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POSTNATAL FATE OF THE ULTIMOBRANCHIAL REMNANTS IN THE RAT THYROID GLAND

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Short Title: Ultimobranchial remnants in rat thyroid

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ABSTRACT

The ultimobranchial follicles (UBFs) are considered embryonic remnants from the ultimobranchial body (UBB). They are follicular structures that vary in size and appearance depending on the age of the rat. The main objective of this article was to study the progressive changes in shape, size and frequency of the UBFs in the postnatal rat, from birth to old-age. To accomplish that objective, a systematic morphometrical and incidental study of the UBF has been carried out in 110 Wistar rats of different ages and both sexes, divided into 3 groups: (1) young rats (5 to 90-day-old); (2) adult rats (6 to 15-month-old), and (3) old rats (18 to 24-month-old). The glands were serially sectioned and immunostained for calcitonin at five equidistant levels. According to our results, UBFs were observed in all thyroid glands but a more exhaustive sampling was occasionally necessary in male rats. In young rats, immature UBFs predominantly appeared while in adult rats, mature UBFs with cystic appearance and variable luminal content prevailed. Furthermore, it was relatively common to find spontaneous anomalous UBFs in old rats, which we have termed as "ultimobranchial cystadenomata". Additionally, in young rats, UBF size significantly increased with age and it was much larger when compared to normal thyroid follicles. Likewise, in adult rats, UBFs were significantly larger than normal thyroid follicles but only in female rats. In general, UBFs in females were also significantly larger than those found in male rats. Finally, the differences in UBF size and apparent frequency between male and female rats, besides a higher incidence in females of UB cystadenomata, have confirmed the existence of a conspicuous sexual dimorphism in regard to the destiny of these embrionary remnants during postnatal thyroid development.

Keywords: Ultimobranchial follicle, ultimobranchial cystadenoma, C cells, rat thyroid gland.

INTRODUCTION

In mammals, the thyroid gland consists of two endocrine cell types, namely, follicular cells and C cells. These two cell populations are of distinct embryonic origins. During development, the thyroid diverticulum, which is derived from the endodermal ephitelium of the ventral pharyngeal floor, moves caudally down along the midline and forms two lateral lobes, thus giving rise to follicular cells. In contrast, the ultimobranchial body (UBB) develops from the fourth pharyngeal pouch and migrates to its final place of residence, the lateral lobes of the thyroid gland. There it gives rise to C cells, which synthesize and secrete calcitonin, a serum calcium-lowering hormone (Fagman and Nilsson, 2011; Westerlund et al., 2008).

However, in a variety of animals, including humans, a third cell population, obviously differing from the aforementioned, has been described. These cells form the lining of follicular structures that have been considered as embryonic remnants of the UBBs. Such structures show interspecies differences and have been described in the literature under several different denominations: “second kind of thyroid follicles”, “ultimobranchial follicles”, “ultimobranchial tubules” or “ultimobranchial cysts”, in rodents (Martin-Lacave et al., 1999; Rao-Rupanagudi et al., 1992; Van Dyke, 1944; Wollman and Neve, 1971a; b); “C-cell complexes”, in dogs (Kameda et al., 1980; Leblanc et al., 1990); “unusual follicles”, in the fox (Srivastav and Swarup, 1982); “ultimobranchial remnants”, in bulls (Ljungberg and Nilsson, 1985); and “solid cell nest” (SCN), in humans (Beckner et al., 1990; Harach, 1988; Harach et al., 1993).

Evidences of the UBB origin of the second kind of thyroid follicles in rodents has been provided by different authors. Specifically, Wollman and Hifler (Wollman and Hilfer, 1977; 1978) demonstrated the development of ultimobranchial follicles (UBFs) from transplants of ultimobranchial outpocketing to kidney capsules, whereas transplants from the ventral outpocketing formed exclusively normal thyroid follicles, with differences between rats and mice related to the characteristics of developed UBF. In contrast, in humans, the ultimobranchial origin of SCN has been extensively discussed. Nevertheless, most evidences support that SCN are vestiges of the UBB as both structures share the same anatomical, morphological and immunohistochemical features, besides the presence of C cells in SCN (Burstein et al., 2004; Harach, 1988; Harach et al., 1993; Janzer et al., 1979; Nadig et al., 1978; Reis-Filho et al., 2003; Rios Moreno et al.).

