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# Study of the effect of luteinizing hormone (LH) in modulating anti-Müllerian hormone (AMH) pathway in human granulosa cells

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## ABSTRACT

Anti-Müllerian hormone (AMH) was initially described for its role in fetal sexual differentiation, but over the past two decades, its importance in adult reproductive physiology has also become increasingly evident. Alongside luteinizing hormone (LH), AMH plays a central role in female gonadal function and reproductive capacity. Secreted by granulosa cells, AMH acts during the initial phases of follicular development by modulating follicular sensitivity to gonadotropins. In contrast, LH primarily exerts its effects during the later stages of follicular maturation, promoting ovulation and luteinization through receptor-mediated signaling in both granulosa and theca cells. Clinical evidence from reproductive disorders such as polycystic ovary syndrome (PCOS) and premature ovarian failure suggests a possible functional interaction between the LH and AMH pathways. Yet, the molecular mechanisms driving this crosstalk are still not fully understood.

Given this background, the study aimed to investigate the regulatory effect of LH on AMH signaling in primary human granulosa lutein cells (hGLCs) isolated from patients undergoing *in vitro* fertilization (IVF). The analysis focused on AMH expression, its type II receptor (AMHRII), SMAD1/5 phosphorylation, and the transcription of AMH-responsive genes: osterix (OSX) and matrix metallopeptidase 2 (MMP2). Quantitative real-time PCR (qPCR) analysis revealed that LH treatment led to a marked downregulation of AMHRII mRNA expression. This reduction was confirmed at the protein level through western blot (WB) and ELISA. In line with these results, AMH-induced SMAD1/5 phosphorylation, also evaluated by WB, was notably reduced.
To investigate the downstream impact of LH on AMH activity, expression levels of OSX and MMP2 transcripts, typically upregulated by AMH, were examined and showed a reduction following LH exposure. Interestingly, this study demonstrated that LH treatment did not affect the amount of endogenously secreted AMH, suggesting that LH exerts its modulatory effect not by altering AMH production, but rather by reducing receptor availability and impairing signal transduction.

Taken together, these findings reveal a functional interaction between LH and AMH in hGLCs, with LH appearing to negatively regulate AMH signaling. This crosstalk could be

crucial for advancing research and developing new treatments for ovarian disorders commonly seen in reproductive medicine.

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# 1. INTRODUCTION

## *1.1 Folliculogenesis: a brief overview*

Ovarian follicles, which consist of oocytes and supporting somatic cells, are the basic structural and functional units of the female reproduction system.

The long process by which a follicle forms, proceeds through several growing stages and develops to eventually release the mature oocyte, is known as *folliculogenesis* and occurs within the female gonad: the ovary [1].

The entire process involves multiple endocrine signals, tightly regulated by the interaction between the hypothalamus, the anterior pituitary gland, and the ovary; collectively referred to as the *hypothalamic–pituitary–ovarian (HPO) axis* [2].

Folliculogenesis is initiated during embryonic development, marked by the migration of primordial germ cells to the genital ridge. Subsequently, germ cell nuclei undergo mitotic division without complete cytokinesis, leading to the formation of a multinucleated syncytium, known as a *germ cell nest*. Through the coordinated actions of germ cell apoptosis and somatic cell migration, individual germ cell nuclei arrested at prophase I become encapsulated by a single layer of flattened granulosa cells (GCs), forming *primordial follicles* and thereby establishing the initial follicle pool [3].

It is well documented that females are born with approximately two million primordial follicles; however, a substantial proportion of these undergoes atresia prior to menarche, resulting in a remaining pool of roughly 400,000 follicles [4].

During the female reproductive years, a cohort of the remaining primordial follicles is recruited each month to undergo follicular growth and maturation, ultimately resulting in the selection of a single dominant follicle and the ovulation of its oocyte, which is then capable of fertilization and embryo development. This process is conventionally divided into two distinct phases: a gonadotropin-independent phase and a gonadotropin-dependent phase [5].

Until the early antral stage, folliculogenesis is traditionally regarded as *gonadotropins-independent* and is regulated locally through paracrine signaling involving various growth factors, such as *anti-müllerian hormone (AMH*), and small molecules secreted by GCs.

The recruitment of primordial follicles triggers morphological changes that lead to their maturation into *primary follicles*: GCs proliferate and differentiate, acquiring a cuboidal shape. Additionally, a glycoprotein-rich envelope known as the *zona pellucida (ZP)* forms between the oocyte and the surrounding granulosa cells.

In secondary follicles, the ZP thickens, and the GCs layers continue to proliferate, closely surrounding the oocyte, which undergoes a rapid increase in volume while remaining in meiotic arrest.

Multiple pockets of fluid-filled lumens form within the GCs, which eventually merge into a single antrum that progressively increases in size. In addition, theca cells (TCs) begin to appear at the periphery of secondary follicles, providing structural integrity to the follicle and producing key endocrine regulatory factors, such as androgens, which serve as substrates for estrogen biosynthesis by GCs [6,7].

As follicles progress to the antral stage, they become responsive to gonadotropins, marking the beginning of the so-called *gonadotropin-dependent phase*. GCs differentiate and begin to express specific membrane receptors for *follicle-stimulating hormone (FSHR)*, while TCs express receptors for *luteinizing hormone (LHCGR)* [5].

The human gonadotropins *follicle-stimulating hormone (FSH)* and *luteinizing hormone (LH)* are glycoprotein hormones secreted by gonadotrope cells of the anterior pituitary in response to stimulation by gonadotropin-releasing hormone (GnRH), a peptide produced by the hypothalamus [8,9].

FSH promotes GCs proliferation, estradiol production, and the selection of growing follicles, whereas LH stimulates androgen synthesis, initiates oocyte meiotic resumption, and supports progression to metaphase II [5]. Additionally, under LH stimulation, TCs undergo extensive extracellular matrix (ECM) remodeling which, together with antral expansion, facilitates follicular rupture and the release of the mature oocyte from the ovary in the final event of folliculogenesis, known as *ovulation* [6].

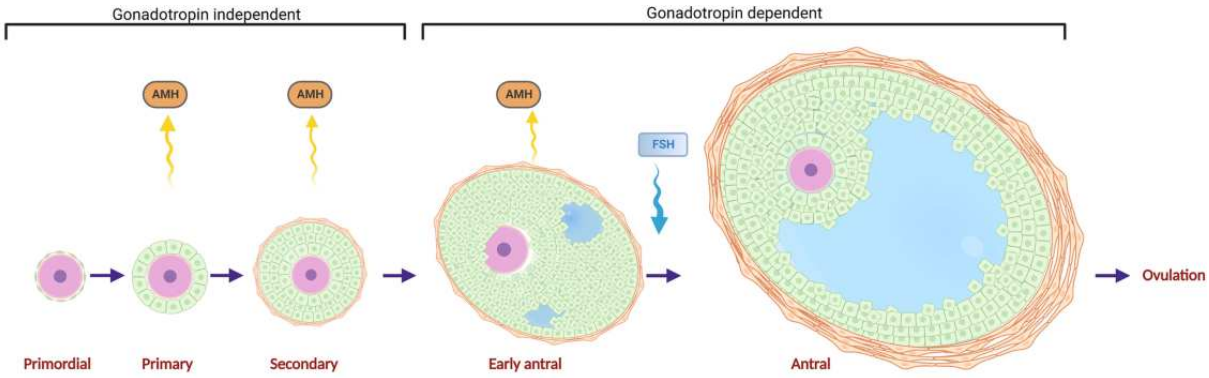



***Fig. 1*** *– Folliculogenesis: distinction between the conventionally defined gonadotropin-independent and gonadotropin-dependent phases (Esencan et al., 2022)*

### *1.2 New perspectives on luteinizing hormone's (LH) role in early follicular development*

Recent evidence highlights a previously underappreciated role of LH in the early stages of ovarian follicular development, particularly in the transition from primordial to preantral and antral follicles. While it was traditionally believed that these early stages of folliculogenesis were independent of gonadotropins, new findings suggest that LH plays a crucial role in accelerating the maturation of non-growing and primary follicles into preantral and antral stages, thereby regulating the rate of follicular progression and recruitment. Remarkably, even small follicles express low levels of functional LH receptors, supporting the hypothesis that LH enhances the transition to the antral stage.

