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GONADOTROPIN ACTION ON TARGET CELLS AND ITS PHARMACOLOGICAL IMPLICATIONS FOR MALE INFERTILITY TREATMENT: IS IT TIME FOR A PHARMA COMPANY TO EMBRACE THE CHALLENGE?

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Recently, I was asked “who are your inspirational and role models in your professional life”, suddenly three ladies appeared as image in front of my eyes, my Professor Manuela Simoni for her leadership style dedicated to scientific rigor and people; my Mother for her resilience to stand up with proud and competency in from of life challenges; finally, my Daughter, she is my muse how so young lady can match clear dreams, insecurity for the future and determination to fight for them.

To these women that serve as role models by embodying qualities that motivate and encourage me both professionally and personally, I dedicate my PhD.

Table of contents

Abstract

Male infertility is one of the most relevant scientific topics in reproduction, and the male factor is responsible of 50% of causes of couple infertility. However, male infertility is rarely treated since assisted reproduction is successful in many cases. This industrial PhD had the objective to build a business case on male infertility treatment thanks to the collaboration between Merck KGaA and Modena & Reggio Emilia University on four main projects: i) to understand thoroughly gonadotropin mechanism of action on target cells/organs using *in vitro* models, aimed at identifying the most effective treatment of the male factor; ii) to identify objective, assessable, clinical endpoints; iii) to evaluate the clinical efficacy of luteinizing hormone (LH) and hCG in patients with hypogonadotropic hypogonadism (HH). Finally, iv) Merck built a business case aimed at evaluating the profitability of entering this therapeutic area, beyond the case of HH, by developing appropriate therapies based on the market potential and unmet medical needs.

In vitro exploration of the human chorionic gonadotropin (hCG) induction of steroid secretion in the mouse cell line mLTC1 by Liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Fanelli *et al.*, 2022) demonstrated that hCG massively stimulates steroid production via both the canonical and backdoor pathway of dihydrotestosterone (DHT) synthesis. Additional analyses aimed at assessing differences between LH and hCG action on Leydig cells, showing that LH and hCG differently impact the expression of steroidogenic genes, reflecting hormone-specificity. Digital droplet PCR analysis revealed that hCG upregulates the *Hsd13b3* gene, while *Hsd3b6* and *2*, as well as *Srd5a2*, are upregulated by LH. Both gonadotropins increased the expression of *Srd5a1*, while only treatment with LH resulted in *Akr1c13, 14* and *20* upregulation (Kruskal Wallis test, $p<0.05$, $n=4$) (manuscript in preparation).

Retrospective *post-hoc* analysis of published data provided evidence that follicle-stimulating hormone (FSH) improves sperm DNA fragmentation (sDF) in idiopathic male patients. Moreover, testosterone (T) levels after treatment are negatively correlated with sDF decrease, suggesting a potential synergic action between the interstitial and tubular testicular compartments (Lispi *et al.*, 2022) and electing T as a potential biomarker of FSH action.

The ongoing clinical study "Pharmacodynamics and safety of human recombinant LH in HH men" aims at assessing whether LH supplementation is efficient as hCG to stimulate steroidogenesis in those cases where endogenous LH activity or production is lacking (EudraCT 2019-004677-12). The statistical hypothesis is non-inferiority of the highest LH dose employed, compared to hCG. Primary endpoint is serum T levels evaluated by LC-MS/MS. Increasing doses of hCG and LH are administered at two-week intervals to obtain dose-response curves of

stimulated serum T levels. Preliminary data demonstrated that LH alone is not able to sufficiently increase T.

Finally, I finalized the “Male Infertility Business Case” for Company discussion and decision. Data generated in this PhD project showed high potential for gonadotropin use to treat HH and idiopathic male infertility. FSH and hCG are target molecules on which the Company could invest to expand indications. The basic research work done here is providing novel evidence on the mode of action of gonadotropins on target cells. This will expand the knowledge on spermatogenesis and steroidogenesis regulation, providing new treatment approaches of male infertility.

L'infertilità maschile è responsabile del 50% delle cause di infertilità di coppia. Tuttavia, poiché la riproduzione assistita è considerata una soluzione efficace, il fattore maschile viene raramente trattato con terapie mirate. Questo PhD industriale ha avuto l'obiettivo di sviluppare un *business case* sul trattamento con gonadotropine dell'infertilità maschile grazie alla collaborazione tra Merck KGaA e l'Università di Modena e Reggio Emilia. Il progetto si è basato su più line di ricerca: i) studiare il meccanismo di azione delle gonadotropine su cellule target utilizzando modelli in vitro; ii) identificare endpoint clinici oggettivi e misurabili; iii) valutare l'efficacia clinica di ormone luteinizzante (LH) e gonadotropina corionica umana (hCG) in pazienti affetti da ipogonadismo ipogonadotropo (Ipo-Ipo); iv) sviluppare un *business case* per valutare la redditività per Merck di espandere il portfolio prodotti nell'area dell'infertilità maschile.

Lo studio in vitro dell'induzione di steroidi con hCG nella linea mLTC1 mediante LC-MS/MS (Fanelli *et al.*, 2022) ha dimostrato che l'hCG è in grado di stimolare la sintesi di *dihydrotestosterone* (DHT) attraverso entrambe ile vie di sintesi note, canonica e *backdoor*. Ulteriori analisi volte a valutare la differenza dell'azione di LH e hCG sulle cellule di Leydig sono state eseguite sulla linea cellulare mLTC1, dimostrando che LH e hCG attivano diversamente l'espressione dei geni steroidogenici, riflettendo una steroidogenesi specificamente ormone-mediata. L'hCG aumenta l'espressione del gene *Hsd13b3*, mentre *Hsd3b6* e *2*, così come *Srd5a2*, sono stimolati dall'LH. Entrambe le gonadotropine aumentano l'espressione di *Srd5a1*, mentre solo l'LH incrementa *Akr1c13*, *14* e *20* (Kruskal Wallis, $p < 0,05$, $n = 4$; *Hprt1* usato come controllo). Questi ultimi dati non sono stati ancora pubblicati.

L'analisi retrospettiva di dati clinici pubblicati ha mostrato che il trattamento con FSH migliora la frammentazione del DNA spermatico (sDF) nei pazienti con infertilità idiopatica. I livelli di testosterone (T) dopo il trattamento con FSH mostrano una correlazione negativa con la riduzione del sDF, suggerendo una potenziale azione sinergica tra i compartimenti interstiziali e tubulari testicolari (Lispi *et al.*, 2022) e candidando il T a potenziale biomarker dell'efficacia del trattamento con FSH.

Lo studio "Farmacodinamica di r-hLH in pazienti con ipogonadismo ipergonadotropo maschile" (EudraCT 2019-004677-12), attualmente ancora in corso, ha lo scopo di valutare se la somministrazione di LH possa essere ugualmente efficace all'hCG (studio di non-inferiorità) nella stimolazione della steroidogenesi, in casi di deficit dell'attività o della produzione di LH endogeno. Endpoint primario dello studio è il livello sierico di testosterone valutato mediante LC-MS/MS. Dosi crescenti di hCG e LH vengono somministrati ogni due settimane per

ottenere una curva dose-risposta dei livelli di T. Dati preliminari hanno dimostrato che LH non è in grado di aumentare sufficientemente il T.

In ultimo, ho finalizzato il "Business case sull'infertilità maschile". I dati forniti da questo PhD hanno mostrato un alto potenziale dell'uso delle gonadotropine per il trattamento di pazienti con ipogonadismo ipogonadotropo e con infertilità maschile idiopatica. FSH e hCG possono rappresentare un potenziale di investimento per l'azienda che potrebbe così ampliare il proprio porfolio prodotti servendo un bisogno terapeutico dovuto alla mancanza di terapie efficaci mirate. Il lavoro di ricerca di base ha generato nuove evidenze sul modo d'azione delle gonadotropine, ampliando le conoscenze sulla spermatogenesi e steroidogenesi e fornendo nuove opportunità terapeutiche per il trattamento dell'infertilità maschile.

Abbreviations

AE	Adverse event
AESI	Adverse events of special interest
AMH	Anti-Müllerian Hormone
CI	Confidence intervals
CP	Clinical pregnancy
CRF	Case report form
CRO	Clinical Research Operation
DHT	Dihydrotestosterone
FSH	Follicle-stimulating hormone
hCG	Human chorionic gonadotropin
HCP	Health Care professionals
HH	Hypogonadotropic hypogonadal
HPLC	High-Performance Liquid Chromatography
ICSI	Intracytoplasmic Sperm Injection
IMP	Investigational Medicinal Product
Im	intramuscolar
IS	Internal standard
IVF	In vitro fertilization
LB	Live birth
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LH	Luteinizing hormone
MAR	Medically Assisted Reproduction
MI	Male Infertility
MRHI	Male Reproductive Health Initiative
NPV	Net Present Value
OS	Ovarian Stimulation
PI	Principal Investigator
PSA	Prostatic specific antigen
QC	Quality control
r-hFSH	recombinant human follicle stimulating hormone
r-hLH	recombinant Luteinizing hormone
SAE	Serious adverse events
sc	subcutaneous
SD	Standard deviation
sDF	Sperm DNA fragmentation
SHBG	Sex hormone binding globulin
TPP	Target Product Profile

1. Introduction

Infertility is characterized by the failure to achieve pregnancy after at least 12 months of regular unprotected sexual intercourse. The inability to have children can be a deeply distressing experience for couples, causing feelings of loss, failure, and isolation. Infertility has significant implications on demographics, economics, and health and it is affecting millions of individuals worldwide (DHS Comparative report no.9, 2004). Even though the issue of infertility is important for sexual and reproductive health and rights, many countries lack adequate policies and services to address it. Tackling infertility is crucial for achieving sustainable development goals related to health, well-being and gender equality.

Understanding the extent of infertility is essential for developing appropriate interventions, monitoring access to quality fertility care, and addressing risk factors and consequences associated with infertility (WHO report, 2023). According to the WHO 2023 Report, which covers data from 1990 to 2021, the global prevalence of infertility is estimated to be approximately one in six individuals, with a lifetime infertility prevalence of 17.5% worldwide (WHO report, 2023). Male factors account for approximately 50% of infertility cases and represent the main contributor in about 20% of cases (Agarwal *et al.*, 2020). These data highlight the significant number of men who may require infertility management and fertility care services. However, availability, accessibility, and quality of interventions for prevention, diagnosis, and treatment of this condition are a challenge.

Recognition of the societal challenge posed by male infertility has led to initiatives such as the Male Reproductive Health Initiative (MRHI) supported by the European Society of Human Reproduction and Embryology (ESHRE), efforts by the European Academy of Andrology to improve awareness, and the World Health Organization's engagement with countries to address infertility within legal and policy frameworks (Guideline Group on Unexplained Infertility *et al.*, 2023; Krausz *et al.*, 2023; WHO report, 2023). Additionally, guidelines for male infertility diagnosis and treatment have been developed by organizations like the American Urological Association/American Society for Reproductive Medicine (AUA/ASRM), the European Association of Urology (EAU) and the European Academy of Andrology (EAA).

Male infertility is a complex condition with various causes, many of which are not thoroughly investigated, managed, nor treated optimally (Esteves and Humaidan, 2023). Diagnostic evaluations for male infertility should consider multiple variables, including congenital and genetic factors, anatomical disorders, hormonal disbalance, ejaculatory dysfunction, and lifestyle habits. Idiopathic infertility affects a significant portion of males undergoing evaluation (more than 40% of male infertility cases), yet the exact etiology of this specific condition remains vague (Punab *et al.*, 2017; Ventimiglia *et al.*, 2021). Traditionally, semen

analysis is used to assess male fertility based on parameters such as sperm count, morphology, motility, and volume according to the WHO laboratory manual (WHO, 2021). However, this approach often neglects crucial dimensions related to paternal age, endocrine functions, sperm DNA quality, lifestyle, and environmental factors (Esteves *et al.*, 2020, 2023a; Murugesu *et al.*, 2022). Clinicians are aware of semen analysis limited prognostic accuracy, and that natural conception might or might not occur across a range of semen analysis values (Kimmins *et al.*, 2023).

The development of Medically Assisted Reproduction (MAR) has provided significant potential for treating infertility. However, despite extensive efforts, approximately 35-40% of couples visiting infertility centers are unable to achieve their goal of having a child and remain childless (Olivius *et al.*, 2002; McLernon *et al.*, 2016). It is worth to note that MAR almost exclusively focuses on the female partner, even when the male factor is identified as the main cause, and the use of MAR techniques such as Intracytoplasmic Sperm Injection (ICSI), has been considered as an efficient therapeutic solution to overcome male factor. According to recent scientific evidence, there is a correlation between male factors and the health conditions of offspring born through ICSI. This finding sparks a debate as to whether sperm quality is underestimated and emphasizes the importance of enhancing sperm quality prior to ICSI. An increasing number of studies suggests that therapeutic interventions may improve sperm quantity/quality and overall male health, ultimately resulting in better reproductive outcomes, even when ICSI is the only option. (Faure *et al.*, 2014; Esteves *et al.*, 2016, 2020, 2023a; Kirby *et al.*, 2016; Salas-Huetos *et al.*, 2017; Samplaski *et al.*, 2017; Vanegas *et al.*, 2017; Ricci *et al.*, 2018; Santi *et al.*, 2018a; Omar *et al.*, 2019; Lira Neto *et al.*, 2021; Persad *et al.*, 2021; Bian *et al.*, 2022; Humaidan *et al.*, 2022).

As mentioned, idiopathic infertility represents more than 40% of male infertility cases, the lack of efficient diagnosis for males with idiopathic infertility, especially those with subtle endocrinological imbalances, is a significant concern. It is widely known that normal spermatogenesis relies on the action of both FSH and LH on the testis. However, despite this knowledge, identifying and addressing these hormonal imbalances in cases of idiopathic infertility remains challenging. This highlights the need for further research, related to FSH and LH action on target cells/tissues, and diagnostic advancements to effectively diagnose and manage male infertility with endocrinological involvement (Oduwole *et al.*, 2021; The Endocrine Society, 2022). Unfortunately, previous attempts to develop and stabilize human Leydig or Sertoli cell cultures in vitro or ex-vivo have been unsuccessful. This limitation has hindered the ability to study the mode of action of FSH, LH, or human chorionic gonadotropin

(hCG) specifically on human cell models. Consequently, reliance on animal models remains necessary, although it may not fully elucidate the complexities observed in the human system and creating a significant knowledge gap.

Meanwhile, hormonal therapy was shown to be promising as a potential treatment in male infertility, particularly in cases of congenital and acquired hypogonadotropic hypogonadism (HH). In HH, the replacement of exogenous hormones can effectively restore spermatogenesis by stimulating the pituitary gland and the gonads (Kliesch *et al.*, 1995; Rastrelli *et al.*, 2014; Santi and Corona, 2017).

Based on the success of hormonal therapy in HH, the same empiric clinical approach has been suggested as a potential treatment to enhance fertility in men with idiopathic oligozoospermia, characterized by low sperm count of unknown origin, or nonobstructive azoospermia (NOA), aiming to replicate the therapeutic approach used for HH. Hormonal therapy in such cases aims to address underlying endocrine imbalances that may be contributing to the infertility (Santi *et al.*, 2015; Laursen *et al.*, 2022).

It is important to note, that the effectiveness of hormonal therapy for idiopathic oligozoospermia is still being studied and the evidence remains mixed. There is a need for more research, including randomized controlled trials, to establish the true benefits and potential risks associated with hormonal therapy in these specific cases (Esteves *et al.*, 2023b) before any MAR procedure or as the main and only therapeutic solution. Designing and conducting randomized clinical trials imply a clear description of target population, defined by homogenous clinical descriptive parameters and diagnostic factor, the last to be translated in clinical outcomes measurable by clear endpoints. Semen analysis is considered as one of the main parameters of idiopathic infertility diagnosis associated with endocrine biomarkers of spermatogenesis such as serum FSH and Inhibin B, while LH and testosterone are biomarkers of endocrine testicular function (Kimmins *et al.*, 2023). However, as mentioned, semen analysis variability represents a significant hurdle to depict robust clinical trials and the main question "which is the pharmacodynamic marker of hormonal treatment efficacy?" remains unreturned. Recently Santi et al. (2023) tried to address the question. The study suggests that the percentage increase of sperm concentration after FSH administration could predict the hormonal treatment efficacy, in terms of pregnancy. The application of mathematical analyses on data distribution identified for the first time a function predicting the sperm concentration increase needed to obtain a pregnancy, in relation to the baseline sperm number. This function may be useful in future studies to assess gonadotropin efficacy in improving male fertility.

In summary, the lack of a clear understanding of male infertility etiology, diagnostic biomarkers, and clinically measurable endpoints, along with the challenges in developing in vitro/ex-vivo research models, have led to empirical treatment approaches for this condition. As a result, there has been reduced interest from health system actors and pharmaceutical companies in investing in research and the development of specific therapies for male infertility. Addressing these knowledge gaps and research challenges is crucial to foster advancements in this field and to improve patient care.

2. Research Purpose and Plan

Pharmaceutical companies play a crucial role in addressing significant unmet medical needs by developing and providing approved therapies. To facilitate this process, collaboration between pharmaceutical companies, clinicians, and researchers is essential. By working together, they can jointly explore effective and safe solutions to address these needs and improve patient outcomes.

Merck KGaA and the University of Modena & Reggio Emilia joined forces to generate biological and clinical evidence to build a business case and to evaluate profitability in investing to expand existing products' indications in male infertility and increase knowledge in gonadotropins mode of action on male target cells and tissues.

- Merck KGaA has developed and produces pharmaceutical preparations of recombinant human FSH, LH, and hCG, which have a wide range of therapeutic indications in female infertility. In addition, r-hFSH also obtained pharmaceutical indications for the congenital and acquired HH male condition when used in combination with hCG, in Europe, US and other countries. HCG is the main, well-established therapy used to treat testosterone deficiency in humans, although this gonadotropin is not physiologically produced in adult males, where hCG is clinically administered as a surrogate for LH activity. Merck's hCG and LH-based products do not have a therapeutic indication for use in males. HCG and LH share more than 80% of amino acid sequence identity, although hCG is highly glycosylated with a half-life longer than LH and exhibits different biological and clinical activity in females (Casarini *et al.*, 2012, 2017, 2018; Riccetti *et al.*, 2017a, 2017b). In males, LH activates steroid synthesis in Leydig cells. However, the pharmaceutical form of human LH, developed after pharmaceutical forms of hCG, is not used to stimulate testosterone production in men, due to limited knowledge of its therapeutic efficacy.
- The Modena and Reggio Emilia University has been working in the field of gonadotropins biology and its clinical implications over 15 years.

The industrial PhD program aimed to gather data on the mode of action of gonadotropins and collect clinical evidence on the use of pharmaceutical forms of hLH and hCG in activating steroid synthesis in adult males, with the goal of addressing knowledge gaps in male infertility. In addition, the aim was also to better understand and evaluate the robustness and effectiveness of highly potential biomarkers in predicting and monitoring the response of males with idiopathic infertility to treatment with hFSH. The data obtained was also utilized to develop a business case and design a possible product development strategy to enhance Merck's existing portfolio in the field of male infertility:

i) **Exploring LH and hCG differentially induced steroid secretion profile of target Leydig cells (mouse cell line).**

The canonical androgen synthesis in Leydig cells involves Δ5 and Δ4 steroids. In particular, dihydrotestosterone (DHT) synthesis in humans occurs from T via Δ5 precursors, whereas the Δ4 pathway, involving androstenedione (A4), is preferred in rodents (Miller and Auchus, 2011). Recently, an alternative route for DHT production, encompassing progesterone (P4) metabolism through 5α and 5α,3α steroids, was discovered in animal model (Tammar Wallaby) and confirmed in humans (Auchus, 2004). It is named "backdoor pathway" and consists in an alternative pathway for DHT synthesis, with a role in both male fetal and adult pathophysiology. A newly developed liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was used to measure 20 steroids and used it to investigate the route of steroid secretion induced by hCG and LH in the mouse Leydig tumor cell line 1 (mLTC1), and increase knowledge on hCG and LH mode of action on mouse Leydig cell line. Unfortunately, Human Leydig cells are not commercially available and the planned experiment could not be confirmed in human *in vitro* model.

ii) **Assess pharmacodynamics and safety of r-hLH (Investigational treatment) and u-hCG (standard treatment) in hypogonadotropic hypogonadal (HH) men**.

