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A Tissue Specific Magnetic Resonance Contrast Agent, Gd-AMH, for Diagnosis of Stromal Endometriosis Lesions: A Phase I Study

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The anti-mullerian hormone (AMH) is a homodimeric glycoprotein member of the transforming growth factor β (TGF-β) superfamily, is secreted by Sertoli cells in the embryonic testes and is responsible of the regression of the mullerian duct. The physiological functions of this protein remain largely unknown, and its expression in human tissues has yet to be completely determined. Firstly, we analyzed AMH expression in human tissues by immunohistochemistry. AMH was distributed in many organs, although with different tissue and cell localization and various expression levels; we also demonstrated strong AMH expression in endometriosis tissues. Secondly, we demonstrated the ability of an anti-AMH antibody, labeled with gadiolinium, to be directly detected by magnetic resonance in small endometriosis lesions (5 mm in diameter) in vivo in a mouse model. In conclusion, our data suggest that based on its expression pattern, AMH may serve to maintain physiological cellular homeostasis in different human tissues and organs. Moreover, it is strongly expressed in endometriosis lesions as a selective tissue specific contrast agent for in vivo detection of stromal endometriosis lesions. The potential significance of these findings could be further validated in a clinical setting.

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The anti-mullerian hormone (AMH) is a homodimeric glycoprotein member of the transforming growth factor β (TGF-β) superfamily, and is secreted by Sertoli cells in the embryonic testes where it is responsible for the regression of the mullerian duct (La Marca et al., 2009). AMH expression in the ovarian follicles starts in the female fetus, around the 32nd week of gestation. AMH expression levels are considered good indicators of a woman's ovarian reserve, as they decline during menopause (Lee et al., 1996). Interestingly, its level is augmented in the serum of women suffering from ovarian polycystosis; this increase does not cause negative organic or biological effects on female organs (Du et al., 2014). Moreover, an anti-cancer function has been proposed for AMH in epithelial tumors in the ovary, and experimental evidence seems to support its cytotoxic effect on tumor cells (La Marca and Volpe, 2007). Considering the very low toxicity of said substance in the human body, the possibility of AMH as an anti-cancer drug is of great clinical interest. Recent studies have demonstrated that AMH, as well as MISRII (one of its receptors), is expressed in the endometrium and endometriosis lesions, where it probably acts as a paracrine signal and negatively regulates cellular viability in the endometrium (Shebl et al., 2009; Wang et al., 2009; Namkung et al., 2012; Carrarelli et al., 2014; Signorile et al., 2014).

"Endometriosis is a recurrent and benign gynecological disorder characterized by the presence of endometrial tissue (glands and stroma) outside the uterus" (Baldi et al., 2008). It is one of the most common diseases in the gynecological field, affecting about 10% of women of reproductive age; its frequency rises to 20–50% in women with fertility problems (Baldi et al., 2008). Endometriosic lesions are localized on the pelvic peritoneum and ovaries, but are commonly found in the sub-peritoneal areas and, more rarely, in any anatomic district, such as pericardium, pleurae, pulmonary parenchyma, spinal cord, and even brain (Giudice and Kao, 2004; Signorile et al., 2009a). The pathogenesis of such disease is still unknown, but the most reliable hypotheses suggest retrograde menstruation and coelomic metaplasia (Benagiano and Brosens, 2006). Recently, the presence of endometriosic lesions in the female fetus has been described; this represents a different pathogenetic theory based upon small defects of embryogenesis during the fine tuning of the female genital system (Signorile and Baldi 2010; Signorile et al., 2009b, 2010b, 2012), probably caused by a mixture of genetic and epigenetic factors (Signorile et al., 2010a; Crispi et al., 2013).

Endometriosis can be diagnosed only when the endometriosis lesions are observed by laparoscopy or laparotomy and after histologic examination of surgically resected lesions (Attaran et al., 2002). In fact, there are no sufficiently sensitive and specific signs, symptoms, or diagnostic tests for the clinical diagnosis of endometriosis (Ballard et al., 2006). Recently, a putative diagnostic marker has been described by our research group (Signorile and Baldi, 2014). However, imaging findings allow us to make a presumptive diagnosis, which can be useful during the differential diagnosis process. Nevertheless, no marker has been described that gives the exact localization of the endometriosic lesions in vivo (Lo Monte et al., 2014). Moreover, several endometriosic lesions can have very reduced sizes (less than 1 cm), making

Abbreviations: Gd, gadolinium; AMH, anti-Mullerian hormone; TGF-β, transforming growth factor β; DTPA, diethylenetriaminepenta-acid; MR, magnetic resonance.