Clinical models of pituitary suppression, such as hypothalamic amenorrhea, hormonal contraception, pregnancy, or GnRH analogue therapy, demonstrate that gonadotropin depletion, particularly LH deficiency, leads to a marked decline in functional ovarian reserve, as reflected by lower levels of anti-Müllerian hormone (AMH) and a reduced antral follicle count (AFC), both key indicators of ovarian function. These effects appear to be reversible once normal gonadotropin levels are restored.

Taken together, these findings suggest that inter-individual differences in antral follicle counts may reflect varying gonadotropin sensitivity during early folliculogenesis, positioning LH as a promising target for therapeutic strategies aimed at optimizing follicular recruitment. However, further research is needed to clarify which specific stages of follicular development respond to LH and to better understand the mechanisms through which LH influences ovarian function and fertility outcomes [10].

## *1.3 Anti Müllerian Hormone (AMH): physiological functions and clinical relevance*

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance, is a dimeric glycoprotein and a member of the *Transforming Growth Factor-Beta (TGF-β) superfamily*.
It was first described in the 1950s by Professor Alfred Jost for its role in the regression of the Müllerian ducts in male fetuses. These ducts are the embryonic precursors of the upper female reproductive tract, including the uterus, fallopian tubes, and the upper portion of the vagina. By inducing their regression, AMH promotes the maintenance and differentiation of the male reproductive tract, whereas, the absence of AMH in female embryos, allows for the full development of Müllerian-derived structures [11].
In female fetuses, AMH is produced by GCs starting at approximately 36 weeks post-conception and continues to be expressed until menopause. However, its expression levels vary depending on the stage of follicular development: AMH production begins in primary follicles, peaks in preantral and small antral follicles and declines in large antral follicles, becoming undetectable in the corpus luteum.
AMH also plays a role in primordial follicle activation. It is widely recognized for suppressing the differentiation of growing follicles, thereby inhibiting the recruitment of primordial follicles and helping to preserve the ovarian reserve by preventing premature depletion [12].
Serum AMH levels correlate with the number of growing follicles, indicating that circulating AMH is primarily of ovarian origin. Since AMH is secreted by growing follicles that have not yet undergone FSH-dependent selection, serum AMH has become a widely used clinical marker of ovarian function, particularly for assessing ovarian reserve and a surrogate for antral follicle count [13].

More recently, AMH and its receptor (AMHRII) have been found in various other tissues, particularly in reproductive organs such as the breasts, uterus, and placenta, as well as in non-reproductive sites like the lungs, motor neurons, and neurons. These findings expand the understanding of the extra-Müllerian effects of AMH beyond its traditional ovarian role, while the exact functions and mechanisms of action of AMH within the ovary remain subjects of ongoing debate [11].

## *1.4 AMH signaling pathways and functional mechanisms*

The human AMH gene is approximately 2.8 kilobase pairs (kbp) in length and is located on the short arm of chromosome 19. It consists of five exons and is transcribed into a monomer. Following dimerization, two monomers, linked by disulfide bonds, form the 140-kDa AMH precursor. This precursor undergoes proteolytic cleavage, generating the N-terminal and C-terminal domains, which remain associated as a non-covalent complex. The N-terminal domain enhances the biological activity of the C-terminal domain, including its affinity for the *AMH type II receptor (AMHRII)*. The C-terminal domain, which contains several cysteine residues (including seven conserved cysteines), exhibits the cysteine knot motif typical of the TGF-β superfamily and shares the highest degree of sequence homology with other family members [14].

As with other TGF-β members, AMH signals primarily through the canonical *small mothers against decapentaplegic (SMAD) pathway*, involving two classes of cell-surfaces serine/threonine kinase receptors, type I and type II, and two groups of intracellular mediators: the receptor-regulated SMADs (R-SMADs) and the common-mediator SMAD (SMAD4) [14].
However, unlike other TGF-β ligands, AMH is unique in binding exclusively to its dedicated, high-affinity AMHRII, also known as MISRII. The AMHRII gene spans ~8 kbp on the long arm of chromosome 12, comprises 11 exons, and encodes a 2.05-kb mRNA, which is translated into a 573-amino-acid protein. The receptor includes two N-glycosylated chains in its extracellular domain (ECD), a single transmembrane domain, and an intracellular serine/threonine kinase domain.

AMHRII is highly specific: mutations in either the AMH or AMHRII gene result in Persistent Müllerian Duct Syndrome (PMDS), characterized by the presence of Müllerian duct derivatives in otherwise virilized males. In mice, AmhrII-null mutants phenocopy AMH-deficient mice, showing both persistence of Müllerian ducts and premature depletion of the ovarian follicle pool [10]. In the ovary, AMHRII is primarily expressed in GCs of growing follicles, particularly in cumulus cells of large antral follicles, with lower expression in mural GCs, theca cells, and ovarian surface epithelial cells.

While the non-covalent AMH complex found in follicular fluid can bind AMHRII *in vitro*, the complex found in serum does not, suggesting the presence of an inhibitory unknown mechanism regulating AMH bioactivity in circulation [16].

Upon AMH binding to AMHRII *in vitro*, the N-terminal domain is cleaved, enabling signal transduction. The C-terminal domain alone has been shown to be more potent than the non-covalent complex in stimulating *SMAD1/5/8* phosphorylation, possibly due to better interaction with *type I receptors: ALK2/3/6*. *In vivo,* AMH first binds to monomeric AMHRII, followed by receptor dimerization and recruitment of a type I receptor, leading to the formation a phosphorilated complex that initiates the SMAD signaling cascade.
Once activated, SMAD1/5/8 associate with *SMAD4*, forming a transcriptional complex that translocates to the nucleus. There, it binds preferentially to GC-rich BMP-responsive elements, inducing transcription of AMH target genes [15].

To date, this AMH–SMAD signaling pathway has been most thoroughly studied in the Müllerian ducts of male fetuses, using transgenic animal models. These studies have identified several AMH-regulated downstream genes involved in Müllerian duct regression, including: *Osterix (Sp7)*, *MMP2 (Matrix Metallopeptidase 2)* and *Wif1 (WNT Inhibitory Factor 1)* [17].
This pathway is negatively regulated by *SMAD6*, an inhibitory SMAD that acts as a final checkpoint, helping to maintain cellular balance [11].

In addition to SMAD-mediated signaling, AMH has also been shown to activate alternative pathways, such as *β-catenin* [17] and *NF-κB* [18], although their integration with the canonical SMAD pathway remains poorly understood [15].