The general clinical question is whether LH supplementation could be as effective as hCG in promoting androgen synthesis, in cases where LH activity is required, such as in men with HH. This clinical trial aims to compare the clinical efficacy of the two molecules in increasing testosterone serum levels. Moreover, the study wishes to design the pharmacodynamic of LH administration, compared to hCG, critically exploring the possibility of replacing non-physiological hCG with LH in a clinical setting of HH patients

iii) **Evaluate the biological/clinical correlation between sDF (sperm DNA Fragmentation), steroidogenesis (Testosterone serum production) and semen parameters in patients treated with FSH**.

As mentioned, idiopathic male infertility accounts for up to 50% of the male infertile population. However, only in Italy there is an approved and reimbursed treatment for this condition. While there is a solid physiological rationale for the use of hFSH in empirically restoring sperm quality in cases of idiopathic male infertility with

altered sperm analysis, there is a significant gap in reliable biomarkers to assess its efficacy. Therefore, considering the unmet clinical need to transition from empirical use to evidence-based treatment in addressing this broad population, the primary objective of this study was to identify potential biomarkers that can be utilized in clinical practice and future clinical studies. The study was aimed to investigate whether there is a correlation between changes in testosterone serum levels and sDF index after FSH administration. Additionally, the study aimed to validate the sDF index as a biomarker for evaluating the effectiveness of FSH administration in cases of male idiopathic infertility.

iv) **Build Business Case on male infertility cases deserving hormonal treatments**.

Pharmaceutical companies face the challenge of evaluating various market and portfolio factors before making critical decisions on portfolio expansion, which often involves significant investments. In the specific context of male infertility, which encompasses scientific gaps like poor knowledge of disease causes, and weak evidence-based data on hormonal treatments, the assessment becomes complex and requires multiple steps.

First, a clear diagnosis of the population that may benefit from treatment with gondotropin needs to be established beyond the scope of congenital HH. Additionally, a business case needs to be developed to assess the profitability of entering this therapeutic area, considering market potential, unmet medical needs, and biological/clinical data.

By leveraging the data from the PhD project and available scientific knowledge, the objective is to build a solid business case that evaluates the feasibility of developing appropriate therapies and determining the potential success in the market. This thorough evaluation is necessary to make informed decisions regarding portfolio expansion in the field of male infertility treatment.

3. Methods and Results

3.1 Investigation of LH and hCG differential steroidogenesis pathway

3.1.1. Exploring the human chorionic gonadotropin induced steroid secretion profile of mLTC1 by a 20 steroid LC-MS/MS panel

The study aimed to investigate the steroid secretion induced by human chorionic gonadotropin (hCG) in the mouse Leydig tumor cell line 1 (mLTC1). A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed to measure 20 steroids. The results showed that hCG stimulation led to increased levels of several steroids including 16OH-progesterone, androstenedione, testosterone, dihydrotestosterone, epitestosterone, progesterone, and 17OH-allopregnanolone. The study also detected traces of 17OH-dihydroprogesterone, androstanediol, and dihydroprogesterone, while some steroids such as androstenediol, 17OH-pregnenolone, dehydroepiandrosterone, pregnenolone, and allopregnanolone did not show any peak. These findings suggest that hCG can induce significant changes in steroid secretion in mLTC1 cells.

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Exploring the human chorionic gonadotropin induced steroid secretion profile of mouse Leydig tumor cell line 1 by a 20 steroid LC-MS/MS panel

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ABSTRACT

The canonical androgen synthesis in Leydig cells involves Δ5 and Δ4 steroids. Besides, the backdoor pathway, eompassing 5α and 5α,3α steroids, is gaining interest in fetal and adult pathophysiology. Moreover, the role of androgen epimers and progesterone metabolites is still unknown. We developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for measuring 20 steroids and used it to investigate the steroid secretion induced by human chorionic gonadotropin (hCG) in the mouse Leydig tumor cell line 1 (mLTC1).

Steroids were extracted from 500 μL supernatants from unstimulated or 100 pM hCG-exposed mLTC1 cells, separated on a Luna C8 100 × 3 mm, 3 μm column, with 100 μM NH4F and methanol as mobile phases, and analyzed by positive electrospray ionization and multiple reaction monitoring.

Sensitivity ranged within 0.012–38.0 nmol/L. Intra-assay and inter-assay imprecision were < 9.1% and 10.0%, respectively. Trueness, recovery and matrix factor were within 93.4–122.0, 55.6–104.1 and 76.4–106.3%, respectively. Levels of 16OH-progesterone, 11-deoxycortisol, androstenedione, 11-deoxycorticosterone, testosterone, 17OH-progesterone, androstenedione, epitestosterone, dihydrotestosterone, progesterone, androsterone and 17OH-allopregnanolone were effectively measured. Traces of 17OH-dihydroprogesterone, androstanediol and dihydroprogesterone were found, whereas androstenediol, 17OH-pregnenolone, dehydroepiandrosterone, pregnenolone and allopregnanolone showed no peak. hCG induced an increase of 80.2–102.5 folds in 16OH-progesterone, androstenedione and testosterone, 16.6 in dihydrotestosterone, 12.2–27.5 in epitestosterone, progesterone and metabolites, 8.1 in 17OH-allopregnanolone and ≤ 3.3 in 5α and 5α,3α steroids.

Abbreviation: 11-DOC, 11-deoxycorticosterone / 21OH-progesterone – 4-pregnen-21-ol-3, 20-dione; 11-S, 11-deoxycortisol – 4-pregnen-17, 21-diol-3, 20-dione; 16OH-P4, 16-hydroxyprogesterone – 4-pregnen-16α-ol-3, 20-dione; 17OH-Allo, 17-hydroxyallopregnanolone – 5α-pregnan-3α, 17-diol-20-one; 17OH-DHP4, 17-hydroxydihydroprogesterone – 5α-pregnan-17-ol-3, 20-dione; 17OH-P4, 17-hydroxyprogesterone – 4-pregnen-17-ol-3, 20-dione; 17OH-P5, 17-hydroxypregnenolone – 5-pregnen-3β, 17-diol-20-one; 3α-diol, androstanediol – 5α-androstane-3α, 17β-diol; 5α-DHP4, dihydroprogesterone – 5α-pregnan-3, 20-dione; 5α-dione, androstanedione – 5α-androstan-3, 17-dione; A4, androstenedione – 4-androsten-3, 17-dione; A5, androstenediol – 5-androsten-3β, 17β-diol; Allo, allopregnanolone – 5α-pregnan-3α-ol-20-one; AN, androsterone – 5α-androstan-3α-ol-17-one; DHEA, dehydroepiandrosterone – 5-androsten-3β-ol-17-one; DHT, dihydrotestosterone – 5α-androstan-17β-ol-3-one; Epi-T, epitestosterone – 4-androsten-17α-ol-3-one; hCG, human chorionic gonadotropin; IS, internal standard; LC-MS/MS, liquid chromatography – tandem mass spectrometry; LH, luteinizing hormone; LLOQ, lower limit of quantification; LOD, limit of detection; mLTC1, mouse Leydig tumor cell line 1; MRM, multiple reaction monitoring; P4, progesterone – 4-pregnen-3, 20-dione; P5, pregnenolone – 5-pregnen-3β-ol-20-one; QC, quality control; RT, retention time; S/N, signal to noise; T, testosterone – 4-Androsten-17β-ol-3-one.

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In conclusion, our LC-MS/MS method allows exploring the Leydig steroidogenesis flow according to multiple pathways. Beside the expected stimulation of the canonical pathway, hCG increased progesterone metabolism and, to a low extent, the backdoor route.

1. Introduction

Leydig cells are the major male androgenic district, accounting for 95% of circulating testosterone (T) and 20% of dihydrotestosterone (DHT) [1]. According to the canonical route, DHT synthesis in humans occurs from T *via* Δ5 precursors, whereas the Δ4 pathway, involving androstenedione (A4), is preferred in rodents (Fig. 1) [2,3]. Recently, an alternative route for DHT production, encompassing progesterone (P4) metabolism through 5α and 5α,3α steroids (Fig. 1), is gaining renewed interest [4,5]. This so-called "backdoor pathway" was discovered in the Tammar Wallaby [6,7] and afterwards confirmed in humans, in which its key role for fetal sex development was hypothesized [8–10]. However, the relevance of this route in adult pathophysiology is still unclear. Leydig androgen profile is further complicated by the presence of epimers, such as 17α-epitestosterone (Epi-T), capable of antagonizing or mimicking T function in different contexts [11,12] (Fig. 1). Evidences also suggest the presence of a complex P4 metabolism, however this has only partly been elucidated. 16OH-progesterone (16OH-P4) has been shown to modulate the P4 receptor and to accumulate in immature testis [13]. Moreover, 21- and 11-hydroxylase activities were described in testis from rodents and in particular human diseases, however, available information are scarce [14–17] (Fig. 1).

The testicular androgen synthesis is naturally stimulated by the luteinizing hormone (LH) and, in clinics, by the human chorionic gonadotropin (hCG), both hormones acting through the same membrane G protein-coupled receptor (LHCGR) [18]. The differential impact of gonadotropins over the canonical and backdoor androgen routes and over P4 metabolism is far from being elucidated.

In such a frame, there is a lack of effective tools to investigate pathological contexts in which classical and/or backdoor steroidogenic pathways might be altered, such as in defects of male fetus masculinization [4], or to evaluate differences between LH- *vs* hCG-induced steroid patterns, which might be relevant in the pharmacological treatment of male reproductive defects [18].

Liquid chromatography – tandem mass spectrometry (LC-MS/MS) is the ideal technology for identifying and quantifying panels of steroids in biological fluids. While this technique has widely been applied to Δ4 androgens, such as T and A4, and C21 steroids, including progestogens, mineralocorticoids and glucocorticoids [19], only a paucity of LC-MS/MS applications to neutral steroids were reported in literature, most often including Δ5 precursors, such as pregnenolone (P5), and DHT [20,21], sometimes 5α,3α steroids, such as allopregnanolone (Allo) [22], and rarely 5α steroids such as 5α-dihydroprogesterone (5α-DHP4) [23,24]. Overall, the panel proposed by these methods do not offer a comprehensive view of the canonical and backdoor pathways, and are often burdened with a complex sample preparation [19].

Here, we developed a LC-MS/MS method to investigate a panel of twenty among the most relevant steroids from Δ5, Δ4, 5α and 5α,3α classes. The method was validated for the application to a model of mouse Leydig cells and used to evaluate the steroid secretion in basal and hCG-stimulated conditions.

2. Materials and methods

2.1. Chemicals

16OH-P4, 11-deoxycortisol (11-S), A4, 11-deoxycorticosterone/21OH-progesterone (11-DOC), T, androstenediol (A5), 17OH-progesterone (17OH-P4), 17OH-pregnenolone (17OH-P5), dehydroepiandrosterone (DHEA), androstanedione (5α-dione), Epi-T, DHT, 17OH-dihydroprogesterone (17OH-DHP4), P4, androstanediol (3α-diol), androsterone (AN), P5, 5α-DHP4, 17OH-allopregnanolone (17OH-Allo), Allo, cortisol, corticosterone, 21-deoxycortisol, 11αOH-progesterone, 11βOH-progesterone, estrone and estradiol were from Steraloids (Newport, RI, USA). T-[2,2,4,6,6–2H5] (d5-T, 98.7% deuterium content) and A4-[2,2,4,6,6–2H5] (d5-A4, 98%) were from Cambridge Isotope Laboratories (Tewksbury, MA, USA); 17OH-P4-[2,2,4,6,6,21,21,21–2H8] (d8–17OH-P4, 98.7%), P4-[2,2,4,6,6,17α,21,21,21–2H9] (d9-P4, >98%) and 11-S-[4-Pregnen-17α,21-diol-3,20-dione-21,21–2H2] (d2–11-S, >98%) were from CDN

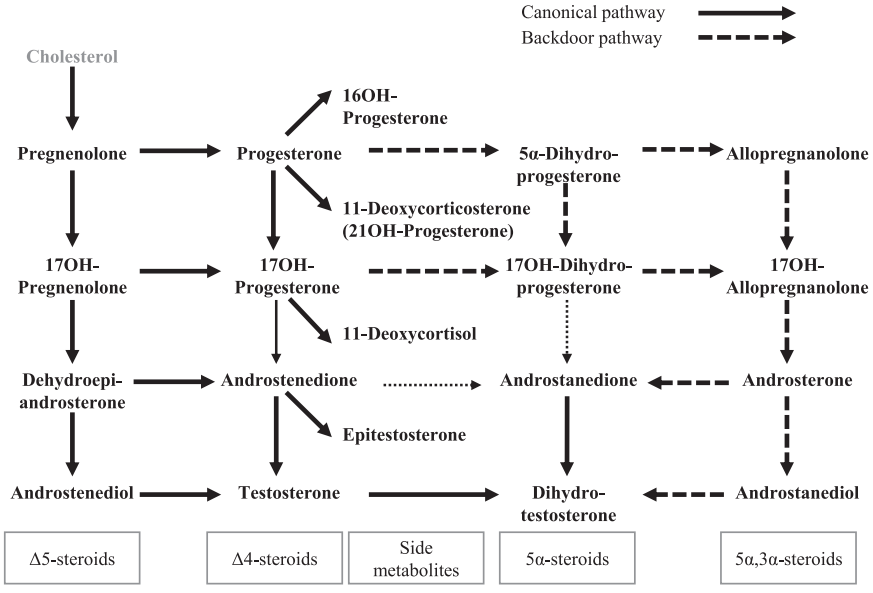

Fig. 1. Scheme of the investigated Leydig steroidogenesis. Continuous lines: canonical pathway; dashed lines: backdoor pathway; bold lines: main flux; thin lines: poor flux.

Isotopes (Pointe-Claire, Canada). Standards and isotopes were provided as lyophilic. LiChroSolv grade methanol, chloroform, N-hexane and ethyl-acetate were from Merck KGaA (Darmstadt, Germany). LC-MS grade ammonium fluoride was from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was produced by MilliQ Gradient A10 system (Burlington, MA, USA). Recombinant hCG (Ovitrelle, Merck KGaA) was provided in injectable saline buffer. The phosphodiesterase 5 inhibitors sildenafil, vardenafil and tadalafil (Sigma-Aldrich) were provided as methanol solution.

2.2. Standard solutions, calibrators and internal standards

Stock solutions were gravimetrically determined by the AX105 DeltaRange® analytical balance (Mettler-Toledo, Columbus, OH, USA) and dissolved in the mg/mL range in methanol, except 17OH-DHP4 and 5α-DHP4 which were diluted in methanol:chloroform (1:1). Working solutions were prepared in methanol from stock solutions. Microman® positive displacement pipettes (Gilson Inc, Middleton, WI, USA) and screw-top (2 mL) borosilicate V-Vials/PTFE-faced caps (Wheaton Industries Inc, NJ, USA) were used. The calibrating mixture was obtained by mixing working solutions at the following concentrations (μmol/L): 16OH-P4, 6.81; 11-S, 2.16; A4, 261.9; 11-DOC, 13.6; T, 26.0; A5, 516.5; 17OH-P4, 4.54; 17OH-P5, 676.7; DHEA, 312.0; 5α-dione, 624.1; Epi-T, 62.4; DHT, 154.9; 17OH-DHP4, 75.2; P4, 4.77; 3α-diol, 769.3; AN, 258.2; P5, 23.7; 5α-DHP4, 47.4; 17OH-Allo, 22.4 and Allo, 23.5. The internal standard (IS) mixture was prepared in 75% methanol with d2–11-S, d5-A, d5-T, d8–17OH-P4 and d9-P4 at 14, 17, 17, 15 and 15 nmol/L, respectively. All were stored at − 20 °C. The day of the assay, 10 μL of the calibrating mixture were diluted in 0.5 mL IS mixture. Eleven further calibrators were obtained by serial dilutions; zero consisted in the IS mixture. Low, medium and high-level quality controls (QCs) were prepared by diluting 50 μL of pure analyte mixtures at proper concentrations in 450 μL of culture medium.

2.3. Cell culture and treatments

The mouse Leydig tumor cell line 1 (mLTC1) was handled as previously described [25,26]. Briefly, mLTC1 cells were cultured in RPMI medium without phenol red, supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 2 mM glutamine, and 1 mM HEPES (Invitrogen, Carlsbad, CA) and maintained at 37 °C and 5.0% CO_2. 3×10^4 cells/well were seeded in 24 multi-well plates 24 h before treatments. Over-night serum-starved cells were washed twice with 37 °C phosphate buffered saline and treated 24 h with 100 pM hCG diluted in RPMI medium without phenol red added with 0.2% bovine serum albumin (Sigma-Aldrich) [27–29]. Control cells were treated with the same solution lacking hCG.

2.4. Sample preparation

Samples were thawed, vortexed and centrifuged at room temperature for 5 min at 5500 g. Five-hundreds μL of each sample and QC were pipetted into 13 × 100 mm Pyrex® tubes (Sigma-Aldrich), spiked with 100 μL IS and vortexed 1 min. Afterwards, tubes were added 0.5 mL of water and vortexed 1 min. Two mL of N-hexane:ethyl-acetate (8:2) were added before tubes were vigorously vortexed for 5 min and centrifuged (5 min, 3000 g, room temperature). The lower aqueous layer was frozen in ice bath, while the upper organic layer was decanted in 12 × 75 mm glass tubes (Laboindustria, Arzergrande, Italy) and dried under nitrogen flow. Samples were reconstituted with 100 μL of 75% methanol and transferred into autosampler glass vials (Agilent Technology, Santa Clara, CA). Each batch included supernatants, calibrators and three QC replicates placed at the beginning, middle and end of the batch.

2.5. Liquid chromatography

The PerkinElmer Series 200 (Waltham, MA, USA) HPLC was used, equipped with the LUNA® C8(2) 100 Å 100 × 3.0 mm, 3 μm column and C8 4 × 2.0 mm guard column (Phenomenex, Torrance, CA, USA), maintained at 45 °C. Solvent A was 100 μM ammonium fluoride in water and solvent B was methanol. The gradient, operated at 0.4 mL/min, started with 45% B, increased to 62% B from 0.3 to 0.6 min and to 78.3% B until 10 min; 100% B was achieved at 10.2 and maintained until 11.2 min, before reconditioning to 45% B until 13 min. The autosampler was set at 8 °C. Injections were performed at 1 and 10 μL.

2.6. Mass spectrometry

Multiple reaction monitoring (MRM) was performed by the API-4000 QTrap triple-quadrupole (Sciex, Framingham, MA, USA) in electrospray positive ionization mode operated at 750 °C and 5500 V. Nebulizing, heating gas (air), curtain and collision activated dissociation gas (nitrogen) were set at 60, 65, 20 psi and "medium", respectively. Data were processed by Analyst v1.7 (Sciex).

2.7. LC-MS/MS method development and validation

The method was validated according to European Medicines Agency guidelines with some modifications [30].

2.7.1. MS/MS detection

MRM transitions were manually optimized by syringe pump infusion at 10 μL/min of pure analyte and isotopes ranging 1–100 μg/mL. Three or more MRM transitions were optimized for each of the 20 validated steroids and for cortisol, 21-deoxycortisol, corticosterone, 11αOH-progesterone, 11βOH-progesterone, estradiol and estrone.

