Histological and Immunohistochemical Study of Calcification Leiomyoma uterus in postmenopausal women

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Abstract

Calcified fibroids, also known as calcified leiomyomas and myomas, are benign tumors located in the uterus. These tumors have become rigid due to calcium deposits inside the mass of smooth muscles and fibrous connective tissues. Fibroids can undergo calcification as they mature and begin to deteriorate. This study utilized histology to analyze 20 cases of total hysterectomy or myomectomy and validate their classification as fibroids. We then fixed all these samples in formalin at a 10% concentration. A histological study has been made by using three types of dyes, hematoxylin and eosin to show the general histological structure, Van Kossa stain to detect the deposition of calcium salts in the tissue, while the immunohistochemical technique has been conducted by using primary and secondary antibodies showing overexpression of osteopontin (OPN) protein within most muscle fibers in fibroids affects the uterine muscle tissue, especially the transversely arranged myofibers compared to the longitudinally arranged myofibers. We found that the overexpression of the osteopontin (OPN) protein in most of the myofibers of the leiomyoma had an effect on the myometrium tissues. Furthermore, there was a notable increase in the expression of OPN in transversally arranged myofibers as compared to longitudinally arranged myofibers.

Key words: leiomyoma, calcified, fibroid, OPN.

Introduction

Calcified fibroids, often referred to as calcified leiomyomas and myomas, are benign tumors in the uterus that have become solid as a result of calcium deposits in the mass of smooth muscles and fibrous connective tissues. Calcified fibroids can be surgically removed through either myomectomy or hysterectomy, both of which are surgical operations. A myomectomy is the

surgical procedure performed to extract a uterine fibroid, whereas a hysterectomy involves the partial or complete removal of the uterus [1]. . Empirical evidence strongly suggests that estrogens and progestogens stimulate tumor development, as fibroids are rarely observed prior to the start of menstruation [2] and tend to undergo reduction in size following menopause. These structures are classified according to their location in respect to the layers of the uterus, namely as subserous, intramural, or submucous [3].

Calcification in the uterus is a dependable indicator of uterine leiomyoma. Its commonly occurs in uterine leiomyomas when there is no blood flow, particularly in women who have gone through menopause. Leiomyomas can exhibit different patterns of calcification, including a mottled or popcorn-like appearance without a clear curved boundary, complete calcification as a solid stone mass with a well-defined outer part, or a high-attenuation border with less calcification in the inner half [4].

Osteopontin (OPN) is an acidic member of the small integrin binding ligand N-linked glycoprotein (SIBLING) family of extracellular matrix proteins/cytokines hypothesized to influence the uterine environment that undergoes extensive post translational modification, including phosphorylation, glycosylation, and cleavage, yielding molecular mass variants ranging in size from 25 to 75 kDa [5].

OPN has been found on epithelial cells and in secretions of the gastrointestinal tract, kidneys, thyroid, breast, uterus, placenta, and testes [6,7,8].

OPN is also expressed by leukocytes, smooth muscle cells, and highly metastatic cancer cells [9,10,11]. OPN has been reported to 1) stimulate cell-cell adhesion, 2) increase cell ECM communication, 3) promote migration of immune cells, osteocytes, and tumor cells, 4) decrease cell death by reducing reactive oxygen

species and nitric oxide production by injured tissues, 5) stimulate immunoglobulin production by B cells, 6) induce changes in the phosphorylation state of focal adhesion kinase and paxillin, 7) stimulate phosphotidylinositol 3-kinase activity, 8) alter intracellular calcium levels, and 9) affect tissue mineralization and pro mote calcium phosphate deposition in bone [12,13]. The study aims to detect calcification leiomyoma uterus, by using histological and immunohistochemical technique to detect the depositions of calcium salts.