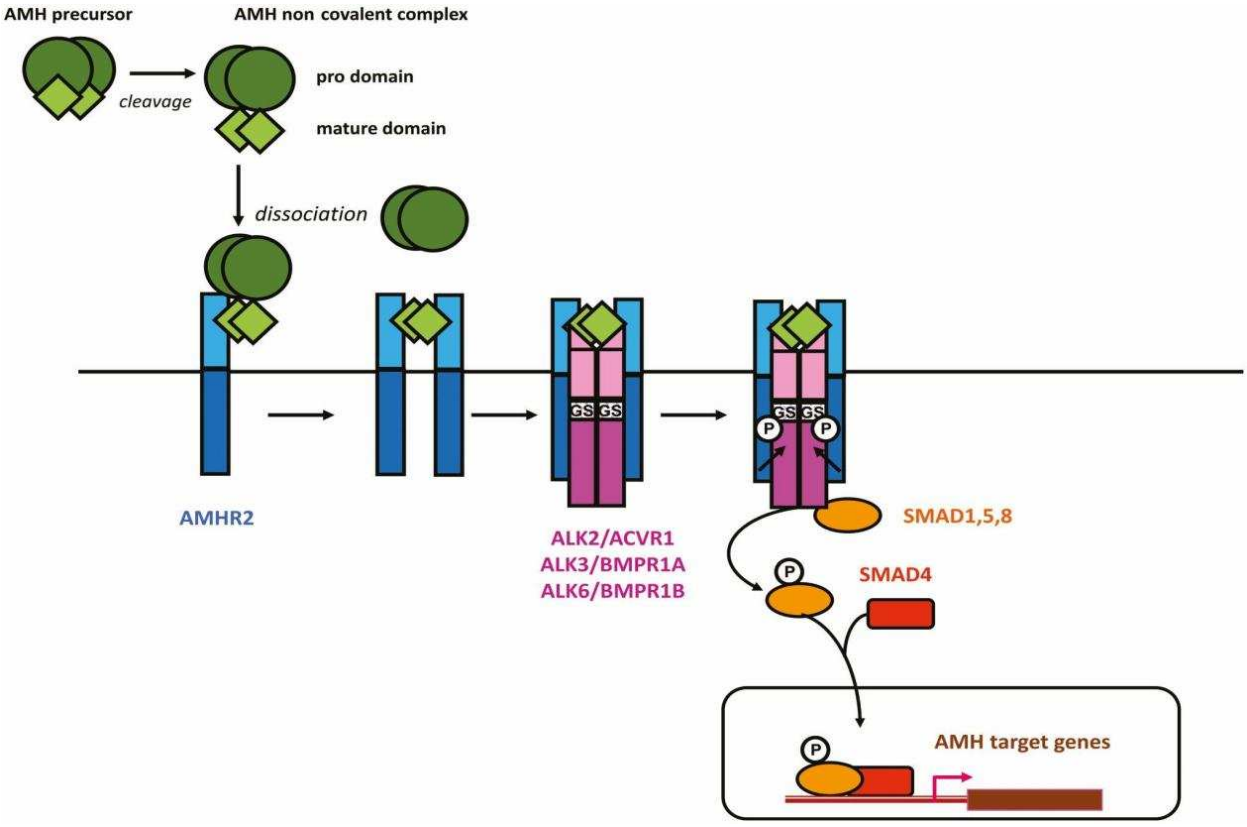



***Fig. 2*** *– AMH canonical signaling pathway (di Clemente et al., 2021)*

## *1.5 Regulatory mechanisms of AMH and AMHRII expression in the ovary*

Although the regulation of AMH and the expression of its receptors remain complex and not yet fully elucidated, different players appear to contribute to the process. These include transcription factors, epigenetic mechanisms, metabolic and inflammatory signals, steroid hormones (such as *estrogens* and *androgens*), and gonadotropins (*LH* and *FSH*) [5,11].

### *1.5.1 Regulation by gonadotropins*

Over the years, numerous studies have explored the connection between AMH and both FSH and LH, with a primary focus on the relationship between AMH and FSH, which has often led to contradictory findings [11]. Across mammalian species, FSH is known to stimulate follicular growth and differentiation, while LH supports final maturation and triggers ovulation [20].

*In vitro* experiments performed using either primary cultures of human granulosa luteal cells (hGLCs) or the mouse luteinized granulosa cell line KK1 demonstrated that the addition of FSH or its second messenger Cyclic Adenosine Monophosphate (cAMP) to the culture medium upregulated AMH mRNA expression at both 24 and 72 hours post-stimulation. The same study also showed that both FSH and cAMP positively influenced the activity of an AMH promoter-reporter construct [21]. Similarly, in bovine GCs, FSH enhanced AMH expression in the presence of low FSH concentrations [22].
However, *in vivo*, most studies have reported an inverse correlation between AMH and FSH levels during controlled ovarian stimulation with FSH. In these conditions, FSH treatment promotes estrogen production, which in turn inhibits AMH expression and alters the composition of the follicular pool by reducing the number of AMH-expressing follicles likely due to the influence of sex steroids and growth factors that mask the direct effects of FSH [23, 24].

The relationship between LH and AMH has been less extensively studied. Only limited data from two studies using hGLCs cultures are available. A major challenge in studying LH's effect lies in its tendency to induce granulosa cell luteinization; high concentrations of LH may mimic pathological conditions such as Polycystic Ovary Syndrome (PCOS) rather than physiological states [11].
These studies showed that, *in vitro*, LH did not affect AMH mRNA or protein expression [25, 26].

The role of both gonadotropins in regulating AMHRII expression has also been investigated in human luteinized GCs. Interesting results indicate that LH downregulates AMHRII mRNA expression, while FSH has no significant effect on receptor expression [26].

In light of these findings, further research is needed to clarify the complex interplay between gonadotropins and the AMH signaling system.

## 2. AIM OF THE STUDY

Over the past two decades, numerous studies have significantly advanced the understanding of the AMH, especially in the female reproductive system. AMH, secreted primarily by GCs of growing follicles, plays a critical role in the regulation of folliculogenesis by inhibiting the initial recruitment of primordial follicles and modulating the sensitivity of growing follicles to FSH. While AMH is widely recognized as a reliable biomarker of ovarian reserve and an essential player in reproductive physiology, the endocrine factors governing its expression and signaling remain not fully understood.

Emerging evidence indicates that gonadotropins, especially FSH and LH, may influence AMH expression at both transcriptional and post-transcriptional levels. In particular LH, traditionally known for its role in triggering ovulation and promoting steroidogenesis, has been suggested to modulate the expression of AMH and its receptor (AMHRII) in granulosa cells. However, the molecular mechanisms underlying this regulation remain largely unclear. Given the physiological overlap and interplay between AMH and LH in the ovary, understanding whether LH directly regulates AMH/AMHRII expression and affects downstream AMH signaling could provide new insights into the tightly regulated hormonal control of follicle development.

This study aims to investigate the role of LH in the regulation of AMH and AMHRII expression and signaling in human granulosa-lutein cell cultures. Specifically, it will be assessed whether LH modulates AMH and AMHRII, both at the mRNA and protein levels and explore the downstream consequences on SMAD1/5 phosphorylation, which are canonical effectors of AMH signaling.

By clarifying the interplay between LH and AMH/AMHRII, this research seeks to contribute to a deeper understanding of the hormonal mechanisms controlling folliculogenesis. Such knowledge is particularly relevant in the context of reproductive disorders like polycystic ovary syndrome (PCOS), where both LH and AMH levels are dysregulated. Additionally, clarifying this regulatory axis could lead to the optimization of ovarian stimulation protocols in assisted reproductive technologies (ART), potentially allowing for more personalized and effective treatment strategies.

# 3. MATERIALS AND METHODS

## *3.1 Isolation of Human Granulosa-Lutein Cells (hGLCs)*

hGLCs were isolated from follicular aspirates collected from women undergoing *in vitro* fertilization (IVF) treatment at the Department of Obstetrics and Gynecology of Modena University Hospital.
Initially, follicular fluids from each patient were pooled and centrifuged (1400 RCF, 5 min, 20–25 °C) to separate the cellular components from the fluid. After removal of the supernatant, the resulting cell pellets were resuspended in a culture medium composed of DMEM/F12 (Dulbecco's Modified Eagle Medium) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (all reagents were purchased from Gibco, Life Technologies Co., Carlsbad, CA, USA) to maintain sterile conditions suitable for cell culture.

Samples were then layered onto a 50% discontinuous Percoll gradient (GE Healthcare, Little Chalfont, UK) and centrifuged (800g, 30 min, 20–25 °C, with minimal acceleration and deceleration) to stratify cellular components. For each sample, the upper layer, enriched in hGLCs, was collected and resuspended in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), a rich source of growth factors and nutrients, 2 mM glutamine (an essential amino acid for cellular metabolism), and the previously described antibiotic mix (all reagents were purchased from Gibco, Life Technologies Co., Carlsbad, CA, USA). The suspension was then centrifuged again (1400 RCF, 5 min, 20–25 °C).

If the sample still contained a high number of red blood cells, the pellet was resuspended in hemolysis buffer (8.29 g/L $NH_2Cl$, 1.0 g/L $NaHCO_2$, 0.0037 g/L EDTA) and incubated for 1 minute to eliminate erythrocyte contamination and improve the purity of the culture. The reaction was stopped by adding fresh culture medium (DMEM/F12 with 10% FBS, 2 mM glutamine, and antibiotics as previously described), followed by a final centrifugation step (1400 RCF, 5 min, 20–25 °C) to remove cell debris.

The resulting hGLCs were then resuspended in fresh culture medium and seeded. Based on the visual evaluation of the pellet volume, cells were plated in Petri dishes of varying sizes (60 mm, 35 mm, or 10 mm), either kept separate by patient or pooled if the individual cell count was insufficient for separate plating.