2.7.2. Selectivity and specificity

The MS cross-interference among steroids included in the panel was verified. In addition, the potential interference from cortisol, 21-deoxycortisol, corticosterone, 11αOH-progesterone, 11βOH-progesterone, estradiol and estrone, sildenafil, vardenafil and tadalafil was tested. Each compound was individually injected. Then, the peak area produced in MRM transitions of monitored analytes and IS was checked. LC gradient was optimized in order to separate analytes showing cross-interference. Quantitative and qualitative MRM transitions were selected as the most sensitive transitions whose ion ratio in tested sample was within ± 20% of ion ratio in analyte standards.

2.7.3. Ammonium fluoride optimization

Ammonium fluoride was added to solvent A at 0, 20, 50, 100 and 200 μM. Peak areas at each level were compared.

2.7.4. Retention time (RT) repeatability, carry-over and IS purity

RT repeatability, accepted within 1% deviation, was evaluated within-run and among-runs across consecutive weeks. Carry-over was determined as the analyte and IS peak area in the blank following the highest calibration point, and was accepted when < 20% of the analyte area at the lower limit of quantification (LLOQ) and when < 5% of the IS area. The presence of unlabeled analytes in IS injections was checked.

2.7.5. Calibration, quantitation range and sensitivity

Three independent calibration curves, each consisting of three replicates of each calibrator, were run in consecutive weeks. The quantitation range was defined by continuous calibration points showing trueness within 85–115% and CV< 15%, with the LLOQ defined as the lowest calibration point showing trueness within 85–115%, CV< 20% and signal-to-noise (S/N) ≥ 5. The limit of detection (LOD) was defined as the lowest analyte amount yielding a S/N ≥ 3.

2.7.6. Recovery and matrix factor

Recovery and matrix factor were evaluated in cell medium containing 100 pM hCG. Fifty μL of methanolic analyte mixture at low, medium and high level were spiked in 450 μL medium before extraction. In addition, 50 μL of the same mixtures were added to 50 μL of 50% methanol and used to reconstitute dried extracts of unspiked medium, or were injected as a matrix-free reference. All were prepared in triplicates. Recovery was calculated as the percentage ratio between peak areas in pre- *vs* post-extraction spiked test samples. Matrix factor was calculated as the percentage ratio between peak areas in post-extraction *vs* matrix-free reference. Deviations from 100% indicated the presence of ion suppression or enhancement. Matrix effect was also tested by post-column infusion. A syringe pump was connected to the LC eluate by a T-piece upstream the ionization source. The analyte mixture in 75% methanol was infused during LC injections of methanol and of extracts of culture media as such or containing 100 pM hCG, or 1 μM sildenafil, vardenafil or tadalafil.

2.7.7. Imprecision and trueness

Five replicates of QCs at low, medium and high levels were injected within the same day and in three independent runs in consecutive weeks. Imprecision was determined as the CV% calculated within run (intra-assay) and among runs (inter-assay). Trueness was calculated as the percentage ratio between the observed and the expected concentration.

2.7.8. Stability

Amounts of steroids in the middle range of the calibration curve were spiked in culture medium and incubated at 37 °C for 0, 1, 4, 8 and 24 h before freezing at − 80 °C. Freshly reconstituted extracts were injected immediately and after 24 h in autosampler at 8 °C. All were tested in triplicate.

2.8. Statistics

Means and standard deviations were computed. Variables were not normally distributed, therefore, values from unstimulated *vs* hCG-stimulated cells were compared by the Wilcoxon test for paired samples (MedCalc, v.18.2.1; Mariakerke, Belgium). $P < 0.050$ was considered statistically significant.

3. Results

3.1. LC-MS/MS method development and validation

3.1.1. LC-MS/MS detection, specificity and selectivity

Compound-dependent parameters are reported in Table 1. A sub-optimal collision energy was selected for A4 and T to avoid signal oversaturation in study samples. Analyte peaks are reported in Fig. 2. Baseline separation was obtained within groups of isobars or cross-interfering compounds including 16OH-P4, 17OH-P4, 11-DOC, 11αOH-progesterone and 11βOH-progesterone; 11-S, corticosterone and 21-deoxycortisol; T, DHEA, 5α-dione and Epi-T; 17OHP5, 17OH-DHP4 and P4; A5 and AN; P5, 5α-DHP4 and 17OH-Allo. Selective detection of 5α-DHP4 and 17OH-Allo was achieved by choosing specific Q3 ions. No interference could be observed from cortisol, estrone, estradiol, sildenafil, vardenafil or tadalafil. Ion ratio consistency was verified in all tested samples.

3.1.2. Ammonium fluoride optimization

One-hundred μM was chosen as the best compromise to optimize sensitivity within the whole panel, increasing the signal to 288–859% in respect to signal at 0 μM. Exception was found for A5 and 3α-diol, whose signal was reduced to 82% and 28%, respectively (Supplemental Fig. 1).

3.1.3. Retention time repeatability, carry-over and IS purity

RT variability was < 0.4% for all analytes. The carry-over was absent or < 0.1% of the area of the highest calibrator. For 11-DOC, Epi-T, DHT and P4, carry-over was occasionally observed up to 20%, 170%, 200% and 16% of the LLOQ, respectively. Therefore, a blank was always injected after the highest calibrator.

3.1.4. Calibration, quantitation range and sensitivity

Curve and quantitation parameters are reported in Table 2. Isotopic

Table 1
Compound-dependent LC-MS/MS detection parameters.

Analyte	Molecular weight	Retention Time	Quantifier MRM				Qualifier MRM			
	(g/mol)	(min)	Q1/Q3	DP	CE	CXP	Q1/Q3	DP	CE	CXP
16OH-Progesterone (16OH-P4)	330.47	4.42	331.4/97.1	110	40	2	331.4/109.1	110	40	2
11-Deoxycortisol (11-S)	346.46	4.44	347.3/109.3	100	35	5	347.3/97.2	100	38	7
Androstenedione (A4)	286.41	5.18	287.3/97.2	90	13 *	5	287.3/109.2	90	14 *	5
11-Deoxycorticosterone (11-DOC)	330.46	5.33	331.4/109.1	130	40	2	331.4/97.2	130	40	3
Testosterone (T)	288.42	5.75	289.2/97.2	80	16 *	4	289.2/109.1	80	17 *	3
Androstenediol (A5)	290.44	5.85	273.4/159.2	60	30	6	273.4/145.2	60	26	6
17OH-Progesterone (17OH-P4)	330.46	5.90	331.4/97.2	80	40	4	331.4/109.2	80	40	2
17OH-Pregnenolone (17OH-P5)	332.48	6.01	315.2/159.2	55	35	9	315.2/91.2	50	75	4
Dehydroepiandrosterone (DHEA)	288.42	6.08	271.3/197.2	65	27	9	271.3/213.2	65	23	10
Androstanedione (5α-dione)	288.42	6.45	289.3/213.3	70	25	7	289.3/161.2	70	25	7
Epitestosterone (Epi-T)	288.42	6.78	289.3/97.2	130	35	6	289.3/109.1	130	35	7
Dihydrotestosterone (DHT)	290.44	6.97	291.3/159.3	110	30	6	291.3/255.3	110	25	3
17OH-Dihydroprogesterone (17OH-DHP4)	332.48	7.02	315.3/111.1	90	25	8	333.3/255.3	90	25	5
Progesterone (P4)	314.46	7.62	315.2/97.1	115	30	2	315.2/109.2	115	35	2
Androstanediol (3α-diol)	292.46	8.08	257.2/161.2	70	20	8	257.2/147.2	70	30	5
Androsterone (AN)	290.44	8.36	273.3/147.2	90	25	10	291.4/199.2	60	30	10
Pregnenolone (P5)	316.48	8.63	317.4/159.3	30	30	11	317.4/255.3	30	15	12
Dihydroprogesterone (5α-DHP4)	316.48	9.31	317.2/85.1	100	20	5	317.2/159.2	100	35	10
17OH-Allopregnanolone (17OH-Allo)	334.49	9.40	317.3/111.2	45	25	5	299.3/135.2	90	30	5
Allopregnanolone (Allo)	318.49	10.68	319.3/257.3	60	20	8	319.3/135.2	60	30	5
d2–11-Deoxycortisol (d2–11-S)	348.46	4.43	349.3/109.2	120	40	5	349.3/97.1	120	40	5
d5-Androstenedione (d5-A4)	291.44	5.14	292.3/100.2	110	35	2	292.3/113.2	110	30	3
d5-Testosterone (d5-T)	293.46	5.68	294.3/100.2	110	40	2	294.3/113.2	110	30	3
d8–17OH-Progesterone (d8–17OH-P4)	338.46	5.84	339.6/100.2	100	40	2	339.6/113.2	100	40	3
d9-Progesterone (d9-P4)	323.52	7.51	324.4/100.2	110	35	2	324.4/113.2	110	40	3

Positive electrospray ionization and 10 eV entrance potential were used for all analytes. MRM: multiple reaction monitoring; DP: declustering potential; CE: collision Energy; CXP: cell exit potential. *For avoiding oversaturation, a sub-optimal CE was selected, inducing about the 10% of the highest achievable signal.

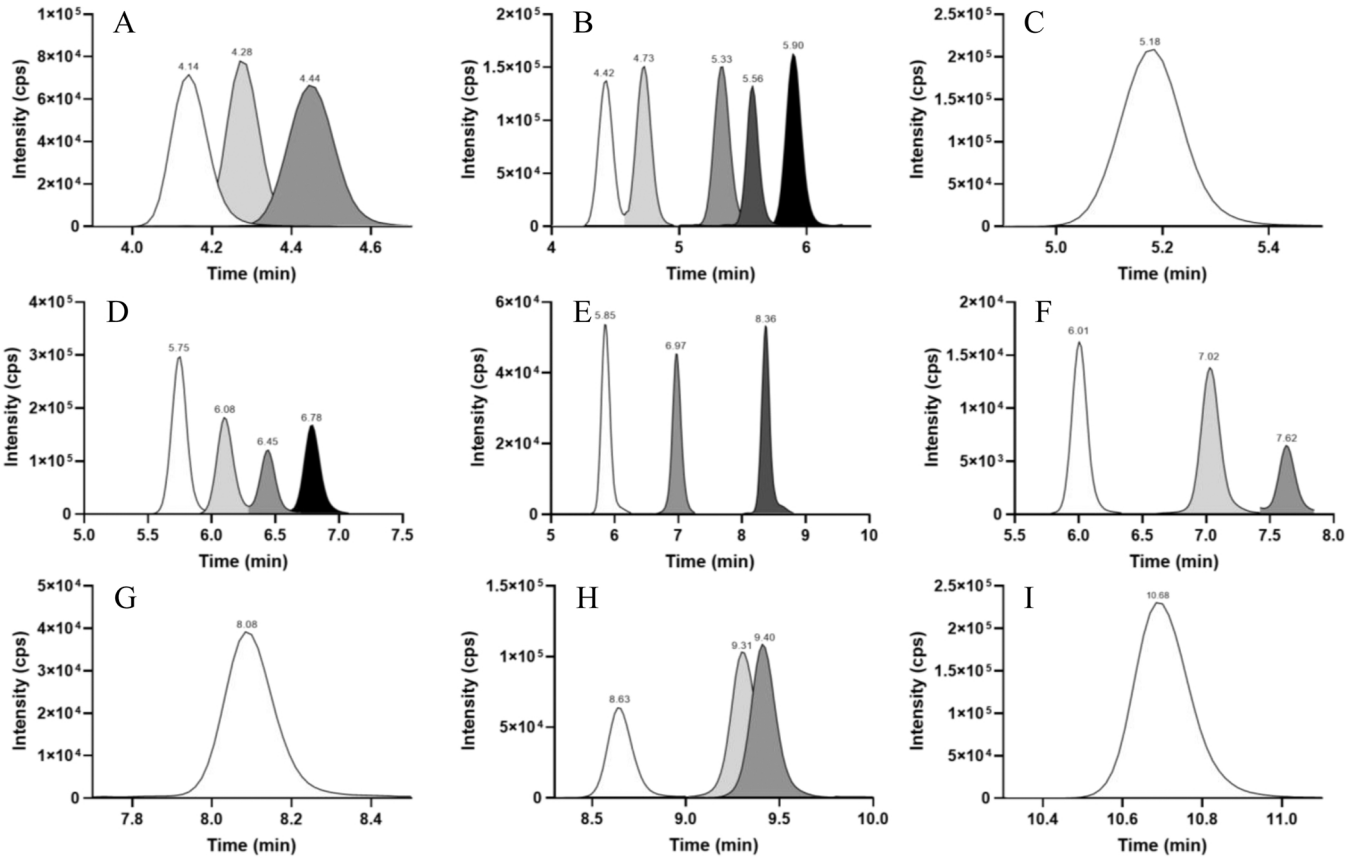

Fig. 2. LC peak separation with focus of cross interfering compounds. A: 21-deoxycortisol (4.14 min), corticosterone (4.28 min) and 11-deoxycortisol (4.44 min); B: 16OH-progesterone (4.42 min), 11αOH-progesterone (4.73 min), 11-deoxycorticosterone (5.33 min), 11βOH-progesterone (5.56 min) and 17OH-progesterone (5.90 min); C: androstenedione (5.18 min); D: testosterone (5.75 min), dehydroepiandrosterone (6.08 min), androstanedione (6.45 min) and epitestosterone (6.78 min); E: androstenediol (5.85 min), dihydrotestosterone (6.97 min) and androsterone (8.36 min); F: 17OH-pregnenolone (6.01 min), 17OH-dihydroprogesterone (7.02 min) and progesterone (7.62 min); G: androstanediol (8.08 min); H: pregnenolone (8.63 min), 5α-dihydroprogesterone (9.31 min) and 17OH-allopregnanolone (9.40 min); I: allopregnanolone.

dilution quantitation was obtained by 1/x weighted linear regression using d2–11-S as IS for 16OH-P4 and 11-S, d5-A for A4 and 11-DOC, d8–17OH-P4 for 17OH-P4, d5-T for A5, T, 17OH-P5, DHEA, 5α-dione and Epi-T, d9-P4 for DHT, 17OH-DHP4, P4, 3α-diol, AN, P5, 5α-DHP4, 17OH-Allo and Allo. Quantitation was performed in 10 μL injections, with some exceptions. Indeed, injections at 1 μL were used for upper calibrators showing loss of linearity due to signal oversaturation for A5, 17OH-P5, DHEA, 5α-dione, 17OH-DHP4, 3α-diol and AN. Moreover, 1 μL injections were used to measure A4 and T, because of their very large concentration in the study samples, and 11-DOC, because of ion suppression from the coeluting A4. Five to seven calibration points were defined for all analytes, except for 17OH-Allo, achieving acceptable performance in four points. The sensitivity in supernatants ranged from 0.012 nmol/L of 11-S to 38.0 nmol/L of 3α-diol.

3.1.5. Recovery and matrix factor

Recovery ranged within 55.6–63.1% for the early eluting analytes 16OH-P4, 11-S and d2–11-S, and within 88.6–101.1% for other compounds. Matrix factor ranged within 94.9–104.7% for all compounds (Supplemental Table 1). The post-column infusion experiment showed a region of signal suppression around 6.5 min which did not impact on RT of any analyte. Among tested drugs, sildenafil caused a slight signal suppression around min 5 (Supplemental Fig. 2).

3.1.6. Imprecision and trueness

Intra- and inter-assay CVs were $< 9.1\%$ and 10.0%, respectively, and trueness within 93.4–122.0% for all analytes at the three tested concentrations (Supplemental Table 2).

3.1.7. Stability

Analyte stability in experimental conditions is shown in Supplemental Table 3. The maximum deviation observed in culture medium after 8 h at 37 °C was 86.7%, whereas in extracts kept at 8 °C for 24 h it was 93.9%.

3.2. Steroid levels in supernatants from unstimulated and hCG-stimulated mLTC1 cell

Steroid levels observed in study samples are reported in Fig. 3 and Supplemental Table 4. Levels within the measurement range were found for 16OH-P4, 11-S, A4, 11-DOC, T, 17OH-P4, 5α-dione, Epi-T, DHT, P4 and AN in both conditions, and for 17OH-Allo in hCG-treated samples. The highest concentrations were achieved by AN (290.5 ± 33.7 nmol/L), 5α-dione (56.4 ± 4.8 nmol/L) and A4 (8.54 ± 0.87 nmol/L) in unstimulated, and by A4 (726.8 ± 88.6 nmol/L), 5α-dione (773.8 ± 163.5 nmol/L) and AN (379.4 ± 80.0 nmol/L) in hCG-treated samples. A trace signal slightly below or above the LOD could be observed for 3α-diol, 5α-DHP4, 17OH-DHP4 and 17OH-Allo. Although a reliable quantitation below the LLOQ is not possible, we reported the concentrations referred to those traces in an attempt to roughly estimate the effect of hCG. Hence, we found that hCG induced a modest increase of AN, 3α-diol, 5α-DHP4, 17OH-DHP4 and 17OH-Allo (1.3–8.1 folds), moderate increase of Epi-T, P4, 5α-dione, 17OH-P4, DHT, 11-S and 11-DOC (12.2–27.5 folds), and a large increase of 16OH-P4, A4 and T

Table 2
Parameters of the calibration curve and assay sensitivity.

Analyte	Internal standard	Calibration points	Range	Slope	Intercept	R^2	LOD		LLOQ				Sensitivity in supernatants
			nmol/L				fmol o.c.	S/N	nmol/L	CV (%)	Bias (%)	S/N	nmol/L
16OH-Progesterone (16OH-P4)	d2–11-S	7	0.187 – 136.2	0.2250 ± 0.0044	0.0119 ± 0.0017	0.9994	0.62	3.7	0.187	10.6	102.1	10.7	0.037
11-Deoxycortisol (11-S)	d2–11-S	6	0.059 – 14.43	0.3887 ± 0.0116	0.0000 ± 0.0000	0.9998	0.30	3.1	0.059	3.8	110.2	8.5	0.012
Androstenedione (A4)*	d5-A4	6	21.6 – 5237	0.0115 ± 0.0006	0.0000 ± 0.0000	0.9999	7.18	4.0	21.5	16.4	103.9	14.7	4.31
11-Deoxycorticosterone (11-DOC)*	d5-A4	6	0.374 – 90.8	0.2597 ± 0.0055	0.0003 ± 0.0005	0.9995	0.13	3.5	0.374	6.6	106.1	12.8	0.075
Testosterone (T)*	d5-T	7	0.713 – 520.1	0.0332 ± 0.0022	0.0000 ± 0.0000	0.9999	5.31	3.6	0.713	12.0	100.4	5.6	0.143
Androstenediol (A5)	d5-T	6	42.5 – 10,329	0.0005 ± 0.0002	0.0000 ± 0.0000	0.9989	318	3.2	42.5	5.2	96.4	5.1	8.50
17OH-Progesterone (17OH-P4)	d8–17OH-P4	7	0.125 – 90.8	0.2610 ± 0.0145	0.0000 ± 0.0000	0.9999	0.95	3.1	0.125	16.7	98.9	5.3	0.025
17OH-Pregnenolone (17OH-P5)	d5-T	6	18.6 – 4512	0.0008 ± 0.0001	0.0000 ± 0.0000	0.9992	141	3.5	18.6	17.7	98.0	5.4	3.71
Dehydroepiandrosterone (DHEA)	d5-T	6	25.7 – 6241	0.0017 ± 0.0006	0.0000 ± 0.0000	0.9994	192	3.3	25.7	16.5	93.0	6.4	5.14
Androstanedione (5α-dione)	d5-T	7	17.1 – 12,482	0.0026 ± 0.000	0.0000 ± 0.0000	0.9997	57.1	4.0	17.1	7.7	94.2	12.4	3.42
Epitestosterone (Epi-T)	d5-T	7	0.190 – 138.7	0.07360 ± 0.0010	0.0000 ± 0.0000	0.9999	0.63	3.6	0.190	4.1	101.2	14.5	0.038
Dihydrotestosterone (DHT)	d9-P4	6	1.42 – 344.3	0.0099 ± 0.0007	0.0000 ± 0.0000	0.9995	8.52	3.4	1.42	14.5	94.1	9.4	0.283
17OH-Dihydroprogesterone (17OH-DHP4)	d9-P4	6	6.19 – 1504	0.0045 ± 0.0007	0.0000 ± 0.0000	0.9997	20.6	3.7	6.19	3.2	83.6	8.8	1.24
Progesterone (P4)	d9-P4	7	0.131 – 95.4	0.1777 ± 0.0106	0.0000 ± 0.0000	0.9999	0.44	4.0	0.131	2.1	102.4	11.3	0.026
Androstanediol (3α-diol)	d9-P4	5	190.0 – 15387	0.0002 ± 0.0000	0.0000 ± 0.0000	0.9980	633	3.5	190	0.1	81.5	7.1	38.0
Androsterone (AN)	d9-P4	7	7.08 – 5165	0.0077 ± 0.0003	0.0000 ± 0.0000	0.9996	23.6	3.5	7.08	11.4	93.8	10.3	1.42
Pregnenolone (P5)	d9-P4	5	5.85 – 474.0	0.0028 ± 0.0003	0.0000 ± 0.0000	0.9993	29.3	3.2	5.85	11.1	94.7	5.9	1.17
Dihydroprogesterone (5α-DHP4)	d9-P4	5	11.7 – 947.9	0.0020 ± 0.0006	0.0000 ± 0.0000	0.9997	39.0	3.6	11.7	3.5	90.4	8.1	2.34
17OH-Allopregnanolone (17OH-Allo)	d9-P4	4	16.6 – 448.4	0.0028 ± 0.0001	0.0000 ± 0.0000	0.9997	55.3	4.6	16.6	2.9	94.1	9.2	3.32
Allopregnanolone (Allo)	d9-P4	5	5.81 – 471.0	0.0038 ± 0.0010	0.0000 ± 0.0000	0.9996	34.7	3.0	5.81	3.3	99.5	5.9	1.16

LOD: limit of detection; LLOQ: lower limit of quantification; o.c.: on column; S/N: signal-to-noise ratio; d2-11-S: d2-11-deoxycortisol; d5A4: d5-androstenedione; d5-T: d5-testosterone; d9-P4: d9-progesterone. *data referred to 1 μL injections.