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localization analysis practically impossible with the currently available methods.

In this study, we have investigated the expression of AMH in adult human and endometriosis tissues, and investigated its feasibility as a tissue specific contrast agent of these lesions in vivo.

Materials and Methods

Normal adult human tissues

A panel of different adult human tissues was used (Human tissue arrays AA9 adult tissues, Superbiochips, Korea). We used two different tissue arrays to check for repeatability of the data. Moreover, endometriosis tissue samples from five patients who underwent surgery for infertility, pelvic pain symptoms, or adnexal masses at the "Centro Italiano Endometriosi" in Rome were included. Written informed consent was obtained from all the subjects before the collection of the tissue samples.

Immunohistochemistry

The immunohistochemical assays were performed as previously described by Signorile et al. (2014): slides were incubated at 4 °C overnight with an affinity-purified rabbit polyclonal immune serum raised against AMH (Abcam, Cambridge, UK) and with a mouse monoclonal antibody for CD10 (clone M7308; Dako Laboratories, Carpinteria, CA) at a 1:100 dilution. Negative controls for each tissue section were prepared by leaving out the primary antiserum. All samples were processed under the same conditions. The expression level of AMH-stained cells per field (250×) at light microscopy was calculated and compared in different specimens by two separate observers (A.B. and P.G.S.) in a double blind fashion and described as: absent (◯); low (●); moderate (●●); high (●●●), based on both the intensity of the staining and the percentage of positive cells. Any disagreement was resolved by reevaluation of the sections and achievement of a consensus between the two observers.

Generation of gadolinium-labeled antibody for AMH (Gd-AMH)

The production of paramagnetic gadolinium (Gd)-labeled polyclonal antibody for AMH was performed essentially as already described (Kornguth et al., 1987). Briefly, the polyclonal antibody for AMH (Abcam, Cambridge, UK) was attached covalently to the chelator diethylenetriaminepenta-acid (DTPA) dianhydride. Successively, this DTPA-antibody complex was added to a mixture containing Gd chloride hexahydrate. All the reagents for this procedure were purchased from Sigma Aldrich (St. Louis, MO).

Xenografts experiments

All procedures involving animals and their care were conducted in conformity with national (D.L. No. 116, G.U., Suppl. 40, Feb. 18, 1992 Circolare No. 8, G.U., July 1994) and international laws (EEC Council Directive 86/609, OJ L 358. 1, Dec 12, 1987 Guide for the Care and Use of Laboratory Animals, United States National Research Council, 1996). Three fragments of 4 mm in diameter of a histologically confirmed biopsy of human stromal endometriosis tissue were implanted in the left flanks of three female nude mice (CD-1 female nude, nu/nu mice, 6–8 weeks old and 22–24 g in body weight, purchased from Charles River Laboratories, Calco, Italy). After implanting endometriosis tissue, performed in total anesthesia, the mice were stabilized for 2 weeks with food and water ad libitum and, limited to the first week, with antibiotic therapy (5% enrofloxacin in the beverage water). On day 14 after endometriosis tissue implants, mice were anesthetized with tiletamine + zolazepam + xylazine in order to perform the imaging studies. The apparatus used was a MR 0.2 Tesla. Total body and abdominal analysis were performed with serial sections of 2 mm. Then, the mice were removed from the apparatus for a second administration of sedative in order to be able to perform the intravenous inoculation (tail vein) of a 10 μl solution of Gd-AMH with gadolinium concentrated at 0.2 mg/ml. Finally, a second series of imaging studies was performed, using the same parameters of the first round of analyses, one hour after the injection of the AMH conjugated with gadolinium.

One week after the experiment, the animals were euthanized and the endometriosis tissues were excised. The biopsy specimens were fixed in 10% buffered-formalin and embedded in paraffin. Sections of 5 μ were stained with haematoxylin-eosin, and haematoxylin-van Gieson. Finally, immunohistochemistry for AMH was performed as previously described.

Animals were monitored for signs of toxicity, including respiratory, gastrointestinal, neurological, dermatological, and hematological symptoms.

Results

AMH localization in adult human tissues

AMH displayed variable tissue distribution and expression levels in the human adult tissues (Table 1). In the integumental system, epithelial cells, either from simple or stratified epithelia, showed high expression for AMH in all the layers of the epitelium. In the skin, we observed high AMH immunoreactivity in hair follicles.