In rodents, particularly in rats, many reports have described the morphology of the UBFs (Martin-Lacave et al., 1999; Rao-Rupanagudi et al., 1992; Van Dyke, 1944; Wollman and Neve, 1971a; b). In general, UBFs were located partially embedded amongst the usual thyroid follicles in the center of the lobe and they were characterized by being lined by two or more layers of squamous cells, having an abundance of desquamated cells in the lumen. Differences related to the UBF size, shape and luminal content with age have been reported (Wollman and Neve, 1971a; b), however, a systematic study of UBF fate during the life span of the rat (0-24 months old) is lacking. Moreover, neither morphometrical analyses nor UBF differential incidences studies between male and female rats have yet to be carried out. Therefore, the main objective of the present study is to describe the UBF developmental progression along the life span of the Wistar rat, in terms of shape, size and frequency, highlighting the possible existence of a sexual dimorphism.

MATERIAL AND METHODS

In the present study, 110 Wistar rats of both sexes and different ages were used. Rats were divided into three groups: (1) 40 young rats of both sexes (5-10-15-20-25-30-60-90 days old); (2) 40 adult rats (20 females and 20 males) of 6-9-12-15 months old and, finally, (3) 30 old rats (15 females and 15 males) of 18-21-24 months old. Each age group consisted of five animals. Rats were anesthetized, and the thyroid gland and the attached parathyroid glands and trachea were removed and processed. All experiments were conducted in accordance with the guidelines proposed in The Declaration of Helsinki (http://www.wma.net) involving the use of laboratory animals. The samples were fixed for 3 h in Bouin's solution and embedded in paraffin. Thyroids were serially sectioned at a thickness of 5 μm, from the superior pole to the inferior pole of the gland, and mounted on slides (15 sections each). The first and last sections were stained with hematoxylin and eosin. At least, five equidistant slides were immunocytochemically stained for calcitonin using a rabbit antibody (DAKO, Glostrup, Denmark) and the peroxidase-antiperoxidase or the LSAB methods (DAKO, Glostrup, Denmark), with 3,3'-diaminobenzidine as chromogen, followed or not by the periodic acid-Schiff (PAS) reaction, and counterstaining with Harris' hematoxylin, as described previously (Conde et al., 1995). Consequently, at least 75 sections were studied per animal. Morphometric analysis of UBFs and normal thyroid follicles areas was also performed by software processing and image analysis (Cell* Imaging Software). The area of normal thyroid follicles was exclusively obtained from those follicles (n=10) which surrounded closely the corresponding UBF. Data were compared using the Student's t-test. P values of less than 0.05 were accepted as significant.

RESULTS

1. *Localization of UBFs in the thyroid gland*

UBFs were found in one or both lobes of the thyroid gland at all ages studied. UBFs occurred singly or in clusters and were mainly located in the central region of each thyroid lobe, intimately related to adjacent thyroid parenchyma and frequently in contact with perivascular connective tissue (Fig.1). In two rats, however, UBFs were detected in an unusual position, specifically, in the connective interstitium between the tracheal cartilage and the thyroid lobe, probably as a result of a UBB emigration defect (Fig.2A). At all ages, thyroid lobes with more than one UBF were found and in most of the cases they were very close or even interconnected.

2. *Morphology of UBFs at different ages*

2.1. Young rats: 5 to 90-day-old

In the youngest rats (5 to 30-day-old), UB remnants were observed to vary in size, appearance, and luminal content, according to these features they were called "immature UBFs". These structures evolved from narrow cellular nests or rods to tubular structures, in 5 to 10-day-old rats, through intermediate stages in which they appeared either as an epithelial pearl-like structure or presented a "mixed follicle" appearance in others (fig. 1A-E). Mixed follicles were follicles partially made by UBF fused to usual thyroid follicles. In the lumen of immature UBFs, some cells and cell debris could be observed (Fig1E). In 60-day-old rats or over, most UBFs were

spheroidal and presented a cystic appearance in whose wall more than one cell layer was always distinguishable. At the periphery they showed cuboidal cells which tended to flatten towards the central lumen. This structure was called "mature UBF" (fig. 1F). By morphometric analysis, UBFs in the young-rat group were found to have a size above normal thyroid follicles, with an average area of 2874±970 vs. 1898±741 μm², highly significant statistically values ($p<0.01$) (Fig.3).