(80.2–102.5 folds).

No peak of 11αOH-progesterone and 11βOH-progesterone, corticosterone, 21-deoxycortisol, cortisol, estrone and estradiol could be found in any tested culture condition (Supplemental Table 5), therefore, these analytes were not included in the panel to ease the practicability of the method.

4. Discussion and conclusions

We developed a LC-MS/MS method to quantify a panel of 20 steroids belonging to the canonical and backdoor androgen pathways, plus P4 metabolites. We validated the method for its application to the study of the mLTC1 model [31]. mLTC1 cells are permanently expressing the murine Lh receptor, which is structurally similar to the human receptor and capable of binding human LH and hCG [32,33]. Most importantly, human ligands trigger steroidogenic signals mainly activating the synthesis of Δ4 hormones [34], although Δ5 steroid production was described as well [35]. However, comparative analyses between mLTC1 and human Leydig steroidogenesis must be considered carefully, as the two models differ for their enzymatic *milieu* [36].

The method overall showed good recovery, precision, trueness and stability, with no relevant matrix effect. Specificity among isobars was achieved by careful LC separation and fragment ion selection. Moreover, to guarantee the reliability of steroid results in the present study and in future *in vitro* as well as *in vivo* studies, we verified the absence of interferences from steroids not included in the panel and from phosphodiesterase 5 inhibitors. As expected, Δ4 steroids exhibited much higher sensitivity than neutral Δ5, 5α and 5α,3α steroids. Notably, the sensitivity of A4 and T was purposely detuned for avoiding signal oversaturation. Previous LC-MS/MS applications to neutral steroids often used derivatization to enhance sensitivity [20–23]. However, given the chemical diversity within our panel, derivatization is hardly practicable and would also complicate the preanalytical stage. As similarly reported [37,38], ammonium fluoride enhanced the signal of 3–9 folds for the overall panel. Unfortunately, it diminished the signal of A5 and 3α-diol. In future, the sensitivity of our method could be ameliorated by moving to a high-end MS instrument. Indeed, a recent study used atmospheric pressure photoionization and a last generation triple quadrupole to measure a similar panel in serum [24].

AN, 5α-dione and A4 were the most abundant steroids secreted by mLTC1 both in unstimulated and hCG-stimulated conditions, although in different relative abundance, *i.e.* 35:6:1 and 1:2:2, respectively. The accumulation of these intermediates could be explained by the fact that they all are substrates of the 17β-HSD3, which was found to be minimally expressed in murine Leydig cells [36]. On the opposite hand, levels of Δ5 steroids were undetectable.

Interestingly, unstimulated cells secreted DHT and T at similar levels. Moreover, T is 20–100 folds less abundant than other DHT precursors 3α-diol and 5α-dione. Notably, hCG induced about 100 fold increase in A4 and T, but only 16.6-fold increase in DHT. A modest increase was also observed in intermediates from the backdoor pathway. However, results about 17OH-DHP4, 3α-diol and 5α-DHP4 are to be taken with caution as only trace levels were found in tested samples.

Taken together, these data suggest that, in absence of gonadotropin, minimal DHT production is, at least in part, maintained through the backdoor pathway. hCG strongly activated the canonical pathway, with an important effect on the generation of T rather than DHT. This may be due to a weak upregulation of 5α-reductase by hCG in mouse Leydig cells, leading to relatively low T-to-DHT conversion rate [39]. Additionally, albeit weak, we observed an effect of hCG on the backdoor

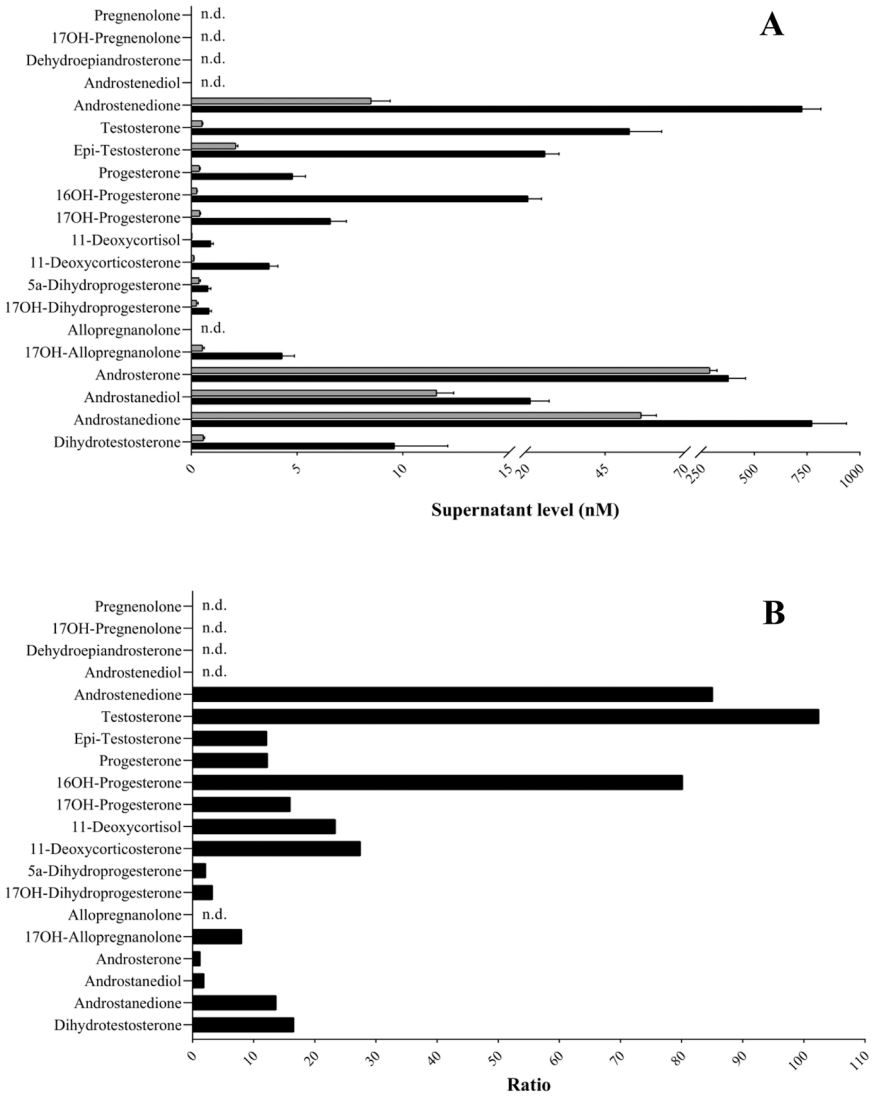

Fig. 3. A: Steroid levels in the culture medium of mouse Leydig tumor cell line 1 in unstimulated condition (grey bars) or upon stimulation with 100 pM human chorionic gonadotropin (black bars). B: Fold increase of steroid levels in culture medium of 100 pM hCG-stimulated compared to unstimulated mouse Leydig tumor cell line 1.

pathway, supporting previous hypothesis about the relevance of the maternal hormone in upregulating the backdoor route during the fetal development [4,5].

Unstimulated mLTC1 cells basally secrete Epi-T levels four times higher than T. Interestingly, hCG enhanced T secretion more than Epi-T, inducing a final T/Epi-T ratio of 2:1. This is consistent with *in vivo* T/Epi-T circulating ratio, increasing during puberty upon the raise in LH secretion [11].

hCG induced about 15-fold elevation of P4 and 17OH-P4 levels. While not corroborating the overall modest increase of backdoor pathway steroids, this finding is consistent with the hCG-induced 25-fold increase of the 21-hydroxylated metabolites 11-DOC and 11-S. The expression of adrenal-specific enzymes in mice testis was previously described [40]. For this reason, in the early stage of method development, we tested the presence of other steroids derived by the 11-hydroxylation of P4, such as 11OHα-P4 and 11OHβ-P4, of 11-DOC, such as corticosterone, of 17OH-P4, such as 21-deoxycortisol, and of 11-S, such as cortisol, and found they were undetectable.

In contrast with previous studies suggesting 16OH-P4 is specific for the primate testis [41], we reported relevant levels of this P4 metabolite in our murine cells. Moreover, in agreement with Storbeck et al. [13], we found 16OH-P4 and 17OH-P4 are secreted in a 1:2 ratio from untreated mLTC1 cells. Surprisingly, the ratio changed to 3:1 upon hCG stimulation. Of note, the extent of the increase in 16OH-P4, about 80-fold, is similar to the increase observed for Δ4 androgens, somehow suggesting a relevant physiologic role of the former. Unfortunately, very little is known about 16OH-P4 [13,41]. We hypothesize that this metabolite may counteract the effect of increasing P4 levels at its receptor, possibly favoring the utilization of the latter as backdoor precursor.

In a previous study in hCG-treated mLTC1 cells, T, A4 and P4 secretion was reported in 1:20:60 relative concentration, respectively [42]. In contrast, in our hands, these steroids are in 11:152:1 proportion, respectively. Such differences may depend on the lower hCG exposure time of 1 h [42] instead of 24 h as here, possibly not allowing the full downstream metabolization of P4.

Finally, in the early development stage we also tested the presence of estrogens in mLTC1 supernatants, and found they were undetectable. It cannot be excluded, however, that an upgrade of instrumental sensitivity would result in measurable levels of both Δ5 and estrogen classes.

In conclusion, our LC-MS/MS method allows exploring the steroidogenesis flow according to multiple pathways. To our knowledge, no previous study has provided such a broad characterization of steroid secretion of Leydig cells *in vitro*. Future studies are needed to explore the

steroid secretion pattern of other Leydig cell lines or primary cultures from animal models and humans, and to characterize how the different routes are modulated by hormones or drugs. In addition, our steroid profiling tool could be applied to the *in vitro* and *in vivo* characterization of diseases featured by a deranged androgen synthesis, such as abnormalities of fetal masculinization [4], or of conditions requiring hCG administration, such as hypogonadic hypogonadism and maldescended testes [18].

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Author contributions

FF and LC conceived, designed and coordinated the study. mL contributed to the study design. MMa, MMe and AT performed the LC-MS/MS experiments. SL performed the cell culture experiments. FF, MMa and LC wrote the manuscript. CP, DS, ML, MS, UP and LC contributed in interpreting results and in writing the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jsbmb.2023.106270.

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3.1.2. LH and hCG differential steroidogenesis pathway on target Leydig cells

3.1.2.1. Chemicals and hormones

The following chemicals were used in the study: 16OH-progesterone (16OH-P4), 11-deoxycortisol (11-S), androstenedione (A4), 11-deoxycorticosterone/21OH-progesterone (11-DOC), testosterone (T), androstenediol (A5), 17OH-progesterone (17OH-P4), 17OH-pregnenolone (17OH-P5), dehydroepiandrosterone (DHEA), androstanedione (5α-dione), 17α-epitestosterone (Epi-T), dihydrotestosterone (DHT), 17OH-dihydroprogesterone (17OH-DHP4), progesterone (P4), androstanediol (3α-diol), androsterone (AN), pregnenolone (P5), 5α-dihydroprogesterone (5α-DHP4), 17OH-allopregnanolone (17OH-Allo), allopregnanolone (Allo), cortisol, corticosterone, 21-deoxycortisol, 11αOH-progesterone, 11βOH-progesterone, estrone, and estradiol. These compounds were obtained from Steraloids (Newport, RI, USA). Additionally, T-[2,2,4,6,6–2H5] (d5-T, 98.7% deuterium content) and A4-[2,2,4,6,6–2H5] (d5-A4, 98%) were sourced from Cambridge Isotope Laboratories (Tewksbury, MA, USA); 17OH-P4-[2,2,4,6,6,21,21,21–2H8] (d8–17OH-P4, 98.7%), P4-[2,2,4,6,6,17α,21,21,21–2H9] (d9-P4, >98%), and 11-S-[4-Pregnen-17α,21-diol-3,20-dione-21,21–2H2] (d2–11-S, >98%) were obtained from CDN Isotopes (Pointe-Claire, Canada). All standards and isotopes were provided in lyophilic form. Solvents including LiChroSolv grade methanol, chloroform, N-hexane, and ethyl-acetate were procured from Merck KGaA (Darmstadt, Germany). LC-MS grade ammonium fluoride was obtained from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was generated using the MilliQ Gradient A10 system (Burlington, MA, USA).

Recombinant hCG (Ovitrelle, Merck KGaA) and LH (Luveris, Merck KGaA) were supplied in injectable saline buffer.

3.1.2.2. Cell Culture and Treatments.

The mLTC1 cell line was handled following previously established protocols (Casarini *et al.*, 2019; Limoncella *et al.*, 2022). In brief, mLTC1 cells were cultured in RPMI medium without phenol red, supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 2 mM glutamine, and 1 mM HEPES (Invitrogen, Carlsbad, CA). The cells were maintained at 37°C and 5.0% CO_2. A seeding density of 3×10^4 cells/well was used in 24 multi-well plates, and the cells were allowed to adhere for 24 h prior to any treatments. Overnight serum-starved cells were washed twice with 37°C phosphate-buffered saline and subsequently treated 24 h with 100 pM hCG or 500 pM LH, as the sub-saturating concentrations calculated for testosterone production in mLTC1 cells (Riccetti *et al.*, 2017a). Gonadotropins were diluted

in RPMI medium without phenol red and added with 0.2% bovine serum albumin (Sigma-Aldrich) (Casarini *et al.*, 2012). Control cells underwent the same treatment with a solution lacking hCG/LH.

3.1.2.3. Sample Preparation

Samples were thawed, vortexed, and centrifuged at room temperature 5 min at 5500 g. Five-hundred µL of each sample and quality control (QC) were pipetted into 13 × 100 mm Pyrex® tubes (Sigma-Aldrich). Subsequently, 100 µL of internal standard (IS) was added, and the mixture was vortexed for 1 minute. Following the initial vortexing, 0.5 mL of water was added to the tubes, and the content was vortexed for an additional 1 minute. Two mL of N-hexane:ethyl-acetate (8:2) were introduced, and the tubes were vigorously vortexed for 5 minutes before being centrifuged (5 min, 3000 g, room temperature). The lower aqueous layer was frozen in an ice bath, and the upper organic layer was carefully decanted into 12 × 75 mm glass tubes (Laboindustria, Arzergrande, Italy). The organic layer was then dried under a nitrogen flow. The dried samples were reconstituted with 100 µL of 75% methanol and transferred into autosampler glass vials (Agilent Technology, Santa Clara, CA, USA). Each batch included supernatants, calibrators, and three QC replicates strategically placed at the beginning, middle, and end of the batch to ensure consistency and accuracy throughout the analysis.

3.1.2.4. Liquid Chromatography

The liquid chromatography setup involved the use of the PerkinElmer Series 200 (Waltham, MA, USA) High-Performance Liquid Chromatography (HPLC) system. This system was equipped with a LUNA® C8(2) 100 Å 100 × 3.0 mm, 3 µm column and a C8 4 × 2.0 mm guard column (Phenomenex, Torrance, CA, USA), which was maintained at 45 ◦C. Solvent A consisted of 100 µM ammonium fluoride in water, while solvent B was methanol. The gradient, operating at 0.4 mL/min, initiated with 45% B, increased to 62% B from 0.3 to 0.6 minutes, and further increased to 78.3% B until 10 minutes. Complete elution with 100% B occurred at 10.2 minutes and was maintained until 11.2 minutes, followed by reconditioning to 45% B until 13 minutes. The autosampler was set at 8 ◦C, and injections were carried out at volumes of 1 and 10 µL.

3.1.2.5. Mass Spectrometry

Mass spectrometry was conducted using the API-4000 QTrap triple-quadrupole system (Sciex, Framingham, MA, USA) in electrospray positive ionization mode, operated at 750°C and 5500 V. The nebulizing, heating gas (air), curtain, and collision-activated dissociation nitrogen were set at 60, 65, 20 psi, and "medium", respectively. Data processing was performed using Analyst v1.7 (Sciex).

3.1.2.6. Statistics

Means and standard deviations, from a total number of 6 independent replicates, were computed. Comparison between untreated vs LH- and hCG-induced steroid production was performed by Kruskal Wallis test. A significance level of $p<0.05$ was considered statistically significant, although lower p values were indicated as well.

3.1.2.7. Results

Steroid Levels in Supernatants from LH- vs hCG-treated mLTC1 Cells

Steroid levels observed in study samples are presented in **Figure 1 and 2** and were partially previously published (Fanelli *et al.*, 2023). Cells were treated 24 h with sub-saturating LH and hCG concentrations, and total steroid production was measured. Untreated samples served as controls. Levels out from the measurement range were found for pregnenolone, 17OH-pregnenolone, DHEA, androstaediol, and allopregnanolone in all samples. The analysis of results revealed gonadotropin-specific steroid pattern. In particular, LH induced higher P4 and dihydroprogesterone levels than hCG (**Figure 1**), while both the gonadotropins induced similar DHT levels. 17OH-Progenterone and all androgens were effectively upregulated by hCG, suggesting that the choriogonadotropin is more potent than LH in activating the pathway for DHT synthesis in the Leydig cells. In particular, DHT was even inhibited by LH treatment, since the levels of this steroid were below those detected in untreated cells. Interestingly, hCG upregulated also backdoor neurosteroids, i.e. Allopreganolone, Androsterone and Androstanediol, whose production is unexpected in the Leydig cell, while they are overall inhibited by LH.

