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The effect of aging on women's mammary gland (histological study by using ImageJ-software technique)

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KEY WORDS:
Mammary glands, Intralobular& Interlobular ducts, Secretory units.

ABSTRACT

Background: The mammary glands are responsible for milk production in infants. It comprises lobes, lobules, secretory alveoli, and ducts for milk transport to the nipples. These structures are surrounded by adipose tissue, which provides insulation and protection to the mammary glands. Objective: To examine mammary gland growth and development from puberty to menopause

Materials and methods: This study involved 60 samples collected from corpses at Baghdad's Morgue Department of the Forensic Medicine Department. The ages of the participants ranged from 15 to over 60 years. The collected samples were divided into three groups: group I (15-25 years), group II (26-45 years), and group III (46-60 years). The samples were fixed in 10% NBF and processed with H&E and PAS staining. **Results:** The number of lobules between groups was counted, and different numbers of lobules between groups were observed. The thickness of the epithelial lining of the secretory units (SU) was also measured. The diameters of the secretory glands were measured between the groups. Thickness of epithelial lining of secretory units. The results were statistically significant (p = 0.001). This study aimed to evaluate the age-related changes in women's mammary glands. Group I had inactive secretory alveoli with fewer and smaller lobules. Group II, representing late pregnancy or lactation, exhibited swollen mammary glands with larger diameters, milk secretions, and more lobules. Group III, corresponding to menopause, displayed numerous alveoli undergoing cell death and was replaced by connective and fat tissues. Conclusion: The histological features of mammary glands transition progressively from active to regressive states as age advances.

Keywords: Mammary glands, Intralobular& Interlobular ducts, Secretory units.

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INTRODUCTION:

The mammary gland is an important organ that experiences dynamic changes throughout a woman's life, from puberty to post-menopause. Most previous studies on histological changes in breast tissue relied on hospital patient samples or animal models, which may introduce biases due to disease conditions or medication effects, potentially affecting result interpretation. In contrast, there is a lack of research utilizing independent samples obtained from forensic autopsies, which presents a unique opportunity to study tissue changes without external interventions. Therefore, this study aims to analyze histological changes in breast tissue across three age stages using independent human samples, addressing a significant research gap. Mammary glands are compound tubuloalveolar glands whose structures change depending on the reproductive status of females. Each breast comprises 15-20 lobes separated by septa of connective tissue and fat cells; each lobe is drained through a single lactiferous duct, then a lactiferous sinus, which opens into the nipple (1,2).

During pregnancy, the mammary glands differentiate into secretory alveoli that produce milk. The alveoli grow and expand during pregnancy for milk production (3).

During pregnancy, the mammary gland undergoes morphological and functional maturation. Each alveolus was lined with simple cuboidal or columnar secretory cells (4,5), for milk secretion. The inactive (resting) mammary gland mainly comprises a ductal system with few alveoli. The stroma comprises dense connective tissue with few smooth muscle bundles separating the lobes (6,7). Mammary gland development depends on the presence of growth hormone and prolactin, whereas estrogen regulates ductal development. During pregnancy, estrogen, progesterone, and prolactin affect tubuloalveolar proliferation (8).

Myoepithelial cells are present between glandular cells and the basement membrane of the alveoli lobules and lactiferous ducts (9). The histological structure of the mammary gland varies depending on the age, sex, and physiological status (10).

Objective: The study aimed to understand how mammary glands develop and the effects of hormonal and genetic factors on mammary gland development. To study the changes in the shape of the glands in different age groups.

Materials and methods:

Sample Collection: This study used 60 mammary gland samples obtained from recently deceased individuals at the Morgue Department of the Forensic Medicine Department in Baghdad, Iraq, between April and September 2024. All samples were collected within 6 to 12 hours after death to minimize autolytic changes that could compromise histological integrity. The selection criteria ensured that the individuals had no pre-existing breast diseases, hormonal disorders, or chronic conditions that could influence tissue structure. Additionally, including samples from forensic cases ensured that tissue morphology was not significantly altered due to prolonged postmortem intervals. The samples were obtained with the consent of the participants' families and an official letter from the Ethics Committee.