2.2. Adult rats: 6 to 15-month-old

In this group, "mature UBFs" were predominant, in most cases with cystic appearance and round (fig.2A-D). Pyknotic nuclei, desquamated cells, and PAS-positive material traces were frequently found in the luminal content. The cells at the outer layer from mature UBFs were generally more flattened than those observed in immature ones. The UBF size was variable according to the rat and age. In general, UBFs in female rats were larger than those found in male rats (Table 1), with an average area of 6205±1781 vs. 5199±2384 μm². Likewise, UBFs in females were significantly larger than normal thyroid follicles, with an average area of 6205±1781 vs. 3839±367 μm², with $p<0.05$. However, in male rats, UBFs were also larger than thyroid follicles, with an average area of 5199±2384 vs. 3581±470, but not statistically significant values (Table 1). Furthermore, UBFs of reduced dimensions, showing morphologies that resembled those observed in young rats, appeared preferently in male rats at any age (Fig.2C).

2.3. *Old rats: 18 to 24-month-old*

In the old-rat group, "mature UBFs" were also predominant (Fig.2E-F). In general, UBFs in female rats were larger than those found in male rats (Table 1), with an average area of 6624±2347 vs. 2843±786 μm² ($p<0.05$). Accordingly, in all ages considered, the female UBF area was superior to that of males (Fig.4). Additionally, UBFs in females were larger than normal thyroid follicles, with an average area of 6624±2347 vs. 4376±370 μm², but not statistically significant. In contrast, in male rats, UBFs were significantly smaller than thyroid follicles, with an average area of 2843±786 vs. 4634±447 ($p<0.01$) (Table 1).

Furthermore, the presence of very large cystic structures was also observed in adult and old rats, sometimes exhibiting a clustered appearance, which we named "UB cystoadenomata" (Fig.2G-I). The UB cystadenomata contained a degenerative material in the lumen, which was organized in concentric layers resembling a sliced onion, with no trace of nuclei, and were scarcely vascularized. Their area ranged from 22,759 μm² to 200,244 μm², with an average area of 83,981±53,682 μm² (Table 2), a considerably greater size than the normal adjacent thyroid follicles (3765± 984 μm², $p<0.01$). These structures were found in 17% of the oldest rats, and were more frequent in females than in males (ratio 2,33:1). Normally, these structures compressed the adjacent thyroid parenchyma which, despite this, maintained its integrity. Nevertheless, there was one single case, a 12-month-old female rat, in which the UB cystadenoma reached such a great size that it practically occupied most of the thyroid lobe, besides exhibiting aggressive morphology (Fig.2I). Furthermore, in two cases, the UB cystadenomata were adjacent to C-cell tumours.

3. Relation of UBFs with thyroid endocrine cells

Small thyroid follicles with a normal appearance could be frequently observed, in close association with the wall of immature UBFs, called "mixed follicles" (Fig1E). In relation to C cells, its presence was only occasional in association with UBFs.

Specifically, C cells and C-cell mitosis were visualized in the wall of those UBFs atypically located in a paratracheal position (Fig.2A), as previously has been mentioned, as well as in scarce immature UBFs, but never in connection with the wall of mature UBFs. Although UBFs were generally located in the interior of the thyroid lobe -the region where C cells predominate, hence the so-called "C-cell area"-, a non specific increase in C-cell numbers could be observed around UBFs at any age.