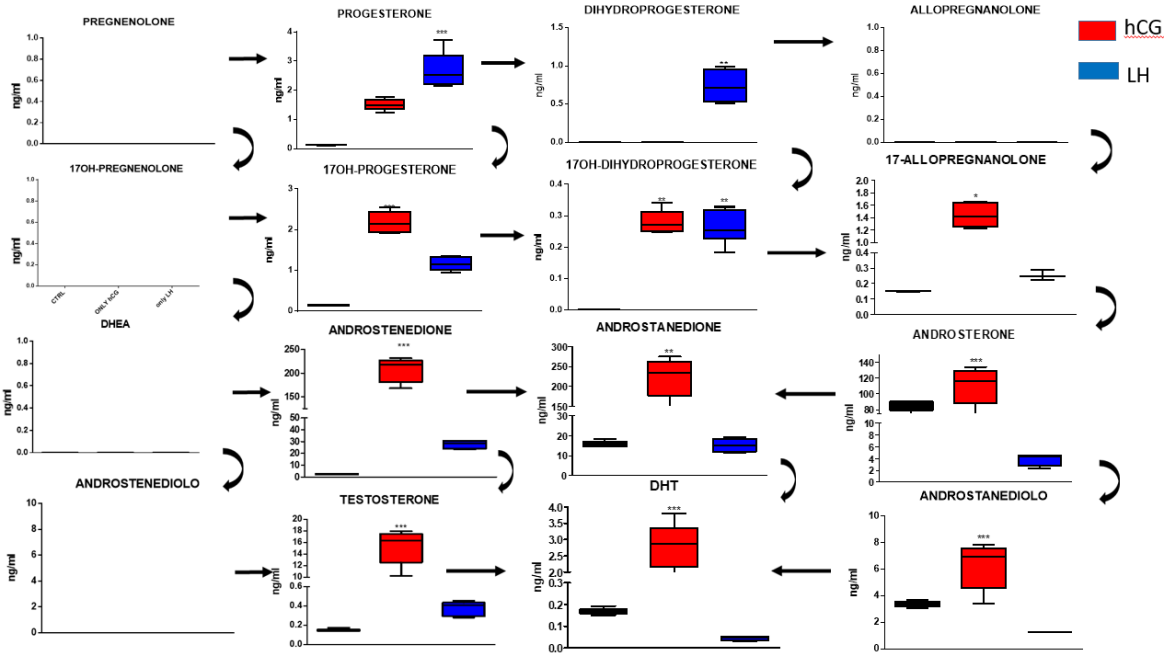

Figure 1. LH/hCG-induced mLTC1 cell steroidogenesis. Cells were treated 24 with 100 pM hCG and 500 pM LH and steroids measured by LC-MS/MS. Data are showed with box and whiskers plot and the statistical analysis performed by Kruskal Wallis test (*statistically different than untreated samples with p<0.05; **p<0.001; ***p<.0001).

Adrenal steroids were also found in media of mLTC1 cells. The production of both deoxicorticosterone ans 11-Deoxicortisol was upregulated by both gonadotropins and achieved higher levels upon treatment by hCG than LH. These steroids were represented in the **Figure 2** as metabolites of Progesterone and 17OH-Progesterone.

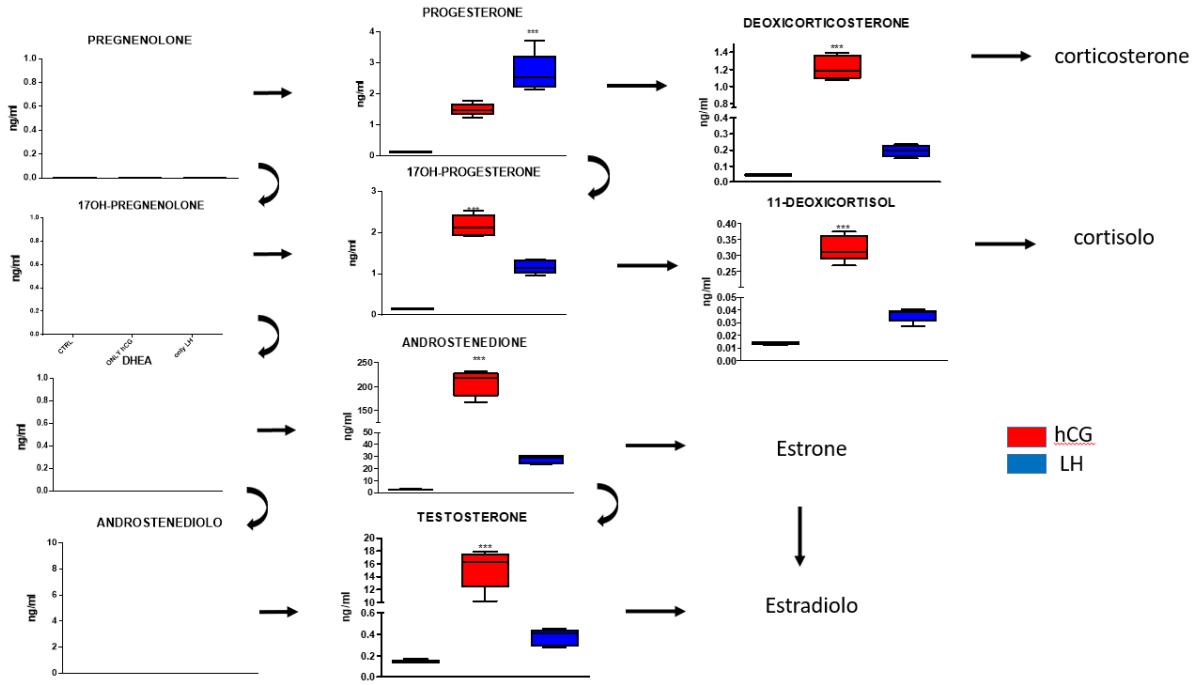

Figure 2. LH/hCG-induced adrenal steroids in mLTC1 cell. Adrenal steroids are shown together with some of those belonging to the "classical" Leydig steroidogenic pathway. Data are showed with box and whiskers plot and the statistical analysis performed by Kruskal Wallis test (***statistically different than untreated samples with p<.0001).

3.2. Assess pharmacodynamics and safety of r-hLH (Investigational treatment) and u-hCG (standard treatment) in hypogonadotropic hypogonadal (HH) men (RHYTHM trial)

3.2.1. Objectives of the study

The main objective of this study is to assess the pharmacodynamics of recombinant LH, comparing the response to LH and hCG in HH patients. Secondary objective is the identification of LH dosages required to restore eugonadism in HH men. Eugonadism is achieved when testosterone serum levels are higher than 3.0 ng/mL (Bhasin *et al.*, 2010). Moreover, a further secondary objective is the evaluation of the suitability of hCG doses currently used in clinical practice.

The results of this study are necessary for future clinical trials to test the hypothesis that, being the physiological hormone, the addition of LH is better than hCG in those stimulation in which LH activity is required. Stimulation of spermatogenesis, which requires several months of treatment, is neither objective of this study, nor an efficacy parameter of short-term LH/hCG action**.** The pharmacodynamics of LH and the comparison of response to LH and hCG are assessed by measuring serum steroid levels by LC-MS/MS. The primary end point is serum testosterone levels in response to increasing doses of LH (pharmacodynamics) and in comparison, with the response to hCG. The statistical hypothesis is non-inferiority of the highest LH dose compared to the hCG dose employed. However, since the hCG dosages used in the clinic are empirical and not evidence-based, we will compare increasing LH dosages with increasing hCG dosages, escalating up to the commonly used dosage of hCG in clinical routine (standard of care).

3.2.2. Study design

This is a multicenter, longitudinal, interventional, randomized, open-label, phase II, clinical trial. The study is designed to characterize recombinant LH pharmacodynamics in men, compared to the standard approach, represented by hCG administration.

The center at the Principal Investigator (PI) site (Unit 1: University of Modena and Reggio Emilia) is responsible to coordinate the study and to prepare the randomization list by permuted blocks, which is sent to each enrolling center. Moreover, Unit 1 is responsible to perform centralized hormone measurements at the end of the study, to coordinate monitoring of centers, to maintain the study database and to analyze the data collected. Finally, the pharmacy at Unit 1 is in charge to receive the IMP, to buy the drug comparator and to package and distribute drugs needed for the study to the other Units.

Patients are screened according to inclusion and exclusion criteria at each Center. During the screening visit, the following evaluations are performed:

Evaluations at the participating center:

- Total testosterone serum levels (using routine immunometric methods)
- Basal hormonal and biochemical assays needed to fulfil inclusion and exclusion criteria
- Scrotal ultrasound for the evaluation of:
  - varicocele presence and degree
  - testicular volume and structure
  - epididymis
- All other imaging analyses needed to fulfil inclusion and exclusion criteria, as well as suggested by the guidelines for pituitary diseases.

Central evaluations at Unit 1:

- Total testosterone serum levels, using LC-MS/MS
- Other hormonal analyses

Eligible patients are enrolled in the study after signing the informed consent. Eligible patients that are under androgen replacement therapy at the time of screening visit are enrolled after three months of testosterone withdrawal. On the contrary, eligible patients who are not treated at the time of screening visit are immediately enrolled.

At Visit 1 (V1), patients are randomized according to the randomization list centrally provided and are allocated to the two following groups:

- Study group
- Control group

Each group undergoes two consecutive phases:

1. Treatment phase
2. Follow-up phase

The first phase will last eight weeks, followed by a follow-up of 4 weeks after treatment withdrawal. Total study duration is 12 weeks. Patients are evaluated two times weekly, during the treatment phase and one time every two weeks during the follow-up phase. The patient is monitored for safety at each visit by the medical staff at PI and participating centers. The patient will remain under supervision for four hours at each visit.

At each visit two blood samples are collected (total of 18ml) in the morning after an overnight fast. The first serum sample is stored at -20°C and sent to Unit 1 at the end of the study for central measurements. The second blood sample is used locally to monitor the treatment efficacy and safety. Overall, the study protocol includes a screening visit followed by 19 visits

(see study scheme). During the study protocol, pharmacovigilance is performed according to Italian regulations.

At each visit the following evaluations are performed:

Evaluations at each participating center:

- Total testosterone serum levels
- LH and FSH serum levels
- Safety profile (as elaborated below)
- Basal hormonal and biochemical assays

Central evaluations at Unit 1:

- Total testosterone serum levels, using LC-MS/MS
- Other sex steroids using LC-MS/MS
- Other hormonal analyses

Biochemical evaluations performed by each center is used to monitor the treatment efficacy and safety, whereas centrally performed analyses are used for the final statistical analysis. Moreover, at the end of treatment phase (V17), and at the end of follow-up phase, scrotal ultrasound is repeated to address possible morphological changes related to endogenous testosterone increase. Pharmacovigilance is carefully considered and evaluated by the Clinical Research Operation (CRO). During each visit, testosterone levels are measured locally and evaluated by clinicians. Whenever testosterone serum levels will reach levels higher than 12 ng/mL, the drug dosage will not be further increased in that patient. The hormonal results are available in about 8 hours.

3.2.3. Study population

All men with acquired HH are evaluated during screening visit for inclusion in the study, according to inclusion and exclusion criteria provided below. Patients enrolled are otherwise healthy and no fragile (such as older patients or patients with cancer) population is involved.

The participating centers are public, secondary or tertiary referral centers specialized in endocrinology across Italy, in a university hospital setting.

The enrolment procedure is followed as described in the previous section. The allocation is following the randomization list. When a participating Center is identified and enrolls a patient, it will send communication thereof to the promoter Center, in which a clinician is identified with only randomization/allocation tasks. The clinician with randomization/allocation tasks will include the patient within the randomization list and will communicate to each participating

Center in which group the patient should be included. All participating centers are committed to conclude patient recruitment and accrual within 24 months from study start.

Since treatment for hypogonadism is usually offered to these patients, no specific difficulty from the side of the participants is expected. Treatment compliance is checked by asking the patients to return the empty drug devices at the next visit. This parameter is confirmed by measuring testosterone, LH or hCG serum levels locally at each visit.

Participants will have the right to withdraw from the study at any time for any reason. Data are collected in eCRF at each visit, even in case of patient withdrawal. Adverse events and serious adverse events (SAE) are registered. Patients will discontinue treatment in case of SAE even if most probably not imputable to the medication. No specific procedure is foreseen for withdrawal from the study**.**

Inclusion criteria

- Male sex
- Age between 18 and 45 years
- Acquired HH forms

HH after neurosurgery for tumors (i.e. pituitary adenoma, including prolactinoma, craniopharyngioma, germinomas, meningiomas, gliomas, and astrocytomas). Infiltrative disease (hemochromatosis, granulomatous disease, histiocytosis, and sarcoidosis), or HH due to pituitary adenoma-related mass effect, in case of cured or controlled hormone hypersecretion

- Documented damage of the gonadotroph cells, by low or undetectable LH (<1.0 IU/L) and FSH (<1.0 IU/L) serum levels
- Total testosterone serum levels below the normal range (lower than 3 ng/mL)
- No androgen replacement therapies in the last three months before enrolment
- No hypersecretion of other pituitary hormones
- Written informed consent

Patients with HH diagnosed in the past and currently under androgen replacement treatment, could be enrolled three months after of treatment withdrawal.

Exclusion criteria

- Congenital HH forms, such as:
  - Combined pituitary hormone deficiency
  - Genetic syndromes (e.g., Prader-Labhart-Willi, CHARGE, Lawrence-Moon-Bardet-Biedl)
- Iatrogenic HH forms, such as traumatic pituitary stalk interruption syndrome, irradiation, high dose corticosteroids, and anabolic steroids

- Drug abuse and major systemic diseases
- Chronic severe liver disease
- Concomitant illnesses which could interfere with the study participation
- Active malignancy diseases
- Known or possible androgen-dependent tumors for example male breast carcinoma or prostatic carcinoma
- Cardiac failure, hypertension, renal dysfunction, migraines, or epilepsy
- Haematocrit <40% or >54%
- Syndromic congenital HH are excluded since these genetic forms of HH could be related to other systemic diseases, which could bias the selection of patients
- Hypersensitivity to any of the component of the two IMPs (including excipients)
- Hypothalamic or pituitary cancer
- Active thrombophlebitis

3.2.4. Intervention

The Investigational Medicinal Product (IMP) is Luveris. The comparator is Gonasi HP. Luveris is provided by Merck. Gonasi HP, is purchased (packages with vials of 250, 1000 and 2000 IU each) by Unit 1. The entire amount of the IMP and comparator is delivered to the coordinating center (according to existing regulations) and distributed in batches to the centers. At the coordinating center, packages containing the number of ampoules sufficient for the patients to be allocated to the study within the next 6 months are prepared and shipped to each center. The coordinating center is responsible for IMP and comparator reception, labelling, packaging and sending to the centers according to existing regulations. All medications to be used in this study will have been manufactured, tested, and released according to current GMP guidelines.

The investigator or authorized staff will have to document the receipt, dispensation, and return of all IMPs received during this study. Records on receipt, use, return, loss, or other disposition of IMPs are maintained. This process is monitored by a CRO during the study. The IMP shipment is provided in two times during the study duration.

All remaining IMPs, used and unused, shall be collected and returned for destruction at the end of the study.

Luveris is self-administered once daily by the patient subcutaneously (sc) in the abdominal skin. Patients are properly trained on how to perform injection and reconstitute the product. Since the sc route is generally preferred by patients, this route is selected for this trial in order to increase treatment compliance. Luveris is package in vial of 75IU and it is dissolved in a vial

of solvent. When higher dosages of Luveris is needed for the protocol (150, 300 and 600), 2, 4 and 8 vials of Luveris are respectively dissolved in a single vial of solvent. In particular, when 300 IU should be used, 4 Luveris vials are dissolved in 2 vials of solvents. When 600 IU should be used, 8 Luveris vials are dissolved in 3 vials of solvents.

Gonasi HP is injected intra-muscularly (im).

At V1 patients are randomized into two different groups:

- Study group
- Control group

The study group will receive the daily sc administration of Luveris with increasing dosages every two weeks (Treatment phase) as follows:

1. Rec-LH 75 IU daily for 2 weeks
2. Rec-LH 150 IU daily for 2 weeks
3. Rec-LH 300 IU daily for 2 weeks
4. Rec-LH 600 IU daily for 2 weeks

The decision to proceed to the next dose level of LH is made by the Study Team [and the investigator] based on safety, tolerability, and preliminary data obtained in at least 5 participants at the prior dose level. If moderate or severe adverse event (SAE) are consistently observed across participants in a group or if unacceptable pharmacological effects, reasonably attributable to LH in the opinion of the investigator are observed in more than 15% of the participants in a cohort, then dose escalation is temporarily halted and no further participants are dosed until a full safety review of the study has taken place. Relevant reporting and discussion with the Medical Monitor and PI will take place before resumption of dosing. The eight weeks of treatment is followed by two weeks of treatment wash-out (follow-up phase).

The control group will receive im Gonasi HP administration, as follows:

- hCG 500 IU two times weekly, for 2 weeks
- hCG 1000 IU two times weekly, for 2 weeks
- hCG 1500 IU two times weekly, for 2 weeks
- hCG 2000 IU two times weekly, for 2 weeks

Hypopituitary patients participating to the study will continue to receive their standard substitution therapy inasmuch needed (thyroxine, hydrocortisone). During the study duration, androgen replacement therapies are not permitted.

Luveris is provided in vials containing 75 IU of lyophilized material + ampoules of solvent. Patients in the study group is instructed to use one vial/d during the first two weeks of treatment, then two in the second two weeks, four in the third two weeks and eight in the last two weeks

of the treatment phase. Up to 3 vials of lyophilized Luveris can be dissolved using only one vial of solvent, whereas 4 Luveris vials are dissolved in 2 solvents. Eight Luveris vials are dissolved in 3 solvents. This will reduce discomfort for the patient. Both IMP and comparator can be either self-administered by the patient or by a third person, as described in the drug instructions. All patients are followed up for further 4 weeks after drug withdrawal according to the study design.

Compliance is monitored by asking the patients to return the empty Luveris and Gonasi HP ampoules and packages at the next visit. In addition, LH/hCG and total testosterone serum levels are measured at each visit before the next LH/hCG injection at each participating center.

3.2.5. Discontinuation criteria (EMEA/CHMP/EWP/5872/03)

The target testosterone serum levels for both study and control groups are 3-12 ng/mL, i.e the normal range in adult males.

Discontinuation criteria are:

For individual patients

- Total testosterone serum levels at next visit, higher than 12 ng/mL
- Haematocrit above 54%
- An increase in serum prostatic specific antigen (PSA) concentration greater than 1.4 ng/ml compared to baseline levels
- SAE even if most probably unrelated to the drug

For part of the trial: no specific criterion, since no interim analysis is planned and no major reasons for discontinuing an arm can be foreseen.

For the entire trial: no specific criterion, since no major reason for discontinuing the entire trial can be foreseen. SAE clearly related to LH or hCG administration to men were never reported so far, considering the available literature on the topic.

3.2.6. Study Outcomes

The primary outcome of the study is total testosterone serum level increase, measured by LC-MS/MS at each visit. Total testosterone serum levels are evaluated both locally at each Unit and centrally, at Unit 1. Local testosterone measurements are used for safety monitoring, whereas central testosterone assays are used for the final analyses. MS-based steroid measurements represent the reference method for steroid specificity and structure (Handelsman and Wartofsky, 2013). The accurate measurement of hormones is the pivot of pharmacodynamics evaluation. Commercial immunoassays directly detect steroid, bypassing

all the original triplet of validity criteria (mainly extraction), sacrificing accuracy and specificity for throughput speed and lower cost (Handelsman and Wartofsky, 2013). MS-based steroid assays resolve the problems of direct steroid immunoassays, improving the accuracy of the measurement (Handelsman and Wartofsky, 2013).