In the muscular system, moderate AMH expression was localized to the cytoplasm of striated muscle fibers, while in the cardiovascular system, very moderate to high AMH immunoreactivity was observed in the heart (Fig. 1A). In the respiratory system, we observed high AMH immunoreactivity in all the layers of the nasal and bronchial mucosa, and in the epithelium of the alveoli (Fig. 1B).

In the gastrointestinal system, high AMH immunoreactivity was observed in the salivary glands and in the exocrine portion of the pancreas (Fig. 1C), whereas the endocrine portion of the gland showed a moderate AMH expression. Moderate expression levels were observed in all the layers of the esophageal epithelium. High AMH immunopositivity was found in the mucosa, in the smooth muscles cells and in the lymphoid cells of stomach, duodenum, small bowel, colon and rectum (Fig. 1D,E). Hepatocytes of the liver displayed a moderate immunopositivity for AMH (Fig. 1F).

In the remainder of the endocrine system, a very low expression level of AMH was detected in the cytoplasm of cells of all the layers of the adrenal gland. Moreover, very low expression levels were observed in the glomerulosa and reticularis regions. In the thyroid gland, a moderate level of AMH expression was detected in the colloid and in the follicular cells.

In the female reproductive system, high AMH expression level was found in the glandular epithelium of both the secretive and proliferative endometrium (Fig. 2A). A high expression level of AMH was also detected in the ectopic endometrium of endometriosis lesions, both in the epithelial and stromal cells (Fig. 2B). A moderate/high AMH immunoreactivity was observed in all the epithelial layers of the cervix. Finally, high AMH expression was found in the breast epithelium (Fig. 2C), salpinx, ovary, and myometrium. In the male reproductive system, a low/moderate AMH expression level was found in the Sertoli cells of the testis and in the epithelium of seminal vesicles.

In theurinary system, AMH was expressed at a low level in the basal portion of both distal and collecting tubules of kidney cortex (Fig. 2D), while in the bladder AMH was expressed at an intermediate level in the epithelium. In the prostate, AMH was expressed at a medium level in the epithelium, whereas smooth muscles expressed low levels of the protein (Fig. 2E).

In the lymphoid system, high AMH immunoreactivity was observed in several tissues, such as lymph nodes, spleen, tonsils, and thymus (Fig. 2F). All neurons and glial cells from different areas of the brain, such as frontal cortex and midbrain,

TABLE 1. AMH protein expression in normal adult human tissues

Tissue		Degree of Expression
Skin		
Hair follicles		●●●
Sweat glands		●●
Sebaceous glands		●●
Epidermis	Basal layers	●●
	Mature layers	●●
Respiratory system		
Bronchus	Epithelia	●●
	Glands	●
Pneumocytes		●●
Mesothelium		○
Gastrointestinal system		
Salivary glands		●●
Esophagus	Epithelia	●●
	Muscles	●●●
Stomach	Epithelia	●
	Muscles	●●
Small intestine		●●
Large intestine		●●
Gall bladder epithelium		○
Liver	Hepathocytes	●
Pancreas	Esocrine	●●
	Endocrine	○
Urinary system		
Kidney	Glomeruli	○
	Proximal tubules	●
	Distal tubules	●
	Collecting ducts	●
Uroepithelium		●
Prostate		●●
Endocrine system		
Thyroid		●●
Adrenal gland	Cortical	●
	Chromaffin	○
Reproductive system		
Breast	Epithelia	●●●
Uterus	Proliferative endometrium	●●●
	Secretive endometrium	●●●
	Ectopic endometrium (endometriosis)	●●●
Salpinx		●●●
Vagina	Epithelia	●●
Testis	Leydig cells	●
	Germ cells	○
Ovary	Granulosa	●●
	Germ cells	○
Cardiovascular system		
Myocardium		●●
Blood and lymphoid tissue		
B lymphocytes		●●●
T lymphocytes		●●●
Spleen		●●●
Thymus lymphocytes		●●●
Nervous system		
Astrocytes		○
Oligodendroglia		○
Microglia		○
Purkinje cells		○
Neurons		○

○, absent; ●, low; ●●, moderate; ●●●, high.

cells of the granular level of the cerebellum displayed a very low or undetectable level of AMH expression.