4. Incidence of UBFs

We considered that a rat contains UB remnants if, at least, they appear in one of the two thyroid lobes. After the analysis of 5 equidistant levels per animal (75 sections in total), an apparently higher incidence in the young rat group was observed (5 to 90-day-old), followed by the groups of adult and old female rats in comparison with male rats of the same age. However, when no UBF was observed in a rat after studying the thyroid lobes in these conditions of sampling, we proceeded to stain additional sections of the same thyroid gland, then the prospect of some UBFs appearing considerably increased, preferentially in male rats. In fact, when we analyzed six completely seriated thyroid glands (3 males and 3 females), we found UBFs in all thyroid lobes. Consequently, we can conclude that all thyroid glands contain UBFs, independently of the age and gender.

DISCUSSION

The developmental progression of UB thyroid remnants throughout the postnatal life of the rat has been studied in the present paper, from the moment of birth to 24 months old, with the aim of checking whether differences are found relating to age and gender. At all ages examined, UBFs could be observed. In younger rats, "immature UBFs" appear that evolve from cellular solid nests and tubular cystic forms through intermediate stages until becoming "mature UBFs", the characteristic cystic and multilayered form with variable luminal content found in adult rats. These UBFs forms are clearly differentiable from normal thyroid follicles, both in structure and size, these being significantly larger than the normal ones at all ages but only in female rats. Furthermore, we have also found a significant difference in the size of the UBFs depending on the age of the rats, UBFs of adult rats being larger than those found in young rats.

Apart from the immature and mature forms of UBFs, a third form of UB remnant has also been found in the oldest rats, which we have designated as "UB cystadenoma". This structure may represent an unusual progression of the previous forms and is characterized by its cystic appearance, the concentric luminal content made of multiple layers of desquamated and anucleated cells, and by its considerable size which compresses the adjacent thyroid parenchyma. The UB cystadenomata are also more frequents in female than in male rats.

This is the first time that a clear sexual dimorphism related to the UB remnants fate has been demonstrated in the rat thyroid gland. Although Wollman & Nève (Wollman and Neve, 1971a; b) meticulously described the UBFs progression in rodents, at both optical and ultrastructural levels, they circumscribed their study to male rats ranging from 2 to 365 days of age (Wollman and Neve, 1971a). Therefore, they could

evaluate neither gender differences nor the existence of the most mature form of the UB remnants, UB cystadenomata.

In relation to the frequency of UBFs in Wistar rats, we have observed this structure in practically all thyroid glands. These results differ from those described by Van Dyke (Van Dyke, 1944) for the Sprague-Dawley rat, who found the following rates: 15%, 34,5% and 61,9% for the same periods of age, more frequent in females than in male rats. Rao-Rupanagudi et al (Rao-Rupanagudi et al., 1992) made a morphological study in Sprague-Dawley rats and found ultimobranchial cysts in one-third of all the animals examined. These last authors, as well as Takaoka et al. (Takaoka et al., 1995), reported, however, that the number of UB cysts decreased with age. A possible explanation of this controversy could possibly be the number of sections observed per thyroid gland. According to our experience, when no UBF was observed in a rat after studying the selected thyroid levels, and we proceeded to stain additional sections of the same gland, the prospect of some UBF appearing significantly increased. Consequently, we coincide with Wollman & Neve (Wollman and Neve, 1971a; b) that UBFs are present in both thyroid lobes of all rats, independently of the age and gender.

In relation to the apparent differences in frequency of UBFs between male and female rats that we and others (Rao-Rupanagudi et al., 1992; Van Dyke, 1944) have observed, the most plausible explanation may be that the size of the UBFs in female rats is significantly greater than that exhibited in male rats, as we have reported in the present manuscript. Consequently, more possibilities to locate UBFs on the selected thyroid sections in female rats, together with the greater ability to distinguish them from normal thyroid follicles are found.

