication. In all women, basal body temperature, luteal serum concentrations of oestradiol and progesterone, and endometrial biopsies, were obtained in the same cycle to assess luteal function according to a scheme of evaluation previously reported (Creus *et al.*, 2002). All control women and a number of infertile patients had participated in previous published studies (Creus *et al.*, 2002; Ordi *et al.*, 2002).

Starting on day 8–10 of the study cycle (depending on the cycle length of the woman), all patients underwent daily transvaginal ultrasonographic evaluation to determine follicular growth using a 5 MHz vaginal transducer attached to an Aloka scanner (Model SSD-620; Aloka Co. Ltd, Tokyo, Japan). The maximum follicular diameter was measured in all patients. Both ovaries were identified, and the largest diameter was measured in both the longitudinal and transverse dimensions in all follicles. The day of ovulation was designated as the day of maximum follicular enlargement, which was followed the next day by sudden disappearance or filling in of this follicle showing loss of clear demarcation of its walls and appearance of intrafollicular echoes (Shoupe *et al.*, 1989; Peters *et al.*, 1992). Ultrasonographic monitoring of ovulation was used in this study because previous studies have shown that the accuracy of histological endometrial dating is best determined when ovulation is detected by this method (Shoupe *et al.*, 1989; Peters *et al.*, 1992). Two endometrial biopsies were performed during a single menstrual cycle in each subject. The patient's chronological day was determined by counting forward from the ovulation day as detected by ultrasonographic scans. The early biopsy (mid-luteal) was performed on ovulation day +7.4 ± 0.5 (range, +6 to +8), whereas the late biopsy was performed on ovulation day +11.2 ± 0.5 (range, +10 to +12). The second biopsy (late luteal) was always performed 4 days after the first biopsy.

Hormones in serum were quantified on the same days as endometrial sampling. All samples were obtained in the fasted state between 08.00 and 10.00 hours, which corresponds to the period of minimal progesterone variability, and adds to the accuracy of the measurement (Filicori *et al.*, 1984).

Endometrial samples

Endometrial samples were divided in two parts. One part was fixed in 10% formalin and embedded in paraffin. The second portion of the tissue was snap frozen in methylbutane (Merck, Darmstadt, Germany) immersed in liquid nitrogen and stored at –70°C until immunolabelling.

Endometrial dating

For endometrial dating 4-µm sections stained with haematoxylin and eosin and periodic acid Schiff stain were evaluated. All endometrial biopsies were evaluated by the same and experienced gynaecological pathologist (JO) according to the histopathological criteria of Noyes *et al.* (1950). The pathologist was blinded with regard to ultrasonographically detected ovulatory day. Endometrial biopsy interpretation was performed using a single-day evaluation whenever possible and when the traditional 2-day spread evaluation method (i.e. day 20–21) was provided, the later day was used for comparison to immunohistochemical assays. An out-of-phase biopsy was defined as a lag of ≥3 days between the chronological and the histological day (Creus *et al.*, 2002; Ordi *et al.*, 2002).

Immunohistochemistry

Immunohistochemical studies were performed using the automated immunohistochemical system TechMate 500™ (Dako Corporation, Carpinteria, CA, USA), using the EnVision system (Dako) as previously reported (Creus *et al.*, 2002). Integrin $\alpha_v\beta_3$ was detected in frozen tissue using a monoclonal antibody (clone LM609, dilution 1:200; Chemicon International, Temecula, CA, USA) and OPN was detected in formalin-fixed, paraffin-embedded tissue, using a polyclonal antibody (Chemicon). Frozen sections (4 µm thick) were fixed for 10 min in acetone at 4°C and dried. Paraffin sections were deparaffinized and rehydrated in xylene and graded alcohols. Peroxidase was blocked for 7.5 min in ChemMate peroxidase-blocking solution (Dako). Then the slides were incubated with the primary antibodies for 30 min and washed in ChemMate buffer solution (Dako). The peroxidase-labelled polymer was applied for 30 min. After washing in ChemMate buffer solution, the slides were incubated with the diaminobenzidene substrate chromogen solution, washed in water, counterstained with haematoxylin, and washed, dehydrated and mounted. As previously reported by the authors and others (von Wolff *et al.*, 2001; Creus *et al.*, 2002), in every case a negative control was performed by omission of incubation with the primary specific antibody. The reactivity of each monoclonal antibody with endometrial glands and surface epithelium, stromal cells and vessels was assessed. The intensity of staining of the endometrial components was evaluated by a semi-quantitative scoring system (0–4) as follows (Creus *et al.*, 1998, 2002; Ordi *et al.*, 2002): absent (–), weak or focal (+), moderate (++), and strong (+++). Endometrial samples were considered as expressing $\alpha_v\beta_3$integrin and/or OPN when these glycoproteins were detected in both endometrial glands and luminal surface epithelium with any intensity of the reaction ranging from weak/focal to strong (Creus *et al.*, 1998, 2002; Ordi *et al.*, 2002).

Hormone assays

Hormones were measured using commercially available kits. Oestradiol and progesterone concentrations in serum were estimated by direct radioimmunoassay (BioMérieux, Marcy l'Etoile, France, for oestradiol; Immunotech International, Marseille, France, for progesterone). Intra-assay and interassay coefficients of variation were <4.5 and <5.5% respectively for oestradiol, and <6.5 and <8.4% respectively for progesterone.

Statistics

Data were analysed with the program STATA version 9.0 (StataCorp 2005; College Station, Texas, USA). The chi-squared test and Fisher's exact test were used as appropriate for comparisons between categorical variables. A *t*-test or Mann–Whitney test was used as appropriate for continuous variables. Results are expressed as means with SEM. The correlation between $\alpha_v\beta_3$ integrin and OPN expression was evaluated using the Spearman's rank correlation coefficient. The association between $\alpha_v\beta_3$ integrin, OPN expression or co-expression of both molecules and patient's clinical condition (fertile versus infertile) or endometrial histological dating (in-phase versus out-of-phase specimens) was assessed (crude and adjusted analyses) by logistic regression models. Results are expressed as odds ratios and 95% confidence intervals.

Results

All menstrual cycles studied in the current investigation were ovulatory according to ultrasonographic criteria and mid-luteal serum progesterone was >10 ng/ml. Accordingly, in all instances the endometrial specimens were noted to be clearly progestational fundal samples. However, 37 out of the 107 (34.6%) mid-luteal biopsies from the study group and four (33.3%) in the control group showed out-of-phase endometria. A late-luteal endometrial biopsy could not be done in seven infertile patients and one of the controls because menses had commenced at the time of the second endometrial sampling. All but one of the infertile patients and all control women had in-phase endometria in the late-luteal biopsy. No inflammatory or reactive change related to the first sampling was detected in any late luteal biopsy.

Tables 1 and **2** summarize data relative to endometrial histology and $\alpha_v\beta_3$ integrin and OPN expression or co-expression on the days of endometrial sampling in the mid-luteal and late-luteal phase biopsies carried out in fertile controls and infertile patients, as well as hormone concentrations. No statistically significant difference between the two groups of women was observed for any parameter studied in either the mid- or the late-luteal phase. Only three infertile women had an out-of-phase endometrium in the late-luteal biopsy and two of them showed mid-luteal expression of both OPN and $\alpha_v\beta_3$ integrin. Similarly, among 97 infertile patients having in-phase late-luteal biopsy, 74.2% and 46.4% expressed OPN and $\alpha_v\beta_3$ integrin, respectively, in the mid-luteal endometrial sample.

As reported in **Tables 3**, **4** and **5**, differences were observed in $\alpha_v\beta_3$ integrin and OPN expression or their co-expression between in-phase and out-of-phase mid-luteal endometria for the whole study population, but endometrial expression of these biochemical markers was unrelated to the patient condition (fertile versus infertile).

Expression of both $\alpha_v\beta_3$ integrin and OPN was closely correlated with histological maturation of the endometrium ($r = 0.885$, $P < 0.001$ for $\alpha_v\beta_3$ integrin; $r = 0.657$, $P < 0.001$ for osteopontin); the latter marker appeared mainly at post-ovulatory days 4–5, whereas $\alpha_v\beta_3$ integrin appeared mainly at postovulatory days 6–7. Both markers were expressed by all endometria dated as post-ovulatory day ≥8. The intensity of their expression also increased from mid-luteal to late post-ovulatory days (**Figures 1** and **2**). These changes in $\alpha_v\beta_3$ integrin and OPN expression occurred irrespective of endometria being in-phase or out-of-phase. However, while a co-ordinately high level of expression of both markers existed from post-ovulatory day 8 onwards, the lack of temporal co-expression of $\alpha_v\beta_3$ integrin and OPN during the implantation window in the endometrial samples studied is shown in **Table 1**, which demonstrates that the simultaneous presence or absence of both markers was observed in only 62.2% (74/119) mid-luteal biopsies. In addition, a significant but weak ($r = 0.315$, $P < 0.001$) correlation between staining intensity for $\alpha_v\beta_3$ integrin and OPN in the mid-luteal phase biopsy was found in the whole study population (**Figure 3**). No differences either in mid-luteal or late-luteal serum concentrations of oestradiol and progesterone were detected among groups when stratified by the expression or not of $\alpha_v\beta_3$ integrin or OPN (data not shown).

No significant differences were detected between groups regarding integrin and OPN expression or co-expression in the mid-luteal phase when patients were grouped according to different aetiologies of infertility (**Table 6**). The same was true when patients who became spontaneously pregnant (n = 34) within 24 months after the study cycle were compared with those who did not get pregnant, irrespective of infertility factors detected (**Table 6**).

Table 1. Endometrial biopsy and epithelial quantitative and qualitative $\alpha_v\beta_3$ integrin and osteopontin (OPN) expression, and their co-expression in infertile and fertile women studied in the mid-luteal phase.

Parameter	*Fertile women (n = 12)*	*Infertile patients (n = 107)*
Endometrial biopsy		
Chronological dating	7.6 ± 0.2	7.3 ± 0.1
Histological dating	5.5 ± 0.5	5.6 ± 0.2
In-phase endometria	8 (66.7)	70 (65.4)
$\alpha_v\beta_3$ integrin expression		
Positive samples	6 (50.0)	51 (47.7)
Mean staining score	0.7 ± 0.2	0.7 ± 0.1
OPN expression		
Positive samples	8 (66.7)	78 (72.9)
Mean staining score	0.9 ± 0.2	1.1 ± 0.2
OPN/$\alpha_v\beta_3$ co-expression		
OPN (–)/$\alpha_v\beta_3$ (–)	3 (25.0)	22 (20.6)
OPN (+)/$\alpha_v\beta_3$ (–)	3 (25.0)	34 (31.8)
OPN (–)/$\alpha_v\beta_3$ (+)	1 (8.3)	7 (6.5)
OPN (+)/$\alpha_v\beta_3$ (+)	5 (41.7)	44 (41.1)
Hormone concentration		
Oestradiol (pg/ml)	138.5 ± 18.3	140.6 ± 15.1
Progesterone (pg/ml)	16.7 ± 2.0	17.9 ± 1.1

Values are means ± SEM or n (%).
There were no statistically significant differences between the two groups.

Table 2. Endometrial biopsy and epithelial quantitative and qualitative $\alpha_v\beta_3$ integrin and osteopontin (OPN) expression and their co-expression in infertile and fertile women studied in the late-luteal phase.

Parameter	*Fertile women (n = 11)*	*Infertile patients (n = 100)*
Endometrial biopsy		
Chronological dating	11.5 ± 0.2	11.3 ± 0.1
Histological dating	11.7 ± 0.2	11.4 ± 0.1
In-phase endometria	11 (100)	97 (97)
$\alpha_v\beta_3$ integrin expression		
Positive samples	11 (100)	100 (100)
Mean staining score	2.5 ± 0.3	2.8 ± 0.1
OPN expression		
Positive samples	11 (100)	100 (100)
Mean staining score	2.3 ± 0.2	2.2 ± 0.1
OPN/$\alpha_v\beta_3$ co-expression		
OPN (–)/$\alpha_v\beta_3$ (–)	0 (0)	0 (0)
OPN (+)/$\alpha_v\beta_3$ (–)	0 (0)	0 (0)
OPN (–)/$\alpha_v\beta_3$ (+)	0 (0)	0 (0)
OPN (+)/$\alpha_v\beta_3$ (+)	11 (100)	100 (100)
Hormone concentration		
Oestradiol (pg/ml)	106.1 ± 19.1	105.1 ± 16.6
Progesterone (pg/ml)	8.2 ± 1.4	9.1 ± 1.5

Values are means ± SEM or n (%).
There were no statistically significant differences between the two groups.

Table 3. $\alpha_v\beta_3$ integrin expression in infertile versus fertile women and in-phase versus out-of-phase mid-luteal endometrial biopsies for the whole study population.

Parameter	*Integrin-positive samples (%)*	*Crude OR (95% CI)*	*P-value (univariate)*	*Adjusted OR*	*P-value (adjusted)*
Patient condition					
Fertile (n = 12)	50.0	1		1	
Infertile (n = 107)	47.7	0.91 (0.28, 3.00)	NS	0.91 (0.22, 3.70)	NS
Endometrial biopsy					
In phase (n = 78)	66.7	1		1	
Out-of-phase (n = 41)	12.2	0.07 (0.02, 0.20)	<0.001	0.07 (0.02, 0.20)	<0.001

CI = confidence interval; NS = not statistically significant; OR = odds ratio.

Table 4. Osteopontin (OPN) expression in infertile versus fertile women and in-phase versus out-of-phase mid-luteal endometrial biopsies for the whole study population.

Parameter	*OPN-positive samples (%)*	*Crude OR (95% CI)*	P-*value (univariate)*	*Adjusted OR*	P-*value (adjusted)*
Patient condition					
Fertile (n = 12)	66.7	1		1	
Infertile (n = 107)	72.9	1.34 (0.38, 4.81)	NS	1.43 (0.37, 5.59)	NS
Endometrial biopsy					
In phase (n = 78)	83.3	1		1	
Out-of-phase (n = 41)	51.2	0.21 (0.09, 0.49)	<0.001	0.21 (0.09, 0.49)	<0.001

CI = confidence interval; NS = not statistically significant; OR = odds ratio.

Table 5. $\alpha_v\beta_3$ integrin and osteopontin (OPN) co-expression in infertile versus fertile women and in-phase versus out-of-phase mid-luteal endometrial biopsies for the whole study population.

Parameter	*OPN/integrin positive samples (%)*	*Crude OR (95% CI)*	P-*value (univariate)*	*Adjusted OR*	P-*value (adjusted)*
Patient condition					
Fertile (n = 12)	41.7	1		1	
Infertile (n = 107)	41.1	0.98 (0.29, 3.28)	NS	1.00 (0.26, 3.81)	NS
Endometrial biopsy					
In phase (n = 78)	56.4	1		1	
Out-of-phase (n = 41)	12.2	0.11 (0.04, 0.30)	<0.001	0.11 (0.04, 0.30)	<0.001

CI = confidence interval; NS = not statistically significant; OR = odds ratio.

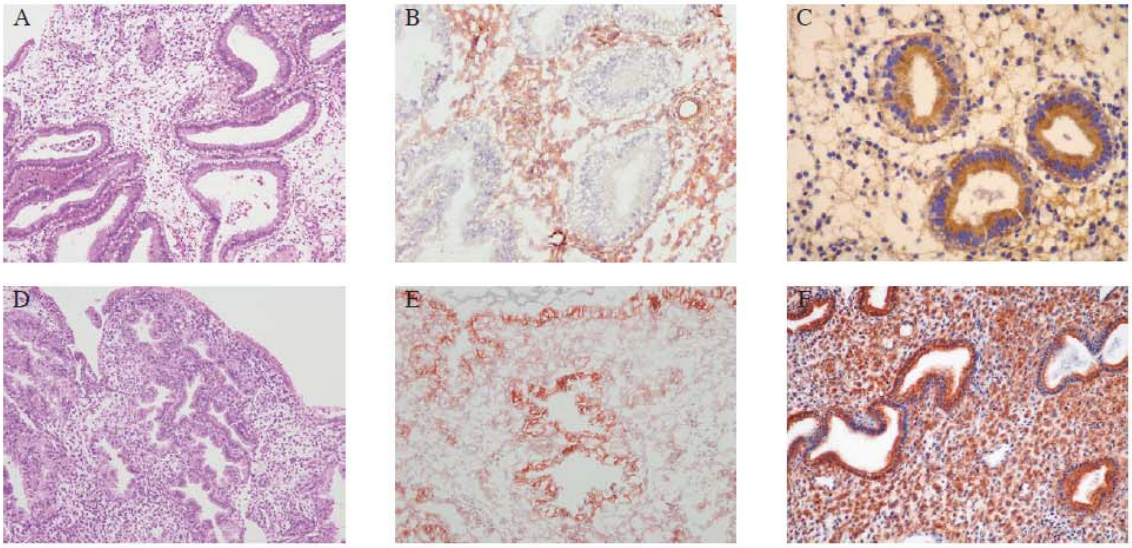

Figure 1. Haematoxylin and eosin staining (**A**,**D**), $\alpha_v\beta_3$ integrin (**B**,**E**) and osteopontin (**C**,**F**) expression in mid- (**A**,**B**,**C**) and late- (**D**,**E**,**F**) luteal endometrial biopsies from the same patient. Note negative staining in endometrial glands in **B**, a weak, supranuclear staining in **C**, and strong glandular immunostaining in **D** and **F**. (**A**,**D**) Haematoxylin and eosin, ×100 magnification. (**B**,**E**) $\alpha_v\beta_3$ integrin, haematoxylin counterstaining, ×200 magnification. (**C**,**F**) Osteopontin, haematoxylin counterstaining, ×200 magnification.