The sample ages ranged from 15 to over 60 years old. The total sample (60) was divided into three groups: group I (15-25years), group II (26-45years), and group III (46-60years).

Sample Preparation and Sectioning: The mammary gland samples were fixed in 10% Neutral Buffered Formaldehyde (NBF) for 24 hours before being processed into paraffin blocks. Sections were cut at 5 µm thickness and stained using Hematoxylin and Eosin (H&E)&Periodic Acid-Schiff (PAS) according to standard histological

protocols (11). Histological staining was performed following standard protocols described by Horobin (11). Slides were examined using a light microscope, and the images were analyzed with ImageJ software for morphometric measurements. For image analysis, microscopic images were captured using a microscope equipped with a camera. Measurements were then conducted using ImageJ software, employing the following methods:

Epithelial Thickness and Gland Diameters: Dimensions were measured in micrometres (µm) using the line tool.

- Collagen Content: The colour threshold function was utilized to highlight regions of collagen in PAS-stained sections, allowing for the calculation of area in square micrometres (μm^2).

To ensure measurement accuracy, 10 randomly selected images per group were manually examined using an optical micrometre. An independent histologist also reviewed the measurements to assess reliability, resulting in a coefficient of variation (CV) of <5%, confirming the reproducibility of ImageJ-based analyses(13).

Statistical Analysis:

All ImageJ-derived data were statistically analyzed using SPSS (version 25, IBM, USA). ANOVA and post-hoc Tukey tests were applied to compare epithelial thickness, glandular diameter, and collagen content across the three age groups(14,15). A Pearson correlation test was performed to examine the relationship between collagen content and glandular regression. A p-value of ≤ 0.05 was considered statistically significant (16,17).

Ethical approval:

The medical medico-legal directorate of the Ministry of Health and Environment in Bagdad, Iraq, and a local ethics commission approved by document number (D. I. A / 1 / A B / K 24/02/2). Document 182 / 7/3 authorized forensic medicine sample collection. This study adhered to ethical standards by the Declaration of Helsinki.

Conflicts of Interest Statement:

The author declares no conflicts of interest regarding the publication of this manuscript.

Result:

The mammary gland in group I (non-pregnant) consists of lobes, each of which is further divided into lobules by connective tissue. These lobules are alveoli, ductulus, and ducts that mature depending on the woman's age and physiological conditions.

The lobules contained connective tissue with few cells. The alveoli were lined with simple cuboidal epithelium and blood vessels and lymphocytes were found between the lobules and the interlobular ducts. The stroma contains many connective tissue cells, macrophages, lymphocytes, a few fat cells, blood cells, and intralobular ducts. The secretory units mature at different stages of secretion and ultimately produce milk during late pregnancy and lactation periods. The lobes were separated by dense connective tissue ducts, blood vessels, nerves, lymphatic vessels, macrophages, and fibroblasts. Abundant collagen fibres, as well as a few fat cells, can also be seen in the stroma. In group I, the alveoli were seen as small buds. Stellate myoepithelial cells are present between the epithelial lining and the basal lamina. The intralobular connective tissues were loose, whereas the lobes were separated by thick dense connective tissue. In this group, the mammary gland is considered immature and contains large amounts of stromal cells, with few fatty tissues in the lobules (Fig.1).

In group II, which is the active stage of late pregnancy and lactation, the gland undergoes large, extensive changes, and starts its activity, through secretion. At this stage, the alveoli expand and have a sac-like appearance, and the epithelial lining of the

alveoli can be observed at various heights. The stages of alveolar secretory maturation also occur at various heights (Fig.2 & Fig.3). Different stages of alveoli can be observed in the lobules. Interlobular connective tissues are composed of plasma cells and lymphocytes. The alveolar lumen is filled with secretory substances (Fig.3).