Materials and Methods

Tissue specimens:

Starting in April 2023, patient samples were (50-60) age exclusively collected at Al Zahra and Al Karama Teaching Hospitals in Wasit Governorate. The specimens of hysterectomy or myoectomy were appropriately labeled, assigned numbers, and preserved in 10% buffered formalin. The gross specimens were analyzed to determine the location, quantity, and degenerative changes of leiomyoma, as well as any associated disorders. Selected portions obtained from an automated tissue processor were extracted and enclosed in paraffin wax. The masses underwent histological sectioning and were regularly stained with hematoxylin and eosin[14]. They were then inspected using a light microscope, and the obtained data led to the diagnosis of fibroid tumor. Subsequently, the Van Kossa stain was employed to identify the presence of calcium deposits inside the smooth muscle mass and fibrous connective tissues. .

Immunohistochemical Technique

We used the immunohistochemical technique with osteopontin markers. We conducted immunohistochemical analyses on 5-m-thick sections of tissue fixed in formalin and embedded in paraffin. Standard procedures were employed. The manufacturer recommended using a buffer that generates heat to extract the epitope. We used the anti-osteoporotic OPN/SPP1 (AKm2A1 antibody) as the main antibody and obtained a positive result, as expected.

Result

Histopathological results

This study analyzed a total of 20 cases of uterine leiomyoma. The patients' ages ranged from 50 to 60 years. The routine histological stain showed fibroids, sometimes called leiomyomas, are non-cancerous growths that develop from the smooth muscle cells in the wall of the uterus, known as the myometrium. As the fibroid grows, the cells differentiate into four distinct cell types: smooth muscle cells, vascular smooth muscle cells, and two distinct subgroups of fibroblasts (Figure 1). The histological finding with Von Kossa's stain also shows areas of intense black to dark brown tissue, which means that calcium has built up in the myofibers of the affected myometrium. The calcium deposition occupied most of the muscular tissue in the affected myometrium layer. However, the fibrous tissue that surrounded the myofibers showed mild calcium deposition compared with calcium deposition observed in muscular tissue in the same area. The calcium deposition primarily occurs at the outer edges of the leiomyoma (Figure 2).