The morphology of UB remnants in rats differs in some aspects from those found in other species such as mice (Wollman and Hilfer, 1978; Wollman and Neve, 1971a; b), dogs (Kameda et al., 1980; Leblanc et al., 1990), shrews (Swarup et al., 1978); foxes (Srivastav and Swarup, 1982), guinea pigs (Juhl, 1981), bulls (Ljungberg and Nilsson, 1985), bisons (Sawicki and Zabel, 1997) and humans (Harach, 1988; Mizukami et al., 1994). Nevertheless, it is generally understood that in early stages of the embryonic development, in all species, these structures constitute the origin of C cells. In fact, we have observed C cells arising from the rat UBFs wall in specific cases (Martin-Lacave et al., 1992). In addition, UB remnants may also be a place where follicular cells could be produced, at least in rats (Conde et al., 1992; Moreno et al., 1989), although it is more controversial in other species, such as humans, where SCNs represent the remnants of the UBB (Williams et al., 1989). However, several authors share the opinion that SCNs contribute to both calcitonin producing cells (C cells) and thyroglobulin-producing cells (follicular cells) to the thyroid gland itself as well as to some specific thyroid tumors, such as the mixed follicular thyroid neoplasia (Cameselle-Teijeiro et al., 1994; Williams et al., 1989). According to the results described by Cameselle et al. (Cameselle-Teijeiro et al., 1994), the male:female ratio of SCNs is 1:6. Nevertheless, other authors, such as Martin et al. (Martin et al., 2000), reported SCNs in 16% of men and 8% of women, therefore it does not allow for any conclusion concerning the relative frequency of SCNs according to sex.

We have identified a new entity arising from the UB remnants in rats, called UB cystadenoma. These structures may represent the final fate of mature UBFs or, more probably, an unusual progression of UBFs, considering their peculiar morphology and

huge size. Similarly, Ljungberg et al. (Ljungberg and Nilsson, 1985) described hyperplastic and neoplastic changes of the UB remnants in bulls, which they termed ultimobranchial carcinomas. These tumors closely resembled an intermediate type of human differentiated thyroidal carcinoma with morphological and inmunohistochemical traits of both medullary and follicular carcinoma, aforementioned mixed follicular thyroid neoplasia, which is probably derived from human UB remnants (Cameselle-Teijeiro et al., 1994).

The sexual dimorphism related to UB remnants destiny in the rat thyroid gland coincides with that reported by us in relation to the frequency of proliferative disorders whose origin is in C cells: both C-cell hyperplasia and C-cell tumours presented a greater incidence in female in comparison with male rats, which increased with age (Martin-Lacave et al., 1999). In regard to the incidence of spontaneous thyroid tumours derived from follicular cells, the data varied according to the rat strain and the authors. Specifically, some researchers found a greater frequency in female than in male rats (Baum et al., 1995; Kaspareit-Rittinghausen et al., 1990) but others described opposite results (Goodman et al., 1979; Goodman et al., 1980). In humans, however, neoplasias derived from follicular cells (follicular adenoma, follicular carcinoma, papillary thyroid carcinoma, anaplastic thyroid carcinoma) as well as C cells (medullary thyroid carcinoma) are more common in women than men (Dionigi et al., 2007; Rosai et al., 1992). The reasons of this sexual dimorphism in thyroid pathology have yet to be discovered.

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Table 1. Areas (μm²) of ultimobranchial follicles (UBF) in comparison with normal thyroid follicles (TF) according to age and gender.

	FEMALE		MALE	
I) ADULT RATS (Age)	UBF Area (μm²)	FT Area (μm²)	UBF Area (μm²)	FT Area (μm²)
6-month-old	3681 ± 4001	3463 ± 454	2087 ± 1412	3145 ± 804
9-month-old	7489 ± 619	4239 ± 886	7859 ± 350*	4010 ± 379
12-month-old	7432± 1681*	3481± 532	5110 ±476*	3204 ±225
15-month-old	6218±2677*	4173 ± 1170	5743± 3136	3967 ± 422
Mean±SD	**6205 ± 1781***	**3839 ± 367**	**5199± 2384**	**3581 ± 470**
II) OLD RATS (Age)	UBF Area (μm²)	FT Area (μm²)	UBF Area (μm²)	FT Area (μm²)
18-month-old	4809 ± 3384	3983 ± 666	1946 ± 1146	4118 ± 484**
21-month-old	5790 ± 2727	4425 ± 96	3414 ± 2431	4864 ± 929
24-month-old	9275 ± 1251*	4720 ± 692	3171 ± 1916	4920 ± 267*
Mean±SD	**6624± 2347**	**4376 ± 370**	**2843 ± 786**	**4634 ± 447****
TOTAL MEAN (6-24-month-old)	**6385 ± 1864***	**4069 ± 467**	**4142 ± 2116**	**4033 ± 702**

N=5 animals/sex/age; SD=standard deviation; *P≤0.05; **P≤0.01

Table 2. Incidences and specific areas of ultimobranchial cystadenomata according to age and gender.