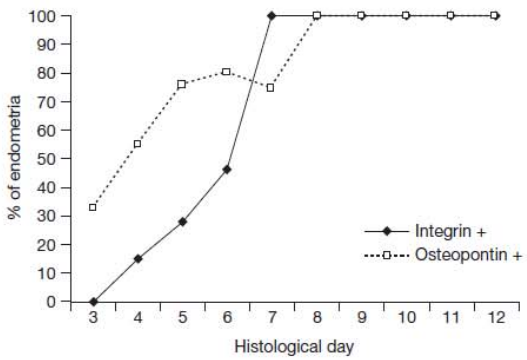

Figure 2. Percentage of endometria showing osteopontin and $\alpha_v\beta_3$ integrin in epithelial cells for each histological day (all women, $n = 119$).

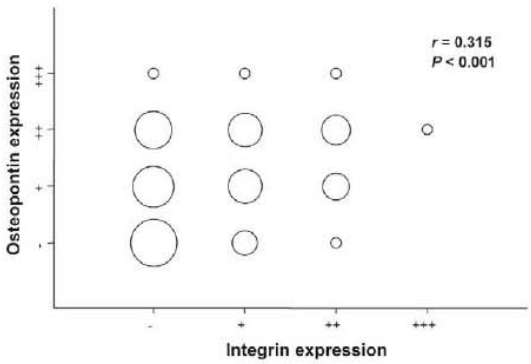

Figure 3. Correlation between staining intensity for osteopontin and $\alpha_v\beta_3$ integrin expression in epithelial cells of mid-luteal endometrial biopsies in the whole group of patients ($n = 119$). In this bubble graph, the area of the symbol is proportional to the number of observations.

Table 6. Mid-luteal endometrial expression of $\alpha_v\beta_3$ integrin and osteopontin (OPN) according to infertility factors detected or whether patients became spontaneously pregnant or not within 2 years after the study cycle.

Infertility factor	n	*No. of positive samples (%)* $\alpha v\beta_3$ *integrin*	*OPN*	*OPN/integrin*
Male factor	30	16 (53.3)	24 (80.0)	14 (46.7)
Unexplained Infertility	22	9 (40.9)	16 (72.7)	8 (36.4)
Endometriosis	24	11 (45.8)	15 (62.5)	9 (37.5)
Tubal	14	8 (57.1)	11 (78.6)	7 (50.0)
Other	17	7 (41.2)	12 (70.6)	6 (35.3)
Pregnant	34	11 (32.4)	26 (76.5)	10 (29.4)
Non-pregnant	73	40 (54.8)	52 (71.2)	34 (46.6)

Discussion

Successful implantation is essential for the establishment of pregnancy. Implantation begins with apposition of the free-floating blastocyst on the uterine luminal epithelium and thereafter, the blastocyst firmly adheres to the uterine wall and the trophoblast migrates across the luminal epithelium (Bulletti *et al.*, 2005; Aplin, 2006; Makker and Singh, 2006). Thus, recently, several adhesion molecules considered essential for uterine competence during implantation have been proposed as markers of endometrial receptivity (Lessey, 2002, 2003). Cell adhesion molecules (i.e. integrins, cadherins, selectins and immunoglobulins) are surface ligands, usually glycoproteins, mediating cell-to-cell adhesion. Integrins are a family of cell adhesion molecules whose expression in the endometrium has been intensively investigated, with integrin $\alpha_v\beta_3$ being the most extensively characterized marker of endometrial receptivity (Lessey 2000a, 2003; Sharkey and Smith, 2003; Hoozemans *et al.*, 2004; Achache and Revel, 2006; Aplin, 2006). In fact, integrin $\alpha_v\beta_3$ first, and more recently OPN, have been proposed as a novel approach to the assessment of uterine receptivity in a variety of infertility states. The following facts would support this contention.

First, whereas a large variety of integrins has been described within the luminal and glandular endometrial epithelium and they are constitutively expressed throughout the entire menstrual cycle, the integrin $\alpha_v\beta_3$ abruptly appears in the endometrial epithelial cells on post-ovulatory days 5 and 6 (Achache and Revel, 2006; Maker and Singh, 2006). Second, it has been suggested that integrins $\alpha_v\beta_3$ and $\alpha_4\beta_1$ are co-expressed only during the putative window of implantation, thus framing the uterine receptivity period. Third, decreased expression of integrin $\alpha_v\beta_3$ during the mid-luteal phase has been reported in patients diagnosed as having unexplained infertility, endometriosis, hydrosalpinges, luteal phase deficiency, and polycystic ovary syndrome (Bulletti *et al.*, 2005; Achache and Revel, 2006; Makker and Singh, 2006). Fourth, clomiphene citrate has a well-known anti-oestrogenic action on the endometrium when given during the first part of the menstrual cycle (Bonhoff *et al.*, 1993). In a recent study (Palomino *et al.*, 2005), delayed secretory endometrial maturation in association with aberrant expression of integrin $\alpha_v\beta_3$ was observed in as many as 38% of mid-luteal phase endometrial samples from normo-ovulatory women treated with clomiphene citrate, a figure which was significantly higher compared with endometrial findings in control subjects. Finally, studies showing maximal expression of both integrin $\alpha_v\beta_3$ and its ligand OPN during the mid to late secretory phase in human endometrial epithelial cells and secretion of OPN into the uterine cavity suggest a role of both factors in the regulation of endometrial function and implantation (Apparao *et al.*, 2001; von Wolff *et al.*, 2001). Both glycoproteins have been detected in the mid-luteal phase on the apical projections of surface (luminal) epithelium known as pinopods (Apparao *et al.*, 2001; Lessey, 2002), thus further suggesting that integrins may be involved in implantation. In fact, the two most cited markers framing the window of implantation are $\alpha_v\beta_3$ integrin expression and pinopod formation in human endometrial epithelium; these are both oestrogen and progesterone dependent, as has been shown in natural and mock hormonal treatment cycles in the donor oocyte model (Nikas, 1999a,b, 2000; Lessey, 2000b,c; Damario *et al.*, 2001).

Against the above background, however, the following facts should be considered. Both the current study and previous work from the authors (Creus *et al.*, 1998) confirm that there is a dynamic expression of several integrins in human endometrium. Thus, it was found that $\alpha_v\beta_3$ integrin expression was closely correlated with histological maturation of endometrium appearing mainly at postovulatory days 6–7 and being expressed by all endometria dated as post-ovulatory day ≥8. Also, the authors have previously reported that $\alpha_4\beta_1$ integrin disappeared in most late-luteal endometrial samples, thus apparently supporting the notion that $\alpha_v\beta_3$ and $\alpha_4\beta_1$ integrins would be co-expressed during the suspected time of maximal uterine receptivity (Lessey, 2002). However, this was true irrespective of endometria being in-phase or out-of-phase and women being fertile or infertile (Creus *et al.*, 1998). Although endometrial integrin expression in polycystic ovary syndrome patients has not been investigated, the current investigation adds

further evidence to previous studies by the authors (Creus *et al.*, 1998; Ordi *et al.*, 2002, 2003) showing that normal or aberrant integrin expression is not associated with specific aetiologies of infertility, mainly endometriosis and unexplained infertility. Finally, in a self-controlled study where each patient served as a control for herself and women were investigated during spontaneous and ensuing treated cycles, it was found that $\alpha_v\beta_3$ integrin expression was not significantly reduced in clomiphene citrate-treated cycles (Creus *et al.*, 2003).

The current investigation shows that OPN either alone, or in combination with its receptor the integrin $\alpha_v\beta_3$, is not a better marker of endometrial function than integrin $\alpha_v\beta_3$. Apart from corroborating the significance (if any) of histological delay, OPN alone or the OPN:$\alpha_v\beta_3$ integrin complex provided no additional useful information beyond that derived from investigating integrin $\alpha_v\beta_3$ alone when patients were considered according to their clinical condition (infertile versus fertile), different infertility factors detected, or to whether infertile patients became spontaneously pregnant or not. In fact, the study shows a lack of temporal relationship in the expression of integrin $\alpha_v\beta_3$ and OPN in mid-luteal phase endometria of both fertile women and infertile patients. This is not surprising considering that these two glycoproteins are differentially regulated. The expression of OPN is primarily stimulated by progesterone, whereas the $\alpha_v\beta_3$ integrin epithelial expression is up-regulated by epidermal growth factor or heparin-binding epidermal growth factor (Apparao *et al.*, 2001; Lessey, 2002; Aplin, 2006). Finally, it should be noted that previous work from this group (Creus *et al.*, 2003) showed a clear dissociation in the temporal expression during the luteal phase of integrin $\alpha_v\beta_3$ and pinopods and questioned the functional significance of the expression/co-expression of the most cited markers postulated to frame the window of implantation. Thus, the proposed co-expression of OPN and integrin $\alpha_v\beta_3$ on pinopods during the mid-luteal phase to underscore the role of these proteins as markers of endometrial receptivity and function (Apparao *et al.*, 2001; Lessey, 2002) seems to be untenable.

It could be argued that there is a discrepancy in the cohort size in this study, which included 107 infertile patients and 12 controls; thus a beta error cannot be excluded. However, as recently stressed (Sharkey and Smith, 2003), many studies on the subject have included only small groups of patients, often lacking suitable fertile controls. All control women included in the present study had proven fertility and regular ovulatory cycles, thus being appropriate for comparative purposes with the study population in the clinical setting. On the other hand, several studies have previously reported the findings of overall mRNA expression (including OPN expression) in endometrial samples (Carson *et al.*, 2002; Kao *et al.*, 2002) or have focused on OPN using northern blots or RNA protection assays (von Wolff *et al.*, 2001). These techniques can be applied to a limited number of cases and are not adequate for studies involving a large number of cases. Moreover, although these techniques are extremely useful in the search for markers of endometrial receptivity, their results need to be confirmed by the evaluation of the functional molecule, which is the protein. Immunohistochemistry allows the analysis of protein expression, providing information on the histological and subcellular distribution of the protein (i.e. glandular epithelium or stroma) and is particularly appropriate for large clinically based studies. In fact, as recently stressed (Pei *et al.*, 2007), immunohistochemistry staining is necessary to access the clinicopathological characteristics of proteins identified by proteomics and genomics analysis.

In conclusion, the present study indicates that although the expression of the OPN:$\alpha_v\beta_3$ integrin complex is closely correlated with histological maturation of endometrium evaluated by histological dating, neither OPN nor $\alpha_v\beta_3$ alone or in combination are useful markers of endometrial functional receptivity. Further studies are warranted to establish the functional significance of the OPN:$\alpha_v\beta_3$ integrin complex in infertility.

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ESTUDIO 2

"Osteopontina e integrina αvβ3 como marcadores de receptividad endometrial: efecto de diferentes tratamientos hormonales"

"Osteopontin and αvβ3 integrin as markers of endometrial receptivity: the effect of different hormone therapies"

Casals G, Ordi J, Creus M, Fábregues F, Carmona F, Casamitjana R, Balasch J

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ARTICLE

Osteopontin and αvβ3 integrin as markers of endometrial receptivity: the effect of different hormone therapies

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Abstract The osteopontin: αvβ3 integrin complex has been proposed as a means of distinguishing receptive from non-receptive endometrium in clinical practice, thus offering new directions for the development of contraceptive approaches targeted to the endometrium as well as a better understanding of occult causes of infertility in women. Histological dating and immunohistochemical study were performed in control and study cycles in seven groups of women including 10 subjects per group and who received clomiphene citrate, ovarian stimulation for IVF, oral contraception, dehydrogesterone for endometrial luteal phase defect, two different regimens of hormone replacement therapy, or no treatment. Ten healthy fertile women served as a general control group. Osteopontin and αvβ3 integrin expression in the human endometrium was closely related to endometrial maturation and this was irrespective of the endometrium being in-phase or out-of-phase and the hormonal treatment (or no treatment) received. In conclusion, immunohistochemical assessment of the endometrium indicates that the use of osteopontin and αvβ3 integrin or the osteopontin: αvβ3 integrin complex as targets for the development of contraceptive approaches or the understanding of the pathogenesis of female infertility offer little benefit compared with simple histological dating.

KEYWORDS: endometrium, hormone treatment, implantation, integrins, osteopontin

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Introduction

The implantation process of the human embryo is a highly complex and orchestrated interaction between the maternal endometrium and the newly formed embryo. On the maternal side, a so-called receptive endometrium is a prerequisite and the window of implantation is defined as the period of endometrial maturation during which the blastocyst can attach to the endometrial epithelial cells and subsequently proceed to invade the endometrial stroma. A number of architectural, cellular, biochemical and molecular events occur in the endometrium within the implantation window (i.e. 6–8 days after ovulation) (Castelbaum et al., 1994; Santoro et al., 2000).

Understanding the factors that contribute to a receptive endometrium is, at present, a pivotal area of research. Among the best-characterized biomarkers for assessment of uterine receptivity are integrins. Different epithelial integrins undergo changes in expression during the luteal phase, being αvβ3 integrin the most extensively studied (Achache and Revel, 2006; Lessey, 2002; Strowitzki et al., 2006). Its ligand osteopontin (OPN) has been shown to bind to several different integrin receptors but the αvβ3 integrin has been recognized as a primary receptor for OPN (Lessey, 2002). In fact, OPN and its receptor αvβ3 integrin have been found to be co-ordinately expressed in the human menstrual epithelium across the menstrual cycle in normally cycling fertile women, both glycoproteins being maximally expressed at the time of the implantation window (Apparao et al., 2001). Thus, a complementary role for these proteins in endometrial function and implantation has been suggested and it has been proposed that an OPN: αvβ3 integrin complex may occur at the apical surface of human uterine luminal epithelium to promote embryo attachment as has been suggested in other species (Lessey, 2002; Makker and Singh, 2006; von Wolff et al., 2001). Accordingly, these markers have been postulated as a means of distinguishing receptive endometrium from non-receptive endometrium in clinical practice, thus offering new directions for a better understanding of occult causes of infertility in women as well as the development of a novel contraceptive approach targeted to the endometrium (Lessey, 2002; Makker and Singh, 2006; Strowitzki et al., 2006).

On the above evidence, an immunohistochemical study was conducted to investigate the effect of exogenous hormonal manipulation on OPN and αvβ3 integrin expression in the endometrium and thus to assess the potential usefulness of these so-called endometrial markers of implantation as targets for contraceptive approaches or fertility-promoting strategies. This was done using a prospective, controlled study design where patients were investigated during spontaneous and ensuing treated cycles.

Materials and methods

Patients, treatments and study cycles

The expression of OPN and αvβ3 integrin in the endometrium of seven groups of patients receiving different treatments as follows. The use of human tissue for research was based on informed consent and was approved by the ethics committee of the hospital.

Clomiphene citrate (group CC)

Ten infertile patients received clomiphene citrate (CC) (Omifin; Laboratorios Effik, Madrid, Spain) 50 mg/day from cycle day 5 to day 9 and were studied in both spontaneous and ensuing stimulated cycles.