### *3.2 Culture of Human Granulosa-Lutein Cells (hGLCs)*

After 48 hours, cell adhesion was assessed via microscopic inspection. Cells were then detached using trypsin (Gibco, Life Technologies Co., Carlsbad, CA, USA) and reseeded into 24-well plates at a density of $10^5$ cells per well. Cultures were maintained for 6 days at 37 °C in a humidified atmosphere with 5% $CO_2$, with medium renewed every 2 days. This culture period allowed the cells to recover from hormonal stimulation received during the IVF protocol and to re-establish endogenous expression of gonadotropin receptors, as previously described [27].

### *3.3 Cell Treatments*

hGLCs were subsequently serum-starved overnight and exposed to the appropriate experimental conditions: either to FBS-free culture medium (negative control) or to recombinant LH at concentrations of 0.1 nM, 1 nM, and 10 nM for 24 or 48 hours. All experiments were conducted using recombinant LH (r-LH; Luveris, by Merck KGaA, Darmstadt, Germany) and recombinant AMH (R&D Systems, Minneapolis, MN 55413, USA).

### *3.4 siRNA Treatments/Trasfection Protocol*

hGLC cells were seeded at $10^5$ cells per well and transfected with 10 nM siRNA targeting LHCGR (ID: s8163; Ambion - Thermo Fisher Scientific) or 10 nM control siRNA, using TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI, USA), according to the manufacturer's instructions.

After 48 hours of siRNA incubation, cells were treated with increasing concentrations of recombinant LH (0.1 nM, 1 nM, 10 nM) or culture medium (negative control) for an additional 24 or 48 hours. Transfected cells were used for subsequent experiments 48 hours after treatment.

### *3.5 RNA Isolation and Gene Expression Analysis*

To evaluate the differential expression of AMH and AMHRII genes following LH treatment, cells were subjected to RNA extraction, reverse transcription, and amplification by RT-qPCR. Wild Type (WT) and LH-siRNA granulosa cells were exposed to 0.1, 1, and 10 nM recombinant LH (Sigma-Aldrich, St. Louis, MO, USA) for 24 hours. Following treatment, total RNA was extracted using the phenol-chloroform method with TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA), in accordance with the manufacturer's instructions.

#### *3.5.1 RNA Extraction*

Total RNA was extracted using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA), a monophasic solution designed for the simultaneous isolation of RNA, DNA, and proteins from biological samples. The RNA extraction protocol, based on the manufacturer's instructions, involved the addition of chloroform, followed by vigorous shaking of the samples and incubation at room temperature for five minutes. Samples were then centrifuged to allow phase separation into a lower phenol–chloroform phase and a clear upper aqueous phase, where RNA is concentrated. The aqueous phase was collected and RNA was precipitated by centrifugation with isopropanol. The resulting RNA pellet was washed with 75% ethanol and subjected to a further centrifugation step. All centrifugations were performed at 4°C. Samples were then allowed to air-dry at room temperature and resuspended in nuclease-free water.

### *3.5.2 Reverse Transcription*

RNA concentration and quality were assessed using the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).
Subsequently, 400 ng of total RNA in a final volume of 20 µL were reverse transcribed into complementary DNA (cDNA) using the High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions.

### *3.5.3 Real-Time PCR*

AMH and AMHRII gene expression were evaluated by real-time PCR (Polymerase Chain Reaction) on an Applied Biosystems Real-Time PCR System using PowerTrack™ SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's recommendations. Amplification reactions were prepared on ice and performed in duplicate using the StepOne Real-Time PCR System (Applied Biosystems, Waltham, MA, USA).
Gene expression was normalized to the endogenous control Ribosomal Protein S7 (RpS7) content using the $2^{-\Delta\Delta Ct}$ method. Primer's specificity was confirmed by melting curve analysis, ensuring the absence of nonspecific amplification. The primer sequences used are listed in *Table 1*.

| Gene | Forward | Reverse |
|---|---|---|
| *AMH* | 5'-TACACCTGGAGGAAGTGACC-3' | 5'-AGGGTACAGCACCAGCAG-3' |
| *AMHRII* | 5′-GAGGTCATGCAGTGGTTTGG-3′ | 5′-GGCTGGCAGTGATAAATCGG-3′ |
| *RPS7* | 5′-AATCTTTGTTCCCGTTCCTCA-3′ | 5′-CGAGTTGGCTTAGGCAGAA-3′ |
| *OSX* | 5′-GCCAGAAGCTGTGAAACCTC-3′ | 5′-GCTGCAAGCTCTCCATAACC-3′ |
| *MMP2* | 5'-GAGCTCTATGGGGCCTCTCC-3' | 5'-CGTCACAGTCCGCCAAATGA-3' |

***Table 1*** *– Primer sequences used in qPCR experiments*

### *3.6 Protein concentration determination*

To evaluate the expression of AMHRII and determine its levels, two main techniques were used: ELISA (Enzyme-Linked Immunosorbent Assay) and Western Blot (WB). These methods not only allow the quantification of the protein but also enable the analysis of its changes in response to treatments with hormonal stimuli, specifically LH.

#### *3.6.1 Protein Quantification - BCA Assay*

Protein concentration was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. The method is based on the Biuret reaction, where the reduction of copper by the sample proteins, in the presence of bicinchoninic acid (BCA), generates a purple complex that absorbs at 562 nm. The intensity of the absorbance is proportional to the amount of protein present in the sample.
A calibration curve was prepared using bovine serum albumin (BSA) with known concentrations ranging from 0 to 2 mg/mL. The working reagent was prepared by mixing Reagents A and B from the kit in a 50:1 ratio and was added to each well of the plate. The plate was incubated at 37°C for 30 minutes. Absorbance was measured at 562 nm using a

plate reader. The protein concentration of each sample was determined by interpolation with the absorbance values from the calibration curve.

### *3.6.2 SDS-page and Western Blotting*

AMHRII protein expression and SMAD 1/5 phosphorylation levels were assessed by Western blotting. AMHRII expression was evaluated after 48 hours of stimulation with 0.1, 1, and 10 nM LH in both Wild-Type and LH-siRNA granulosa cells. Phospho-SMAD (pSMAD) levels were analyzed following short-term exposure (15 and 30 minutes) to 100 ng/mL AMH in both untreated control cells and those pre-stimulated for 48 hours with 10 nM LH.

Treated cells were detached from culture plates and resuspended in lysis buffer (50 mM Tris-EDTA, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 0.1% Triton X-100), supplemented with protease and phosphatase inhibitors (Halt™ Protease and Phosphatase Inhibitor Cocktail, Thermo Fisher Scientific, Waltham, MA, USA). Protein samples were mixed with 4X Bolt™ LDS Sample Buffer and 10X Bolt™ Sample Reducing Agent (Thermo Fisher Scientific), which contained a reducing agent to denature the proteins and a Coomassie Blue dye to visualize the proteins as they separated in the gel. Samples were then boiled at 100°C for 5 minutes to ensure complete protein denaturation.

Equal amounts of protein (20 µg per sample) were separated on 4-12% SDS-polyacrylamide gels (SDS-PAGE) at 70 V for 10 minutes, and then increased to 140 V for the remaining duration of the run. Proteins were separated according to their molecular weight and transferred onto a nitrocellulose membrane (Thermo Fisher Scientific) using blotting. The transfer was performed in a transfer buffer (200 mM Tris, 0.2% SDS, and 1.5 M glycine) inside a transfer chamber. The blotting was conducted at a constant voltage of 100 V for 75 minutes on ice.

After transfer, the membranes were blocked for 2 hours at room temperature with a blocking solution containing 2% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) in Tris-buffered saline with 0.05% Tween® 20 (TBST), to prevent non-specific binding between the membrane and the primary antibody. The membranes were then incubated overnight at

4°C with the following primary antibodies: *mouse anti-AMHRII* (1:800, Novus Biologicals, USA; NBP1-05491), *rabbit anti-pSMAD 1/5* (1:500, Cell Signaling Technology, Danvers, MA, USA; #9516S), *mouse anti-β-actin* (1:5000, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany; A3854), and *rabbit anti-vinculin* (1:1000, Thermo Fisher Scientific, #700062).