Imaging studies

Fragments of human connective solid endometriosic tissue (max diameter about 5 mm), collected from three different patients during laparoscopic surgery were transplanted subcutaneously in the left side of three female nude mice (see also the method section). Both in the total body study (wherein subcutaneous capitation is found in the inoculation site in the caudal vein) and in some cross sections, Gd-AMH capitation was highlighted in the site of transplanting the endometriosis tissue (Fig. 3A,B). Interestingly, in the cross section of an animal before the treatment, the subcutaneous mass with no capitation signs was clearly visible. (Fig. 3C,D). Similar results were obtained with all the animals analyzed. After the imaging experiment, the animals were euthanized after one day to explant the ectopic tissues. The animals remained active and survived 7 days after injection with no changes in behavior and there was no observed toxicity. The excised ectopic tissues were analyzed by histology and immunohistochemistry. These examinations confirmed that the transplant histological aspect was that of a connective solid endometriosic tissue. Moreover, by means of immunohistochemical examination, it was demonstrated that such transplanted tissues expressed CD10, a marker of endometriosic tissue, and the codifying protein for AMH (Fig. 3E,F).

Discussion

Previous observations based on mRNA levels have demonstrated that AMH is differently expressed in various tissues. Studies of AMH immunohistochemical expression have been performed in the female genital system and in human testis, documenting localization in different cell populations and structures (Rey et al., 2003). However, little is known about the localization of AMH in other tissues, and little to no information is available for human tissues (La Marca et al., 2009). In order to gain additional insight into the pathophysiology of AMH-linked disorders, we undertook a study to characterize the expression patterns of AMH in adult human tissues. We observed that AMH was ubiquitously expressed in many human organs and tissues. Intense AMH immunoreactivity was observed in both striated and smooth muscle fibers. In addition, moderate expression levels were observed in both simple and stratified epithelia in all the organs examined in our study. Finally, high expression levels for AMH were detected in the female genital system and especially in endometriosis lesions, confirming previous observations (Signorile et al., 2014; Carrarelli et al., 2014). These data suggest that AMH is probably involved in the maintenance of a differentiated cell state in some tissues such as muscles and epithelia. Moreover, as demonstrated by recent functional studies, AMH expression suggests a role for this protein in cellular viability both in eutopic and ectopic endometrium (Wang et al., 2009; Namkung et al., 2012; Signorile et al., 2014).

Still nowadays the one and only effective therapeutic strategy for endometriosis is the surgical removal of the endometriosic lesions. Indeed, there is no resolving pharmacological therapy and all the pharmacological treatments used by the medical-scientific community only relieve symptoms (Bulun, 2009). However, the success of the surgical procedure is substantially based upon the possibility of displaying in vivo all the endometriosic lesions. Since the disease is generally multicentric and often microscopic, the surgeon cannot eliminate all disease foci (Fuldeore et al., 2011). Therefore, it is necessary to detect procedures which would allow a precise depiction regarding the localization and the size of the disease's different foci in the patient, so as to be able to diagnose and intervene in the most effective way in patients with endometriosis (Kennedy et al., 2005). Moreover, is currently not possible to detect endometriosis spots on peritoneal surface, on bowel bladder, in vagina, and in the pelvic tissue in the retro-peritoneum (Lo Monte et al., 2014).

MR imaging offers excellent spatiotemporal resolution without exposure to harmful radiation or the need for specialized radiochemistry equipment (Weissleder, 2006). Recently, it has been demonstrated for MR high sensitivity, specificity, positive and negative predictive values, and accuracy in the prediction of the locations and extension of the disease in patients with deep pelvic endometriosis (Grasso et al., 2010). The scope of the present work was to overcome the problems associated to the detection of the endometriosis formations and to improve the diagnostic performance of MR analysis. The results produced, indeed, suggest that AMH can be used as an