IVF (group IVF)

Ten oocyte donors undergoing ovarian stimulation with recombinant human FSH (Gonal F; Merck-Serono, Madrid, Spain) under pituitary suppression for IVF. For the specific purpose of this investigation, the luteal phase was supported with micronized vaginal progesterone (Progeffik; Laboratorios Effik) (600 mg/day for 12 days and commencing on the day after oocyte retrieval). Three to four months after the IVF cycle, these patients underwent endometrial and blood samplings during a spontaneous ovulatory control cycle.

Oral contraception (group OC)

Ten normal healthy women requesting contraception and receiving a low-dose oral contraceptive containing 30 μg of ethinyl oestradiol plus 150 μg of desogestrel (Microdiol; Organon, Barcelona, Spain) and undergoing endometrial samplings in both spontaneous and ensuing oral contraception-treated cycles. Treatment was commenced on cycle day 3.

Dehydrogesterone (group DHG)

Ten infertile patients with endometrial luteal phase defect as defined by delayed endometrial maturation (≥3 days) in two separate cycles and treated with dehydrogesterone (Duphaston; Duphar Nezel, Barcelona, Spain) (20 mg/day for 12 days, starting 2 days after ovulation). The second spontaneous menstrual cycle showing delayed endometrial maturation was used for comparative purposes in this study.

Hormone replacement therapies

Twenty women with primary or secondary ovarian failure (serum FSH and LH >40 IU/l and serum oestradiol <30 pg/ml) and receiving standard hormone replacement therapy in the form of oestradiol valerate (Progynova; Schering, Madrid, Spain) (6 mg daily during 28 days per month) and micronized vaginal progesterone (Progeffik) (600 mg/day administered from days 15 to 28 of the oestrogenic treatment). Group HT1 comprised these 20 women. For an ensuing cycle, group HT1 was divided randomly and prospectively into two subgroups of 10 patients: group HT2, who received oestradiol valerate treatment for 14 days but oestrogen treatment was stopped during the artificial luteal phase and only micronized vaginal progesterone was administered for another 14 days and group HT3, who were given the standard therapy with also episodic progesterone administration during the artificial follicular phase on days 8 and 11 (200 mg of micronized vaginal progesterone) to mimic premature luteinization.

No treatment (group NT)

Ten infertile patients with out-of-phase endometria (delayed endometrial maturation ≥3 days) in the first study

cycle and undergoing a second endometrial study in the consecutive spontaneous cycle receiving no treatment.

According to the above study design, each woman acted as her own control for endometrial markers of implantation. However, considering that experimental subjects were mostly infertile or menopausal women, an additional group of 10 fertile healthy women was also included (mean parity 1.5, range 1–3; age 29–40 years, mean ± SEM 32.9 ± 1.0) who were undergoing tubal sterilization and served as a general control group. These control women had regular menstrual cycles (28–32 days) and were taking no medication. The mean age of study patients was 33.2 ± 1.6 years (range 24–39) and all had regular ovulatory menstrual patterns every 26–33 days. A number of both infertile patients and controls had participated in previous published studies (Casals et al., 2008; Creus et al., 2002; Ordi et al., 2002). In all groups of recruited normally ovulating women, basal body temperature, luteal serum concentrations of oestradiol and progesterone and endometrial biopsies were used in the same cycle to assess luteal function according to a scheme of evaluation previously reported (Casals et al., 2008; Creus et al., 2002).

Commencing on days 8–10 of the study cycle (depending on the cycle length of the woman) patients underwent daily transvaginal ultrasonographic evaluation of the follicular growth using a Eccocee SAA-340A/EF unit (Toshiba, Tokyo, Japan) equipped with a 5–7 MHz endo-vaginal probe (PVF-641VT). The maximum follicular diameter was measured in all patients. Both ovaries were identified and the largest diameter was measured in both the longitudinal and transverse dimensions in all follicles. The day of ovulation was designated as the day of maximum follicular enlargement, which was followed the next day by sudden disappearance or a filling in of this follicle showing loss of clear demarcation of its walls and intrafollicular echoes (Peters et al., 1992; Shoupe et al., 1989). Ultrasonographic monitoring of ovulation was used because previous studies have shown that the accuracy of histological endometrial dating is best determined when ovulation is detected by that method (Peters et al., 1992; Shoupe et al., 1989).

Two endometrial biopsies were performed during two menstrual cycles (control and untreated in group NT and control and treated in the remaining study groups) in each experimental subject and in a single menstrual cycle in control fertile women. The patient's chronological day was determined by counting forward from the ovulation day as detected by ultrasonographic scans. The early biopsy (mid-luteal) was performed on ovulation day +6 to +8 (mean 7.3 ± 0.6) whereas the second biopsy (late luteal) was always performed 4 days after the first biopsy (mean 11.4 ± 0.6). The day of oocyte retrieval was designated day 14 in ovarian stimulation cycles, while hormone replacement therapy cycles were studied on days 7–8 after the commencement of progesterone treatment and 4 days later. In oral contraception-treated cycles, endometrial samplings were obtained on cycle days 21–22 and 4 days later.

Hormones in serum were quantified on the same days as endometrial sampling. All samples were obtained in the fasted state between 8 and 10 a.m., which corresponded to the period of minimal progesterone variability in spontaneous menstrual cycles and added to the accuracy of the measurement (Filicori et al., 1984). In patients receiving premature progesterone administration (group HT3) during hormone replacement therapy cycles, additional blood samples were obtained 4–6 h after each dose of vaginal progesterone administered during the artificial follicular phase in order to evaluate serum progesterone at the steady-state concentrations previously reported in pharmacokinetic studies (Miles et al., 1994).

Endometrial samples

Endometrial samples were divided into two parts. One of them was fixed in 10% formalin and embedded in paraffin. The second portion of the tissue was snap frozen on methylbutane (Merck, Darmstadt, Germany) immersed in liquid nitrogen and stored at −70°C until immunolabelling.

Endometrial dating

For endometrial dating, 4 μm sections stained with haematoxylin and eosin and periodic acid–Schiff stain were evaluated. All endometrial biopsies were evaluated by the same and experienced gynaecological pathologist (JO) according to the histopathological criteria of Noyes et al. (1950). The pathologist was blinded with regard to ultrasonographically detected ovulatory day. Endometrial biopsy interpretation was performed using a 1-day evaluation whenever possible and when the traditional 2-day evaluation method (i.e. days 20–21) was provided, the later day was used for comparison to immunohistochemical assays. An out-of-phase biopsy was defined as a lag of three days or longer between the chronological and the histological day (Creus et al., 2002; Ordi et al., 2002).

Immunohistochemistry

Immunohistochemical studies were performed using the automated immunohistochemical system TechMate 500 (Dako, Carpinteria, CA, USA), using the EnVision system (Dako) as previously reported (Casals et al., 2008; Creus et al., 2002). Integrin αvβ3 was detected in frozen tissue using a monoclonal antibody (clone LM609, dilution 1:200; Chemicon, Temecula CA, USA) and OPN in formalin-fixed, paraffin-embedded tissue, using a polyclonal antibody (Chemicon). Frozen sections (4 μm thick) were fixed for 10 min in acetone at 4°C and dried. Paraffin sections were deparaffinized and rehydrated in xylene and graded alcohols. Peroxidase was blocked for 7.5 min in ChemMate peroxidase-blocking solution (Dako). Then the slides were incubated with the primary antibodies for 30 min and washed in ChemMate buffer solution (Dako). The peroxidase labelled polymer was applied for 30 min. After washing in ChemMate buffer solution, the slides were incubated with the diaminobenzidine substrate chromogen solution, washed in water, counterstained with hematoxylin, washed, dehydrated and mounted. As previously reported (Creus et al., 2002; von Wolff et al., 2001), in every case a negative control was performed by omission of incubation with the primary specific antibody. The reactivity of each monoclonal antibody with endometrial glands and surface epithelium, stromal cells and vessels was assessed. The intensity of staining of the endometrial components was evaluated

by a four-point semi-quantitative scoring system as follows (Creus et al., 2002; Ordi et al., 2002): absent (−), weak or focal (+), moderate (++) and strong (+++). Endometrial samples were considered as expressing αvβ3 integrin and/or OPN when these glycoproteins were detected in both endometrial glands and luminal surface epithelium with any intensity of the reaction ranging from weak/focal to strong (Casals et al., 2008; Creus et al., 2002; Ordi et al., 2002).

Hormone assays

Hormones were measured using commercially available kits. Oestradiol and progesterone in serum were estimated by a competitive chemiluminescent assay (ADVIA Centaur CP System; Siemens Healthcare Diagnostics). The sensitivity was 10 pg/ml for oestradiol and 0.15 ng/ml for progesterone and the inter-assay coefficients of variation were 5% and 5.4%, respectively.

Statistics

Data were analysed using the Statistical Package for Social Sciences version 10.0 (SPSS, Chicago, IL, USA). The Mann–Whitney *U*-test, chi-squared test and Wilcoxon matched-pairs signed-ranks test were used as appropriate with Bonferroni correction for multiple comparisons. Results are expressed as means ± SEM. The level of significance was set at $P \leq 0.05$.

Results

All spontaneous menstrual cycles included in the present study were ovulatory according to ultrasonographic criteria and mid-luteal serum progesterone concentration >10 ng/ml. A late luteal endometrial biopsy could not be done in seven of the 140 cycles in experimental subjects because menses had commenced at the time of the second endometrial sampling. In all instances, the endometrial specimens were noted to be clearly progestational fundal samples. When dysynchronous glandular and stromal endometrial development was found, endometrial maturation was defined according to the most advanced elements in either the glands or stroma (Meyer et al., 1999). However, the glands were never more advanced than the stroma. This was especially evident in OC and HT3 groups, which showed a markedly decidualized stroma with glands with an underdeveloped or atrophic appearance. However, the apparent delayed glandular development did not preclude the expression of potential markers of endometrial receptivity (Figure 1) and thus, histological dating according to glandular differentiation would not change the results with respect to markers of implantation in the present study. No inflammatory or reactive change related to the first sampling was detected in any late luteal biopsy.

Mid-luteal hormonal concentrations are presented in Table 1. As expected, oestradiol and progesterone serum

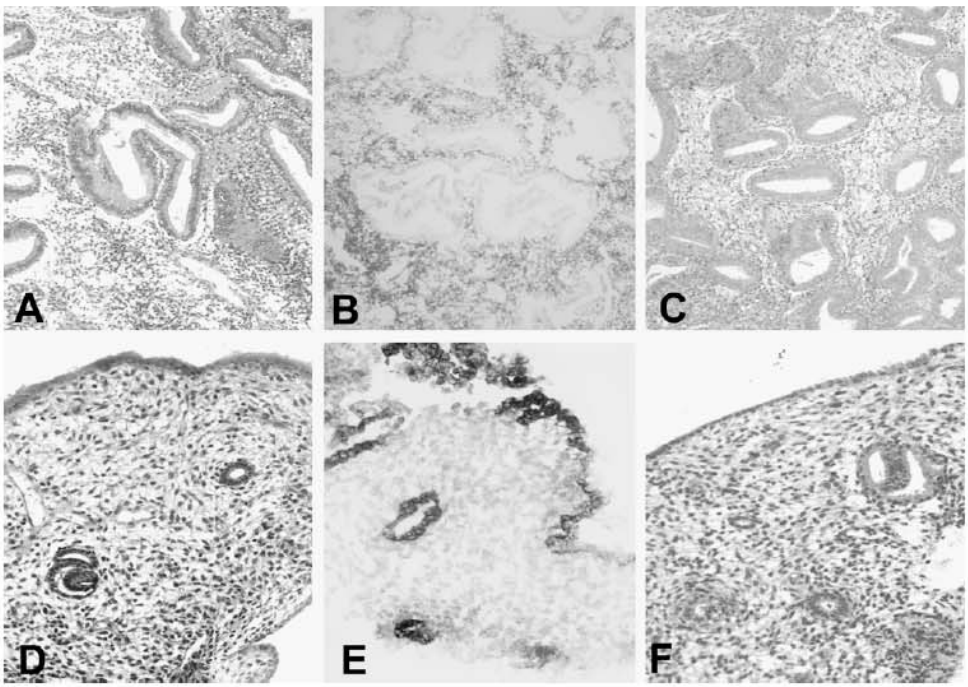

Figure 1 Histological appearance, immunostaining for αvβ3 integrin and osteopontin in mid-luteal endometrial specimens from (A–C) spontaneous and (D–F) treatment cycles in a woman receiving oral contraceptives. Endometrial dating in the spontaneous cycle (A) corresponds to a post-ovulatory day +4 and in treatment cycle (D), marked decidual change of the stroma (post-ovulatory day +13) and small inactive-appearing glands are seen (haematoxylin and eosin, original magnification ×100). Immunostaining for αvβ3 integrin shows the absence of glandular staining in the spontaneous cycle (B) and staining both in stroma and glands in treated cycle (E) (αvβ3 integrin, Envision, haematoxylin counterstaining, original magnification ×200). Immunostaining for osteopontin shows the glandular staining in the spontaneous cycle (C) and staining both in stroma and glands in treated cycle (F) (osteopontin, Envision, hematoxylin counterstaining, original magnification ×200).

Table 1 Serum hormone concentrations in the mid-luteal phase in control and treated cycles in the seven experimental groups studied and in control fertile women.

Group	Estradiol (pg/ml)	Progesterone (ng/ml)
Clomiphene citrate		
Control cycle ($n = 10$)	159 ± 17[a]	19 ± 3[b]
Treated cycle ($n = 10$)	**383 ± 47[a]**	**45 ± 11[b]**
IVF		
Control cycle ($n = 10$)	151 ± 16[c]	18 ± 3[d]
Treated cycle ($n = 10$)	**901 ± 98[c]**	**79 ± 26[d]**
Oral contraceptive pill		
Control cycle ($n = 10$)	161 ± 16[e]	17 ± 3[f]
Treated cycle ($n = 10$)	**10 ± 0[e]**	**1 ± 0[f]**
Hormone replacement therapy		
Control cycle (HT1) ($n = 20$)	**265 ± 52[g]**	13 ± 1
Treated cycle (HT2) ($n = 10$)	**12 ± 1[g,h]**	13 ± 1
Treated cycle (HT3) ($n = 10$)	**298 ± 46[h]**	12 ± 1
Dehydrogesterone		
Control cycle ($n = 10$)	148 ± 13	17 ± 1
Treated cycle ($n = 10$)	141 ± 18	17 ± 2
No treatment		
First cycle ($n = 10$)	138 ± 13	15 ± 2
Second cycle ($n = 10$)	142 ± 12	14 ± 2
Fertile women ($n = 10$)	146 ± 21	18 ± 2

Values are mean ± SEM.
[a–h]Figures with common superscripts are significantly different ($P < 0.05$). Bold denotes figures significantly different ($P < 0.05$) from values in fertile women. HT1 = oestradiol valerate 28 cycle days and micronized vaginal progesterone from days 15 to 28 of the oestrogenic treatment; HT2 = first cycle treated as for HT1 and ensuing cycle treated with oestradiol valerate treatment for 14 days and micronized vaginal progesterone for another 14 days; HT3 = same treatment as HT1 with also micronized vaginal progesterone on days 8 and 11.

concentrations were significantly increased in cycles treated with ovarian stimulants or supplemented with oestradiol or progesterone mimicking the natural cycle and reduced in oral contraception-treated cycles (all $P < 0.05$). For patients in group HT3, serum progesterone concentrations after premature progesterone administration on cycle days 8 and 11 were 7.1 ± 0.9 ng/ml and 8.6 ± 1.0 ng/ml, respectively.