The following day, after washing in TBST, membranes were incubated for 2 hours at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-mouse and goat anti-rabbit, 1:10,000; Thermo Fisher Scientific, G21040 and G21234, respectively). Signal detection was carried out using an enhanced chemiluminescence (ECL) solution (Amersham Corp., Arlington Heights, IL, USA). Images were acquired using the ChemiDoc™ XRS system (Bio-Rad Laboratories, CA, USA). Densitometric analysis was performed using Image Lab software (Bio-Rad), with normalization to β-actin or vinculin as loading controls.

### *3.6.3 ELISA*

AMH levels secreted into the culture medium by hGCs exposed to recombinant LH (0.1, 1, and 10 nM) for 24 hours were measured using an Enzyme-Linked Immunosorbent Assay (ELISA). The cultured supernatants were collected by centrifugation (20,000 g for 10 minutes) and stored at −80°C until further analysis. The ELISA was performed using a commercially available AMH ELISA Kit (Signalway Antibody, Maryland 20770, USA), following the manufacturer's instructions.

This kit provided a standard curve, which was constructed using a series of known AMH concentrations included in the kit. These known standards were processed alongside the samples to generate a standard curve, allowing the concentration of AMH in the unknown samples to be determined by comparison of their Optical Density (OD) values with the curve. OD values were measured at 450 nm using a plate reader. The hormone concentrations were then normalized by dividing the obtained values by the corresponding cell number, ensuring that the data accurately reflect the amount of AMH secreted per cell. This normalization step is essential for accounting for any variations in cell density between the different experimental conditions.

### *3.7 Statistical Analysis*

All data are presented as mean ± standard error of the mean (SEM), providing a measure of the variability around the mean for each experimental group. Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA), a software widely used for statistical analysis and graphical representation of data.

To determine whether there were significant differences between the experimental groups, the results were analyzed using the nonparametric Kruskal-Wallis test, which is appropriate when the data do not follow a normal distribution or when the assumptions of parametric tests are not met. The Kruskal-Wallis test is a rank-based method that compares the medians of the groups and is suitable for small sample sizes or data that are not normally distributed. Following the Kruskal-Wallis test, Dunn's post-hoc test was applied to identify which specific groups differed from each other. A p-value of $< 0.05$ was considered statistically significant, indicating that the observed differences between groups were unlikely to have occurred by chance.

# 4. RESULTS

## *4.1 LH treatment does not affect AMH gene expression or protein secretion in hGLCs*

The initial step in our investigation aimed to assess the impact of LH stimulation on AMH expression in human granulosa cells by evaluating both gene transcription and protein secretion of AMH in response to increasing doses of LH. To this end, hGLCs were incubated for 24 hours with exogenous LH at concentrations of 0.1, 1, and 10 nM, or with culture medium alone as a negative control.

Quantitative real-time PCR was performed to analyze AMH mRNA levels, while secreted protein concentrations in the culture medium were measured using ELISA.

The results obtained revealed that LH treatment does not significantly influence AMH gene expression in hGLCs. As shown in *Figure 3A*, AMH transcript levels remained comparable across all LH-treated groups and the untreated control. AMH mRNA levels were quantified using the $2^{-\Delta\Delta Ct}$ method and normalized to the untreated control group, which was set as the baseline (value = 1).
These findings were corroborated at the protein level: as illustrated in *Figure 3B*, AMH concentrations measured in the culture medium did not significantly differ between treated and untreated samples, confirming that LH does not modulate AMH secretion in these cells under these conditions. Statistical analysis of protein levels was performed using the Kruskal–Wallis test, which confirmed the absence of significant differences ($p \geq 0.05$).

Collectively, these findings indicate that, under the tested experimental conditions, LH does not modulate AMH production or secretion in human granulosa cells.

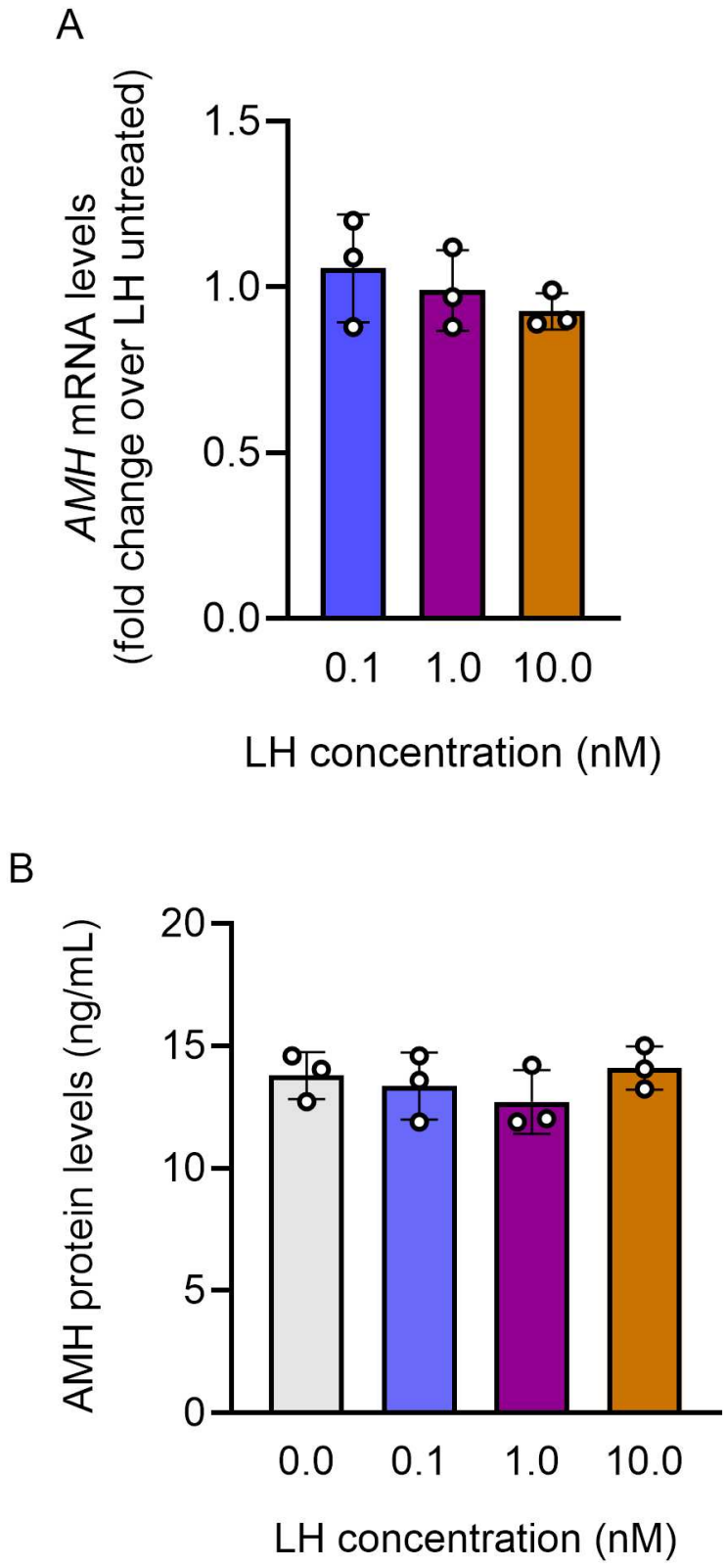



***Fig. 3*** *- Analysis of AMH mRNA and protein levels in hGLCs.* ***A*** *Real-time PCR analysis of AMH mRNA expression in hGCs.* ***B*** *ELISA analysis of AMH protein levels in the cells' culture medium.*

### *4.2 LH treatment downregulates AMHRII expression at both gene and protein levels in hGLCs*

Following the evaluation of LH effects on AMH production in human granulosa cells, the potential regulatory role of LH on the expression of the anti-Müllerian hormone type II receptor (AMHRII) was investigated, as this receptor mediates AMH signaling at the cellular level. AMHRII expression was assessed both at the transcriptional and protein levels to determine whether LH modulates AMH responsiveness through regulation of its receptor.