Secondary outcomes:

- Inhibin B
- Free testosterone serum levels, calculated through the measurement of sex hormone binding globulin (SHBG)
- Serum LH, FSH, anti-Müllerian hormone by immunoassay
- Red blood cell count
- Markers of liver and renal function
- Markers of coagulation
- 1,25 and 25 Hydroxy-vitamin D serum levels, Insulin-like factor 3
- Testicular volume measured at baseline and at the end of treatment and follow-up phases.
- Safety and tolerability as determined by AE, SAE reporting, vital signs, ECG, Concomitant medication, laboratory parameters should be included in each panel (e.g., for haematology, chemistry, urinalysis) and evaluation of anti-Human-LH Antibodies formation in the serum

Both primary and secondary endpoints are evaluated during the screening visit and at each of the following 19 visits. Testicular volume is assessed locally at each visit by a physician blind to the patient group allocation. An aliquot of serum is kept at -20°C after each visit for centralized hormone determination at the end of the study. The lab performing the assays is blind to the patient allocation**.**

3.2.7. Study Design

The study has a prospective, interventional design. Thus, consecutive patients attending the participating Units are enrolled according to inclusion and exclusion criteria. The study design includes the randomization of eligible patients in study and control groups, treated with Luveris and Gonasi HP, respectively. Patients' allocation will follow the random sequence generated at Unit 1 before the start of the study. The list is generated using permuted blocks, considering at maximum 10 patients for each Unit. Moreover, being a pharmacodynamics, phase II clinical trial, a placebo-group is not foreseen. The study, includes a group receiving the "standard" comparator, in order to reveal possible qualitative differences in the biological response.

Considering the different frequency and route of drug administration in the two groups, a double-blind design is not possible. Indeed, the study is open-label, since each patient is aware of the LH or hCG dosage to be used. Similarly, clinicians involved in the study is not blinded to allocation. The following study personnel is kept blinded to the treatment:

- The central lab in which LC-MS/MS assays is performed
- The clinician involved in scrotal ultrasound evaluations
- The statistician who finally will perform the statistical analysis

There are practical and scientific reason for the choice of the drug and the methodology proposed. Concerning recombinant LH (r-hLH), Luveris is the only LH approved for human use. The dosage was chosen according to the scientific evidence of different LH and hCG action at the molecular level, as well as *in vivo*, in women (Santi *et al.*, 2017a). The dosage and the frequency of administration were chosen according to previous experience existing and considering the half-life of LH. Daily injections are appropriate because clinical practice in women undergoing ART or with HH demonstrates the efficacy of LH daily protocols. In our previous experience, LH was administered as single daily bolus, reaching a significant testosterone increase. We considered that physiological LH secretion is pulsatile. Veldhuis et al., (2009) compared constant to pulsatile infusion of r-hLH in 19 healthy men previously treated with GnRH antagonist. These two LH administration patterns reached a similar testosterone increase, suggesting that pulsatile LH administration is not necessary to stimulate Leydig cells activity. Moreover, in this clinical trial, a daily LH dosage of 112.5 IU resulted in maximum testosterone level of 485±114 ng/dl, which falls within the physiological range. Similarly, in our case report, eugonadism was restored in a HH man treated daily with 75 IU of LH. Thus, much lower LH dosages than expected seem to be sufficient to increase testosterone serum levels into the normal range. Therefore, we will test different LH dosages, starting from that expected to be the minimum efficient one (75 IU), to the maximum dose (600 IU). The later dose is expected to induce testosterone serum levels within the upper half of the physiological range.

The comparator drug is the hCG “Gonasi HP” (IBSA, Lugano, Switzerland). Gonasi HP type and dosage were chosen according to the literature. the rationale to choose this drug as a reference is that Gonasi HP is currently the unique hCG preparation approved for use in male HH in Italy.

The increasing hCG dosages has been selected for the control group. Indeed, an evidence-based gold standard indicating fixed hCG dosage does not exist so far, and the current standard of care provides injections of 1500-2000 IU two times weekly. However, the experience of

standard care shows a high heterogeneity of response and, generally, the dosage must be reduced when the treatment continues for several months. Thus, the standard hCG dosages used in clinical practice is empirical and a protocol with escalating hCG dosages is necessary to provide accurate comparison with LH.

Data are gathered with the following modalities.

At the participating Units:

- patient's clinical information including safety and tolerability as determined by AEs, SAE reporting, vital signs, ECG, concomitant medication, laboratory parameters should be included in each panel (e.g., for haematology, chemistry, urinalysis) and evaluation of anti-Human-LH Antibodies formation in the serum
- basal hormonal assessment
- collection and storage of 2 serum samples (18 ml) at -20°
- packaging and shipment of the samples to the centralized laboratory site

Each Unit has received from the Unit 1 a detailed investigator manual and a study kit containing tubes, labels and boxes for sample storage and shipment. All modalities of data collection are explained in an investigator meeting before starting the study.

Each participating Unit follows and treat patients during the entire duration of the trial according to the study design and patients' allocation. All Units possess an institutional accreditation and have a quality management in place.

At Unit 1 (Coordinating center):

- patient's clinical information including safety and tolerability as determined by AE, SAE reporting, vital signs, ECG, concomitant medication, laboratory parameters should be included in each panel (e.g., for haematology, chemistry, urinalysis)
- basal hormonal assessment
- collection and storage of 2 serum samples (18 ml) at -20°
- at the end of the study the center will receive all stored serum samples from the Units and provide centralized hormone analysis through LC-MS/MS. In addition, all other parameters indicated in the section "outcomes" are measured in the hormone laboratory using validated techniques
- keep the overall database
- perform data analysis

All clinical and hormonal data are recorded in appropriate electronic case report forms (eCRFs) provided by an experienced CRO. These data, together with hormonal parameters assessed

centrally and eCRFs data will represent the dataset for the final analysis. The quality of the data is checked by monitoring (site visits) by an independent party, the selected CRO.
Follow-up of the patients will last four weeks after drug withdrawal to check for return of the values to baseline. We planned a possible lost-to-follow-up of 10%.

3.2.8. Sample size calculation

There is very little experience with recombinant LH in male HH. Veldhuis et al. (2009) showed that daily administration of 112.5 IU/day (787.5 IU/week) resulted in serum testosterone levels close to the lowest limit of the normal range in young men treated with a GnRH antagonist. We recently reported one case of a HH man in whom daily administration of 75 IU of Luveris or Gonasi HP (525 IU/week) resulted in similar total testosterone serum levels (3.46 ng/ml by hCG vs 2.49 ng/ml by LH, Mann–Whitney: p=0.245 (Santi *et al.*, 2017b). Thus, from a clinical point of view, it is reasonable to approach our pharmacodynamics study with a non-inferiority approach. A non-inferiority trial evaluates whether the effect of a new drug is not unacceptably worse than the effect of the active comparator (Althunian *et al.*, 2017).
To design a non-inferiority trial, the identification of the non-inferiority margin is needed. This is the largest clinically acceptable difference between the test drug and the active comparator (Acuna *et al.*, 2019). To calculate the non-inferiority margin, we evaluated previous published works. Since our trial does not provide a placebo arm, indirect comparison between LH and placebo was performed considering previous studies. We identified a previously published work in which LH was administered to normal men in comparison to both hCG and placebo. As required by European Medicine Agency guidelines for non-inferiority trials (Rothmann *et al.*, 2003; Agency EM, 2006), we created "historical" confidence intervals (CI). Since the non-inferiority margin must demonstrate both the efficacy and the acceptable efficacy relative to the active comparator (Agency EM, 2006), we evaluated the literature, searching for studies in which LH was compared to placebo (establishing efficacy – CI2) and to hCG (establishing efficacy related to the comparator –CI3). In this setting, LH determined a testosterone raise from baseline higher than placebo. The 95% confidence interval (95%CI) of the delta testosterone (difference between peak and baseline testosterone levels) was calculated for LH (1.75; 1.85 ng/mL) (CI1) and for placebo (0.89; 1.51 ng/mL) (CI2). In order to calculate the expected testosterone raise in our setting, we calculated the 95%CI of testosterone increase when LH was compared to hCG (1.41; 1.79 ng/mL) (CI3). Thus, we calculate the non-inferiority margin for our sample size calculation, combining 95% CIs obtained in previous studies. In particular, we considered the difference between the lower bound of CI2 and the

lower bound of CI3. The final cut-off used was 0.52 ng/mL. Moreover, since Cailleaux-Bounacer et al. (2008) directly compared urinary hCG 5000 IU and increasing recombinant LH (75, 150, 225 IU) in a single administration, we considered the testosterone peaks reached. The testosterone peak obtained for hCG group was 31.4% higher than LH (10.98 + 3.49 ng/mL versus 7.53 + 1.73 ng/mL). A preserved fraction of 50% was clinically considered for the calculation of the sample size in our LH group. Thus, the fixed margin method was used to calculate the 95%CI expected for our trial (Rothmann *et al.*, 2003). Combining this result, with means + standard deviations testosterone levels obtained in previous work, the sample size was calculated, considering an α-error of 0.05, a statistical power of 0.90, and the difference between independent means, using Mann-Whitney's *U*-test. The calculated sample size was 15 patients for each group. In this way, considering a drop-out rate of 10%, the overall number of patients to be enrolled is 32. The total number of patients to be enrolled are divided in the coordinating center and in the four participating centers. Each Center will enroll a maximum of 10 patients, until the sample size is reached.

3.2.9. Statistics

The final analysis is performed considering all data collected during the study. Primary analyses are performed using data obtained by central lab measurements.

The primary outcome is the change of testosterone serum levels after r-hLH (in comparison to the standard treatment by hCG). A dose-response curve for LH is created by plotting LH dosages against total testosterone serum levels to calculate maximum response (Top), the slope (Hill slope), and the drug concentration that provokes a response halfway between baseline and maximum (EC50). The EC50 generally represents the potency of a drug. Moreover, we will calculate the percentage of LH changes needed to obtain a specific testosterone increase, using the Wilcoxon-rank-sum test.

We will then compare the response to the four different LH dosages and to hCG. Finally, steroid profiles obtained by Luveris and Gonasi HP are compared. For these analyses, data are expressed as median [quartiles] when non-normally distributed, and as mean ± standard deviation (SD) when normally distributed. Kolmogorov-Smirnov test will assess the parameter distribution. Data are analysed through ANOVA univariate or Kruskal Wallis test on the basis of normal or not-normal distribution, respectively. On the contrary, categorical data are analysed by Chi-square or Fisher exact tests. Paired t-test is used to compare normal parameters while Wilcoxon Signed Ranks Test is used for non-parametric factors. Finally, correlations among variables are evaluated by Pearson or Spearman's coefficient. Since testosterone serum

levels are measured both centrally with LC-MS/MS and locally by commercially available kits, the comparison between these two analytical methods is performed.
AE and SAE are analysed in a descriptive way. The AE and SAE occurrence (if any) are related to Luveris or Gonasi HP dosages used, applying polynomial regression analyses. Moreover, the AE and SAE frequency are compared to testosterone serum levels reached in both study and control groups.

3.2.10. List of participating centers:

- Unit 1: Unit of Endocrinology of Modena: Prof. Manuela Simoni, PI: Study coordination and logistics, treatment allocation, monitoring (through and experienced CRO), recruiting patients (n=10), data collection, dataset creation and data analysis.
- Unit 2: Department of Experimental Medicine, Section of Medical Pathophysiology, Food Science and Endocrinology, Sapienza - University of Rome (Prof. Francesco Lombardo): patient enrolment and treatment (n=10).
- Unit 3: Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico (Prof. Giovanna Mantovani): patient enrolment and treatment (n=10).
- Unit 4: Division of Endocrinology, Diabetes and Metabolism, Department of Medical Science, University of Turin, Turin (Prof. Ezio Ghigo): patient enrolment and treatment (n=10).
- Unit 5: Dipartimento di Medicina Clinica e Chirurgia, Sezione di Endocrinologia, Università degli Studi di Napoli “Federico II”, Naples, Italy (Prof. Rosario Pivonello): patient enrolment and treatment (n=10).

At the date of December 2023, only Unit 1, 3 and 5 have been initiated. Unit 2 is currently waiting for Ethical Committee approval. On the contrary, Unit 4 will not participate in the study.

3.2.11. Adverse Events and Serious Adverse Events

The only risk related to short-term use of hCG and, presumably, LH in men is excessive increase of testosterone due to overtreatment, mainly increase in hematocrit and PSA, which, however, require chronic treatment. At the dosage proposed here hCG is usually sufficient to restore and maintain testosterone levels within the normal range. hCG is given at the maximal cumulative dose of 4000 IU/week, while the dosage of LH is from 525 to 4200 IU/week. From experimental models 6-8 IU of LH are necessary for a bioequivalent response in vivo (Choi and Smitz, 2014). However, LH action in vitro on cAMP production is much faster and LH is five-times less potent than hCG at equimolar doses (Casarini *et al.*, 2012). For these reasons it is not expected

that LH will overstimulate testosterone production at any of the dosages employed. In any case, serum testosterone levels are monitored at bi-weekly intervals. The duration of the treatment is limited to 8 weeks since this time-interval could be enough to evaluate the testosterone increase. Moreover, patients are followed up so that no major adverse events due to excessive testosterone production are expected. These events are recorded as adverse events of special interest (AESI): gynecomastia, acne, liver enzymes increase, increase of blood pressure.

In particular, the AE reporting period for safety surveillance is beginning when the participant is initially included in the study (date of first signature of informed consent) and continues until End of Study Visit. Any serious AE (SAE) assessed as related to study intervention is recorded and reported. At each study visit, the participant is queried on changes in his condition. During the reporting period, any unfavorable changes in the participant's condition is recorded as AEs, regardless of the reporting by the participant or observation by the Investigator. Complete, accurate and consistent data on all AEs experienced for the duration of the reporting period are reported on an ongoing basis in the appropriate section of the CRF. All SAEs and all nonserious AEs of special interest are additionally documented and reported. All SAEs ongoing at the End of Study Visit are monitored and followed up by the Investigator until stabilization or until the outcome is known, unless the participant is documented as "lost to follow-up". Reasonable attempts to obtain this information is made and documented.

When specifically required by regulations and guidelines, the principal investigator is provided with appropriate Safety Reports directly to the concerned lead IEC/IRB and to maintain records of these notifications.

The AE detection considers any untoward medical occurrence in a participant administered a pharmaceutical product, regardless of causal relationship with this treatment. Therefore, an AE can be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal product, regardless of its relation with the medicinal product. The AE severity is classified as:

- Mild: The participant is aware of the event or symptom, but the event or symptom is easily tolerated
- Moderate: The participant experiences sufficient discomfort to interfere with or reduce his or her usual level of activity
- Severe: Significant impairment of functioning: the participant is unable to carry out his or her usual activities

Investigators will also systematically assess the causal relationship of AEs to study intervention (including any other non-study interventions, radiation therapy, etc.) using the following definitions:

- Unrelated: Not reasonably related to the study intervention. AE could not medically (pharmacologically/clinically) be attributed to the study intervention under study in this clinical study protocol. A reasonable alternative explanation must be available.
- Related: Reasonably related to the study intervention. AE could medically (pharmacologically/clinically) be attributed to the study intervention under study in this clinical study protocol.

Abnormal laboratory findings and other abnormal investigational findings should not be reported as AEs unless they are associated with clinical signs and symptoms, lead to study intervention discontinuation or are considered otherwise medically important by the Investigator.

A SAE is any untoward medical occurrence that at any dose:

- results in death
- is life-threatening
- requires inpatient hospitalization or prolongs an existing hospitalization
- results in persistent or significant disability or incapacity
- is a congenital anomaly or birth defect
- is otherwise considered to be medically important

In the event of any new SAE occurring during the reporting period, the Investigator will immediately (within a maximum of 24 hours after becoming aware of the event) inform the regulatory agency**.**

3.2.12. Preliminary Results on pharmacodynamics and safety of r-hLH (Investigational treatment) and u-hCG (standard treatment) in hypogonadotropic hypogonadal men.

Up to now, seven patients have been enrolled within the RHYTHM clinical trial, five enrolled at UNIMORE (Unit 1), one at UNIMI (Unit 3) and one at UNINA (Unit 5) Centers. Preliminary data are available for the five patients enrolled at UNIMORE Center.

Table 1 summarizes patients' baseline characteristics.

Table 1. Baseline characteristics of five patients enrolled at Unit 1 of the RHYTHM trial.

	Study group (n=4)	**Control group (n=1)**
Testosterone (ng/mL)	0.95 ± 0.81	2.40
LH (IU/L)	0.33 ± 0.15	2.20
FSH (IU/L)	0.55 ± 0.33	0.90
Estradiol (pg/mL)	9.25 ± 0.50	0.90
AMH (ng/mL)	14.08 ± 11.52	-
Inhibin B (ng/mL)	74.73 ± 51.58	-

[Footnotes to Table 1: AMH: Anti-Mullerian Hormone; FSH: Follicle-stimulating hormone; LH: luteinizing hormone]

Four patients were enrolled in study group and one in control group. Testosterone serum levels were not significantly different between study and control group (0.95 ± 0.81 *versus* 2.40 ng/mL, p=0.208). Similarly, no differences at baseline were observed for both LH (0.32 ± 0.15 *versus* 0.90 IU/L, p=0.062) and FSH serum levels (0.52 ± 0.33 versus 0.90 IU/L, p=0.385) (**Table 1**).

Although control group includes only one patient so far, testosterone serum levels increased during treatment phase, reaching levels higher than the lower limit of reference range (**Figure 3**).

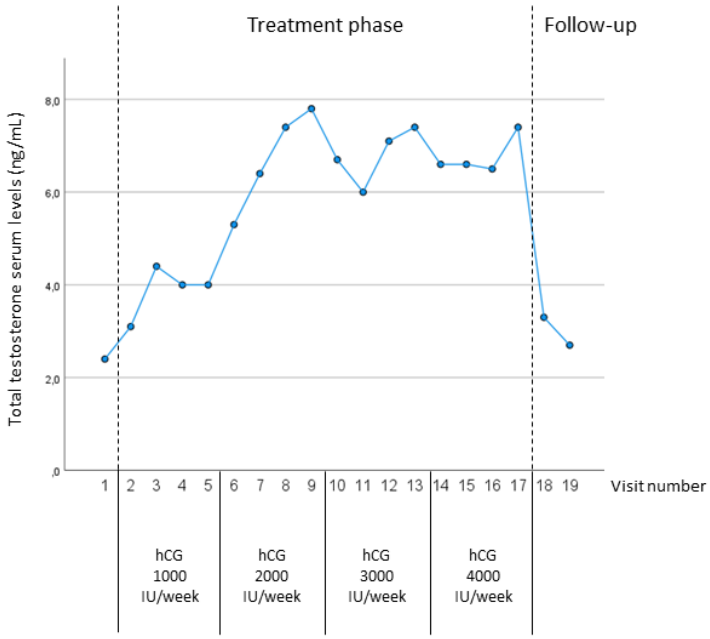

Figure 3. Testosterone serum levels in one patient enrolled in control group. The x-axis reports individual visits performed during the treatment phase and follow-up phase, respectively. The y-axis reports testosterone serum levels in ng/mL.

In the study group, testosterone serum levels did not significantly increase during LH administration (Wilcoxon test, p=0.994) (**Figure 4**).

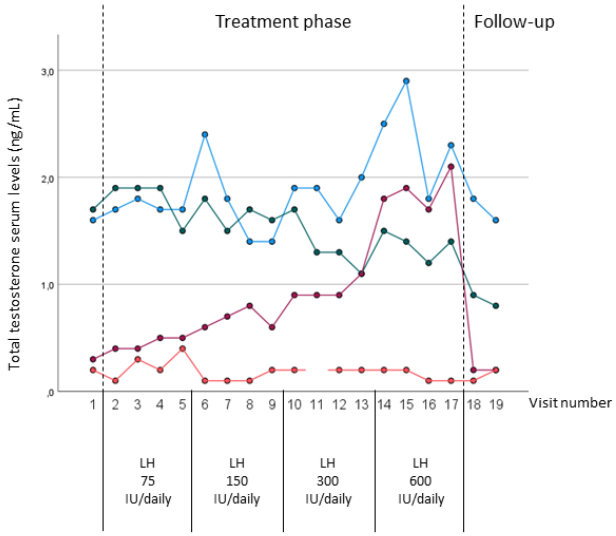

Figure 4. Testosterone serum levels in four patients enrolled in study group. Each line represents testosterone serum levels detected in a single patient. The x-axis reports individual visits performed during the treatment phase and follow-up phase, respectively. The y-axis reports testosterone serum levels in ng/mL.