Rat age/Incidence	Female	Male
9-month-old F: 0/5; M: 1/5	-	57,964 µm²
12-month-old F: 1/5; M: 1/5	200,244 µm²	32,817 µm²
15-month-old F: 2/5; M: 0/5	103,404 µm² 22,759 µm²	-
18-month-old F: 2/5; M: 1/5	34,238 µm² 88,004 µm²	70,112 µm²
21-month-old F: 1/5; M: 0/5	128,828 µm²	-
24-month-old F: 1/5; M: 0/5	101,443 µm²	-
F: 7/30; M: 3/30 Mean±SD	96,988±59,532 µm²	53,631±19,021 µm²
Total mean±SD	83,988±53,682 µm²	

F=female; M=male; N=5 animals/sex/age; SD=standard deviation.

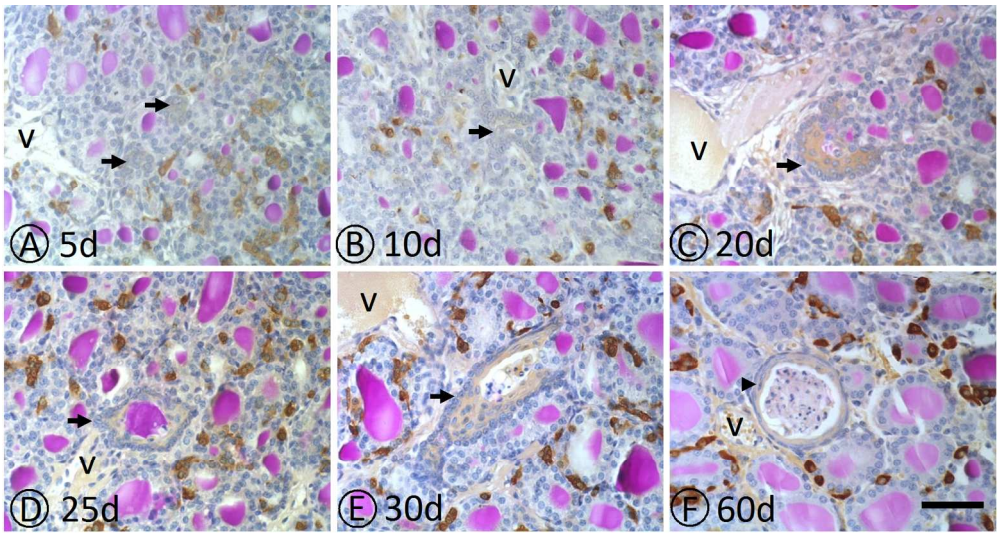
Figure legends

Figure 1. Progressive UBF transition in the rat thyroid gland from immature (5 to 25-day-old) to mature forms (60-day-old). Immature UBFs (see arrows) consist of solid cellular nests (A, 5d), narrow rods (B, 10d), epithelial pearls (C, 20d), tubular structures with luminal content (D, 25d) and "mixed follicles" (E, 30d). However, mature UBFs (see arrow head) are cystic structures with a multilayered wall and cellular detritus in the lumen (F, 60d). In general, UBFs are located in contact with perivascular connective tissue around a major vein (see V). In all photomicrographs, C cells are labelled for calcitonin (in brown colour). d=days of age; Bar=80 μm.