Human granulosa cells were incubated for 48 hours with either increasing doses of LH (0.1, 1, and 10 nM) or culture medium (negative control). AMHRII mRNA levels were quantified via real-time PCR and normalized to untreated control values. As shown in *Figure 4A*, LH treatment resulted in a dose-dependent reduction in AMHRII transcript levels. A significant (Kruskal-Wallis test; $p < 0.05$) decrease was observed at 1 nM LH, which became well-defined at 10 nM, with mRNA expression reduced by approximately 60–65% relative to controls. Even at the lowest concentration (0.1 nM), a reduction in transcript levels was observed, though not statistically significant.

To verify whether this effect was directly mediated through LH receptor (LHCGR-Luteinizing Hormone/Choriogonadotropin Receptor) signaling, RNA interference was employed to selectively silence LHCGR expression in hGLCs using siRNA. This approach allows for the specific inhibition of receptor-mediated signaling, thereby clarifying the dependency of AMHRII modulation on LHCGR activation.
*Figure 4A* shows that, in LHCGR-silenced hGLCs, LH treatment had no significant effect on AMHRII transcript levels at any concentration tested (Kruskal-Wallis test; $p \geq 0.05$), indicating that the observed downregulation of AMHRII is LHCGR-dependent.
Results were calculated using the $2^{-\Delta\Delta Ct}$ method, with RPS7 gene expression as the reference, and normalized to LH/siRNA untreated samples, which were set to a value of 1 (not shown in the figure).

Consistent with transcript-level findings, AMHRII protein expression was examined by Western Blot analysis following 48-hour LH treatment. As shown in the Western blot images in *Figure 4B*, the band corresponding to AMHRII in cells treated with LHCGR-targeting

siRNA appears markedly less intense compared to that of non transfected cells, indicating a decreased abundance of the receptor upon LHCGR knockdown. In contrast, the β-actin band, used as a loading control, exhibits comparable intensity across all samples, confirming equal protein loading and excluding technical artifacts as a source of the observed differences. This supports the conclusion that the downregulation of AMHRII is a specific biological effect of LHCGR silencing rather than an experimental inconsistency.

The densitometric semi-quantification analysis shown in *Figure 4C*, representing the AMHRII/β-actin ratio, quantitatively confirms that treatment with 10 nM LH significantly reduces receptor expression (Kruskal-Wallis test; $p < 0.05$), with an approximate 60% decrease compared to untreated cells. Moreover, LHCGR silencing via siRNA prevented LH-induced AMHRII downregulation, as no significant difference was observed in this condition ($p \geq 0.05$). Results were normalized as a percentage relative to LH/siRNA untreated samples, which were set at 100% (data not shown).

These results show that LH acts through LHCGR reducing AMHRII levels in human granulosa cells, both at the gene and protein level. LH lowers AMHRII expression reducing granulosa cells sensitivity to AMH, thus regulating AMH signaling during follicle development.

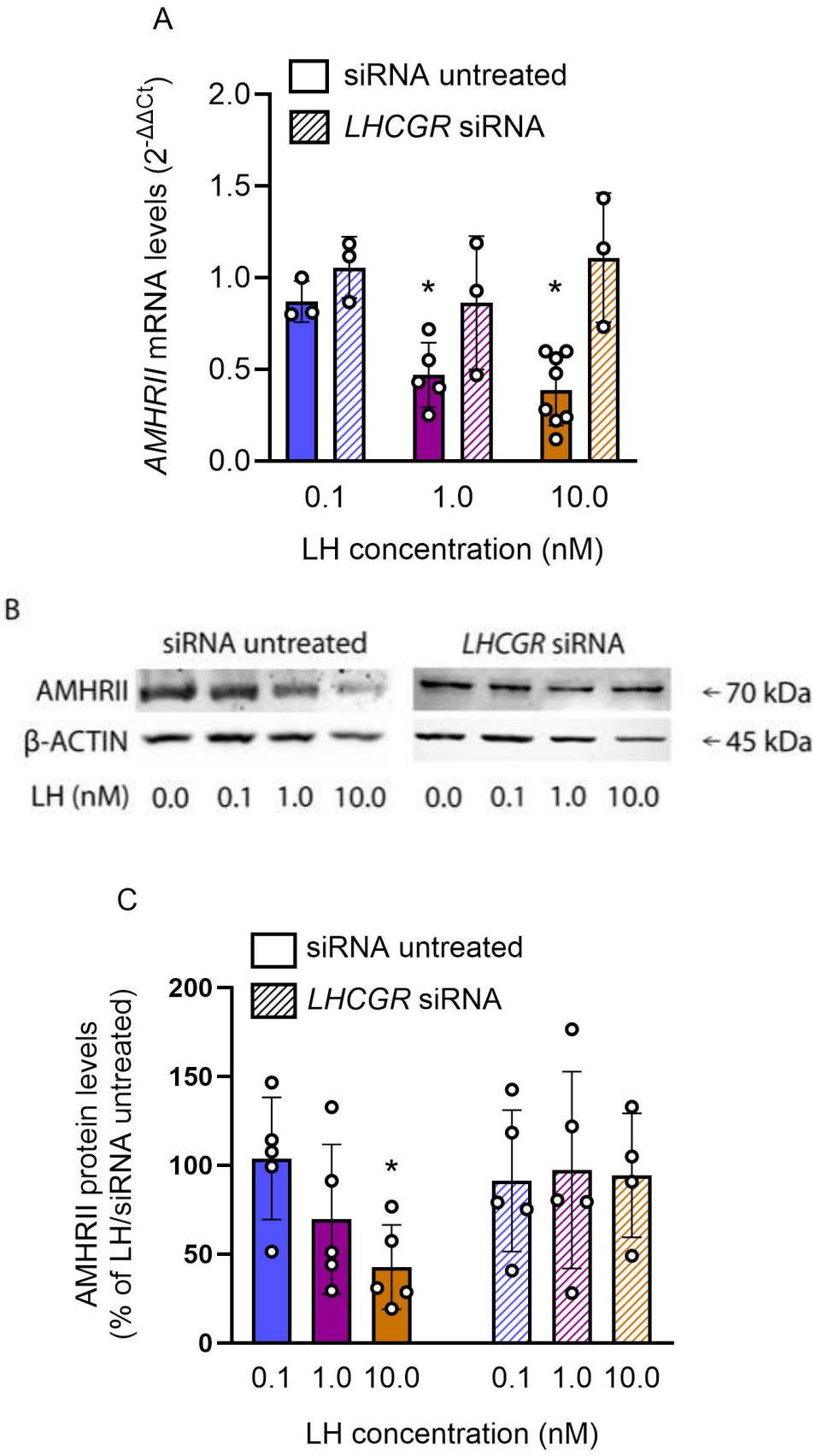



***Fig. 4*** *- Analysis of LH-induced inhibition of AMHRII expression in hGLCs at both mRNA and protein levels.* ***A*** *Real-time PCR analysis of AMHRII mRNA expression in hGLCs. * = significantly different from LH-untreated samples (Kruskal–Wallis test, $p < 0.05$).* ***B*** *Western blot analysis of AMHRII protein levels in hGLCs. β-actin was used as a loading control.* ***C*** *Densitometric quantification of AMHRII Western blot bands. * = significantly different compared to LH-untreated samples (Kruskal–Wallis test, $p < 0.05$).*

### *4.3 LH treatment inhibits AMH-mediated SMAD 1/5 phosphorylation in hGLCs*

Following confirmation of AMHRII expression and functionality in hGLCs, and its downregulation upon LH exposure, the potential impact of LH on downstream AMH signaling was examined. AMH pathway activation in granulosa cells is known to involve phosphorylation of SMAD1/5 proteins, which act as key intracellular mediators of the AMH signaling cascade [15].

To investigate whether LH modulates this pathway, hGLCs were pre-treated with 10 nM LH for 48 hours, followed by short-term stimulation with recombinant AMH (100 ng/mL) for 15 minutes. The control group included cells unexposed to LH and untreated with AMH.