Moreover, in the study group, LH serum levels significantly increased during treatment (Wilcoxon test, p<0.001), confirming the correct gonadotropin administration during the treatment phase as provided by the study protocol (**Figure 5**).

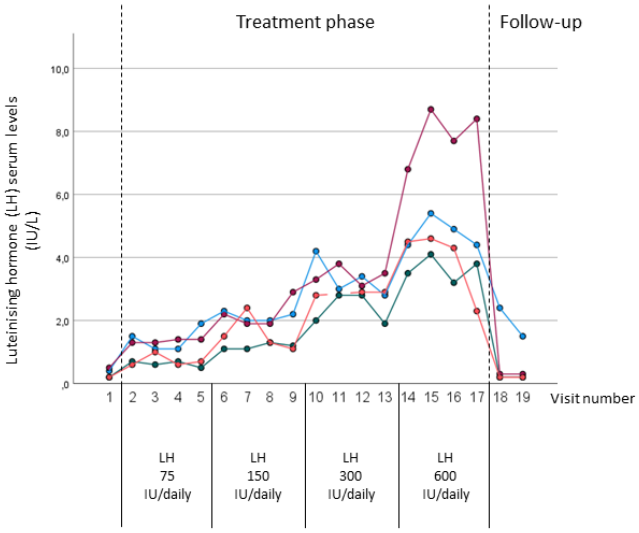

Figure 5. Luteinizing hormone (LH) serum levels (IU/L) in four patients enrolled in study group. Each line represents LH serum levels detected in a single patient. The x-axis reports individual visits performed during the treatment phase and follow-up phase, respectively. The y-axis reports LH serum levels in IU/L.

3.3. Testosterone Serum Levels Are Related to Sperm DNA Fragmentation Index Reduction after FSH Administration in Males with Idiopathic Infertility

This study aimed to evaluate the relationship between testosterone levels and sperm DNA fragmentation (sDF) index change after FSH administration in male idiopathic infertility. The analysis of three trials involving 251 patients confirmed the beneficial effect of FSH on spermatogenesis, with a significant decrease in sDF of 20.2% from baseline after FSH treatment. While there was no correlation between sDF and testosterone levels at baseline, a significant correlation was observed after three months of FSH treatment. Testosterone levels and patient age also correlated with sDF. The effectiveness of FSH treatment in improving sDF was related to testosterone levels and male age. The study suggests that FSH administration may promote communication or interactions between Sertoli cells and Leydig cells.

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Testosterone Serum Levels Are Related to Sperm DNA Fragmentation Index Reduction after FSH Administration in Males with Idiopathic Infertility

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Abstract: Purpose: Although a robust physiological rationale supports follicle stimulating hormone (FSH) use in male idiopathic infertility, useful biomarkers to evaluate its efficacy are not available. Thus, the primary aim of the study was to evaluate if testosterone serum levels are related to sperm DNA fragmentation (sDF) index change after FSH administration. The secondary aim was to confirm sDF index validity as a biomarker of FSH administration effectiveness in male idiopathic infertility. Methods: A retrospective, post-hoc re-analysis was performed on prospectively collected raw data of clinical trials in which idiopathic infertile men were treated with FSH and both testosterone serum levels and sDF were reported. Results: Three trials were included, accounting for 251 patients. The comprehensive analysis confirmed FSH's beneficial effect on spermatogenesis detected in each trial. Indeed, an overall significant sDF decrease ($p < 0.001$) of 20.2% of baseline value was detected. Although sDF resulted to be unrelated to testosterone serum levels at baseline, a significant correlation was highlighted after three months of FSH treatment ($p = 0.002$). Moreover, testosterone serum levels and patients' age significantly correlated with sDF ($p = 0.006$). Dividing the cohort into responders/not responders to FSH treatment according to sDF change, the FSH effectiveness in terms of sDF improvement was related to testosterone and male age ($p = 0.003$). Conclusion: Exogenous FSH administration in male idiopathic infertility is efficient in reducing sDF basal levels by about 20%. In terms of sDF reduction, 59.2% of the patients treated were FSH-responders. After three months of FSH administration, a significant inverse correlation between sDF and testosterone was detected, suggesting an association between the FSH-administration-related sDF improvement and testosterone serum levels increase. These observations lead to the hypothesis that FSH may promote communications or interactions between Sertoli cells and Leydig cells.

Keywords: FSH; sperm DNA fragmentation index; idiopathic male infertility; testosterone

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1. Introduction

1.1. Idiopathic Male Infertility

The contributing role of the male partner in infertile couple is currently well demonstrated and estimated to be causative in about 50% of cases [1]. Recently, the International Committee for Monitoring Assisted Reproductive Technologies (ICMART) provided a new definition of hypogonadotropic hypogonadism that fits well with the male infertility

status, namely, "Gonadal failure associated with reduced gametogenesis and reduced gonadal steroid production due to reduced gonadotropin production or action" [2]. Male idiopathic infertility is defined as the clinical condition in which spermatogenesis is altered without a known cause [3–5]. This condition should be considered distinct from unexplained male infertility, defined as infertility of unknown origin but with normal sperm parameters [5]. Male idiopathic infertility management remains complex and challenging since an aetiological therapy cannot be proposed. Several empirical treatments have been suggested, trying to mirror the therapeutic approach applied to hypogonadotropic hypogonadism [6–8]. Among these, the exogenous administration of follicle stimulating hormone (FSH) shows a strong physiological rationale when steroidogenesis is not compromised, aiming at stimulating spermatogenesis [1,7,9]. Although FSH administration is suggested by several scientific societies' guidelines, this hormonal approach is currently used only in few countries worldwide due to national regulations about drug reimbursement [1,7,9,10].

1.2. FSH Administration in Male Idiopathic Infertility

In male idiopathic infertility, FSH administration could be considered either as a replacement therapy or as an attempt to boost spermatogenesis. Indeed, FSH-mediated testicular overstimulation activates spermatogenesis and, theoretically, may improve sperm quality, as reported in 21 clinical trials and four meta-analyses [11–14]. The results of these meta-analyses are obviously affected by the high heterogeneity of included trials and the selection of pregnancy rate as primary endpoint. Although pregnancy rate is clearly the most meaningful outcome when evaluating an infertile couple, it may be affected by both male and female factors, irrespective of FSH action on the male partner. Therefore, FSH action on male cannot be assessed without considering the different confounders. An overall pregnancy rate increase (OR: 2.09; CI95%: 1.46, 3.01) in infertile couples in which FSH was administered to the male partner was detected. However, the calculated number needed to treat was high, showing that 10 to 18 men should be treated to achieve one pregnancy, spontaneously and following the assisted reproductive technique (ART), respectively [13,15]. In addition, only a limited increase in conventional sperm parameters was detected after FSH administration [13,15], confirming the debate on low accuracy of conventional semen analysis to estimate hormonal treatment efficacy. Thus, the first crucial question about FSH efficacy is to evaluate which could be the most appropriate endpoint.

1.3. Endpoints to Assess Treatment Efficacy Are Not Satisfactory

Alongside sperm concentration improvement, several randomized controlled clinical trials (RCTs) showed sperm DNA damage reduction in men treated with FSH [16]. This sperm quality parameter was evaluated by the sperm DNA fragmentation (sDF) index, which is a simple and reliable tool, currently suggested as a co-endpoint in male infertility work-up [17,18]. Infertile men showed a higher sDF index compared with fertile ones, and FSH administration reduces sDF by about 4.24% in idiopathic infertility [16,17]. This relative sDF improvement was higher than the sperm concentration increase reported in the aforementioned meta-analysis (2.66 million/mL; CI95%: 0.47, 4.84). However, it is still not clear whether sDF improves after FSH administration only in men with altered baseline levels. Similarly, we need to understand if sDF could be considered as a marker of FSH efficacy, discriminating between patient "responders" and "non-responders" to FSH treatment.

It is well established that in order to identify markers to evaluate FSH efficacy in male idiopathic infertility, RCTs remain the best methodological approach. However, since RCTs are very difficult in this field due to the large amount of resources, time and expertise required, it has been suggested that the re-analysis of raw RCTs-derived data could give an answer to new study questions [19].

1.4. Study Objectives

Since FSH acts on Sertoli cells, researchers focused their attention to the seminiferous testicular compartment, without considering the interstitial counterpart. However, aiming to identify new markers of FSH effectiveness, the potential correlation between semen parameters and steroids after FSH stimulation is still an unexplored aspect with potential relevant clinical impact. Thus, the aim of our study was the evaluation of the relationship between sDF and testosterone serum levels in men treated with FSH. We wished to explore whether a communication/interaction between the two cell compartments of the testes (Sertoli and Leydig cells) could be found by exogenous FSH administration.

Moreover, secondary aims were (i) to confirm FSH efficacy in terms of sDF improvement and (ii) to evaluate whether testosterone serum levels could predict sDF change after FSH administration.

2. Materials and Methods

2.1. Systematic Literature Search

A retrospective *post-hoc* analysis using Individual Patients' Data (IPD) from previously published clinical trials was conducted. Thus, the study consisted of two steps: in the first one, a systematic search of the literature was performed; in the second step, data derived from the clinical trials identified were analysed.

During the first step, a comprehensive systematic literature search for English-language articles in MEDLINE (PubMed) and EMBASE was conducted. The literature search was performed using the following keywords: male infertility, couple infertility, FSH, FSH administration, sperm DNA fragmentation index, sDF and testosterone. The Boolean functions AND and OR were used to combine keywords.

The following inclusion criteria were established before the literature search: (i) clinical trials (ii) in which the male partner of infertile couples was treated with FSH and both (iii) sDF index and (iv) testosterone serum levels were reported. Considering the rich literature on FSH application in male idiopathic infertility, keywords and inclusion criteria were set to detect only those studies in which both testosterone and sDF after treatment were reported.

Men enrolled showed idiopathic infertility, with one or more sperm parameters altered, in whom no specific causes of male infertility were detected. Retrospective studies were not included. No other inclusion/exclusion criteria were provided.

The corresponding author of each eligible trial was contacted to obtain raw data. When the author accepted, IPD were collected, considering the following specific endpoints: sDF index; testosterone serum levels (measured both before and after FSH administration); patient's age; body mass index (BMI); FSH dosages; treatment duration; conventional semen analysis parameters (such as sperm concentration, total sperm count, progressive sperm motility, total sperm motility and sperm morphology); hormonal evaluations (such as FSH, luteinizing hormone (LH), inhibin B, sex hormone binding globulin (SHBG) and anti-Mullerian hormone (AMH)); couple infertility duration; number of pregnancies obtained, both spontaneous and after assisted reproduction (if available).

IPD obtained by each study were combined in a single dataset and descriptive analyses were conducted.

2.2. Identification of FSH Administration Efficacy in Men with Idiopathic Infertility

The final dataset generated on IPD extracted following the systematic literature search was evaluated to reach primary and secondary endpoints. In detail, the primary endpoint was the potential correlation between testosterone serum levels and sDF change after FSH treatment. Secondary endpoints were (i) sDF decrease after FSH administration and (ii) to determine whether testosterone serum levels could predict the sDF decrease after FSH administration.

2.2.1. Endpoints' Definitions

As previously reported, the efficacy of FSH administration is associated with sDF index decrease. This improvement was recorded in each study considered alone (or "as a unit"). Here, we analysed IPD to quantify the comprehensive sDF decrease after FSH administration, comparing pre- and post-treatment values.

Similarly, the pre- and post-FSH treatment change in secondary endpoints was evaluated.

In order to better define a successful treatment, the entire cohort was divided into responders and non-responders to FSH. Responders were empirically defined as men in whom the sDF index decreased by at least 20% (relative decrease) of baseline levels after treatment. This threshold was empirically adopted to highlight a clinically significant sDF reduction.

2.2.2. Statistical Analysis

Statistical procedures were applied to the dataset to reach the secondary objectives of the study.

In order to determine the correlation between testosterone serum levels and sDF change after FSH treatment, correlation analyses were performed, combining testosterone serum levels and sDF at baseline and after FSH administration. Data were first analysed for distribution with Kolmogorov–Smirnov test and correlations were assessed using Pearson's or Spearman's methods for normally or not-normally distributed data, respectively. Correlation analyses were performed by considering anthropometric variables, hormones, semen parameters and sDF index, and by applying Bonferroni adjustment. Since 14 variables were considered, $p < 0.003$ was considered for statistical significance in correlation analyses. Moreover, multivariate stepwise linear regression analyses were performed, using sDF index as the dependent variable and testosterone, FSH, LH, SHBG, inhibin B, AMH, FSH treatment duration, patient's age and BMI as independent parameters. In order to correct potential confounders, multiple models were used to yield total-effect estimates for covariates [20].

Moreover, to determine the change in hormone levels to classify a man with and without significant sDF decrease, the entire cohort was considered by dividing patients into responders and non-responders, according to sDF change after FSH administration. Then, logistic regression analyses were performed using the responders/non-responders classification as the dependent variable. Conventional semen parameters, patients' age, BMI, FSH dosage, treatment duration and hormones collected after FSH administration were included among co-variates. Logistic regression analyses were repeated considering baseline parameters to identify potential predictors of FSH effectiveness. In this setting, to graphically show the connection/trade-off between clinical sensitivity and specificity for every possible cut-off for a predictor of FSH effectiveness, receiver operating characteristic (ROC) curves were generated.

Statistical analysis was performed using the "Statistical Package for the Social Sciences" software for Windows (version 27.0; SPSS Inc., Chicago, IL, USA). For all comparisons, $p < 0.05$ was considered statistically significant.

3. Results

Among 21 published studies investigating FSH administration to the male partner of infertile couples, three studies were selected (Figure S1) according to the inclusion criteria specified above [21–23]. Table 1 summarizes the study characteristics.

Table 1. Characteristics of clinical trials included in the analysis in which male partners of infertile couples were treated with follicle stimulating hormone (FSH).

Author	Year	Study Design	FSH Type	FSH Scheme	Inclusion Criteria	Number of Patients (n)		Age (Years) (Mean ± SD)	
						Study Group	Control Group	Study Group	Control Group
Colacurci et al.	2012	Prospective longitudinal, randomized, case-control	r-FSH	150 IU on alternate days for 90 days	FSH 1–7 IU/L, LH 1–8 IU/L, T 3–10 ng/mL	65	64	31.6 ± 3.1	33.6 ± 3.5
Simoni et al.	2016	Prospective longitudinal, case-control	r-FSH	150 IU on alternate days for 90 days	FSH ≤8 IU/l, normal LH and T, homozygous FSHR p.N680S N or S genotype, sDF index >15%	66	-	36.4 ± 4.7	-
Colacurci et al.	2018	Prospective longitudinal, case-control	r-FSH	150 IU on alternate days for 90 days	FSH 1–8 IU/L, sperm count >10 million, total sperm motility 5–25%	111	-	36.1 ± 4.7	-

FSHR: follicle stimulating hormone receptor; N: asparagine; r-FSH: recombinant follicle stimulating hormone; S: serine; SD: standard deviation; sDF: sperm DNA fragmentation; T: testosterone.

Finally, 251 patients were overall considered. Table 2 summarizes baseline patients' characteristics.

Table 2. Baseline patient's characteristics. Data are expressed as median (interquartile range).

Variables	Reference Ranges *	Baseline Values
Age (years)	-	35.0 (6.0)
BMI (kg/m^2)	-	25.5 (3.5)
FSH (IU/L)	1–8	3.1 (1.9)
LH (IU/L)	1–8	2.9 (1.4)
Prolactin (ng/mL)	3–13	7.9 (4.0)
Testosterone (ng/mL)	>3.0	4.0 (1.7)
Semen volume (mL)	>1.5	2.2 (1.8)
Sperm concentration (million/mL)	>15	24.0 (66.5)
Total sperm number (million)	>39	64.3 (237.1)
Progressive motility (%)	>32	20.8 (9.0)
Normal morphology (%)	>4	14.0 (9.0)
Sperm DNA fragmentation index (%)	-	18.0 (12.5)
Sex hormone binding globulin (nmol/L)	-	32.0 (16.1)
Inhibin B (pg/mL)	-	137.3 (70.0)
Anti-Mullerian hormone (ng/mL)	-	3.7 (3.4)
Smokers *n* (%)	-	120 (47.8)

BMI: body mass index; FSH: follicle stimulating hormone; LH: luteinizing hormone. * Reference ranges for semen analysis were evaluated considering the V edition of the World Health Organization manual for semen analysis [24].

3.1. Are Testosterone Serum Levels Correlated to sDF Decrease after FSH Administration?

At baseline, sDF was not significantly related to testosterone serum levels, which, in turn, were only directly related to LH serum levels (Spearman's correlation analysis: Rho 0.362, $p < 0.001$) (Table S1). FSH serum levels were directly related to both LH ($p < 0.001$) and inhibin B serum levels ($p < 0.001$) (Table S1).

After FSH treatment, sDF was inversely and significantly correlated to testosterone serum levels (Rho −0.327, $p = 0.002$) (Supplementary Table S2). Moreover, testosterone serum levels remained statistically significantly correlated with LH (Rho 0.272, $p = 0.004$) (Table S2).

In order to identify how study variables correlated with both sDF and testosterone serum levels after FSH administration, multivariate stepwise linear regression analysis was performed. The sDF index obtained after three months of FSH administration was directly related to testosterone serum levels and inversely to patients' age ($p = 0.006$) (Table 3).

Table 3. Multivariate stepwise linear regression analyses using sperm DNA fragmentation index after three months of follicle stimulating hormone (FSH) treatment as dependent variable. Bold characters reported statistically significant parameters.

	Not Standardized Coefficients		Standardized Coefficient	t	*p*-Value	95% Confidence Interval	
	Beta	Standard Error	Beta			Lower Limit	Upper Limit
(Constant)	9.988	10.565	-	0.945	0.348	−11.112	31.088
Testosterone	−3.277	1.722	−0.258	−2.054	**0.003**	−6.781	−0.931
Age	4.291	1.155	0.220	1.931	**0.004**	0.410	2.311
BMI	0.188	0.183	0.078	1.025	0.307	−0.174	0.549
FSH	−0.004	0.423	−0.001	−0.011	0.992	−0.839	0.830
LH	0.438	0.493	0.074	0.888	0.375	−0.535	1.411
SHBG	0.031	0.062	0.049	0.502	0.617	−0.091	0.154
Inhibin B	−0.001	0.009	−0.008	−0.092	0.927	−0.018	0.016
AMH	0.214	0.169	0.096	1.269	0.206	−0.119	0.547
FSH duration	0.293	0.215	0.146	1.365	0.177	−0.136	0.722

AMH: anti-Mullerian hormone; BMI: body mass index; FSH: follicle stimulating hormone; LH: luteinizing hormone; SHBG: sex hormone binding globulin.

3.2. What Is the Overall sDF Decrease after FSH Administration?

The comprehensive analysis confirmed the FSH beneficial effect on spermatogenesis detected in each trial. Indeed, increasing the sample size, this new analysis highlighted an overall significant sDF decrease ($p < 0.001$) of 20.2% of the baseline value (Table 4). Moreover, FSH administration significantly increased inhibin B ($p = 0.006$), AMH ($p = 0.001$) and testosterone ($p = 0.001$) serum levels (Table 4).

Table 4. Comparison between baseline and after follicle stimulating hormone (FSH) administration, considering both hormone and semen parameters, using Mann–Whitney *U*-test. Data are expressed as mean ± standard deviation. Bold characters reported statistically significant parameters.