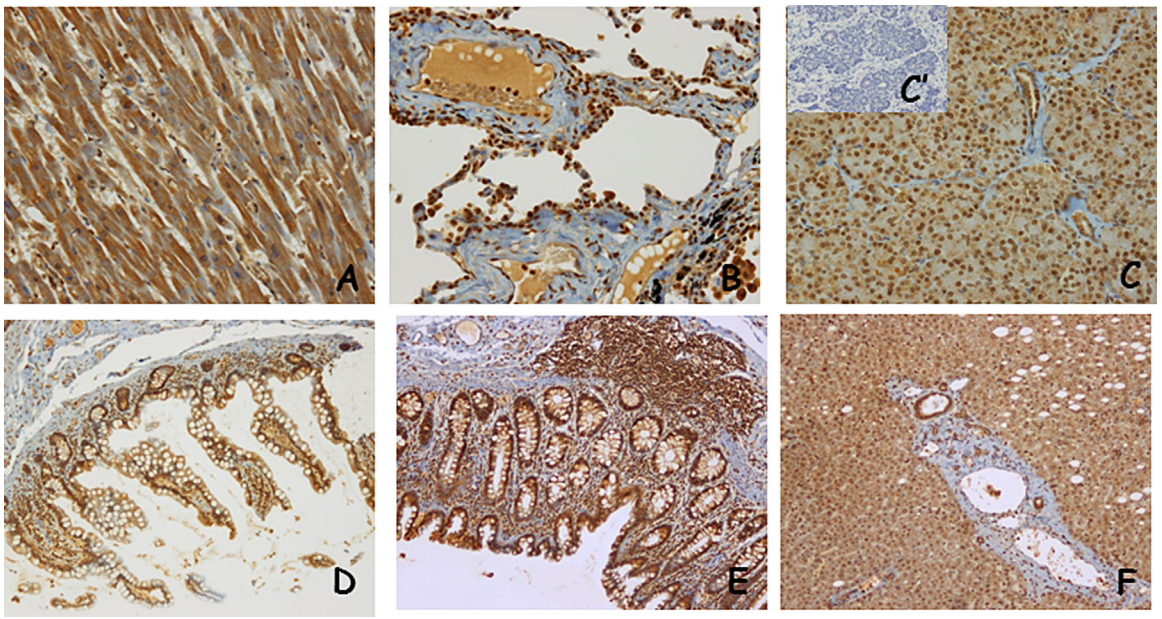

Fig. 1. A) AMH high expression in the myocardial fibers; B) moderate AMH expression in the pneumocytes; C) AMH expression in the exocrine portion of the pancreas; C') Negative control, performed leaving out the primary antibody; D) AMH low expression in the duodenum; E) moderate AMH expression in the colon; F) AMH expression in the liver. Original magnification ×20.

effective cellular target to allow in vivo detection of the exact localization of the endometriosic tissue. Moreover, considering the small size of the transplants (around 5 mm in diameter), these data also suggest that the compound can be used advantageously for localizing small foci of endometriosis and to eventually detect residual disease after surgical remove. It should be also highlighted the fact that the mice did not show changes in behavior and there was no observed toxicity.

Additional experiments on a significantly larger group of animals are required in order to better define the exact

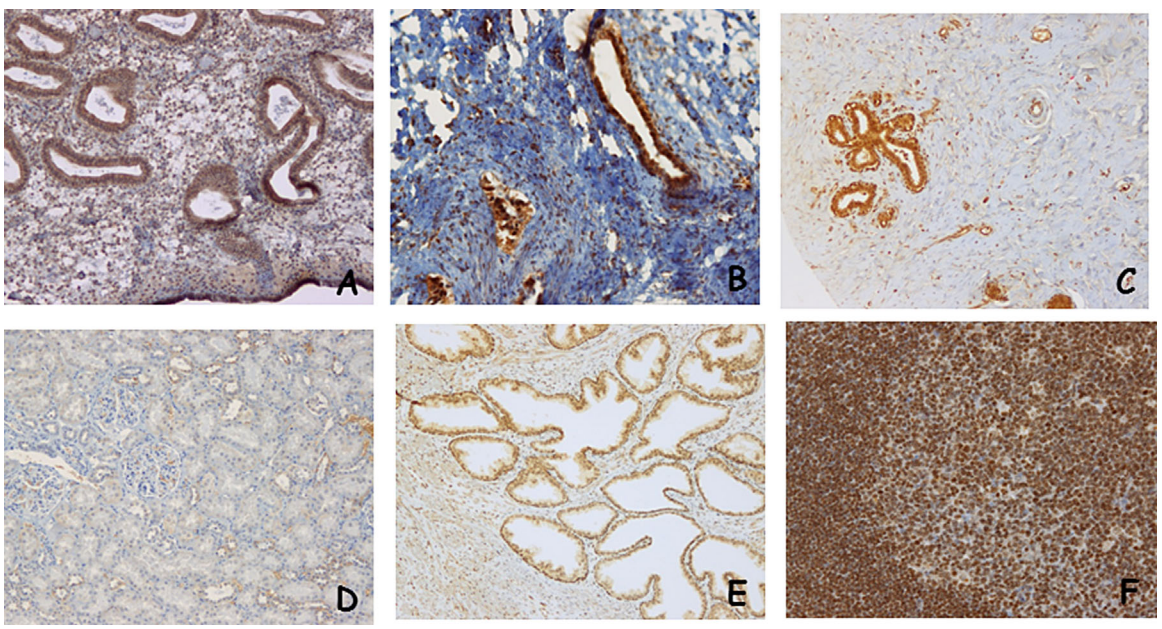

Fig. 2. A) moderate to high expression of AMH in the proliferative endometrium; B) high AMH expression in endometriosis lesions; C) moderate to high AMH expression in the epithelium of the breast ducts and acini; D) low AMH expression in the kidney; E) moderate AMH expression in the prostatic glands; F) high AMH expression level in a lymph node. Original magnification ×20.