As shown in *Figure 5A*, Western Blotting analysis revealed that AMH stimulation induced a clear increase in pSMAD1/5 levels in cells not pre-exposed to LH, confirming functional activation of the AMH pathway. In contrast, cells pre-treated with LH showed markedly reduced phosphorylation of SMAD1/5 following AMH exposure, suggesting that LH interferes with AMH downstream signaling. The vinculin band, used as a loading control, showed consistent intensity across all lanes, confirming equal protein loading and excluding technical variability as a source of the observed differences.

Western blot analysis was followed by densitometric quantification of pSMAD1/5 bands, shown in *Figure 5B*, which confirmed a significant decrease in AMH-induced SMAD1/5 activation in LH pre-treated cells. This reduction was statistically significant (Kruskal–Wallis test, $p < 0.05$) compared to AMH untreated samples unexposed to LH.

These results support the findings that LH diminishes AMH signaling effectiveness by downregulating AMHRII expression, thereby attenuating the phosphorylation of SMAD1/5 in response to AMH, as also previously shown in *Figure 4*.

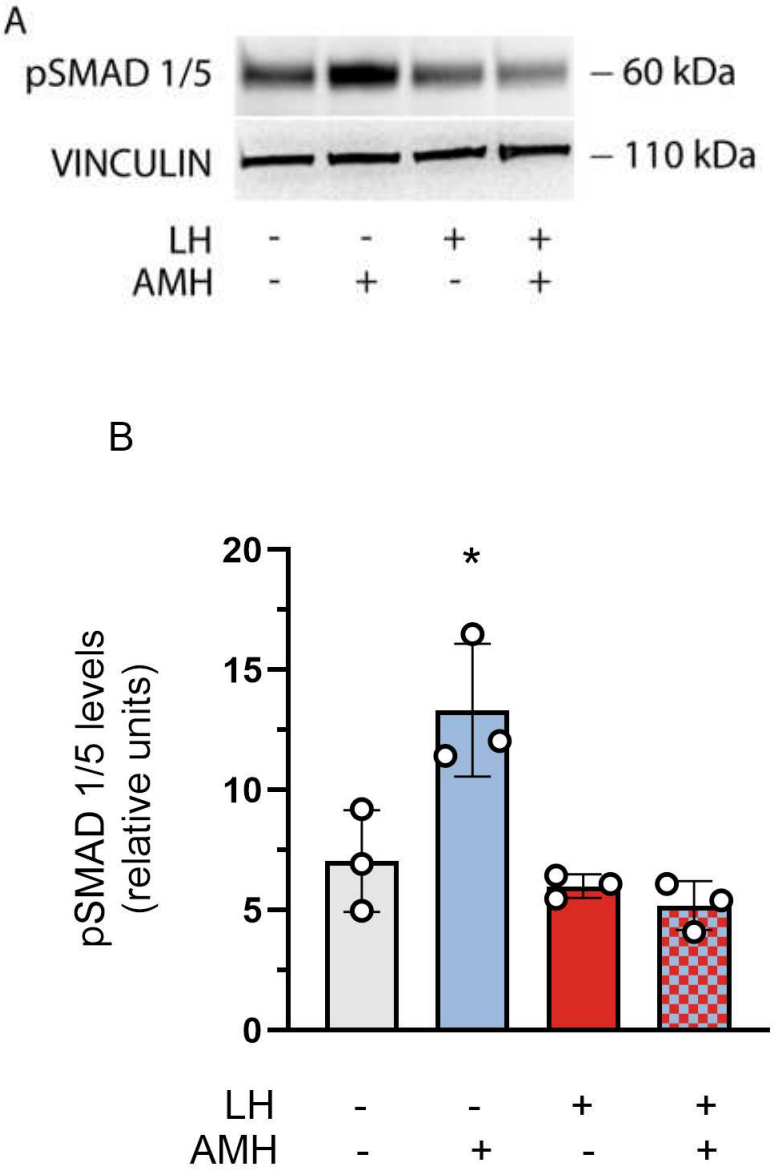



***Fig. 5*** *- Analysis of LH-induced inhibition of AMH-mediated SMAD1/5 phosphorylation in hGLCs.* ***A*** *Western blot analysis of AMH-induced pSMAD activation. Vinculin was used as a loading control.* ***B*** *Densitometric analysis of pSMAD Western blot bands. * = significantly different compared to AMH untreated samples unexposed to LH (Kruskal-Wallis test, $p < 0.05$)*

### *4.4 LH treatment prevents AMH-mediated gene expression in hGLCs*

Previous results demonstrated that LH exposure leads to downregulation of AMHRII expression (*Figure 4*) and impairs AMH-induced SMAD1/5 phosphorylation (*Figure 5*), suggesting that AMH signaling is functionally compromised by LH. Since SMAD1/5 activation drives the transcription of several known AMH target genes [17], the next step was to investigate whether the LH-induced disruption of receptor expression and signaling also affects AMH-mediated transcriptional responses.

To this end, granulosa cells were pre-treated with 10 nM LH for 48 hours, followed by a 24 hour stimulation with 100 ng/mL recombinant AMH. The expression levels of two known AMH-responsive genes, MMP2 and OSX, were measured using real-time PCR and normalized to RPS7 as a housekeeping gene. Results were calculated using the $2^{-\Delta\Delta Ct}$ method and expressed as fold changes relative to the untreated condition (LH- / AMH-), which was set as baseline (value = 1), not shown in the figure.

As shown in *Figure 6A*, AMH treatment significantly upregulated MMP2 mRNA levels in LH-untreated cells, confirming transcriptional activation of this target gene. A similar trend was observed for OSX expression (*Figure 6B*), which was also significantly induced by AMH in the absence of prior LH exposure.
However, when granulosa cells were pre-treated with LH, no significant induction of either MMP2 or OSX was observed in response to AMH, indicating that LH pre-treatment abolishes the AMH-mediated gene expression response.
Statistical analysis using the Kruskal–Wallis test confirmed the significance of these observations ($p < 0.05$ for AMH-treated vs untreated in the absence of LH; $p \geq 0.05$ in LH-treated cells).

These findings further support the hypothesis in which LH, through AMHRII downregulation, impairs the downstream transcriptional activity of AMH, leading to a functional suppression of its target gene expression.

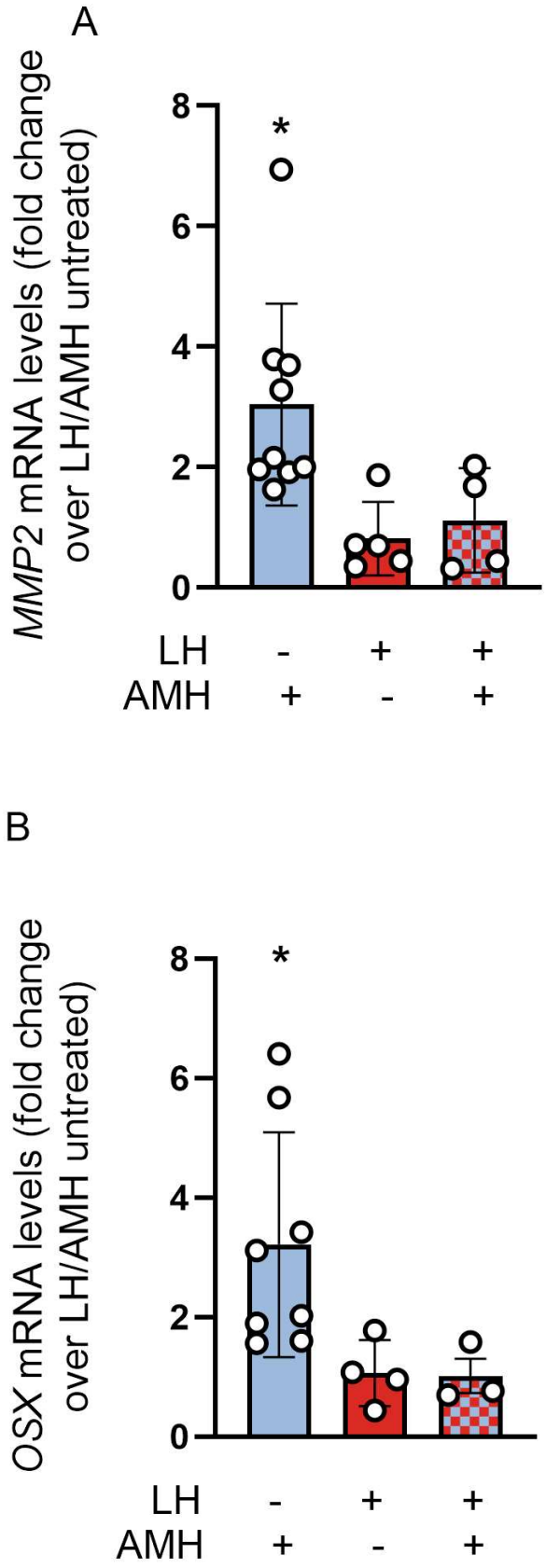



***Fig. 6*** *- Analysis of LH modulation of AMH-induced gene expression levels.* ***A*** *Real-time PCR analysis of MMP2 mRNA expression in hGLCs.* ***B*** *Real-time PCR analysis of OSX mRNA expression in hGLCs. * = significantly different compared to LH/AMH untreated samples (Kruskal-Wallis test, $p < 0.05$).*