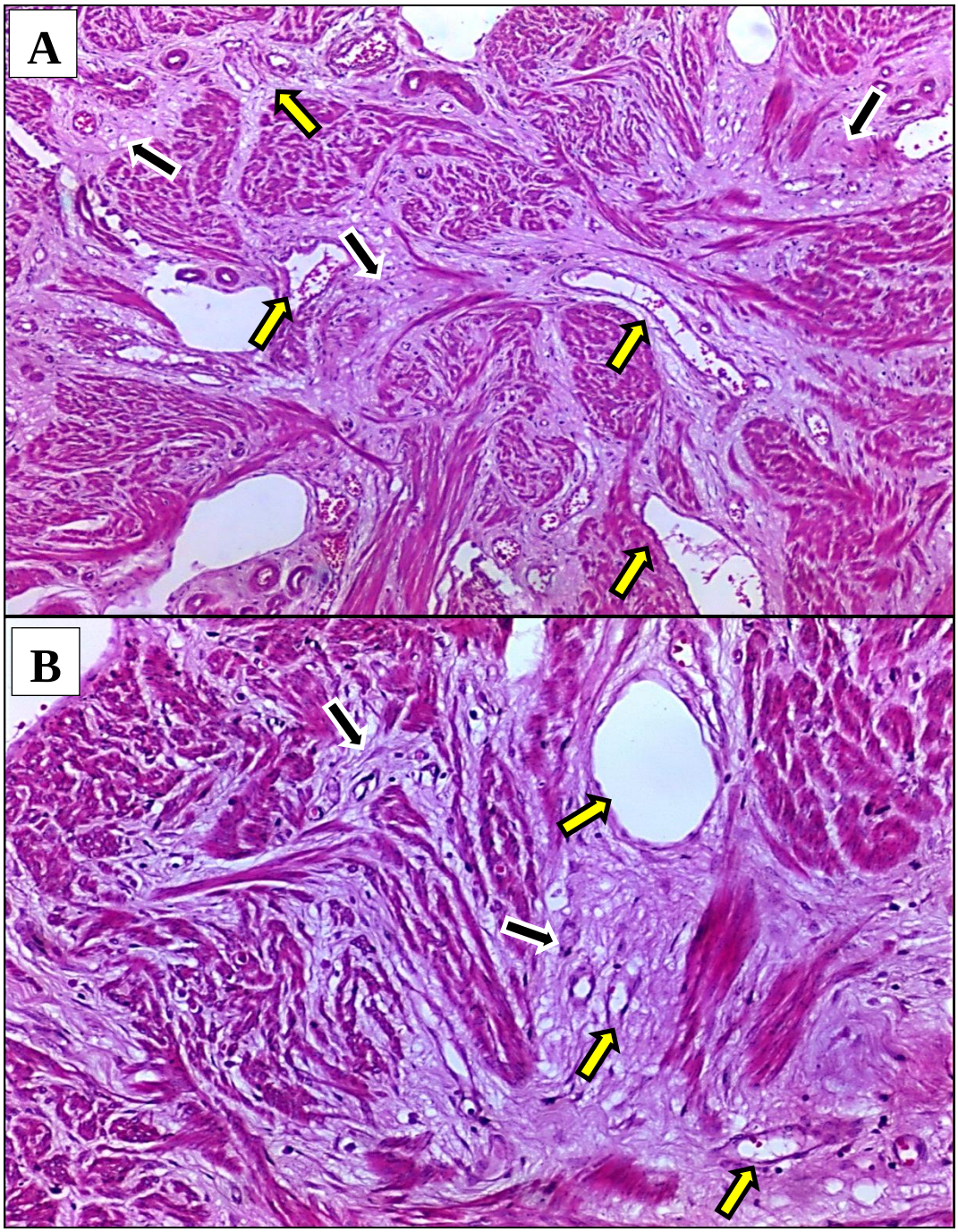