As expected, in all study groups, chronological dating of endometrial biopsies (day of sampling) was the same in control and treated cycles (data not shown). Histological dating, OPN and αvβ3 integrin expression in spontaneous and treatment (groups CC, IVF, OC, DHG, HT2, HT3) or untreated (group NT) endometrium specimens of experimental subjects and control ovulatory cycles in fertile women are presented in Figures 2–6. Histological dating was similar in spontaneous (post-ovulatory day 6.7 ± 0.5) and treatment (post-ovulatory day 5.4 ± 0.9) cycles in group CC and, also, neither OPN nor αvβ3 integrin expression was significantly changed in CC-treated cycles (data not shown). Ovarian stimulation for IVF caused advanced histological dating (post-ovulatory day 5.8 ± 0.5 versus 7.3 ± 0.2) and increased OPN and αvβ3 integrin expression (Figure 2). All endometrial specimens from oral contraception-treated cycles were consistent with advanced histological dating (post-ovulatory day 6.5 ± 0.6 versus 12.3 ± 0.7) and αvβ3 integrin but not OPN expression (Figure 3). Parameters of endometrial morphology and function in group HT1 were similar to those in fertile women (Figure 4) thus stressing the validity of HT1-treated cycles as controls for the two subgroups of patients receiving different forms of hormone replacement therapy in ensuing cycles. No differences were found between control and treatment cycles in group HT2 with respect to the three endometrial variables investigated. However, premature administration of progesterone during the follicular phase (group HT3) was associated with advanced histological dating (post-ovulatory day 5.8 ± 0.6 versus 12.0 ± 0.1) and increased αvβ3 integrin but not OPN expression (Figure 4). Dehydrogesterone treatment in patients having retarded endometrial maturation in spontaneous cycles was associated with advanced endometrial dating (post-ovulatory day 3.7 ± 0.2 versus 5.7 ± 0.5) and increased expression of both OPN and αvβ3 integrin (Figure 5). Histological dating was advanced in group NT during the second study cycle (where six out of 10 patients had in-phase endometrial biopsies) as compared with the first out-of-phase endometrial specimens (post-ovulatory day 3.3 ± 0.2 versus 4.3 ± 0.3) in the 10 women. This was associated with an increased OPN but not αvβ3 integrin endometrial expression (Figure 6).

As shown in Figure 7, αvβ3 integrin expression was closely related to histological maturation of the endometrium

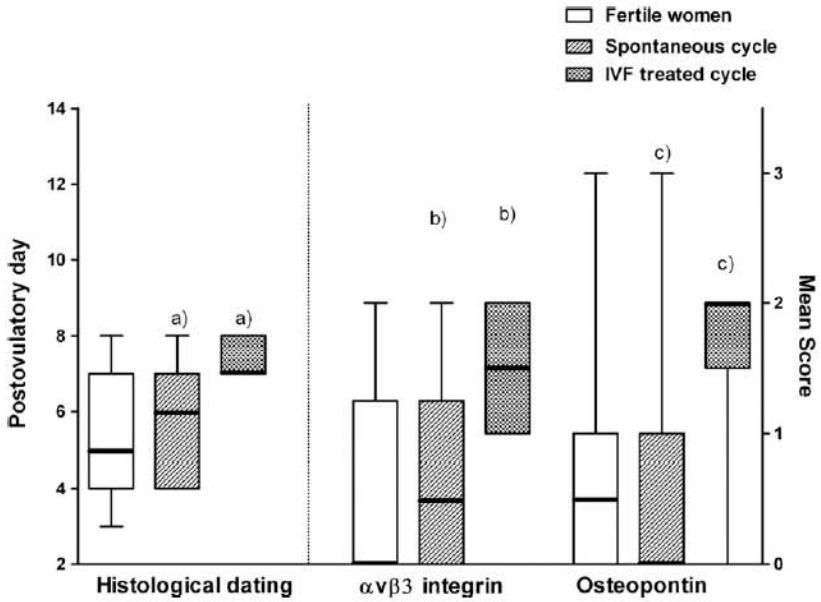

Figure 2 Histological dating, αvβ3 integrin and osteopontin expression in spontaneous cycles in control fertile women and spontaneous and treatment mid-luteal endometrium specimens in the IVF-treated group. Each box represents the middle 50% of the data (25—75% range). The central horizontal line represents the median. Whiskers represent the 10—90% range of data. Statistically significant differences are indicated with common superscripts, all $P < 0.05$.

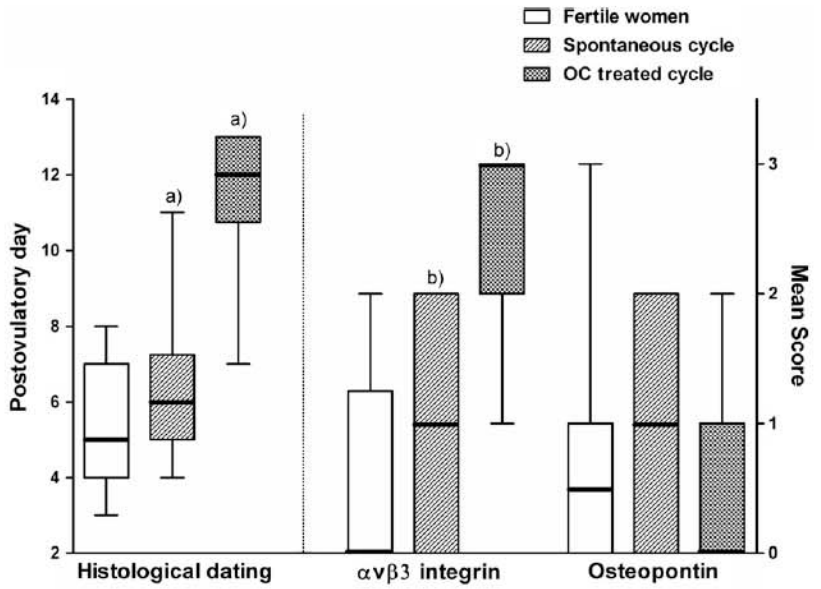

Figure 3 Histological dating, αvβ3 integrin and osteopontin expression in spontaneous cycles in control fertile women and spontaneous and treatment mid-luteal endometrium specimens in the oral contraception (OC)-treated group. Each box represents the middle 50% of the data (25—75% range). The central horizontal line represents the median. Whiskers represent the 10—90% range of data. Statistically significant differences are indicated with common superscripts, all $P < 0.05$.

appearing mainly at post-ovulatory days 6—7 and being expressed by all endometria dated as post-ovulatory day ≥8. In contrast with the expression of αvβ3 integrin, OPN was already expressed in 40—50% of endometria dated as post-ovulatory day 3—4, was observed in 80—100% of endometrial biopsies dated as post-ovulatory days 5 to 11 and was much less frequently observed in endometria dated as post-ovulatory day >11 (Figure 7). These changes in OPN and αvβ3 integrin expression occurred irrespective of endometria being in phase or out of phase and obtained both in spontaneous or treated cycles. No significant differences were found between spontaneous and treatment cycles in

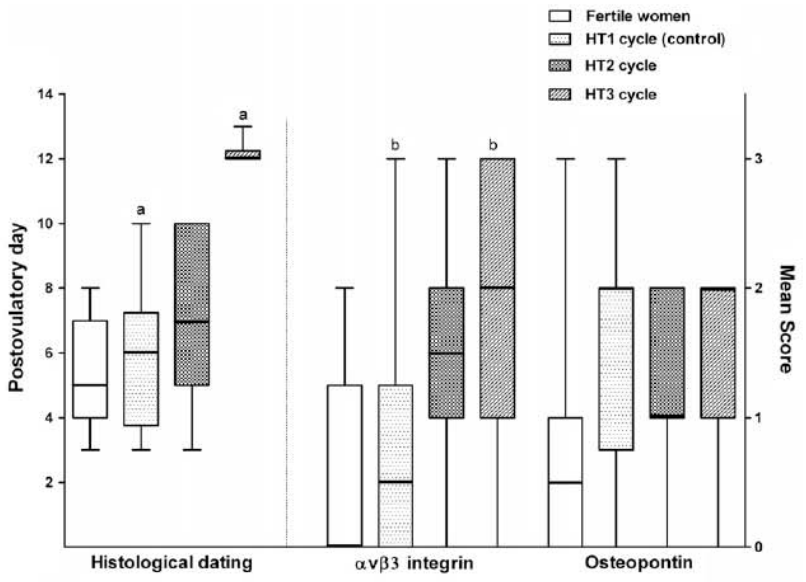

Figure 4 Histological dating, αvβ3 integrin and osteopontin expression in spontaneous cycles in control fertile women and control (group HT1) and treatment (group HT2, group HT3) mid-luteal endometrium specimens in patients receiving hormone replacement therapy. Each box represents the middle 50% of the data (25–75% range). The central horizontal line represents the median. Whiskers represent the 10–90% range of data. Statistically significant differences are indicated with common letters: a = $P < 0.001$; b = $P < 0.05$.

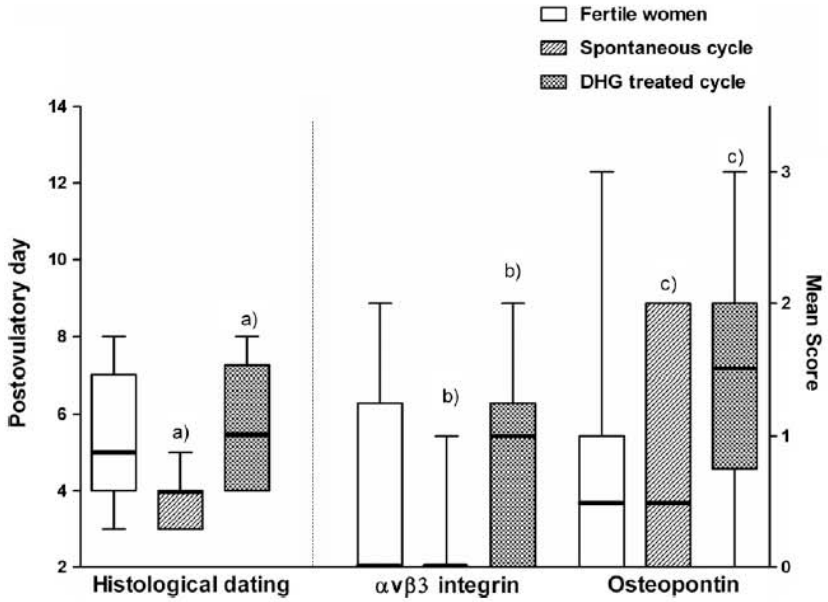

Figure 5 Histological dating, αvβ3 integrin and osteopontin expression in spontaneous cycles in control fertile women and spontaneous and treatment mid-luteal endometrium specimens in the dehydrogesterone (DHG)-treated group. Each box represents the middle 50% of the data (25–75% range). The central horizontal line represents the median. Whiskers represent the 10–90% range of data. Statistically significant differences are indicated with common superscripts, all $P < 0.05$.

the seven experimental groups studied with respect to the expression of endometrial markers or serum steroid concentrations in the late luteal phase biopsy (data not shown).

The co-expression of OPN and αvβ3 integrin in the seven experimental groups of patients is presented in Table 2. The simultaneous presence or absence of both endometrial markers was observed in 71.7% and 76.7% of control and treated cycles. Corresponding figures for the first and second study cycles in group NT were 60% and 50%, respectively.

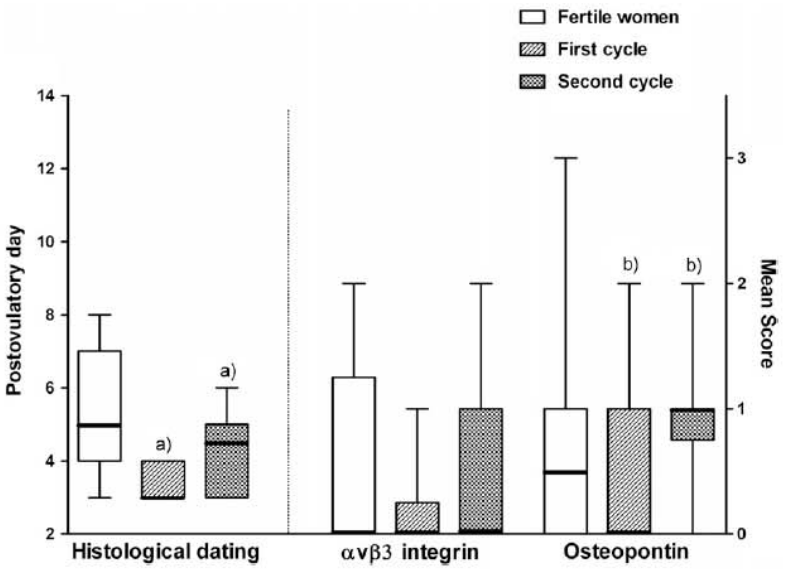

Figure 6 Histological dating, αvβ3 integrin and osteopontin expression in spontaneous cycles in control fertile women and spontaneous and treatment mid-luteal endometrium specimens in the not-treated group. Each box represents the middle 50% of the data (25–75% range). The central horizontal line represents the median. Vertical lines represent the 10–90% range of data, as indicated by the small horizontal lines. Statistically significant differences are indicated with common superscripts, all $P < 0.05$.

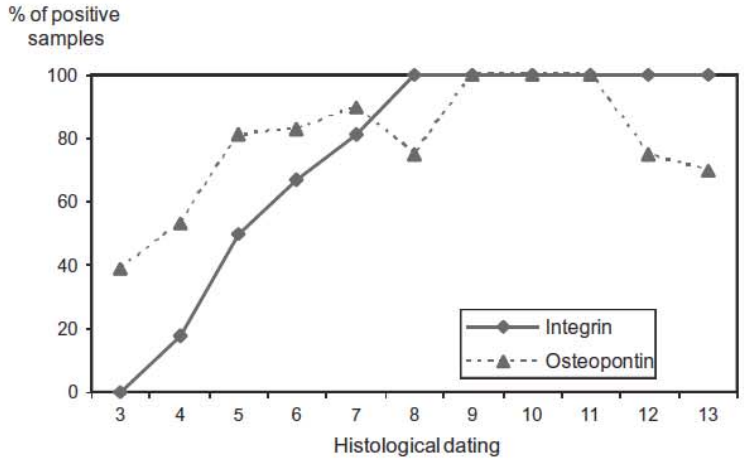

Figure 7 Temporal patterns of endometrial αvβ3 integrin and osteopontin expression over the luteal phase in the endometrial samples studied ($n = 133$). Percentage of endometria showing αvβ3 integrin and osteopontin for each histological day is presented.

Discussion

Traditionally, histological assessment and morphological features of luteal endometrial samples were used to investigate the human endometrium as a fertility-determining factor (Noyes et al., 1950). Using this diagnostic tool, adverse effects on endometrial secretory patterns have been reported in women using oral contraceptives, in patients receiving ovarian stimulation with clomiphene citrate or gonadotrophins for IVF and in those given hormone replacement therapy (Habiba et al., 1998; Makker and Singh, 2006; Strowitzki et al., 2006). Similarly, the administration of progesterone in the follicular phase of an artificial cycle (as a model of premature luteinization) has been reported to impair endometrial development (Ezra et al., 1994). In contrast, luteal oestradiol depletion in hormone replacement therapy cycles does not seem to adversely affect the morphological developmental capacity of the endometrium (Younis et al., 1994). Dehydrogesterone can be successfully used to induce normal endometrial maturation in patients diagnosed as having luteal phase deficiency (Balasch et al., 1982) but an association between the treatment for delayed maturation endometria and pregnancy in infertile patients is lacking (Balasch et al., 1986, 1992). However, it has become clear that normal endometrial histology does not reflect normal endometrial function or receptivity and, thus, a number of biomarkers of normal endometrial development and receptivity have recently

Table 2 Osteopontin (OPN) and αvβ3 integrin epithelial co-expression in control and treated cycles in the seven experimental groups studied in the mid-luteal phase.

Group	n	OPN/αvβ3 co-expression			
		OPN−/αvβ3−	OPN+/αvβ3−	OPN−/αvβ3+	OPN+/αvβ3+
Clomiphene citrate					
Control cycle	10	1 (10)	3 (30)	1 (10)	5 (50)
Treated cycle	10	1 (10)	2 (20)	2 (20)	5 (50)
IVF					
Control cycle	10	3 (30)	2 (20)	1 (10)	4 (40)
Treated cycle	10	1 (10)	1 (10)	1 (10)	7 (70)
Oral contraceptive pill					
Control cycle	10	1 (10)	0 (0)	1 (10)	8 (80)
Treated cycle	10	0 (0)	0 (0)	4 (40)	6 (60)
Hormone replacement therapy					
Control cycle (HT1)	20	4 (20)	6 (30)	0 (0)	10 (50)
Treated cycle (HT2)	10	1 (10)	1 (10)	1 (10)	7 (70)
Treated cycle (HT3)	10	1 (10)	0 (0)	0 (0)	9 (90)
Dehydrogesterone					
Control cycle	10	5 (50)	3 (30)	0 (0)	2 (20)
Treated cycle	10	2 (20)	2 (20)	0 (0)	6 (60)
No treatment					
First cycle	10	5 (50)	3 (30)	1 (10)	1 (10)
Second cycle	10	2 (20)	5 (50)	0 (0)	3 (30)

Values are number or number (%).
HT1 = oestradiol valerate 28 cycle days and micronized vaginal progesterone from days 15 to 28 of the oestrogenic treatment; HT2 = first cycle treated as for HT1 and ensuing cycle treated with oestradiol valerate treatment for 14 days and micronized vaginal progesterone for another 14 days; HT3 = same treatment as HT1 with also micronized vaginal progesterone on days 8 and 11.

been proposed (Makker and Singh, 2006; Sharkey and Smith, 2003; Strowitzki et al., 2006).