# 5. DISCUSSION AND CONCLUSIONS

Folliculogenesis represents a fundamental process in female reproductive biology, ensuring the cyclical maturation and release of oocytes throughout the reproductive lifespan. However, its regulation remains only partially understood, particularly in the context of early follicular development. Despite decades of research, a substantial gap persists in identifying the precise hormonal and molecular mechanisms that govern the recruitment and progression of follicles from the primordial pool. Traditionally considered independent of gonadotropins, the initial phases of folliculogenesis are now being re-evaluated in light of growing evidence suggesting a functional role for luteinizing hormone (LH) even at these early stages [10].
At the same time, the expression and signaling of anti-Müllerian hormone (AMH), a key regulator of ovarian reserve, remain subject to conflicting regulatory inputs, including those exerted by gonadotropins [11, 21–26]. In this intricate physiological context, there is an urgent need to better define the roles of LH and AMH in follicular recruitment and maturation, not only to advance our biological understanding but also to improve the management of fertility and reproductive health in clinical settings.

In recent years, growing attention has been directed towards AMH in the field of ovarian physiology, particularly following the demonstration of its secretion by granulosa cells in the female gonad. Initially considered a hormone with male-specific developmental roles, AMH has since been recognized as a central regulator of female reproductive function, expanding our understanding of its biology beyond its canonical role in Müllerian duct regression [11]. While its inhibitory action on follicle-stimulating hormone (FSH) at the pre-antral stages is well established [5], its function during the antral stages remains less clearly defined.

Interestingly, studies in primate follicles have suggested that AMH may exert a dual function, promoting follicular growth at the pre-antral stage, while acting as a negative regulator during the antral phase [28, 29]. This duality has been supported by findings in human primary granulosa lutein cells, where AMH was shown to suppress FSH-induced steroidogenesis by downregulating key steroidogenic enzymes [30], reinforcing the hypothesis of an antagonistic relationship between AMH and FSH during the antral stage. Conversely, the interplay between AMH and LH is far less understood.

LH plays a crucial role in the antral stage, driving androgen synthesis, dominant follicle selection, ovulation, and luteinization [20]. Recent hypotheses have proposed a potential involvement of LH even earlier, in the late pre-antral or early antral stages, where it may support the recruitment of the antral follicle pool through mechanisms that remain to be elucidated [10]. This emerging perspective challenges the long-standing view that FSH is the sole driver of antral follicle recruitment [20], and opens new avenues for reproductive therapies, where LH supplementation could enhance clinical outcomes in infertility treatments [31].

Within this framework, the present study aimed to investigate the functional relationship between LH and AMH signaling pathway in human granulosa lutein cells, with particular attention to the expression and intracellular signaling of the AMH receptor AMHRII. The initial focus was placed on assessing whether LH directly modulates the expression of AMH itself, both at the mRNA and protein levels. Under the tested experimental conditions, no significant changes in AMH transcript or protein expression were observed following LH treatment. This result led to the hypothesis that LH might instead exert its regulatory action downstream, possibly by affecting AMH signaling responsiveness through modulation of its receptor AMHRII.

Starting from this hypothesis, both transcript and protein levels of AMHRII were analized in hGLCs exposed to increasing concentrations of LH. The results demonstrated a clear dose-dependent downregulation of AMHRII mRNA, in agreement with previous studies [26], and a similar trend was observed at the protein level. These findings suggest that LH negatively modulates AMH signaling by reducing receptor availability on the cell surface.
To confirm that this effect was mediated by the LH receptor (LHCGR), was performed an siRNA-mediated knockdown of LHCGR in hGLCs. The silencing of LHCGR prevented LH-induced AMHRII downregulation, indicating that the observed effects were specifically dependent on LH receptor activation.

Subsequently, it was analyzed whether reduced AMHRII expression could impair AMH downstream signaling, based on the well established evidence that AMH activates the canonical SMAD pathway via phosphorylation of SMAD1/5 [15]. Interestingly, when cells were pre-treated with LH prior to AMH exposure, a marked reduction in pSMAD1/5 levels

was observed, supporting the hypothesis that LH impairs AMH signaling by limiting receptor expression and downstream pathway activation.

Finally, to further investigate the effect of LH on the AMH pathway, the transcript levels of two well known AMHRII downstream target genes, OSX and MMP2 [17], were analyzed. In both cases, a similar decrease in gene expression was observed following LH treatment, further supporting the hypothesis that LH negatively modulates AMH signaling by impairing receptor-mediated transcriptional activation.

Altogether, these findings indicate that LH may exert a dual role in the antral follicle: on one hand, it directly promotes granulosa cell survival through intracellular prosurvival pathways [32], on the other, it indirectly enhances follicular maturation by suppressing AMH activity, which is known to antagonize FSH-induced follicle growth at this stage [33]. This hypothesis offers a new perspective on the regulatory crosstalk between gonadotropins and AMH in the ovarian environment, suggesting that LH may adjust follicular responsiveness to FSH by modulating AMHRII expression levels. This is particularly relevant given the growing clinical use of AMH and antral follicle count (AFC) as biomarkers of ovarian reserve and predictors of ovarian response to stimulation protocols [10].

The clinical implications of these findings may extend to infertility treatments, where understanding the balance between LH and AMH could help refine gonadotropin administration protocols to enhance both oocyte quantity and quality. Indeed, while FSH remains the principal driver of antral follicle recruitment and maturation, the suppressive action of AMH may constitute a limiting factor in certain contexts. These data suggest that LH, by downregulating AMHRII expression, might relieve this inhibitory brake, thereby indirectly supporting FSH action and improving follicular progression. Furthermore, the possibility of manipulating the LH-AMH axis could represent a novel therapeutic approach in patients with suboptimal ovarian response or in conditions characterized by altered AMH levels, such as polycystic ovary syndrome.

Despite these promising data, the present study has some limitations that need to be acknowledged. The experimental model was based on primary human granulosa lutein cells, which, although of ovarian origin and expressing relevant markers such as LHCGR and AMHRII, do not fully recapitulate the functional complexity and metabolic state of antral

granulosa cells *in vivo*. Additionally, the limited number of biological replicates restricted the statistical significance of some findings. Moreover, the absence of systemic endocrine and paracrine factors in the *in vitro* setting may limit the translatability of results to physiological or clinical scenarios.

Future experiments will aim to overcome these limitations by increasing sample size and testing additional markers of AMH pathway activity. It would also be valuable to analyze the expression of other components of the SMAD pathway and to investigate the transcriptional activation of downstream AMH target genes under different hormonal conditions.

In conclusion, this study provides new insights into the molecular interplay between LH and AMH in hGLCs. It was demonstrated that LH exposure leads to a dose-dependent downregulation of AMHRII at both the transcript and protein levels and that this event correlates with impaired activation of the AMH-dependent SMAD1/5 pathway. These findings support the existence of a regulatory network between LH and AMH in the ovary and suggest that LH may act not only as a direct promoter of follicular maturation but also as an indirect modulator of the AMH signaling axis. While further studies are necessary to validate and expand these results, this work represents an important step toward clarifying the functional interdependence of gonadotropins and local ovarian factors in human folliculogenesis.

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