Figure 2. Developmental progression of UBFs in male and female rats from 6 to 24-month-old. In male rats (♂), either mature and immature forms of UBFs are indistinctly observed (see arrows), ranging from an unusual peripherically located UBF with C cells in the wall (A, 6m), a characteristic mature form (B, 9m) to an immature UBF (C, 15m). In female rats (♀), however, mature UBFs of increasing sizes are observed (see arrows), ranging from typical cystic structures of variable luminal content (D, 6m; E, 18m; F, 24m) up to the appearance of characteristic ultimobranchial cystadenomata (G, 18m; H, 21m). The cystadenoma found in a 12-month-old rat (I), which was stained with Hematoxylin-Eosin, exhibits a more aggressive morphology by invading the adjacent thyroid parenchyma (see arrow head). At all ages, UBFs usually occur in the interior of the thyroid lobe embedded among normal thyroid follicles (see TF). In all photomicrographs, C cells are labelled for calcitonin (in brown colour). m=months of age; CCT=C-cell tumour; CT=connective tissue; SM=skeletal muscle; TF=usual thyroid follicle; V=vein; Long and small bars=80 μm.

Figure 3. Progression of the UBF area, in comparison with the area of normal thyroid follicles (TF), with age in young rats (5 to 90-day-old). As can be observed, UBFs are larger than thyroid follicles at all ages studied.

Figure 4. Progression of the UBF area in relation to the gender and age in adult and old rats (6 to 24-month-old). A clear sexual dimorphism related to the size of UBFs is observed.



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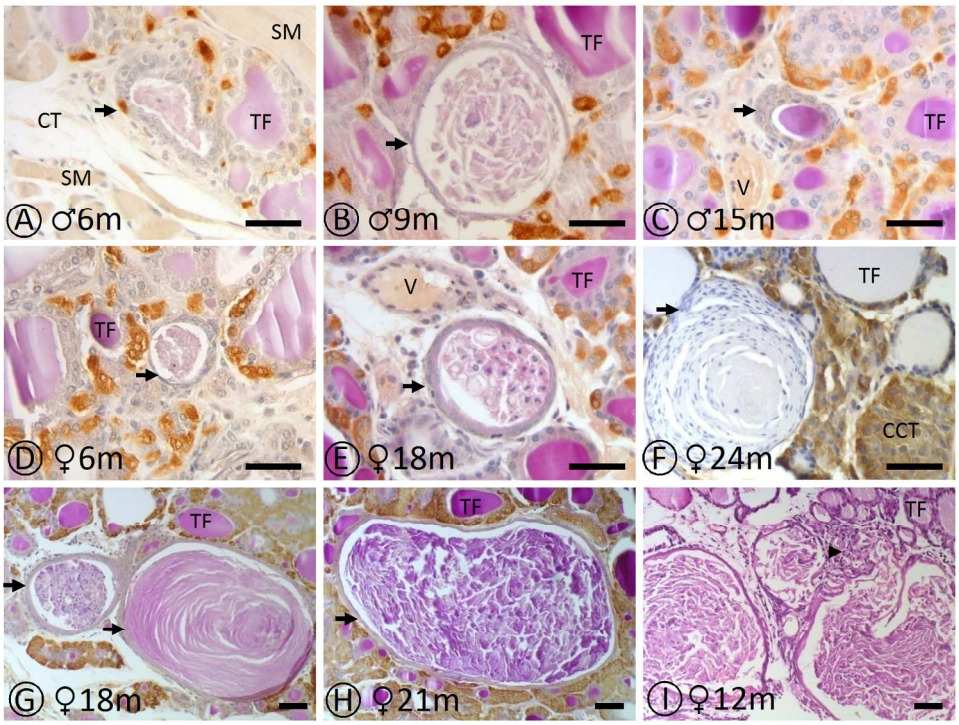

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179x132mm (300 x 300 DPI)