In the third group (group III), which included menopausal women, the mammary gland underwent involution. The secretory alveoli start atrophies, some disappear, and only a few ducts persist. Dilation of the remaining ducts was observed.

The stromal connective tissue became dense, and the deposition of fat cells was evident. The abundance of blood vessels, macrophages, and lymphocytes was also characteristic (Fig.4). The number of lobules was calculated using a morphometric comparison of the three groups. The mean ± standard deviation in group I was (64.39 ± 39.25), in group II was (135.1 ± 88.58), and in group III was (35.78 ± 22.00).

The thickness of the lining of epithelial secretory glands between the three groups measurements of the first group were (66.77 ± 23.37) µm, the second group (88.25± 20.43) µm, and the third group (41.45± 8.1) µm, and results were of significant value=0.001. As for the measurement of the diameters of the secretory glands, they were in the first group (260.452 ± 76.32) µm, the second group (505.231 ± 97.85) µm, and the third group (85.878 ± 41.30) µm, and the results were significant at p. value ≤ 0.05 (Fig.5), as in Table 1.

PAS staining was used to determine the type of arrangement and aggregation of glycoprotein materials in the mammary glands of different age groups, and the different glycoproteins and glycogen components between different age groups were quite clear. In Group I, the PAS reaction was weak and appeared in the form of small, few clusters of eosinophils in the mammary gland (Fig.6); in Group II, a large highly eosinophilic aggregation of glycoprotein was seen distributed all over the secretory unit. The PAS reaction was strong (Fig.7). In Group III, the reaction of PAS decreased in the secretory units, but it strongly reacted in the connected tissues containing more adipose tissue, macrophages, fewer fibroblasts, and less connective tissue loss between the lobules (Fig.8). The amount of collagen in the connective tissue of each group was measured using ImageJ software. The mean deviation ± SD was calculated for the first group, which was (85753 ± 44462) micrometres; the second group was (117396 ± 41035) micrometres, and the third group was (65498 ± 37742) micrometres, as shown in (Fig.6), Table 1. There was a clear difference between the groups with a statistical significance of 0.02, as the collagen levels in groups I and II were higher, whereas the collagen levels in group III, which contained an abundance of adipose tissue instead of connective tissue, were lower, The morphometric analysis of the mammary gland was conducted to evaluate differences in the number of lobules, secretory gland diameters, and collagen content among the three age groups. The analysis revealed statistically significant variations across the groups ($p \leq 0.05$), as in Table. 1.

Table 1: Morphometric data for the number of lobules, secretory gland diameters, and collagen content across age groups.

Groups	Number of Lobules (Mean ± SD)	Diameter of Secretory Glands (µm, Mean ± SD)	Collagen Content (µm², Mean ± SD)
Group I	64.39 ± 39.25	260.452 ± 76.32	85753 ± 44462
Group II	135.1 ± 88.58	505.231 ± 97.85	117396 ± 41035
Group III	35.78 ± 22.00	85.878 ± 41.30)	65498 ± 37742

As summarized in Table 1, Group II showed the highest number of lobules, averaging 135.1 ± 88.58, indicating an active secretory state during the reproductive years. In

contrast, Group III exhibited the lowest collagen content (65498 ± 37742 µm²), which is consistent with glandular regression occurring after menopause. The quantitative findings are further supported by the histological observations shown in Figures (1- 4). These figures illustrate the histological characteristics observed across the three groups. They highlight distinct structural features, including variations in connective tissue density, glandular activity, and the distribution of adipose tissue

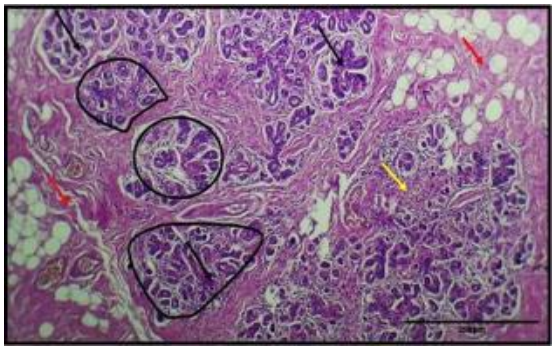

Fig.1: Group II : Histological section of mammary gland in Group II showing lobules (black circle), secretory unit (black arrow), dense interlobular connective tissue (red arrow), and loose intralobular connective tissue (yellow arrow) (H&E, 40X).