A receptive endometrium is characterized by abundant secretory activity (Noyes et al., 1950). In women, OPN has been associated with glandular epithelial cells during the secretory phase and in increasing concentrations in uterine secretions during the midsecretory to late secretory phase during which implantation would be expected to occur (Apparao et al., 2001; von Wolff et al., 2001). In fact, OPN is transcriptionally regulated by progesterone and is an acidic extracellular matrix glycoprotein; it binds especially to αvβ3 integrin and both glycoproteins are involved in cell adhesion and migration (Apparao et al., 2001). Thus, at present, they are being intensively studied in infertility patients (Cho et al., 2009; DuQuesnay et al., 2009; Wei et al., 2009) but the need for prospective clinical controlled studies has been stressed (Sharkey and Smith, 2003).

Therefore, the current study provides new useful information on the subject. The effect of different hormone treatments on the endometrial expression of those two markers of implantation has been studied using a prospective, self-controlled study design where patients served as their own controls and were investigated during spontaneous and ensuing treated (or untreated) cycles. Both in CC and HT2 groups, mean histological dating in spontaneous and ensuing treated cycles was similar and no effect on OPN and αvβ3 integrin expression was observed. In contrast, in the IVF, OC, HT3 and DHG groups, as well as in patients in group NT, there was a significant advance in endometrial histological dating and this was associated with a significant increase in both OPN and αvβ3 integrin expression (groups IVF and DHG), in OPN but not αvβ3 integrin increased staining (group NT) or in αvβ3 integrin but not OPN expression (groups OC and HT3). Overall, these results clearly indicate that the expression of these two putative biomarkers of implantation in the human endometrium is a process closely related to endometrial maturation and this is irrespective of the endometrium being in-phase or out-of-phase and the hormonal treatment (or no treatment) received. In addition, the current investigation shows that a high level of co-ordinate expression of OPN and αvβ3 integrin expression existed on post-ovulatory days 9–11 but there was a lack of temporal co-expression of these markers over the luteal phase in the endometrial samples studied (**Figure 7**). Remarkably, again this was true irrespective of endometria being in-phase or out-of-phase and coming from spontaneous or treated cycles.

The above results should not be surprising considering that that these two glycoproteins are differentially regulated. The expression of OPN is primarily stimulated by progesterone, whereas the αvβ3 integrin epithelial expression is up-regulated by epidermal growth factor or heparin-binding epidermal growth factor (Apparao et al., 2001; Lessey, 2002). This may also explain why the OPN: αvβ3 integrin complex provided no additional useful information beyond that derived from investigating OPN alone or integrin αvβ3 alone as markers of endometrial function.

It could be argued that the αvβ3 integrin has been reported to be a versatile receptor of other extracellular matrix proteins such as vitronectin and fibronectin (García et al., 2004; Hoozemans et al., 2004). On the other hand, leukaemia inhibitory factor (LIF), an interleukin-like cytokine, seems to be a critical regulator of the endometrial function and meets also the profile of a potential marker of implantation (Hoozemans et al., 2004; Kondera-Anasz et al., 2004; Minas et al., 2005). Endometrial expression of those factors was not investigated in the current study. Even so, previous work has clearly shown that OPN is the most highly up-regulated extracellular matrix adhesion molecule in the human endometrium as it becomes receptive to implantation (Makker and Singh, 2006). In contrast, while human studies indicate that fibronectin and vitronectin are present in the decidua and experimental work suggests that they may promote adhesion of preimplantation embryos, the role of these molecules as markers of endometrial receptivity in the human remains to be determined (García et al., 2004; Hoozemans et al., 2004; Mangale and Reddy, 2007; Ruck et al., 1994). Notably, simultaneous immunohistochemical determination of integrins (αvβ3, α4β1) and LIF in the endometrium of healthy fertile women has shown that these two widely cited markers of implantation were present in the luteal phase but do not have synchronous expression; αvβ3 integrin was highly expressed in luminal and glandular epithelium from day 22–28 while LIF was present in both luminal and glandular epithelium showing weak, erratic expression (i.e. without a definite luteal phase pattern) (Acosta et al., 2000). In addition, there is no clear evidence for alterations in LIF secretion or expression in subfertile women (Sharkey and Smith, 2003). Therefore, it is unlikely that analysis of vitronectin, fibronectin and LIF expression would provide additional useful information beyond that derived from the current study.

Global gene expression profiling in human endometrium has identified OPN but not integrin αvβ3 as being markedly up-regulated during the receptive phase for implantation (Aghajanova et al., 2008). On the other hand, several studies have previously reported the findings of overall mRNA expression (including OPN expression) in endometrial samples (Aghajanova et al., 2008) or have been focused in osteopontin using northern blots, RNA protection assays (von Wolff et al., 2001) or quantitative PCR (Cho et al., 2009; DuQuesnay et al., 2009). However, many genes are expressed during the window of implantation, but their functional roles remain unclear (Wei et al., 2009) and although these techniques are extremely useful in the search of markers of endometrial receptivity, their results need to be confirmed by the evaluation of the functional molecule, which is the protein. As recently stressed, the simplest and most efficient way to study gene expression is the identification of specific proteins with antibodies applied in capture assays, flow cytometry, immunocytochemistry and immunohistochemistry (Serafini et al., 2009; Uhlen and Ponten, 2005), as mRNA recomposition, modification and processing alters functional protein production (Azad et al., 2006).

Immunohistochemistry enable the pathologist to extract additional information from fixed, deparaffinized specimens and to provide data critical to optimal clinical management of the patient. There is currently a wealth of applications of this technique to gynaecological pathology, in lesions that include neoplastic and non-neoplastic conditions (Yaziji and Gown, 2001). Immunohistochemistry, as used in this study, allows the analysis of protein expression, providing information on the histological and subcellular distribution of the protein (i.e. glandular epithelium or stroma) and is particularly appropriate for clinically based studies. In fact, as previously stressed (Pei et al., 2007), immunohistochemistry staining is necessary to access the clinicopathological characteristics of proteins identified by proteomics and genomics analysis.

In conclusion, immunohistochemical assessment of the endometrium is a potentially valuable method for the evaluation of the receptive endometrial state. Using such a technique, the present study indicates that the use of OPN and integrin αvβ3 or the OPN: αvβ3 integrin complex as targets for the development of contraceptive approaches or the understanding of the pathogenesis of female infertility offer little benefit compared with simple histological dating.

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ESTUDIO 3

"Patrón de expresión de la osteopontina y la integrina αvβ3 durante la ventana de implantación en pacientes estériles con estadios iniciales de endometriosis"

"Expression pattern of osteopontin and αvβ3 integrin during the implantation window in infertile patients with early stages of endometriosis"

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ORIGINAL ARTICLE *Reproductive biology*

Expression pattern of osteopontin and αvβ3 integrin during the implantation window in infertile patients with early stages of endometriosis

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BACKGROUND: To study endometrial receptivity in terms of osteopontin (OPN) and αvβ3 integrin expression and co-expression in infertile women with early stages of endometriosis.

METHODS: We investigated the immunohistochemical expression and co-expression of OPN and αvβ3 integrin in the endometrium of 20 infertile patients with Stage I or II endometriosis as the only detectable cause of infertility, 20 infertile patients with unexplained infertility and 20 fertile women undergoing tubal sterilization. Two endometrial biopsies were performed during a single menstrual cycle (postovulatory Day +7 to +8 and 4 days later) in each subject.

RESULTS: No statistically significant differences regarding OPN and αvβ3 integrin expression were found between infertile patients with endometriosis and the two control groups. There was no significant correlation between OPN and αvβ3 integrin staining intensity in the mid-luteal phase biopsies in any of the groups studied.

CONCLUSIONS: Endometrial OPN and αvβ3 integrin expression or co-expression is not impaired during the window of implantation in patients with Stage I–II endometriosis. Further studies are needed to determine whether these results imply normal endometrial receptivity in such patients or add to the increasing uncertainty about the clinical value of assessing the endometrium with these markers of implantation.

Key words: endometrium / endometriosis / implantation / integrins / osteopontin

Introduction

A significant association between infertility and minimal-to-mild endometriosis has been reported in the literature (Trinder and Cahill, 2002). Numerous investigations have been performed to study fertility impairment in patients with endometriosis and different mechanisms have been proposed to explain why fertility is affected in these women: altered folliculogenesis, poor oocyte quality, reduced fertilization, abnormal embryogenesis and also decreased embryo implantation capacity (Gupta *et al.*, 2008).

The reduced implantation capacity in endometriosis patients is difficult to explain because of a lack of understanding about the normal physiologic mechanisms of embryo implantation. Moreover, successful implantation depends both on a good quality embryo and a receptive endometrium and, at present, there is controversy as to whether reduced implantation in patients with endometriosis is due to altered oocyte/embryo quality or endometrial inadequacy (Garrido *et al.*, 2002). Interestingly, a previous meta-analysis (Barnhart *et al.*, 2002) suggested that the potential negative effect of endometriosis on IVF is not exclusively on the receptivity of the endometrium but also on the development of the oocyte and embryo.

One of the mechanisms proposed for impaired implantation in endometriosis patients involves the abnormal expression of cellular adhesion molecules in the eutopic endometrium. αvβ3 Integrin and its extracellular matrix ligand, osteopontin (OPN) are two of the best-characterized endometrial receptivity biomarkers. These two glycoproteins have been found to be co-ordinately expressed in the human endometrium throughout the menstrual cycle in normally

cycling fertile women (Apparao et al., 2001). The maximal expression of these two molecules during the implantation window in human endometrial epithelial cells and secretion of OPN into the uterine cavity suggests a role of both factors in the regulation of endometrial function and embryo implantation (Apparao et al., 2001; Von Wolff et al., 2001). For this reason, the study of these two markers has been proposed as a means of distinguishing receptive from non-receptive endometrium in clinical practice and as a new method to investigate the impaired endometrial receptivity in certain groups of infertile patients (Lessey, 2002; Makker and Singh, 2006; Strowitzki et al., 2006).

Different authors (Lessey et al., 1994; Ordi et al., 2003; Odagiri et al., 2007; Cho et al., 2009; Wei et al., 2009) have studied the endometrial expression of αvβ3 integrin or OPN in endometriosis patients with controversial results. On the other hand, to our knowledge there are no previous controlled investigations on the simultaneous expression of these two endometrial receptivity markers in endometriosis patients. Therefore, the present study was aimed to investigate endometrial expression and co-expression of αvβ3 integrin and OPN in women with early stages of endometriosis.

Materials and Methods

Patients and study cycle

We investigated the expression of αvβ3 integrin and OPN in the endometrium of 20 infertile patients diagnosed by laparoscopy with Stage I or II endometriosis (American Fertility Society, 1985) as their sole detectable cause of infertility (END group). We included two control groups: 20 infertile patients with unexplained infertility (UNEX group) and 20 fertile women who were undergoing tubal sterilization and had no evidence of endometriosis (FERT group). This latter group were healthy women who had at least one child and had no history of infertility or miscarriage. Unexplained infertility patients had a normal infertility work-up including, in addition to endometrial biopsy, a semen analysis, mid-luteal serum progesterone and prolactin determination, an hysterosalpingogram and laparoscopy. According to the ESHRE Guidelines (1996), standard investigations aimed to evaluate infertility in a couple include laboratory assessment of ovulation, evaluation of tubal patency and semen analysis. Thus, unexplained infertility is a term applied to an infertile couple whose standard investigations (semen analysis, tubal patency and laboratory assessment of ovulation) yield normal results (ESHRE, 1996). However, for the specific purpose of this study couples were diagnosed as having unexplained infertility only once laparoscopy was performed and the presence of endometriosis excluded. The mean age of the women with endometriosis, unexplained infertility and those who were fertile was 31.5 ± 0.7, 32.1 ± 0.5 and 34.9 ± 0.9 years (mean $\pm$ SEM), respectively. All the women (both fertile and infertile) included in our study had regular menstrual cycles (27–32 days) and were taking no medication. A number of both infertile patients and controls had participated in previously published studies (Casals et al., 2008, 2010). The sample size was decided arbitrarily but in keeping with previous studies on the subject (Odagiri et al., 2007; Wei et al., 2009).

In all women, basal body temperature, luteal serum concentrations of estradiol and progesterone and endometrial biopsies were obtained in the same cycle to assess luteal function according to a previously reported (Casals et al., 2008) scheme of evaluation. Commencing on Days 8–10 of the study cycle (depending on the cycle length of the woman) patients underwent daily transvaginal ultrasonographic evaluation of follicular growth using a Toshiba Eccocee SAA-340A/EF unit (Toshiba Co., Tokyo, Japan) equipped with a 5–7 MHZ endo-vaginal probe (PVF-641VT). The maximum follicular diameter was measured in all patients. Both ovaries were identified and the largest diameter was measured in both the longitudinal and transverse dimensions in all follicles. The day of ovulation was designated as the day of maximum follicular enlargement, which was followed the next day by sudden disappearance or filling in of this follicle showing loss of clear demarcation of its walls and intrafollicular echoes (Shoupe et al., 1989; Peters et al., 1992). We used ultrasonographic monitoring of ovulation because previous studies have shown that the accuracy of histological endometrial dating is best determined when ovulation is detected by that method (Shoupe et al., 1989; Peters et al., 1992).

Two endometrial biopsies were performed during a single menstrual cycle in each subject. The chronological day of each patient was determined by counting forward from the ovulation day as detected by ultrasonographic scans. The early biopsy (mid-luteal) was performed on ovulation Day +7 to +8, whereas the second biopsy (late luteal) was always performed 4 days after the first biopsy. For the specific purpose of this study, endometrial evaluation was performed in all women as a part of a routine infertility work-up and always before laparoscopy. It should be stressed that a study by Daya (1996) on pregnancy loss showed that a relationship between miscarriage and endometriosis was only found in the patients studied before laparoscopy not after. The use of human tissue for research was based on informed consent and was approved by the Ethics Committee of our hospital.

Hormones in serum were quantified on the same day as endometrial sampling. All samples were obtained in the fasting state between 08.00 and 10.00 h corresponding to the period of minimal progesterone variability in spontaneous menstrual cycles, thereby providing added accuracy to the measurement (Filicori et al., 1984).

Endometrial samples

Endometrial samples were divided into two parts: one part was fixed in 10% formalin and embedded in paraffin and the second tissue sample was snap frozen in methylbutane (Merck, Darmstadt, Germany) immersed in liquid nitrogen and stored at −70°C until immunolabelling.

Endometrial dating

For endometrial dating, 4-μm sections stained with haematoxylin and eosin and periodic acid-Schiff stain were evaluated according to the histopathological criteria of Noyes et al. (1950). All endometrial biopsies were evaluated by the same experienced gynaecological pathologist (J.O.) who was blinded with regard to the ultrasonographically detected ovulatory day and the study group. Endometrial biopsy interpretation was performed using a single-day evaluation whenever possible and when the traditional 2-day spread evaluation method (i.e. Day 20–21) was provided, the latter day was used for comparison with immunohistochemical assays. An out-of-phase biopsy was defined as a lag of ≥3 days between the chronological and the histological day (Creus et al., 2002; Ordi et al., 2002).