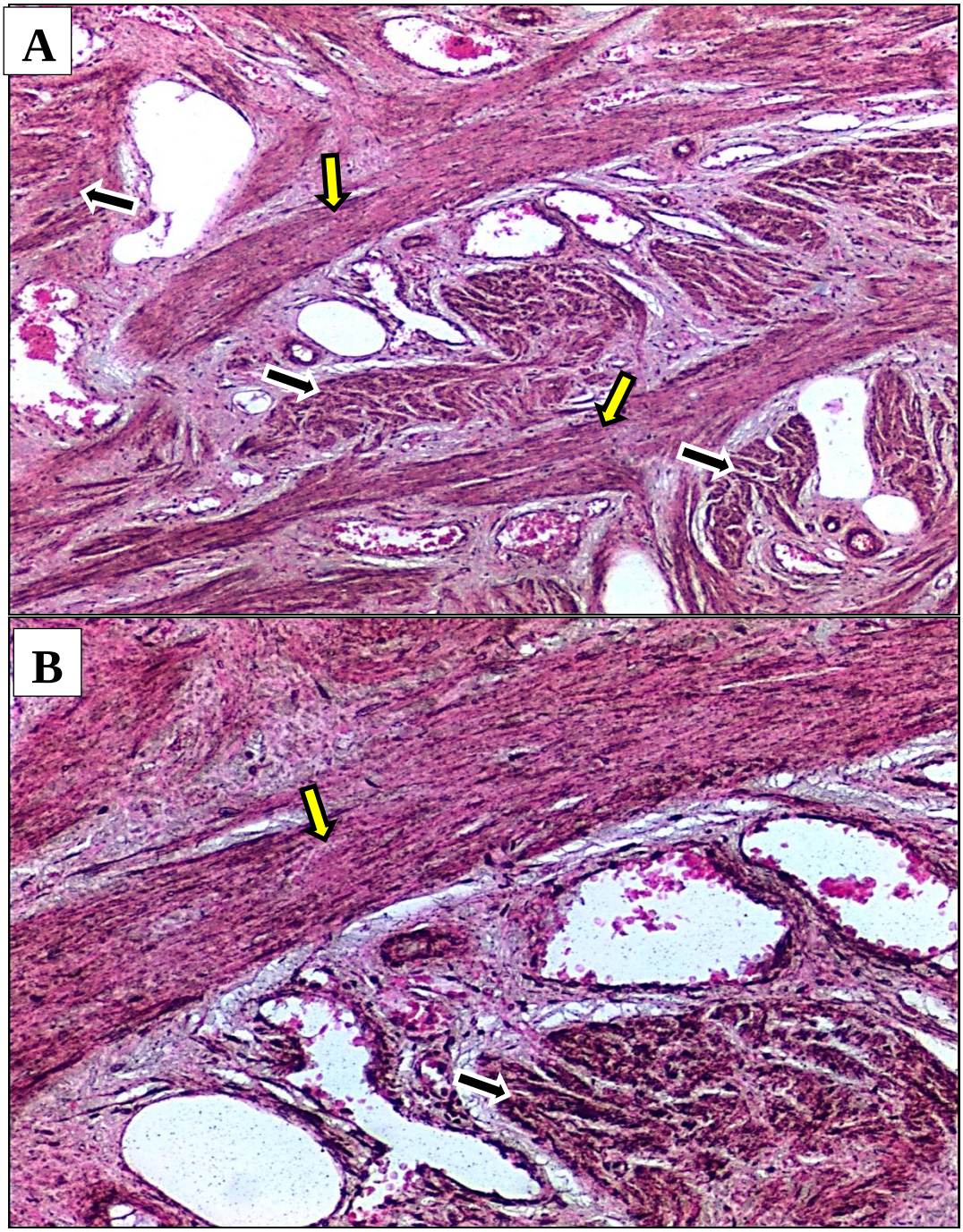