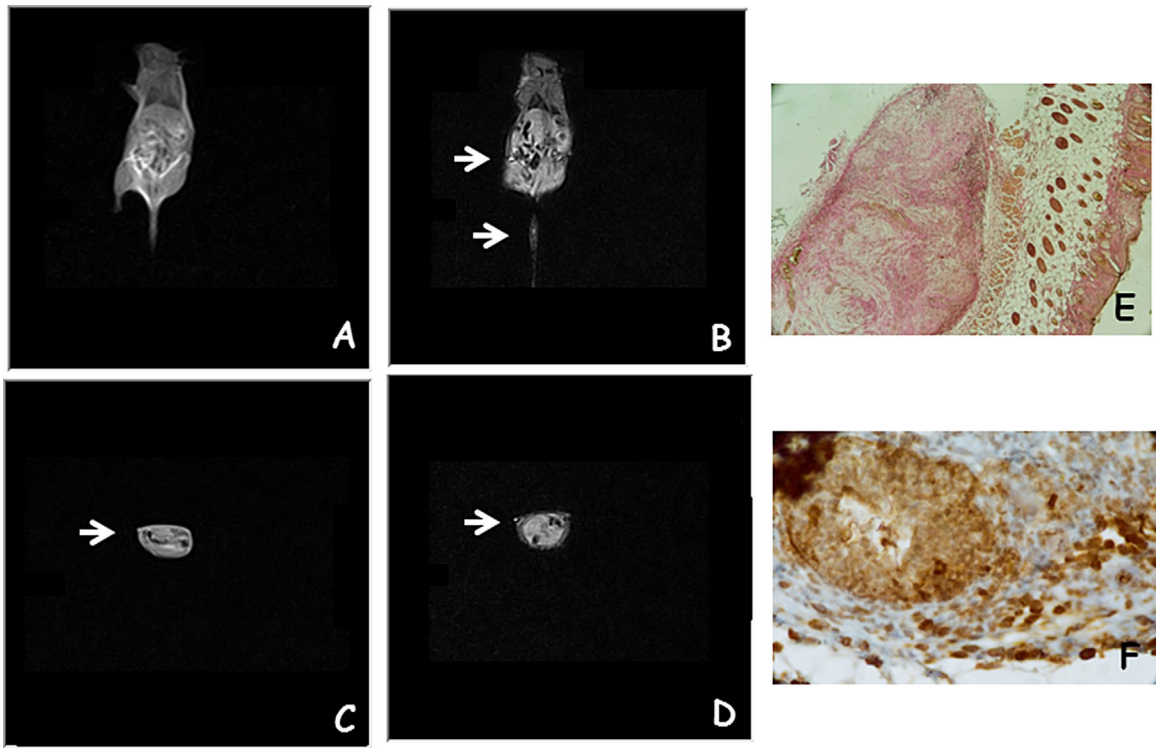

Fig. 3. A: MR total body analysis of a xenograft, before the injection of the GD-AMH. B: MR total body analysis of a xenograft, after the injection of the GD-AMH: the abdominal site of the ectopic implant of endometriosis tissue. C: RM regional analysis of the abdominal area through serial section before the injection of the GD-AMH: the white arrow indicates the ectopic implant. D: MR regional analysis of the abdominal area through serial section after the GD-AMH: the white arrow indicates the specific signal for the ectopic implant. E: Histologycal examination of the excised ectopic implant localized under the skin, original magnification X20. F: Immunohistochemical expression of AMH in the endometriosis glands of the implant, original magnification ×40.

experimental conditions for the use of Gd-AMH in MR studies. Nevertheless, the data presented suggest the use of such compound for diagnosing in vivo endometriosis and for localizing and/or evaluating the entity of the endometriosis lesions in affected patients. The potential significance of these findings deserves to be validated in a clinical setting.

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