Immunohistochemistry

Immunohistochemical studies were performed with the automated immunohistochemical system TechMate 500 ™ (Dako Co., Carpinteria, CA, USA), using the EnVision system (Dako) as previously reported (Creus et al., 2002; Casals et al., 2008, 2010). Integrin αvβ3 was detected in frozen tissue using a monoclonal antibody (clone LM609, dilution 1:200; Chemicon Int., Temecula CA, USA,) and OPN in formalin-fixed, paraffin-embedded tissue, using a polyclonal antibody (Chemicon). Frozen sections (4 mm thick) were fixed for 10 min in acetone at 4°C and dried. Paraffin sections were deparaffinized and rehydrated in xylene and graded alcohols. Peroxidase was blocked for 7.5 min in

ChemMate peroxidase-blocking solution (Dako). Then the slides were incubated with the primary antibodies for 30 min and washed in ChemMate buffer solution (Dako). The peroxidase-labelled polymer was applied for 30 min. After washing in ChemMate buffer solution, the slides were incubated with the diaminobenzidine substrate chromogen solution, washed in water, counterstained with haematoxylin, washed, dehydrated and mounted. As previously reported by our group and others (Von Wolff *et al.*, 2001; Creus *et al.*, 2002), a negative control was performed in every case by omission of incubation with the primary specific antibody. The reactivity of each monoclonal antibody with endometrial glands and surface epithelium, stromal cells and vessels was assessed. The intensity of staining of the endometrial components was evaluated by a semi-quantitative scoring system (0–4) used in our previous publications (Creus *et al.*, 2002; Ordi *et al.*, 2002): absent (−), weak or focal (+), moderate (++) and strong (+++; Fig. 1). As there is frequent expression of OPN and αvβ3 integrin in the endometrial stroma with minor variations, the intensity of the staining was evaluated as the intensity observed in the epithelial surface and glands where variations were evident. Endometrial samples were considered as expressing αvβ3 integrin and/or OPN when these glycoproteins were detected in both endometrial glands and luminal surface epithelium with the intensity of the reaction ranging from weak/focal to strong (Creus *et al.*, 2002; Ordi *et al.*, 2002; Casals *et al.*, 2008, 2010). The whole biopsy was analysed in each sample for immunohistochemical expression. As some variation was observed in intensity between different areas in a single biopsy, each biopsy was scored as the highest intensity observed in at least 30% of the glands.

To further evaluate OPN and αvβ3 integrin expression in the endometrium, the H-Score method was also used as recommended by others (Lessey *et al.*, 1995a,b). This immunohistochemical semi-quantitative method consists of a sum of the percentages of positively stained cells multiplied by a weighted intensity of staining: H-Score = $\Sigma Pi(i+1)$, where Pi is the percentage of stained cells in each intensity category (0–100%), and i is the intensity indicating weak ($i = 1$), moderate ($i = 2$) or strong staining ($i = 3$; Budwit-Novotny *et al.*, 1986; Lessey *et al.*, 1995b). A large study of endometrial adenocarcinoma have previously reported a low intraobserver ($r = 0.983$; $P = 0.00001$) and interobserver ($r = 0.994$; $P = 0.00001$) differences for H-Score method (Budwit-Novotny *et al.*, 1986).

The correlation between the H-Score method and the scoring system used in our previous studies on the subject was also analysed in the current investigation.

Hormone assays

Hormones were measured using commercially available kits. Estradiol and progesterone concentrations in serum were estimated by a competitive chemiluminescent assay (ADVIA Centaur CP System; Siemens Healthcare Diagnostics, Tarrytown, NY, USA). The sensitivity was 10 pg/ml for estradiol and 0.15 ng/ml for progesterone and the inter-assay CVs were 5 and 5.4%, respectively.

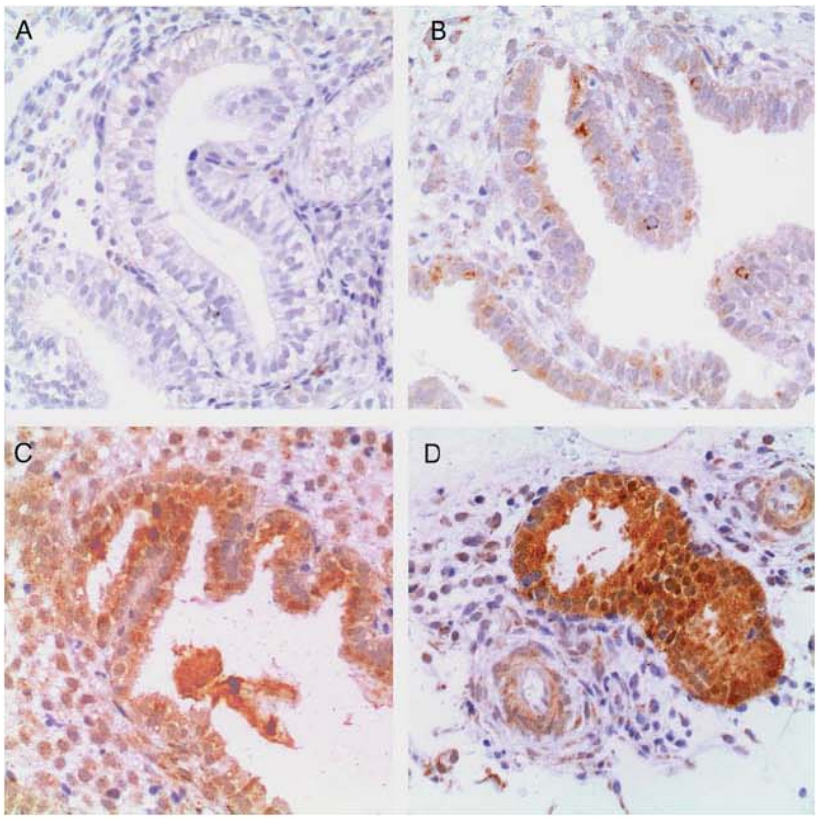

Figure 1 Immunohistochemistry of OPN in endometrial specimens: (**A**) no expression detected in epithelial cells; (**B**) focal immunostaining detected in the glandular epithelium; (**C**) moderate immunostaining and (**D**) strong immunostaining.

Statistical analysis

Data were analysed with SPSS statistical software (Release 15.0, SPSS, Inc., Chicago, IL). The χ^2 test and Kruskal–Wallis test were used as appropriate. The correlation between histological dating, αvβ3 integrin and OPN expression and between the H-Score method and our previously used scoring system was evaluated using the Spearman rank correlation coefficient. Results are expressed as means ± SEM. The level of significance was set at $P \leq 0.05$.

Results

All menstrual cycles studied in the current investigation were ovulatory according to ultrasonographic criteria and mid-luteal serum progesterone >10 ng/ml. The endometrial specimens were noted to be clearly progestational fundal samples in all instances. A late-luteal endometrial biopsy could not be done in four patients in the END group, in four in the UNEX group and in one in the FERT group because menses had commenced at the time of the second endometrial sampling. No inflammatory or reactive change related to the first sampling was detected in the second biopsy in any patient.

Tables I and II summarize the data related to endometrial histology and αvβ3 integrin and OPN expression in the mid-luteal and late-luteal phase endometrial biopsies carried out in the END, UNEX and FERT groups, as well as mid-luteal and late-luteal serum hormone concentrations. No statistically significant differences were found between the three groups studied with respect to histology, expression of endometrial markers evaluated with both staining intensity evaluation methods or hormonal parameters in either the mid- or the late-luteal phase. Histological dating, αvβ3 integrin expression and OPN expression in endometrial samples in the three groups are presented in Fig. 2 (mid-luteal biopsy) and Fig. 3 (late-luteal biopsy).

When the whole group of endometrial samples included in this study were considered, the H-Score method and our previously reported scoring system were highly correlated in evaluating the immunohistochemical staining intensity: $r = 0.96$ for integrin and $r = 0.91$ for OPN in the mid-luteal phase, $r = 0.71$ for integrin and $r = 0.84$ for OPN in the late-luteal phase ($P < 0.0001$ in all cases). This provides support to our previous studies.

Endometrial co-expression of αvβ3 integrin and OPN during the implantation window is shown in Table I. The simultaneous presence or absence of both markers was observed in only 60% (36/60) of mid-luteal biopsies with no differences between the three groups studied. There was no significant correlation between αvβ3 integrin and OPN staining intensity in the mid-luteal phase biopsies in any of the groups studied (Fig. 4).

Discussion

OPN is related to cell adhesion and inflammation and, therefore, this molecule and its ligand αvβ3 integrin may be involved in the pathogenesis of endometriosis. On the other hand, OPN has been found to be consistently up-regulated in the endometrium during the window of implantation in different studies of the transcriptome (Kao *et al.*,

Table I Endometrial biopsy and epithelial quantitative and qualitative αvβ3 integrin and OPN expression and their coexpression in the three groups studied in the mid-luteal phase.

Parameter	Endometriosis ($n = 20$)	Unexplained infertility ($n = 20$)	Fertile controls ($n = 20$)	*P* value
Endometrial biopsy				
Chronological dating	7.3 ± 0.2	7.3 ± 0.1	7.5 ± 0.1	NS
Histological dating	5.6 ± 0.4	5.4 ± 0.4	5.6 ± 0.4	NS
In-phase endometria	14 (70.0)	11 (55.0)	12 (60.0)	NS
αvβ3 integrin expression				
Positive samples	10 (50.0)	8 (40.0)	7 (35.0)	NS
Mean staining score	0.7 ± 0.2	0.5 ± 0.2	0.6 ± 0.2	NS
H-Score	0.5 ± 0.1	0.4 ± 0.2	0.6 ± 0.2	NS
OPN expression				
Positive samples	14 (70.0)	13 (65.0)	14 (70.0)	NS
Mean staining score	1.3 ± 0.2	1.3 ± 0.2	1.1 ± 0.2	NS
H-Score	1.1 ± 0.2	0.9 ± 0.2	0.8 ± 0.2	NS
OPN/αvβ3 coexpression				NS
OPN (−)/αvβ3 (−)	4 (20.0)	6 (30.0)	5 (25.0)	
OPN (+)/αvβ3 (−)	6 (30.0)	6 (30.0)	8 (40.0)	
OPN (−)/αvβ3 (+)	2 (10.0)	1 (5.0)	1 (5.0)	
OPN (+)/αvβ3 (+)	8 (40.0)	7 (35.0)	6 (30.0)	
Hormone concentrations				
Estradiol (pg/ml)	153.2 ± 13.9	143.3 ± 14.5	139.3 ± 12.9	NS
Progesterone (pg/ml)	20.2 ± 2.8	19.2 ± 1.6	17.8 ± 1.6	NS

Values are expressed as mean ± SEM or *n* (%).

Table II Endometrial biopsy and epithelial quantitative and qualitative αvβ3 integrin and OPN expression and their coexpression in the three groups studied in the late-luteal phase.

Parameter	Endometriosis (n = 16)	Unexplained infertility (n = 16)	Fertile controls (n = 19)	P value
Endometrial biopsy				
Chronological dating	11.2 ± 0.2	11.3 ± 0.1	11.5 ± 0.1	NS
Histological dating	11.3 ± 0.2	11.2 ± 0.2	11.7 ± 0.2	NS
In-phase endometria	16 (100)	16 (100)	19 (100)	NS
αvβ3 integrin expression				
Positive samples	16 (100)	16 (100)	18 (94.7)	NS
Mean staining score	2.6 ± 0.2	2.9 ± 0.1	2.6 ± 0.2	NS
H-Score	3.2 ± 0.2	3.4 ± 0.2	3.2 ± 0.3	NS
OPN expression				
Positive samples	16 (100)	16 (100)	19 (100)	NS
Mean staining score	2.3 ± 0.1	2.1 ± 0.1	2.3 ± 0.2	NS
H-Score	2.4 ± 0.2	2.2 ± 0.1	2.6 ± 0.2	NS
OPN/αvβ3 coexpression				NS
OPN (−)/αvβ3 (−)	0 (0)	0 (0)	0 (0)	
OPN (+)/αvβ3 (−)	0 (0)	0 (0)	1 (5.3)	
OPN (−)/αvβ3 (+)	0 (0)	0 (0)	0 (0)	
OPN (+)/αvβ3 (+)	16 (100)	16 (100)	18 (94.7)	
Hormone concentrations				
Estradiol (pg/ml)	122.2 ± 11.1	125.9 ± 15.6	93.0 ± 13.7	NS
Progesterone (pg/ml)	10.9 ± 1.2	11.6 ± 1.0	8.8 ± 1.2	NS

Values are expressed as mean ± SEM or n (%).

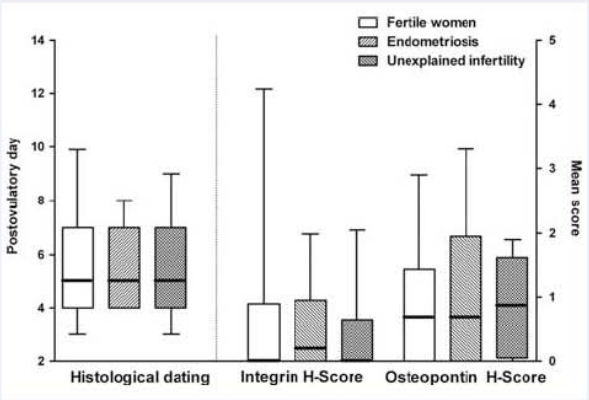

Figure 2 Box-and-whisker plot showing histological dating and αvβ3 integrin and OPN expression in patients with endometriosis, unexplained infertility and fertile women in the mid-luteal phase. Each box represents the middle 50% of the data (25–75% range). The central horizontal line represents the median. Vertical lines represent the 10–90% range of data, as indicated by the small horizontal lines. No significant differences were found among the three groups studied.

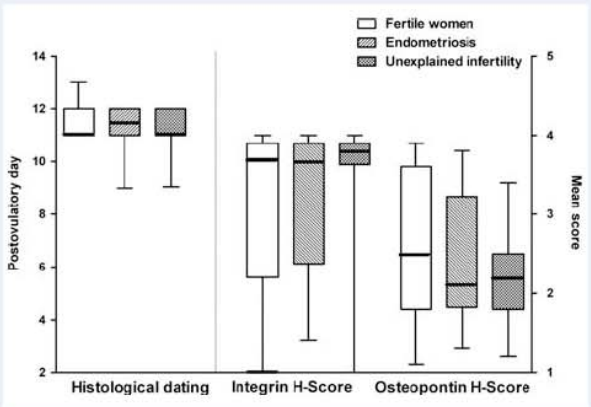

Figure 3 Box-and-whisker plot showing histological dating and αvβ3 integrin and OPN expression in patients with endometriosis, unexplained infertility and fertile women in the late-luteal phase. Each box represents the middle 50% of the data (25–75% range). The central horizontal line represents the median. Vertical lines represent the 10–90% range of data, as indicated by the small horizontal lines. No significant differences were found among the three groups studied.

2002; Borthwick *et al.*, 2003; Riesewijk *et al.*, 2003; Mirkin *et al.*, 2005). For these reasons, in recent years OPN and its ligand αvβ3 integrin have been intensively studied in reproductive medicine in general (Quenby *et al.*, 2007; Franchi *et al.*, 2008; DuQuesnay *et al.*, 2009) and in endometriosis in particular (Odagiri *et al.*, 2007; Cho *et al.*, 2009; Wei *et al.*, 2009).

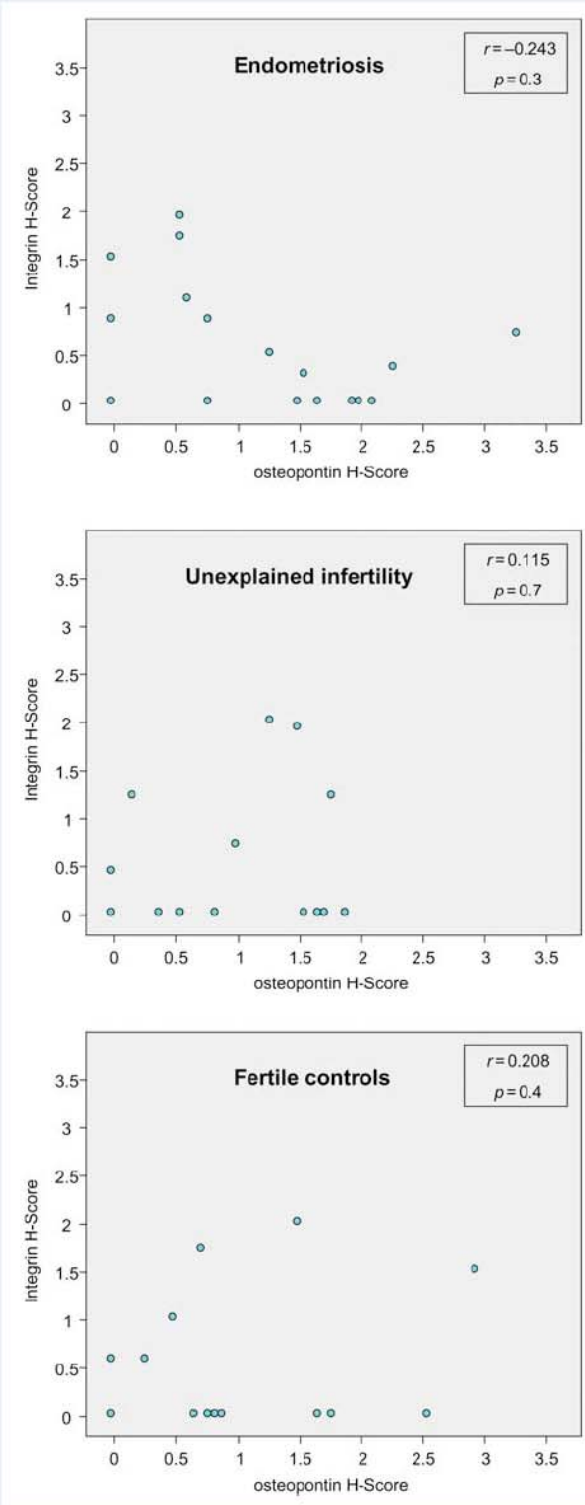

Figure 4 Correlation between staining intensity for OPN and αvβ3 integrin expression in epithelial cells of mid-luteal endometrial biopsies in patients with endometriosis, unexplained infertility and fertile women.