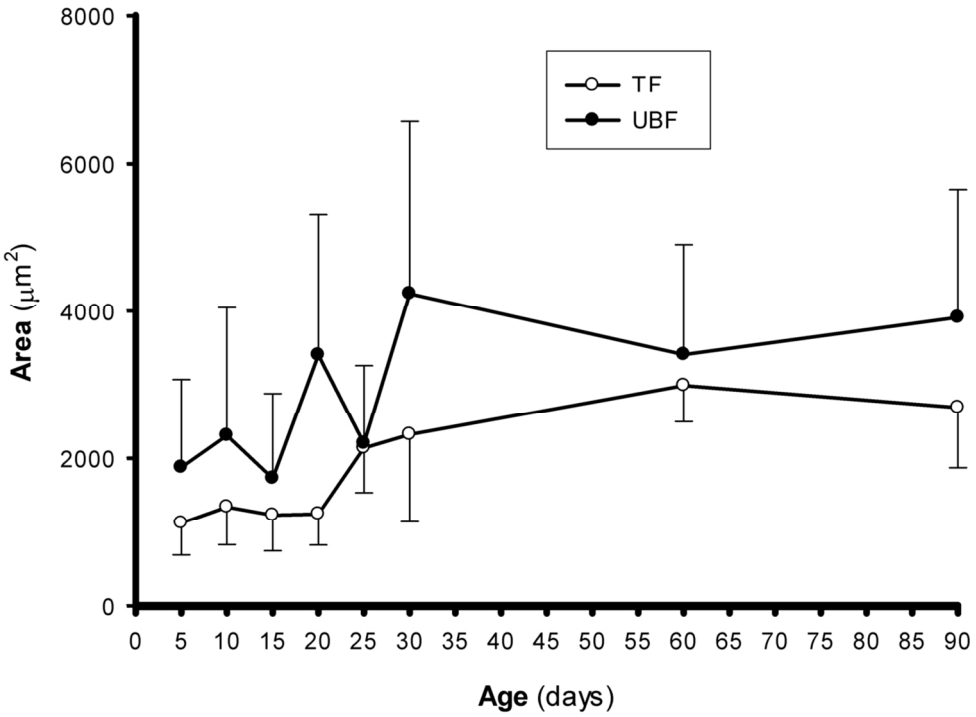
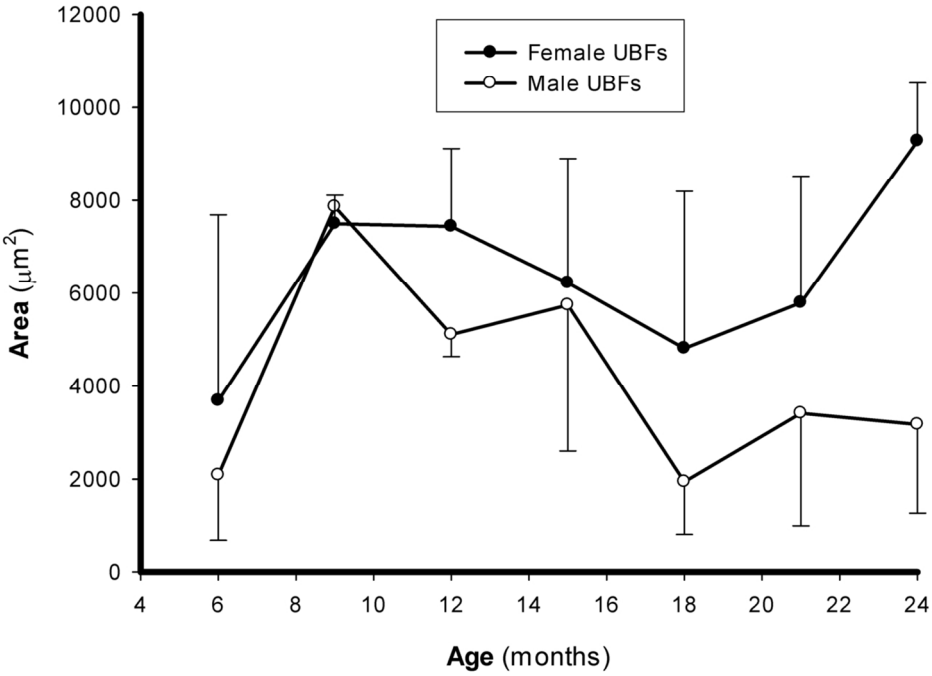


Figure 3. Progression of the UBF area, in comparison with the area of normal thyroid follicles (TF), with age in young rats (5 to 90-day-old). As can be observed, UBFs are larger than thyroid follicles at all ages studied.
114x86mm (300 x 300 DPI)



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116x83mm (300 x 300 DPI)