Figure 1: Photomicrograph of the uterus of 50-60 years-old woman.

A&B/ Uterus Fibroid. The expansion of fibrous tissue that surrounded the muscular tissue led to reduce the affected muscular tissue area fibrous tissue (black arrow). Also, the angiogenesis

activity was observed significantly in affected area of myometrium, where many blood vessels (yellow arrow). **H&E. A: 40x and B: 100x.**



Figure(2) A&B/ Uterus fibroid. Note the black to dark brown color-stained tissue (black arrow), indicating the deposition of calcium in myofibers of the affected myometrium. Where,

the calcium deposition occupied most of muscular tissue in the affected myometrium layer. However, the fibrous tissue (yellow arrow) that surrounded the myofibers showed mild calcium deposition compared with calcium deposition that observed in muscular tissue in same area. **Von Kossa stain. A: 40x and B: 100x.**

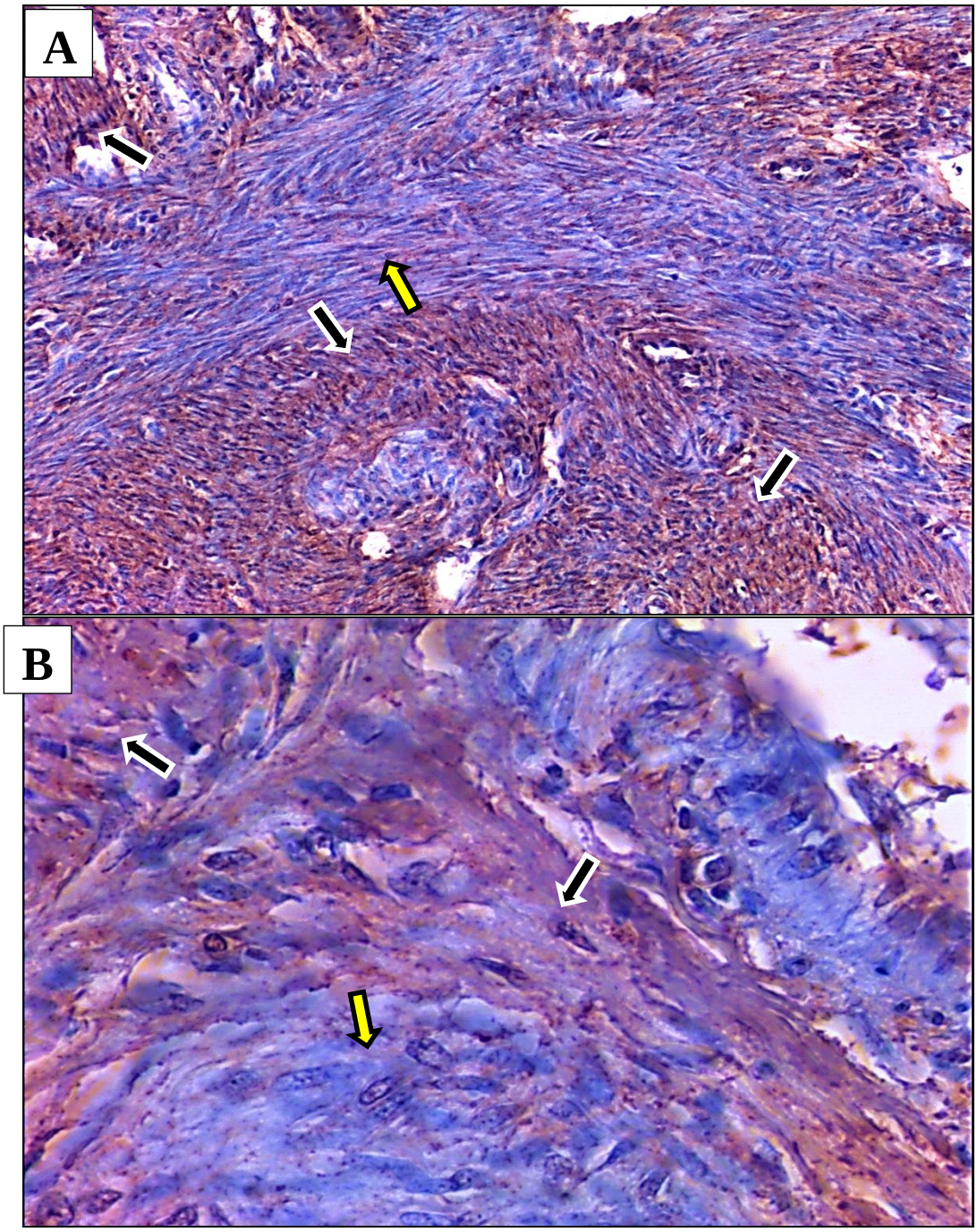


Figure 3: Photomicrograph of uterus of 50-60 years-old woman

A&B/ Uterus fibroid (leiomyoma). Note the overexpression of osteopontin (OPN) protein within most of the myofiber of leiomyoma affected myometrium tissues. However, the overexpression of OPN was observed significantly in transvers arranged myofibers (black arrow)

compared with longitudinal arranged myofibers (yellow arrow). **DAB and Hematoxylin. A: 100x**

Immunohistochemical results

The immunohistochemical approach was used to test for elevated levels of osteopontin protein in fibroid calcifications, a positive result will be shown by a strong reaction with the osteopontin-specific stain. Osteopontin will be clearly seen as a separate mark in regions of calcification inside the fibroid uterus, The immunohistochemical showed the heightened expression of this protein in those regions. The outcome can manifest as either dark or clearly defined spots or regions on a tissue backdro. Note the overexpression of osteopontin (OPN) protein within most of the myofibers of leiomyoma affected myometrium tissues. However, the overexpression of OPN was observed significantly in transvers arranged myofibers compared with longitudinal arranged myofibers (Figure3)

Discussion

Leiomyoma is a prevalent gynecological condition that frequently occurs in women of childbearing age. The tumor is a non-cancerous growth that arises from smooth muscle and consists of different quantities of connective tissue [15]. The tumor is believed to be dependent on estrogen and it is commonly observed that most cases retreat following menopause [16]. The histopathological examination of a leiomyoma shows intersecting bundles of spindle-shaped cells with blunt spindle nuclei and cytoplasm that stains pink with eosin. As the leiomyomas increase in size, they surpass the blood supply and undergo numerous degenerative alterations [17]. Calcification usually takes place in the final phases of a fibroid's life cycle [18].