Endometrial expression of OPN has been studied by immunohistochemistry in a rat endometriosis model and in human endometriosis patients and controls (Odagiri *et al.*, 2007). This study showed that, in humans, the staining pattern of OPN in endometriotic lesions was similar to that in eutopic endometrium in the secretory phase of control specimens. Other investigators demonstrated that OPN mRNA expression in eutopic endometrium was significantly increased in patients with endometriosis compared with that in controls (Cho *et al.*, 2009). In contrast with these findings, a recent immunohistochemical study revealed decreased OPN expression in the late secretory phase endometrium in patients with endometriosis. However, OPN expression was similar in endometriosis and control groups in the mid-secretory phase (Wei *et al.*, 2009). On the other hand, an earlier report on patients with endometriosis indicated that αvβ3 integrin expression appeared to be reduced while OPN expression remained unaffected. This author suggested reduced OPN binding to the surface epithelium, but only in cases where αvβ3 integrin was decreased (Lessey, 2002). Finally, our immunohistochemical analysis of OPN and its ligand αvβ3 integrin did not show different expression of these two markers between eutopic endometrial specimens of endometriosis patients and control groups without endometriosis, either when these two markers were studied alone or in combination. Discrepancies between different studies could be explained by the following facts.

First, discordant results are often obtained in endometriosis investigations because of the heterogeneity of this disorder, with patients with different stages of endometriosis often being included in the same study group (Gupta *et al.*, 2008). To avoid this circumstance, the inclusion of patients in the current study was restricted to patients with early stages of endometriosis. Second, it has been stressed that appropriate controls without the disease are required to study endometrial receptivity in endometriosis (Garrido *et al.*, 2002). This was done in our study which included fertile women without endometriosis as well as a second control group of patients with unexplained infertility. On the contrary, patients with pathologies such as cervical carcinoma *in situ* (Odagiri *et al.*, 2007), myomas or benign ovarian cysts (Cho *et al.*, 2009) have been included as controls in some cases, although the authors stressed this limitation in their studies. Third, endometrial samples obtained at different times during the menstrual cycle have been included in the same study group in some investigations (Odagiri *et al.*, 2007; Cho *et al.*, 2009), but we and others have previously demonstrated the cyclic changes in the endometrial expression of integrins and OPN along the menstrual cycle (Lessey *et al.*, 1992; Creus *et al.*, 1998; Apparao *et al.*, 2001; Von Wolff *et al.*, 2001; Casals *et al.*, 2008). Finally, the analysis of mRNA or protein could produce discrepant results because all the mRNA expressed may not always be translated into protein (Cho *et al.*, 2009).

Although immunohistochemical studies have low sensitivity to detect minor variations and semi-quantitative scoring systems used to evaluate these studies have some limitations, they have been extensively used not only in reproductive medicine studies but also in many other areas. On the other hand, both intra- and inter-observer validation may be necessary in studies using immunohistochemical

evaluation of the endometrial markers investigated. In this respect, it is to note that the H-Score used in this investigation has been reported as having low intra-observer and inter-observer differences (Budwit-Novotny *et al.*, 1986).

In addition, as recently stressed, the simplest and most efficient way to study gene expression is the identification of specific proteins with antibodies applied in capture assays, flow cytometry, immunocytochemistry and immunohistochemistry (Uhlen and Ponten, 2005; Serafini *et al.*, 2009), as mRNA recomposition, modification and processing alters functional protein production (Azad *et al.*, 2006). Immunohistochemistry enables the pathologist to extract additional information from fixed, deparaffinized specimens and to provide data critical to optimal clinical management of the patient. There is currently a wealth of applications of this technique to gynaecologic pathology, in lesions that include neoplastic and non-neoplastic conditions (Yaziji and Gown, 2001). Immunohistochemistry, as used in this study, allows the analysis of protein expression, providing information on the histological and subcellular distribution of the protein (i.e. glandular epithelium or stroma) and is particularly appropriate for clinically based studies. In fact, as previously stressed (Pei *et al.*, 2007), immunohistochemistry staining is necessary to access the clinicopathological characteristics of proteins identified by proteomic and genomic analysis.

It could be argued that repetitive endometrial biopsies may have an impact on subsequent endometrial findings. Thus, recent data suggest that injury to the endometrium causes biological events that appear to return the endometrium to a more normal and fertile state (Almog *et al.*, 2010). In addition, an early paper by Castelbaum *et al.* (1994) using two biopsies in the same month also showed correction of a histologic delay, possibly illustrating catch-up, following an initial out of phase biopsy. Although a mechanical effect of the first biopsy in inducing endometrial differentiation in the second biopsy cannot be completely excluded, it is unlikely. The following facts support this contention. First, we and others (Castelbaum *et al.*, 1994; Creus *et al.*, 1998; Acosta *et al.*, 2000; Ordi *et al.*, 2002, 2003) have found no inflammatory or reactive changes consistent with a previous biopsy site when performing two endometrial biopsies during a single menstrual cycle for luteal phase evaluation. Second, we have previously reported (Creus *et al.*, 1998; Ordi *et al.*, 2002, 2003; Casals *et al.*, 2008) that normal or aberrant integrin expression is not associated with specific aetiologies of infertility, mainly endometriosis and unexplained infertility. Third, we have also shown that the expression of the OPN:αvβ3 integrin complex is closely correlated with histological maturation of the endometrium evaluated by histological dating, but neither OPN nor αvβ3 alone or in combination are useful markers of endometrial functional receptivity (Casals *et al.*, 2008, 2010). In fact, neither mid-luteal histological evaluation nor αvβ3 integrin expression in mid- or late-luteal endometrial biopsy specimens correlated with outcome for subsequently untreated infertile women (Ordi *et al.*, 2002). Finally, the normal pattern of pinopode expression, one of the most cited markers postulated to frame the window of implantation, has been established on the basis of sequential endometrial biopsies performed in normal menstruating women (Nikas, 1999a,b).

It could be claimed that if biomarkers like integrins and OPN are evidence of a biochemical defect, only a subset of infertile or endometriosis patients would appear to be affected. Given the high rate of out-of-phase endometrium in both fertile and infertile women (Coutifaris *et al.*, 2004; Murray *et al.*, 2004), inclusion of these samples would tend to dilute the true negative biopsy (those that were in phase). For this reason Lessey *et al.* (1994) studied αvβ3 integrin expression in endometriosis patients including only in phase biopsies. However, no differences were detected in integrin and/or OPN expression between the three groups of patients in our study irrespective of considering in phase mid-luteal biopsies alone, out-of-phase mid-luteal biopsies alone, in phase late-luteal biopsies alone, out-of-phase late-luteal biopsies alone (data not shown) or the whole group of histological samples investigated.

Studies such as ours, which showed no impairment of potential markers of endometrial receptivity, could be in line with different authors suggesting that infertility in endometriosis patients is not related to an inadequate endometrial environment affecting endometrial receptivity but is due to decreased oocyte quality (Pellicer *et al.*, 1994; Sung *et al.*, 1997). The oocyte donation model has been used for this purpose in several studies. A large retrospective analysis compared reproductive outcomes in oocyte recipients with and without endometriosis and demonstrated no adverse effects of this disease on implantation rates, even when recipients were subdivided by stage of endometriosis (Sung *et al.*, 1997). Other authors prospectively compared oocyte donors with endometriosis with recipients who had endometriosis and found reduced pregnancy and implantation rates when the oocytes came from donors with endometriosis but normal rates when only the recipients had endometriosis (Pellicer *et al.*, 1994). The same group confirmed these findings in recipients with Stage III–IV endometriosis (Díaz *et al.*, 2000).

According to our results, it may be concluded that there is no impairment in endometrial receptivity markers in patients with mild stages of endometriosis if OPN and αvβ3 integrin are proved to be accurate markers of uterine receptivity. However, we and others have reported data providing uncertainty about the value of integrins and OPN in assessing endometrial receptivity in the clinical setting (Creus *et al.*, 2002, 2003; Ordi *et al.*, 2002; Thomas *et al.*, 2003; Casals *et al.*, 2008, 2010). On the other hand, the number of patients in our study is relatively low, and thus a type II error cannot be excluded.

In conclusion, the results of the present study show that OPN and αvβ3 integrin expression or co-expression during the window of implantation are not impaired in patients with Stage I–II endometriosis. Whether these results imply normal endometrial receptivity markers in such patients or add to the increasing uncertainty about the clinical value of assessing the endometrium with these markers of implantation remains to be shown.

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Authors' roles

G.C., J.O. and J.B. conceived and designed the study; G.C., M.C. and F.F. contributed to patient recruitment, ultrasonographic monitoring of ovulation and endometrial biopsies; J.O. performed the histopathological and immunohistochemical analysis; R.C. performed the

hormonal analysis; G.C., J.O., F.C. and J.B. contributed to interpreting the data and redacted the manuscript. All the authors revised the paper and approved the final version to be published.

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Conflict of interest

None declared.

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5. DISCUSIÓN GENERAL

El proceso de implantación embrionaria humano requiere un diálogo entre el endometrio y el blastocisto. Durante el periodo en el que el blastocisto debe implantarse, el tejido endometrial exhibe unas características determinadas que definen su estado de receptividad para la implantación. La mucosa endometrial es receptiva durante un periodo restringido del ciclo menstrual, llamado "ventana de implantación" (Strowitzki y cols., 2006). Por tanto, en el contexto de la valoración de la fertilidad de la mujer, resulta crítico poder determinar el momento de transición a la fase de receptividad y las características que definen a la misma.

El endometrio desarrolla una serie precisa de cambios morfológicos que tienen lugar bajo el influjo hormonal del ovario, mediante la secreción de estrógenos y progesterona, hasta alcanzar su estado de receptividad. Estos cambios morfológicos fueron descritos ya en el 1950 por Noyes, Hertig y Rock y han constituido la base para evaluar la función endometrial y los defectos progestacionales durante más de medio siglo (Noyes y cols., 1950). Sin embargo, el datado histológico a partir de la biopsia endometrial ha sido cuestionada no solo por sus limitaciones en cuanto a su pobre relación con la fertilidad de la mujer (tal como se ha comentado en el apartado de justificación de esta Tesis Doctoral), sino también por las limitaciones intrínsecas a la práctica de la misma, que motivaron el establecimiento de las siguientes recomendaciones:

a)) El datado cronológico (día del ciclo de la mujer, que se correlacionará con el datado histológico obtenido según los parámetros de Noyes) debe determinarse contando los días de forma "anterógrada" a partir del día de la ovulación, y no de forma "retrógrada" desde el primer día de la siguiente menstruación (Shoupe y cols., 1989; Peters y Wentz, 1992; Lessey y cols., 2000).

b) Una de las principales limitaciones en la interpretación de las biopsias endometriales es la variabilidad interobservador, por lo que se ha

determinado que deben existir como mínimo 3 días de desfase entre el datado cronológico e histológico para considerar una biopsia como "fuera de fase" (Lessey y cols., 1992; Castelbaum y Lessey, 1995).

c) Las biopsias tomadas durante el periodo premenstrual pueden ser discrepantes con las obtenidas durante la fase lútea media y, si bien pueden ser adecuadas para establecer el diagnóstico de insuficiencia luteínica, no lo son para valorar la fase de receptividad uterina. Por tanto, las muestras endometriales deben obtenerse en fase lútea media (Blasco, 1994; Castelbaum y cols., 1994; Lessey y cols., 1995, 2000).

Estas recomendaciones se han tenido en cuenta en el diseño de los estudios incluidos en la presente Tesis Doctoral, tal como se detalla en el apartado de Material y Métodos de cada uno de los artículos. Debemos destacar que todas las muestras endometriales fueron valoradas por el mismo patólogo ginecológico (Dr. Jaume Ordi) sin que conociera previamente el grupo de estudio al que pertenecía cada paciente ni el teórico día ovulatorio determinado por ecografía. Por otra parte, se utilizó el control ecográfico seriado del desarrollo folicular para establecer el día de la ovulación (a partir del cual se programaron las tomas endometriales), ya que estudios previos han demostrado una mayor precisión en el datado endometrial cuando la ovulación se determina mediante este método (Shoupe y cols., 1989; Peters y cols., 1992).

A pesar de las limitaciones de la valoración clásica de la biopsia endometrial mediante los parámetros morfológicos, el estudio del endometrio continua siendo crucial en la evaluación de la fertilidad de la mujer, por lo que en la última década se ha intensificado la investigación de nuevos marcadores o métodos para determinar el estado de receptividad endometrial.

Una de las técnicas que ha suscitado mayor interés es la valoración ecográfica y por Doppler de la morfología y vascularización endometrial. Sus principales ventajas son el carácter no invasivo de la técnica y la posibilidad de estudio del endometrio en

el mismo ciclo de tratamiento, aspecto fundamental en las pacientes sometidas a Fecundación *in vitro*. Uno de los parámetros ecográficos más estudiados es el grosor endometrial. No obstante, pese a ser un parámetro fácil de determinar, su valor pronóstico de cara a determinar la probabilidad de embarazo es bajo (Yuval y cols., 1999; De Geyter y cols., 2000; Kovacs y cols., 2003; Zhang y cols., 2005). La textura ultrasonográfica del endometrio también se ha estudiado como factor pronóstico para conseguir la gestación. En este sentido, Check y cols. (2000, 2003) observaron una tasa de embarazo significativamente superior cuando el endometrio valorado en fase lútea media presentaba un patrón homogéneo hiperecogénico. Por otra parte, la valoración del flujo sanguíneo endometrial y subendometrial mediante Doppler tiene aún un papel poco claro en la predicción de la implantación y la gestación (Ng y cols., 2007; Cakmak y Taylor, 2011). Finalmente, un estudio controlado de nuestro centro (Puerto y cols., 2003) que fue motivo de una Tesis Doctoral (Puerto, 2001) demostró un escaso valor clínico-práctico de la valoración rutinaria del endometrio por ecografía y Doppler en el pronóstico de la implantación embrionaria en pacientes de Fecundación *in vitro*.

Dentro del espectro de técnicas no invasivas, el análisis de ciertas proteínas endometriales como la inhibina-A o la glicodelina A en plasma o suero tampoco ha resultado útil para predecir el estado funcional del endometrio (Mackenna y cols., 1993; Balasch y Vanrell, 1987; Balasch y cols., 1996). Por otro lado, se ha propuesto el análisis de las secreciones uterinas obtenidas mediante lavado uterino o aspiración de secreciones endometriales como técnica de fácil aplicación y que podría proporcionar información útil sobre la función endometrial de forma menos invasiva que el estudio del tejido endometrial mediante biopsia (Beier-Hellwig y cols., 1989; Li y cols., 1993; van der Gaast y cols., 2003).

No obstante, la aparición de nuevas tecnologías ha potenciado la investigación de nuevos marcadores moleculares, inmunológicos y morfológicos en el tejido

endometrial obtenido mediante biopsia. El desarrollo de un endometrio receptivo requiere de la estrecha colaboración de un número importante de factores que parecen estar regulados de forma muy precisa y que podrían mediar las interacciones entre el blastocisto y el endometrio. Así, técnicas como la inmunohistoquímica han permitido identificar diferentes marcadores endometriales, algunos de ellos presentes en la superficie del epitelio luminal representando así elementos importantes en los procesos de aposición, adhesión y fijación del embrión sobre el endometrio, mientras que otros han sido identificados en la matriz extracelular del estroma endometrial y constituyen factores involucrados en el proceso de invasión trofoblástica.