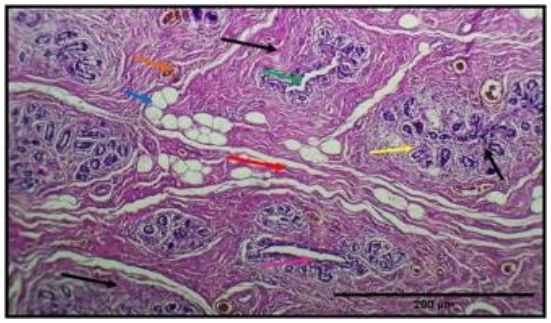

Fig.2 : Group I : Histological section of mammary gland in Group I showing dense connective tissue (red arrow), loose connective tissue (yellow arrow), intralobular ducts (green arrow), blood vessels (brown arrow), and adipose tissue (blue arrow) (H&E, 40X).

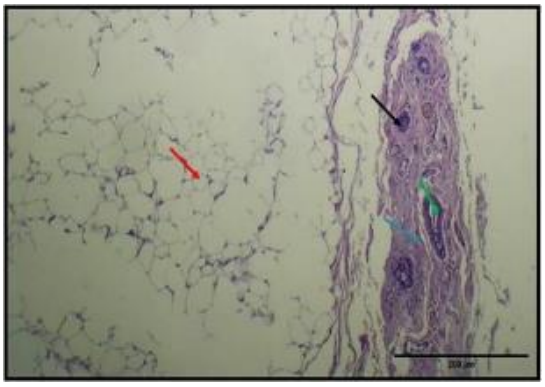

Fig.3:Group III : Histological section of mammary gland in Group III showing lobules (black arrow), dense interlobular connective tissue with abundant adipose tissue (red arrow), loose connective tissue (blue arrow), and intralobular ducts (green arrow) (H&E, 40X).

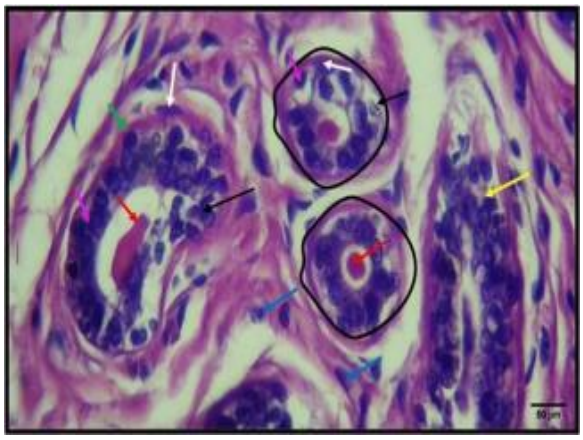

Fig.4: Group II ,Histological section of mammary gland in Group II showing secretory units (black circle), milk secretion (red arrow), basement membrane (green arrow), myoepithelial cells (white arrow), simple cuboidal epithelial cells (pink arrow), macrophages (blue arrow), and interlobular ducts (yellow arrow) (H&E, 400X).