The prevalence of calcified degeneration is higher among individuals in the postmenopausal age range, Black women, and in pedunculated subserous fibroids [16, 17]. Generally, leiomyomas undergo reduction in size after menopause as a result of the lack of estrogen stimulation. Research studies have suggested that estrone, insulin-like growth factor, and epidermal growth factor facilitate the development of leiomyoma in individuals who have reached menopause [19].

The fibroid experiences impaired blood flow, resulting in ischemia and degenerative alterations. Calcium deposition takes place near the outside edge of the leiomyoma, as reported by [16]. Increased levels of osteopontin in fibroid uterine calcification may be associated with many physiological and pathological processes, such as tissue healing, inflammation, and fibrosis, which have been observed in multiple tissues. [20]. According to Lund et al. [21], OPN serves as a regulator of biomineralization and a strong inhibitor of vascular calcification. The osteopontin protein is commonly associated with both calcification and macroscopic calcification. Several other proteins, including but not limited to bone morphogenetic protein-2a (BMP-2a), matrix Gla protein, and osteopontin itself, have also been implicated in calcium deposition in bone and other tissues (22; 23, 24). Osteopontin (OPN) was first discovered in osteoblasts as a matrix protein that regulates mineralization. OPN has recently been investigated as a versatile protein that is increased in several acute and chronic inflammatory disorders, including wound healing, fibrosis, autoimmune illness, and atherosclerosis. OPN exhibits significant upregulation at locations characterized by atherosclerotic plaques, particularly those that are linked to macrophages and foam cells. OPN is commonly recognized as a chemical that promotes inflammation and the

development of atherosclerosis in the setting of this disease. This information is consistent with Cho *et al*., [25]. OPN plays a negative regulatory role in vascular calcification (VC), which is strongly associated with chronic and active inflammation. It acts as an inhibitor of calcification and a promoter of decalcification.

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Conclusion

The calcification of uterine fibroids can greatly affect reproductive health, while elevated levels of OPN may indicate the existence of inflammatory processes or pathological changes in the body.

Reference

1.Khan AT, Shehmar M, Gupta JK. Uterine fibroids: current perspectives. Int J Womens Health. 2014;6:95-114.

2. Donnez J, Dolmans MM. Uterine fibroid management: from the present to the future. Human Reproduction Update, 2016 Nov 20; 22(6): 665-86.

3. Ibrar F, Riaz S, Dawood NS, Jabeen A. Frequency of fibroid uterus in multipara women in a tertiary care centre in Rawalpindi. Journal of Ayub Medical College Abbottabad, 2010 Sep 1; 22(3): 155-7

4. Walsh J.W., Rosenfield A.T., Jaffe C.C., Schwartz P.E., Simeone J., Dembner A.G., Taylor K.J. Prospective comparison of ultrasound and computed tomography in the evaluation of gynecologic pelvic masses. AJR Am. J. Roentgenol. 1978;131:955–960.

5-Greg A. Johnson, Robert C. Burghardt, Fuller W. Bazer, Thomas E. Spencer, Osteopontin: Roles in Implantation and Placentation, Biology of Reproduction, Volume 69, Issue 5, 1 November 2003, Pages 1458–1471.

6- Johnson GA, Spencer TE, Burghardt RC, Bazer FW. Ovine osteopontin. I.Cloning and expression of mRNA in the uterus during the peri-implantation period. Biol Reprod 1999; 61:884–891.

7- Luedtke CC, McKee MD, Cyr DG, Gregory M, Kaartinen MT, Mui J, Hermo L. Osteopontin expression and regulation in the testis, efferent ducts, and epididymis of rats during postnatal development through to adulthood. Biol Reprod 2002; 66:1437–1448.

8- Johnson GA, Burghardt RC, Spencer TE, Newton GR, Ott TL, Bazer FW. Ovine osteopontin II. Osteopontin and alpha(v)beta(3) integrin expression in the uterus and conceptus during the peri-implantation period. Biol Reprod 1999; 61:892–899.

9. Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaideh S, Rittling SR, Denhardt DT, Glimcher MJ, Cantor H.Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. Science 2000; 287:860–864.

10. Malyankar UM, Scatena M, Suchland KL, Yun TJ, Clark EA, Gia chelli CM. Osteoprotegerin is an alpha v beta 3-induced, NF-kappa B dependent survival factor for endothelial cells. J Biol Chem 2000;275:20959–20962.

11. Hotte SJ, Winquist EW, Stitt L, Wilson SM, Chambers AF. Plasma osteopontin: associations with survival and metastasis to bone in men with hormone-refractory prostate carcinoma. Cancer 2002; 95:506-512

12. Miyauchi A, Alvarez J, Greenfield EM, Teti A, Grano M, Colucci S, Zambonin-Zallone A, Ross FP, Teitelbaum SL, Cheresh D, Hruska KA. Recognition of osteopontin and related peptides by an v 3 integrin stimulates immediate cell signals in osteoclasts. J Biol Chem 1991; 266:20369–20374.

13- Goldberg HA, Hunter GK. The inhibitory activity of osteopontin on hydroxyapatite formation in vitro. Ann N Y Acad Sci 1995; 760:305–308.

14-Bancroft JD, Suvarna KS, Laytonn C, (2018). Bancroft's Theory and Practice of Histological Techniques. Established itself as the standard reference for histo-technologists and laboratory scientists, p96.

15- Singh RP, Patarca R, Schwartz J, Singh P, Cantor H. Definition of a specific interaction between the early T lymphocyte activation 1 (ETA-1) protein and murine macrophages in vitro and its effect upon macrophages in vivo. J Exp Med 1990; 171:1931–1942.

16- Ozdemır O, Atalay CR, Sarı ME, Sen E, Nebıoglu M.Postmenopausal calcified pedunculated large subserous leiomyoma: A case report. Obstet Gynecol Cases Rev 2015;2:54.

17- Samal SK, Rathod S, Rani R, Anandraj R. An unusual presentation of a severely calcified subserous leiomyomain a postmenopausal woman: A case report. Int J Reprod Contracept Obstet Gynecol 2014;3:463-5

18-Williams ARW. Uterine fibroids - what's new?. F1000Res. 2017;6:2109. doi:10.12688/f1000research.12172.1

19. Singh K, Prasad D, Pankaj S, Suman S, Kumar A, Choudhary V, et al. Postmenopausal massive subserous calcified fibroid – Acase report. J Evol Med Dent Sci 2014;3:2255-7.

20. Pagel, C.N., Wasgewatte Wijesinghe, D.K., Taghavi Esfandouni, N. et al. Osteopontin, inflammation and myogenesis: influencing regeneration, fibrosis and size of skeletal muscle. J. Cell Commun. Signal. 8, 95–103 (2014). https://doi.org/10.1007/s12079-013-0217-3

21. Lund, S.A., Giachelli, C.M. & Scatena, M. The role of osteopontin in inflammatory processes. J. Cell Commun. Signal. 3, 311–322 (2009). https://doi.org/10.1007/s12079-009-0068-0

22- Watson KE, Boström K, Ravindranath R, Lam T, Norton B, Demer LL. TGF-beta 1 and 25-hydroxycholesterol stimulate osteoblast-line vascular cells to calcify. J Clin Invest. 1994;93:2106-2113.

23- Shanahan CM, Cary NRB, Metcalfe JC, Weissberg PL. High expression of genes for calcification-regulating proteins in human atherosclerotic plaques. J Clin Invest. 1994;93:2393-2402.

24-Fitzpatrick LA, Severson A, Edwards WD, Ingram RT. Diffuse calcification in human coronary arteries: association of osteopontin with atherosclerosis. J Clin Invest. 1994;94:1597-1604.

25-Cho, HJ., Cho, HJ. & Kim, HS, 2009. Osteopontin: A multifunctional protein at the crossroads of inflammation, atherosclerosis, and vascular calcification. Curr Atheroscler Rep 11, 206–213