En las primeras fases de la implantación embrionaria, principalmente en los procesos de adhesión e invasión, que requieren interacciones célula-célula y célula-matriz extracelular, la participación de las moléculas de adhesión celular es fundamental. Dentro de este grupo, las integrinas han sido una familia de moléculas extensamente estudiadas como marcadores de receptividad endometrial. En concreto, la integrina αvβ3 ha sido objeto de mayor estudio ya que su expresión endometrial delimita el inicio de la ventana de implantación (Lessey y cols., 1992; Young y Lessey, 2010). Estudios inmunohistoquímicos endometriales realizados durante el ciclo menstrual determinaron que la expresión de esta integrina presentaba un incremento súbito en las células epiteliales luminales y glandulares en fase lútea media (Lessey y cols., 1992; Lessey y cols., 1994a). La función de la integrina αvβ3 depende esencialmente de la unión de ciertos ligandos, caracterizados estructuralmente por presentar la secuencia tripeptídica RGD. La osteopontina, que contiene esta secuencia y se considera su principal ligando, fue identificada inicialmente en el hueso y allí, mediante su unión a la integrina αvβ3, interviene en procesos de adhesión y señalización celular (Flores y cols., 1992; Zimolo y cols., 1994).

Diferentes hechos hacen pensar que la interacción entre ambas moléculas está implicada en la regulación de la función endometrial y en el proceso de implantación

embrionaria. En primer lugar, el estudio simultáneo de la integrina αvβ3 y la osteopontina en el endometrio humano reveló una expresión coordinada de ambas moléculas durante el ciclo menstrual, con un incremento marcado durante la fase lútea media, es decir, cuando tiene lugar la implantación del embrión (Apparao y cols., 2001; Von Wolff y cols., 2001). Además, la osteopontina es secretada a la cavidad uterina donde podría interactuar con el embrión que va a implantar, contribuyendo de esta forma al diálogo materno-embrionario (Von Wolff y cols., 2001). Ambas glicoproteínas también están presentes en las proyecciones apicales de la superficie endometrial llamadas pinópodos durante el periodo de receptividad uterina (Apparao y cols., 2001; Lessey, 2002a). Por otra parte, ambas moléculas se expresan también de forma sincrónica en las células trofoblásticas y, de nuevo, esto sugiere un papel crucial en el proceso implantatorio (Daiter y cols., 1996). Estudios oncológicos han aportado evidencia indirecta sobre la capacidad invasiva de la osteopontina, lo que sugiere que esta molécula podría estar implicada no solo en las primeras fases de la implantación, si no también en los procesos de invasión del trofoblasto (Oates y cols., 1996, 1997). Por último, la osteopontina también podría participar de forma destacada en la regulación de la compleja red de citoquinas derivadas de las células inmunológicas (Von Wolff cols., 2001).

En resumen, la interacción de la osteopontina con su receptor, la integrina αvβ3, podría tener un papel destacado en la adhesión celular, migración y en la modulación de la respuesta inmunológica, aspectos clave en la implantación embrionaria. Por tanto, se planteó el estudio simultáneo de ambos marcadores como método para distinguir el endometrio receptivo del no receptivo en la práctica clínica, para mejorar el conocimiento de determinadas causas de esterilidad y para el desarrollo de métodos contraceptivos dirigidos al endometrio (Lessey, 2002a; Makker y Singh, 2006; Strowitzki y cols., 2006).

En general, el estudio individual de los marcadores de receptividad endometrial nos ha permitido mejorar nuestros conocimientos sobre la función endometrial y los procesos de implantación embrionaria. No obstante, mediante esta estrategia aún no se ha conseguido identificar un marcador útil de forma individual como indicador de un endometrio receptivo. En la última década, los estudios de genómica y proteómica endometrial han permitido el estudio simultáneo de gran número de genes o proteínas, con lo que el número de potenciales marcadores de receptividad endometrial ha crecido de forma exponencial, pero la identificación de marcadores útiles y la aplicabilidad de estas técnicas a la práctica clínica están siendo complejas. No obstante, el hecho de que uno de los marcadores ya relacionados previamente con el proceso de implantación embrionaria como la osteopontina se haya identificado en la mayoría de estudios de genómica como molécula que experimenta un incremento marcado de su expresión durante la fase lútea media apoya de nuevo su posible papel crucial en el establecimiento de la receptividad endometrial (Aghajanova y cols., 2008). Por todo ello, el estudio endometrial de la osteopontina y su receptor, la integrina αvβ3, se ha intensificado en los últimos años (Cho y cols., 2009; DuQuesnay y cols., 2009; Wei y cols., 2009).

Sin embargo, la utilidad de la integrina αvβ3 y la osteopontina como marcadores de receptividad endometrial ha sido cuestionada por algunos autores. Por un lado, estudios de nuestro grupo han puesto de manifiesto que la expresión de integrinas, junto con la formación de pinópodos endometriales, otro marcador intensamente estudiado, es un proceso relacionado con el grado de maduración endometrial independientemente de que el endometrio esté "en fase" o "fuera de fase" y la expresión de estos marcadores es coincidente a mitad de la fase secretora pero existe una clara disociación temporal en su co-expresión durante la fase lútea (Creus y cols., 2002). Tampoco se detectaron diferencias en la expresión endometrial de integrina αvβ3 entre mujeres fértiles y pacientes estériles ni entre diferentes causas de esterilidad y su expresión no guardó relación con la posterior consecución o no de la

gestación (Creus y cols., 2002; Ordi y cols., 2002). Por otra parte, otros autores no observaron diferencias significativas en la expresión de las integrinas α4β1, α1β1 y αvβ3 durante la ventana de implantación entre un grupo de pacientes que realizaron FIV por patología tubárica, endometriosis o esterilidad desconocida respecto a otro grupo de pacientes sometidas a ICSI por factor masculino y un grupo control de mujeres fértiles (Thomas y cols., 2003). Estos datos contrastan con los de otros autores que hallaron una expresión alterada de la integrina αvβ3 en pacientes con endometriosis, hidrosálpinx y esterilidad de causa desconocida (Lessey y cols., 1994b, 1995; Meyer y cols., 1997). En cuanto a la osteopontina, se ha demostrado a través de experimentación animal que los ratones mutados que no expresan esta glicoproteína siguen siendo fértiles (Liaw y cols., 1998; Rittling y cols., 1998). Por último, existen grandes discrepancias entre los diferentes estudios que han analizado estos marcadores en muestras procedentes de pacientes con endometriosis (Lessey, 2002b; Ordi y cols., 2003a; Odagiri y cols., 2007; Cho y cols., 2009; Wei y cols., 2009).

Por tanto, si bien existe una dependencia hormonal y una ciclicidad en la expresión endometrial de la integrina αvβ3 y la osteopontina, no existe una relación de causa-efecto convincente entre su presencia o ausencia y sus efectos sobre la fertilidad. Los estudios que configuran el cuerpo doctrinal de esta Tesis Doctoral aportan importante información en este sentido y se resume a continuación.

El ***estudio 1*** aporta datos relevantes acerca del potencial valor del análisis conjunto de la integrina αvβ3 y la osteopontina en la misma biopsia endometrial en mujeres fértiles y en pacientes estériles. No se detectaron diferencias estadísticamente significativas entre ambos grupos ni en el análisis de los parámetros histológicos, ni en la valoración de ambos marcadores tanto de forma individual como conjunta. Por otra parte, al estudiar todas las biopsias obtenidas en fase lútea media en el conjunto global de pacientes, se observaron diferencias en la expresión de la integrina αvβ3, la osteopontina y en la coexpresión de ambos marcadores entre biopsias endometriales

"en fase" (sin retardo madurativo) y "fuera de fase" (con maduración retardada), pero la expresión o coexpresión de estos marcadores no estuvo relacionada con la fertilidad o esterilidad de la mujer.

Debemos destacar que se observó una correlación entre la expresión de la integrina αvβ3 y de la osteopontina con el grado de maduración endometrial: mientras que la osteopontina aparece principalmente a partir de los días 4-5 post-ovulación, la integrina αvβ3 lo hace a partir de los días 6-7 y ambos marcadores se expresan en todas las muestras datadas a partir del día 8 post-ovulación. Estos cambios en la expresión de ambos marcadores ocurrieron independientemente de que el endometrio estuviera "en fase" o "fuera de fase". No obstante, el grado de coexpresión de ambos marcadores (presencia o ausencia simultánea de ambos) durante la ventana de implantación fue relativamente bajo y la correlación entre la intensidad de la expresión de integrina αvβ3 y osteopontina en fase lútea media fue significativa pero débil.

El ***estudio 1***, por otra parte, apoya de nuevo los resultados obtenidos en trabajos previos de nuestro grupo, según los cuales la expresión de integrina no se asociaba a ninguna etiología específica de esterilidad (Creus y cols., 1998; Ordi y cols., 2002, 2003a). Los datos del estudio actual lo hacen extensivo a la expresión de osteopontina y a la coexpresión de ambos marcadores. Por último, y también de acuerdo con una publicación previa de nuestro grupo (Ordi y cols., 2002), la expresión o coexpresión de estos marcadores no guardó relación con la instauración posterior de la gestación, tras el seguimiento durante 2 años del grupo de pacientes estériles que no recibieron tratamiento alguno relacionado con su esterilidad durante el periodo de control. En resumen, la expresión endometrial de osteopontina o la coexpresión de ésta con la integrina αvβ3 no nos proporciona más información respecto el estudio individual de la integrina en la valoración de las pacientes en función de su fertilidad o esterilidad, la etiología de la esterilidad o su pronóstico de fertilidad ulterior.

Si la osteopontina y la integrina αvβ3 fueran marcadores de receptividad endometrial útiles en la práctica clínica, el efecto de diferentes tipos de terapéuticas hormonales encaminadas a potenciar la fertilidad de la mujer o a establecer un mecanismo anticonceptivo debería quedar reflejado en su expresión endometrial. El ***estudio 2*** de esta Tesis Doctoral se desarrolló en base a esta hipótesis. Para ello se eligieron diferentes fármacos que incluían el citrato de clomifeno, la hiperestimulación ovárica controlada con gonadotrofinas bajo supresión hipofisaria para Fecundación *in vitro*, diferentes formas de terapia hormonal sustitutiva como método de preparación endometrial para la recepción embrionaria, la dehidrogesterona como terapéutica de los defectos progestacionales del endometrio y los contraceptivos orales. A destacar que este estudio controlado se diseñó de forma que cada paciente actuara como su propio control. Los resultados indican que la expresión de osteopontina y integrina αvβ3 estuvo íntimamente relacionada con el grado de maduración endometrial, de forma similar al estudio 1, y esto fue cierto independientemente de que el endometrio estuviera "en fase" o "fuera de fase" y se observó tanto en ciclos espontáneos como en ciclos sometidos al estímulo de un tratamiento hormonal. Por otra parte, aún cuando existió un alto grado de coincidencia en la expresión de ambos marcadores para los días 9-11 post-ovulación, hubo una patente falta de coexpresión temporal de dichos marcadores a lo largo de la fase lútea en las muestras estudiadas.

Esta falta de coexpresión temporal de osteopontina e integrina αvβ3 durante la fase lútea observada en los estudios 1 y 2 podría explicarse por los diferentes mecanismos de regulación a los que están sometidos estos marcadores: mientras que la expresión endometrial de la osteopontina está estimulada por la progesterona, la integrina αvβ3 está regulada por el epidermal growth factor o el heparin-binding epidermal growth factor (Apparao y cols., 2001; Lessey, 2002a). Esto también explicaría el hecho de que el análisis combinado de los dos marcadores no proporcione más información útil sobre el estado funcional del endometrio respecto su estudio individual.

Por último, la endometriosis aún hoy plantea una serie de cuestiones sin resolver y, entre ellas, la incógnita sobre qué mecanismo es el responsable de la esterilidad en estas pacientes, principalmente en los estadios iniciales de la enfermedad, donde no existe una distorsión anatómica de los genitales como causa evidente de esterilidad. Entre los posibles mecanismos implicados, se ha propuesto que una expresión anómala de ciertos marcadores de receptividad endometrial podría conducir a una disminución de la capacidad de implantación embrionaria en las pacientes con endometriosis. Con el objetivo de avanzar en el conocimiento de la receptividad endometrial en estas pacientes, en el ***estudio 3*** se investigó la expresión endometrial de la osteopontina y la integrina αvβ3 en pacientes con estadios iniciales de endometriosis y se comparó con un grupo de pacientes con esterilidad de origen desconocido y otro de pacientes fértiles. De hecho, otros autores habían estudiado anteriormente la expresión endometrial de la integrina αvβ3 o la osteopontina en pacientes con endometriosis con resultados controvertidos (Lessey y cols., 1994b; Ordi y cols., 2003a; Odagiri y cols., 2007; Cho y cols., 2009; Wei y cols., 2009), pero nuestro trabajo fue el primer estudio controlado en el que se analizó la expresión simultánea de ambos marcadores en estas pacientes.

De nuevo, no se detectaron diferencias estadísticamente significativas entre los tres grupos de estudio ni en el análisis de los parámetros histológicos, ni en la valoración de ambos marcadores tanto de forma individual como conjunta. El grado de coexpresión de estos marcadores durante la ventana de implantación fue bajo. Tampoco se observó una correlación significativa entre la intensidad de la expresión de la osteopontina y de la integrina αvβ3 en las muestras obtenidas en fase lútea media de ninguno de los grupos estudiados. Por tanto, los resultados obtenidos en este estudio indican que la expresión y coexpresión de la osteopontina y la integrina αvβ3 durante la ventana de implantación no se encuentran alteradas en las pacientes con estadios iniciales de endometriosis.

En este mismo trabajo se analizó la intensidad de la expresión de ambos marcadores utilizando el H-Score, un sistema que ha demostrado presentar baja variabilidad intra-observador e inter-observador (Budwit-Novotny y cols., 1986). Además, se comprobó que existe un alto grado de correlación entre los resultados obtenidos mediante el H-Score y el sistema semicuantitativo utilizado en los **estudios 1 y 2**, lo que apoya la validez de los mismos.

6. CONCLUSIONES

1. La expresión de osteopontina e integrina αvβ3 en el endometrio humano viene condicionada por el grado de maduración del endometrio establecido por el datado histológico y ello es independiente de que el endometrio presente un estado de maduración histológica adecuada o que se detecte un retardo madurativo.

2. El endometrio humano presenta un elevado nivel de expresión de osteopontina e integrina αvβ3 a partir del día 8-9 post-ovulación, pero existe una falta de coexpresión temporal de ambos marcadores durante la ventana de implantación.

3. No existen diferencias ni en la frecuencia ni en la intensidad de expresión o de coexpresión de osteopontina e integrina αvβ3 entre mujeres con fertilidad probada y pacientes estériles.

4. La expresión o coexpresión de osteopontina e integrina αvβ3 endometriales no se relacionan ni con la causa de esterilidad de la mujer ni con la consecución ulterior de la gestación.

5. La administración de diferentes hormonas con efectos probados sobre el endometrio (inductores de la ovulación, estimulación ovárica para Fecundación in vitro, gestágenos, terapia hormonal sustitutiva, anticonceptivos orales) modifica la expresión de osteopontina e integrina αvβ3 sólo en tanto en cuanto son capaces de influir sobre el estado de maduración histológica de la mucosa endometrial.

6. Las pacientes con estadios iniciales de endometriosis (grados I y II) no presentan una alteración en la expresión y coexpresión endometrial de osteopontina e integrina αvβ3 durante la ventana de implantación en

comparación con mujeres fértiles controles y pacientes con esterilidad de origen desconocido.

Como consecuencia de todo ello y a modo de conclusión general de interés clínico-práctico, podemos afirmar que no existe una relación causa-efecto entre la presencia o ausencia de estos dos marcadores endometriales (osteopontina e integrina $\alpha v\beta 3$) durante la denominada "ventana de implantación" y la esterilidad de la mujer asociada o no a la presencia de endometriosis. Por tanto, el interés de su uso rutinario en la valoración de la paciente estéril no puede confirmarse de acuerdo con los resultados de esta Tesis Doctoral.

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