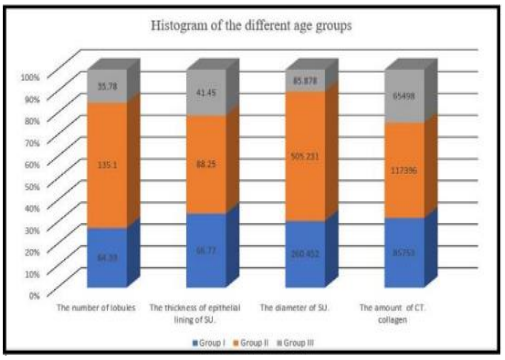

Fig.5 : Histogram showing different age groups measured using ImageJ software. Statistical significance ($p \leq 0.05$) was observed among the groups.

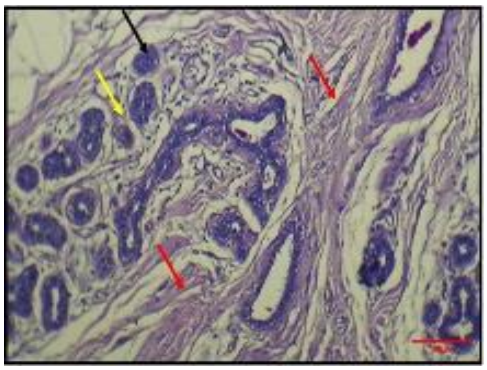

Fig.6: Group I: Histological section of mammary gland in Group I showing lobules (black arrow), dense connective tissue (red arrow), and loose connective tissue (yellow arrow) (PAS, 100X).

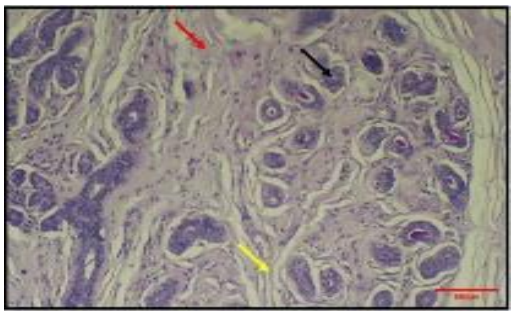

Fig.7:Group II: Histological section of mammary gland in Group II showing lobules (black arrow), dense connective tissue (red arrow), and loose connective tissue (yellow arrow) (PAS, 100X).

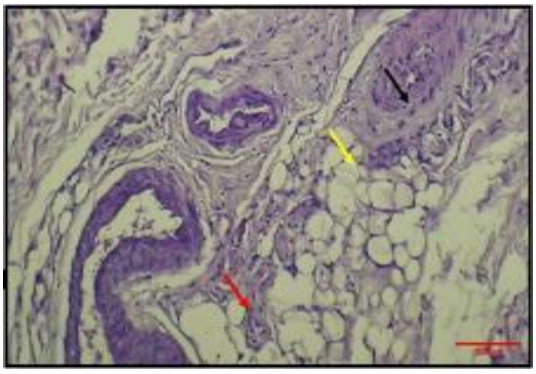

Fig.8 :Group III: Histological section of mammary gland in Group III showing lobules (black arrow), dense connective tissue (red arrow), and loose connective tissue (yellow arrow) (PAS, 100X).

Discussion:

This study provides a new perspective on how mammary gland structure changes with age after death, emphasizing the progressive remodelling of glandular and stromal tissues. Unlike studies that utilize biopsy samples from living individuals, the use of postmortem tissue eliminates the confounding effects of circulating hormonal fluctuations. This approach enables a clearer assessment of age-related structural changes.

Previous research on in vivo mammary gland biopsies by (4,5) has shown that hormonal surges significantly affect lobuloalveolar proliferation and secretory activity. However, the current study, which utilizes postmortem specimens, confirms that even without active hormonal influence, connective tissue remodelling, and alveolar atrophy continue as intrinsic age-related changes. Notably, the decline in collagen density and alveolar structures observed in postmenopausal samples aligns with findings from Lin et al. (10), highlighting the impact of estrogen withdrawal on glandular involution.

ImageJ offers a reliable and reproducible method for conducting histomorphometric analysis, allowing for assessing age-related

changes in mammary gland structure. Unlike manual measurements, ImageJ enables high-throughput quantification of epithelial thickness, secretory gland diameter, and collagen deposition while minimizing observer bias.

everal previous studies, such as those by Lin et al. and Shams et al. (10,18), have validated the use of ImageJ for morphometric analysis in breast histology, confirming its accuracy in measuring glandular structures. However, some limitations exist, including the challenges associated with segmenting overlapping histological components, particularly in PAS-stained sections. Future research should explore machine learning-based image analysis to improve segmentation accuracy. The correlation between collagen content and glandular atrophy ($p = 0.02$) is consistent with the findings of Russo and Russo (23), who reported a progressive decline in collagen density after menopause. This reduction may contribute to the increased breast adiposity and ductal atrophy observed in postmenopausal women, further supporting the role of estrogen depletion in mammary gland involution.The present study highlighted significant histological changes in the mammary gland across different age groups. In group I (15-25 years), The mammary gland in this group exhibited immature structures with small, inactive lobules. The reduced glandular activity and smaller lobules observed can be explained by the lower levels of reproductive hormones, such as estrogen and progesterone, during this age range, where the gland remains immature and inactive, preparing for future reproductive activity. This is consistent with the findings by Shams. et.al (18), who reported similar structural characteristics in pre-reproductive age groups.

In group II (26-45 years), This group exhibited the most active phase of the mammary gland, characterized by larger lobules and heightened secretory activity. The proliferation and expansion of alveolar cells during this phase are closely associated with the hormonal influences of estrogen, progesterone, and prolactin, which promote lobuloalveolar development and milk production during both pregnancy and lactation (19,20)). These findings are in agreement with Alekseev (20), who documented the impact of hormonal surges on glandular activity during reproductive years. The presence of milk secretion and highly eosinophilic glycoproteins further confirms the active state of the gland, as supported by Watson (21). The secretory units are with secretory substances. In this stage, mammary glands secrete milk. Mammary glands during pregnancy are affected by many hormones such as progesterone, prolactin, estrogen, and placental lactogen (16). These hormones cause the proliferation of alveoli lining cells and then the intralobular ducts (22). In Group III, the menopausal group, the mammary gland underwent significant regression. This regression was characterized by the atrophy of secretory alveoli, dilation of ducts, and increased deposition of adipose tissue. These changes reflect the decline in estrogen and progesterone levels following menopause, which leads to glandular regression and remodelling of the stromal tissue. This decline is consistent with findings by Russo and Russo (23), who highlighted the effects of hormonal cessation on the structure of the mammary gland. Additionally, the observed reduction in collagen density and the increase in adipose tissue in this group may contribute to various breast-related conditions, such as ductal atrophy or increased susceptibility to breast cancer (24-26). The results confirm that histological changes in the mammary glands are not solely dependent on hormonal fluctuations

but also represent intrinsic age-related transformations. Previous studies by Stingl et al.(25) have reported that, even without active hormonal influence, significant connective tissue remodelling and alveolar atrophy continue with ageing. This aligns with the present study, which observed structural modifications across different age groups, notably the transition from a highly glandular state in reproductive years to an adipose-rich composition post-menopause.

The amount of collagen in the connective tissue was notably high, particularly in the first and second groups. This increased collagen content can be attributed to these groups' larger quantities of loose and dense connective tissue. These findings are consistent with the observations made by Urmila (27), who noted that the epithelial cells surrounding the connective tissue were very active when stained PAS(28).

Additionally, this research innovates by employing the ImageJ technique to measure collagen and analyze histological changes across various age stages. This adds a quantitative dimension that improves the accuracy of histological assessment.

Conclusion:

The mammary gland undergoes significant changes across different life stages, including alterations in lobules, connective tissue ratio, fat tissue, and gland activity, which peaks during pregnancy and lactation and ceases in menopause. The use of ImageJ-assisted morphometric analysis strengthens the study's methodology. Future research should include immunohistochemical and molecular techniques to further understand the mechanisms driving these changes.

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