Variables	Baseline	After FSH Administration	*p*-Value
sDF index (%)	18.9 ± 8.6	15.8 ± 6.5	**<0.001**
Testosterone (ng/mL)	4.3 ± 2.2	4.9 ± 1.8	**0.001**
FSH (IU/L)	3.3 ± 1.5	5.6 ± 2.1	**<0.001**
LH (IU/L)	3.1 ± 1.4	2.9 ± 1.3	0.098
Prolactin (ng/mL)	8.2 ± 3.6	8.3 ± 3.8	0.783
SHBG (nmol/L)	32.0 ± 12.6	32.5 ± 12.3	0.629
Inhibin B (pg/mL)	154.5 ± 69.6	175.4 ± 79.9	**0.006**
Anti-Mullerian hormone (ng/mL)	4.3 ± 2.8	5.4 ± 4.0	**0.001**
Semen volume (mL)	2.8 ± 2.5	3.1 ± 1.3	0.058
Sperm concentration (million/mL)	59.8 ± 63.3	95.4 ± 107.2	**<0.001**
Total sperm number (millions)	217.7 ± 522.8	323.3 ± 420.2	**0.019**
Progressive motility (%)	20.3 ± 7.5	39.8 ± 16.2	**<0.001**
Normal morphology (%)	16.0 ± 11.5	21.3 ± 13.3	**<0.001**

FSH: follicle stimulating hormone; LH: luteinizing hormone; sDF: sperm DNA fragmentation; SHBG: sex hormone binding globulin.

3.3. Are Testosterone Serum Levels Predictive of Responders and Non-Responders, Defined on sDF Decrease after FSH Administration?

Here, we empirically defined FSH-responders as men who achieved an sDF reduction of at least 20% of baseline values after three months of FSH administration. With this definition, the effectiveness rate was 59.2% (148 patients out of 251). Responders showed higher testosterone serum levels after FSH treatment ($p = 0.014$) (Table 5); however, conventional semen parameters were not significantly different between responders and non-responders (Table 5).

Table 5. Comparison between responders and non-responders based on sperm DNA fragmentation index, considering parameters after three months of follicle stimulating hormone (FSH) administration using Mann–Whitney *U*-test. Data are expressed as mean ± standard deviation. Bold characters reported statistically significant parameters.

	Non-Responders	Responders	*p*-Value
Number of patients n (%)	102 (40.8)	148 (59.2)	-
Age (years)	36.1 ± 4.5	34.9 ± 5.3	0.070
sDF index (%)	17.2 ± 6.1	15.5 ± 7.0	**0.007**
Testosterone (ng/mL)	4.5 ± 1.7	5.1 ± 1.9	**0.014**
FSH (IU/L)	5.7 ± 2.3	5.5 ± 2.0	0.362
LH (IU/L)	3.1 ± 1.4	2.7 ± 1.2	0.125
Prolactin (ng/mL)	9.5 ± 4.6	7.6 ± 3.1	0.114
SHBG (nmol/L)	31.5 ± 12.7	33.0 ± 11.9	0.389
Inhibin B (pg/mL)	162.6 ± 74.5	182.8 ± 80.8	0.121
AMH (ng/mL)	5.1 ± 3.6	5.6 ± 4.3	0.404
Semen volume (mL)	2.9 ± 1.2	3.2 ± 1.3	0.129
Sperm concentration (million/mL)	81.6 ± 96.2	104.0 ± 112.8	0.140
Total sperm number (millions)	263.2 ± 381.0	360.7 ± 439.9	0.100
Progressive motility (%)	40.8 ± 15.5	39.7 ± 16.6	0.440
Normal morphology (%)	19.5 ± 13.7	22.2 ± 13.1	0.141

AMH: anti-Mullerian hormone; FSH: follicle stimulating hormone; LH: luteinizing hormone; sDF: sperm DNA fragmentation index; SHBG: sex hormone binding globulin.

Logistic regression analyses were performed to highlight markers and predictors of response to FSH treatment. The first analysis used all parameters detected after FSH administration as cofactors/covariates, showing a significant relationship between response and both testosterone and male age serum levels (Table 6). To identify potential thresholds of these variables, two ROC analyses were generated using testosterone and age as test variables. Neither testosterone (area under the curve—AUC = 0.584, $p = 0.124$) nor age (AUC = 0.537, $p = 0.373$) displayed significant thresholds.

The second analysis considered all parameters detected at baseline as cofactors/covariates. A significant predictive role of FSH efficacy was detected only for sDF ($p = 0.002$) (Table 7). An ROC analysis was performed, setting the response as the dependent variable and sDF as the test variable. The ROC generated showed a significant threshold (AUC = 0.754, $p < 0.001$) of 16.75% (sensitivity 75.7%, specificity 69.1%) (Figure 1). When patients with baseline sDF higher than 16.75% were selected, 72.7% (104 patients) showed an sDF decrease of at least 20% of baseline sDF levels, with an average decrease of 5.23% (from 24.1 ± 7.2% to 18.9 ± 5.9%, $p < 0.001$). Interestingly, in this subgroup, a significant total testosterone serum levels increase was detected (from 4.2 ± 2.3 to 4.9 ± 2.0 ng/mL, $p = 0.003$). Similarly, a significant inhibin B and AMH increase after FSH administration was confirmed ($p = 0.034$ and $p = 0.001$, respectively).

Table 6. Logistic regression analysis to predict patients with sperm DNA fragmentation index improvement. Bold characters reported statistically significant parameters.

	B	Standard Error	Wald	*p*-Value	Exp (B)	95% Confidence Interval	
						Lower Limit	Upper Limit
Intercept	−0.298	4.671	1	0.949	-	-	-
sDF	0.027	0.054	0.247	0.619	1.027	0.924	1.143
Age	3.387	0.385	2.163	**0.005**	1.066	1.006	1.399
FSH	−0.11	0.196	0.314	0.575	0.896	0.610	1.316
Prolactin	0.107	0.081	1.725	0.189	1.113	0.949	1.305
LH	0.387	0.289	1.793	0.181	1.472	0.836	2.592
Testosterone	4.121	0.305	3.982	**0.015**	3.822	1.670	4.521
SHBG	0.002	0.035	0.002	0.964	1.002	0.935	1.073
Inhibin B	−0.002	0.004	0.297	0.586	0.998	0.989	1.006
AMH	−0.045	0.085	0.275	0.600	0.956	0.809	1.130
Semen volume	−0.31	0.423	0.538	0.463	0.733	0.32	1.680
Sperm concentration	−0.002	0.012	0.038	0.846	0.998	0.975	1.021
Total sperm number	0.001	0.003	0.043	0.835	1.001	0.994	1.007
Sperm motility	0.028	0.023	1.575	0.210	1.029	0.984	1.075
Sperm morphology	−0.014	0.028	0.237	0.626	0.986	0.933	1.043
BMI	−0.063	0.100	0.396	0.529	0.939	0.772	1.142
Smoking	0.731	0.699	1.096	0.295	2.078	0.528	8.171

AMH: anti-Mullerian hormone; BMI: body mass index; FSH: follicle stimulating hormone; LH: luteinizing hormone; SE: standard error; SHBG: sex hormone binding globulin.

Table 7. Predictive markers: logistic regression analysis performed using sperm DNA fragmentation index improvement as dependent variable and all parameters obtained at baseline as independent ones.

	B	Standard Error	Wald	*p*-Value	Exp (B)	95% Confidence Interval	
						Lower Limit	Upper Limit
Intercept	12.098	5.698	1	0.034	-	-	-
sDF	−0.206	0.066	9.792	**0.002**	0.814	0.715	0.926
Age	−0.129	0.073	3.101	0.078	0.879	0.762	1.015
FSH	−0.447	0.320	1.95	0.163	0.64	0.341	1.198
Prolactin	0.169	0.103	2.693	0.101	1.184	0.968	1.448
LH	0.137	0.302	0.205	0.650	1.147	0.634	2.074
Testosterone	0.287	0.33	0.754	0.385	1.332	0.697	2.544
SHBG	−0.07	0.04	3.09	0.079	0.932	0.862	1.008
Inhibin B	−0.006	0.006	0.914	0.339	0.994	0.982	1.006
AMH	−0.046	0.144	0.100	0.752	0.955	0.720	1.268
Semen volume	0.113	0.389	0.084	0.772	1.119	0.523	2.397
Sperm concentration	−0.002	0.017	0.013	0.908	0.998	0.965	1.032
Total sperm number	0.001	0.005	0.032	0.858	1.001	0.992	1.010
Sperm motility	0.103	0.06	2.937	0.087	1.108	0.985	1.246
Sperm morphology	0.040	0.041	0.92	0.337	1.040	0.960	1.128
BMI	−0.255	0.126	4.111	0.043	0.775	0.605	0.992
Smoking	1.385	0.738	3.524	0.060	3.995	0.941	16.962

AMH: anti-Mullerian hormone; BMI: body mass index; FSH: follicle stimulating hormone; LH: luteinizing hormone; SE: standard error; SHBG: sex hormone binding globulin.

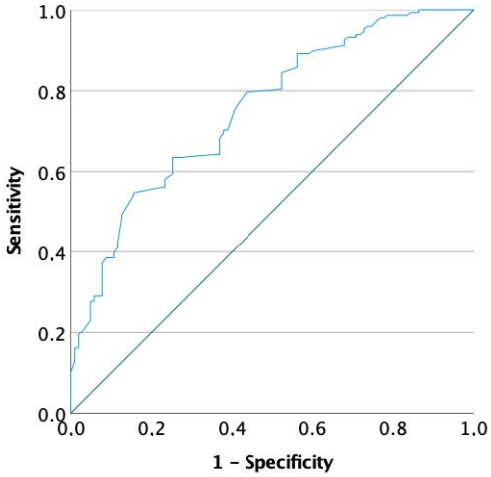

Figure 1. Receiver operating curve (ROC) analysis using responders as dependent variable and sperm DNA fragmentation (sDF) index as test variable.

4. Discussion

This re-analysis of published clinical trials data investigating FSH administration in male idiopathic infertility highlights a novel aspect of male infertility management. Alongside the expected amelioration of conventional semen quality and an overall 20% decrease of sDF baseline index, exogenous FSH stimulation induces an increase in testosterone, inhibin B and AMH serum levels. Both inhibin B and AMH are Sertoli cell products, reflecting the proliferative status of the testicular germinative epithelium [25,26]. Thus, their increase after FSH administration is not unexpected. On the contrary, the testosterone rise after FSH administration is unexpected and detected here for the first time, suggesting an action of FSH on testicular function more complex than thought so far. Indeed, we could speculate that FSH boosts spermatogenesis throughout a direct action on Sertoli cells and also an indirect effect involving the testis interstitial compartment. This finding is in line with previous demonstrations of the capability of the supraphysiological FSH stimulation to sustain spermatogenesis even in the absence of LH action [27]. Indeed, a quali-quantitative normal spermatogenesis was reported in a hypophysectomised man presenting an activating FSH receptor mutation, suggesting that a strong FSH action alone could support the LH/testosterone function [27].

Our new analysis showed for the first time the global testicular action (not limited to the spermatogenic compartment) of FSH chronically administered to infertile men. As confirmed, after three months of FSH administration, sperm quality (in terms of sDF index) correlated with testosterone serum levels, highlighting the association between the FSH-related sDF improvement and the increase in testosterone serum levels. Accordingly, both multivariate linear and logistic regression analyses identified a strong correlation between testosterone increase and sDF decrease after FSH treatment. This finding is totally novel since the literature on this topic is entirely silent. This innovative result could open new perspectives in the way of evaluating responses to FSH treatment in male idiopathic infertility. This new correlation, although interesting, is far from being directly transposed to clinical practice. Specific, properly designed prospective trials must be designed to understand the real clinical application of the finding. From a physiological point of view, the strict connection between seminiferous and interstitial testicular compounds is expected, since effective spermatogenesis requires both FSH action and adequate intratesticular testosterone levels [28]. However, this link has been generally underestimated in clinical practice, since intratesticular testosterone assessment is very complex, requiring testicular biopsy or sampling [29]. In addition, testosterone measured in the peripheral blood correlates poorly to its intratesticular levels, which are estimated to be at least 100 times higher [30]. For these reasons, several attempts have been made to identify surrogate markers of intratesticular testosterone levels [29]. Among these, serum 17-hydroxyprogesterone (17-

OHP) and Insulin-like factor 3 (INSL3) have been proposed [30,31]. These hormones were demonstrated as able to predict intratesticular testosterone levels after human chorionic gonadotropin (hCG) stimulation [32,33]. However, both 17-OHP and INSL3 did not correlate with testosterone intratesticular levels at baseline, only after hCG treatment. This result mirrors the sDF–testosterone correlation detected only after FSH administration in this study. Thus, the connection of sDF–testosterone is novel in the field of human reproduction, although some suggestion of such a correlation is provided in other fields. For example, Wood et al. described improvements in both testosterone serum levels and sDF index six months after bariatric surgery [34]. In our analysis, we speculate that both testicular compartments, seminiferous and interstitial, tend to realign only after overstimulation induced by exogenous gonadotropins. However, as neither intratesticular testosterone levels nor their surrogate markers are available in our analysis, we cannot provide conclusive explanations of the FSH action at intratesticular level.

Putting together three different cohorts of patients treated with the same regimen of recombinant FSH for three months, we highlight an overall 20% decrease of sDF baseline index with the current therapeutic approach. In particular, we detect a FSH efficacy rate of about 59.2%, considering the sDF index decrease. This relevant result could be directly translated into clinical practice. Hitherto, many direct tests have been suggested to predict sperm capability to penetrate the oocyte, such as sperm–zona binding ratios and zona pellucida-induced acrosome reaction tests [35,36]. Similarly, indirect variables could be measured in seminal plasma with the same objective, such as phospholipase, sperm acrosin, fructose and neural alfa-glucosidase [37–39]. In this setting, the sDF index provides an informative and reliable measure of the real fertilization capability [40–43]. SDF is the end result of the action of multiple factors that induce single- or double-strand DNA breaks in the sperm genome [44], due to oxidative stress [45], apoptosis [46,47], impaired chromatin remodelling [48] and environmental agents, such as toxicants, drugs and radiation [49–51]. As a result, high levels of sDF index reflect an impaired semen quality [17] and a reduced fertility [16]. Sperm DNA integrity is crucial for embryo development and successful pregnancy outcome, and sDF values are inversely related to the chances of achieving natural pregnancy [52,53]. Accordingly, increased sDF index was comprehensively detected in infertile compared with fertile men in a recent meta-analysis, which proposed an sDF threshold of 20% to discriminate between fertile and infertile men (AUC: 0.84, $p < 0.001$, sensitivity 79%, specificity 86%) [16]. The proven relevance of sDF assessment in human fertility justified the insertion of its measurement within the latest edition of the World Health Organization (WHO) laboratory manual for the examination and processing of human semen [54]. In the current re-analysis, sDF index significantly decreased after FSH treatment concomitantly with the increase in conventional semen parameters, demonstrating an overall FSH-induced improvement in sperm quality, which could be measured through this relatively new tool. However, the correlation between sDF and conventional semen parameters is still controversial [55–57]. Here, sDF is correlated to sperm progressive motility after FSH administration, in line with previous studies depicting the highest sDF index in men presenting the lowest percentage of motile sperms [58,59]. A connection between sDF and sperm motility could be hypothesized since these two parameters share a marked detrimental susceptibility to reactive oxygen species (ROS). Indeed, oxidative stress is assumed to be the most relevant causative factor contributing to sDF [17], while sperm motility is acquired during the long sperm transit through the epididymis, resulting more likely vulnerable to ROS damage [47].

Subgrouping our cohort in responders and non-responders to FSH treatment considering an sDF decrease threshold of 20% of its basal level could be useful to identify markers and predictors of FSH efficacy. With this approach, only two markers of FSH efficacy are identified, testosterone serum levels and male age, although ROC analyses were not able to identify useful thresholds for clinical practice. On the contrary, sDF amelioration after FSH therapy is predicted only by sDF basal levels, with a significant threshold of 16.75%. Since no differences between responders and non-responders were demonstrated

by conventional semen parameters, we could speculate that semen analysis is not really accurate to evaluate male fertility status.

Our results should be carefully considered due to several limitations. First, we combined raw data of three clinical studies, putting together a large cohort of patients for this research topic, yet still limited to elaborate definitive conclusions. Second, a relatively high heterogeneity among trials should be expected. Although all three trials used recombinant FSH at the same dosage (150 IU every other day) for the same treatment period (three months), inclusion criteria of each trial may have differed. Indeed, Simoni et al. enrolled only men with sDF >15% at baseline, while Colacurci et al. did not consider sDF index as an inclusion criterion. Thus, enrolled populations were not completely homogeneous. In addition, in all studies, testosterone serum levels were measured by immunometric assays and not by liquid chromatography-mass spectrometry, which is the gold standard for steroid measurement [60].

5. Conclusions

In conclusion, our analysis reports for the first time an association between testosterone levels rise and sDF improvement, after exogenous FSH administration in men with idiopathic infertility. Both testosterone and male age levels represent FSH-effectiveness markers, although clinically applicable thresholds were not identified. These results deserve further consideration, but broaden the vision on male idiopathic infertility management, suggesting that fertility status and treatments response should not be barely limited to conventional semen parameters assessment. On the other hand, sDF baseline levels are predictors of FSH response, and those men presenting basal sDF index > 16.75% are expected to be more sensitive to FSH-induced sDF amelioration. How this improvement could translate into clinically relevant outcomes, i.e., pregnancy rates, remains to be investigated.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines10102599/s1, Figure S1: PRISMA diagram for study flow chart; Table S1: Correlation analyses among available variables at baseline; Table S2: Correlation analyses among available variables after follicle stimulating hormone (FSH) administration.

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3.4.Male Infertility Business case

A business case is a document that outlines the justification for initiating a business project or investment. It presents the rationale, benefits, costs, and risks associated with the proposed initiative. A well-developed business case helps decision-makers evaluate the viability and potential returns of a project, enabling them to make informed choices about allocating resources and approving investments. It typically includes information such as the project objectives, market analysis, financial projections, timeline, and implementation strategy. The business case serves as a tool to support decision-making and secure buy-in from stakeholders by providing a clear understanding of the expected outcomes and value proposition of the project.

Specifically in this business case decision-making should be based on the Net Present Value (NPV) obtained with the model developed. NPV is a financial metric used to evaluate the profitability of an investment or project. NPV calculates the difference between the present value of cash inflows and the present value of cash outflows over a specified time. By discounting future cash flows back to their present value using an appropriate discount rate, NPV assesses the value that an investment adds to a Company or project.

A positive NPV indicates that the investment is expected to generate more cash inflows than outflows, potentially creating value for the Company or project. A negative NPV suggests that the investment is not expected to generate sufficient returns to cover the initial investment and related costs.

NPV is widely used in capital budgeting and investment decision-making to determine the economic viability and financial attractiveness of projects or investments. It helps in comparing different investment options and selecting the most financially advantageous one.

3.4.1. Business case model Assumptions

As mentioned, Male Infertility represents a complex landscape due to significant scientific gaps impacting the business assumptions. However, thanks to evidence generated from this PhD and other recent scientific publications it was possible to develop a model for understanding the complex landscape of male infertility and its impact on business decisions on following assumptions:

- Identification of the target population for different gonadotropins treatment combinations in male infertility.

- Reliable scientific evidence supporting the therapeutic use, efficacy, and safety of gonadotropins in the target populations.
- Consideration of the clinical development and Health Authority costs and timing required to obtain indications in male infertility for the use of gonadotropins in the target population.
- Estimating the volume of sales based on the target population, any barrier (Assisted Reproduction Technique, cultural barrier, and competitive landscape).
- Estimation of the net selling price of the products once the new indication becomes available.
- Considering the average cost of goods, which includes the production costs of each product.
- Taking into account the costs associated with product launch and marketing.

Due to confidentiality of some Company information and data, not all the contents of the present business case are reported and discussed in this PhD.

Target population - To evaluate Male Infertility (MI) target population the general disease landscape was analyzed according to available scientific literature (updated up to September 2023) and health governance bodies published MI epidemiology, to identify and isolate the infertile males deserving hormonal treatment (**Figure 6**)

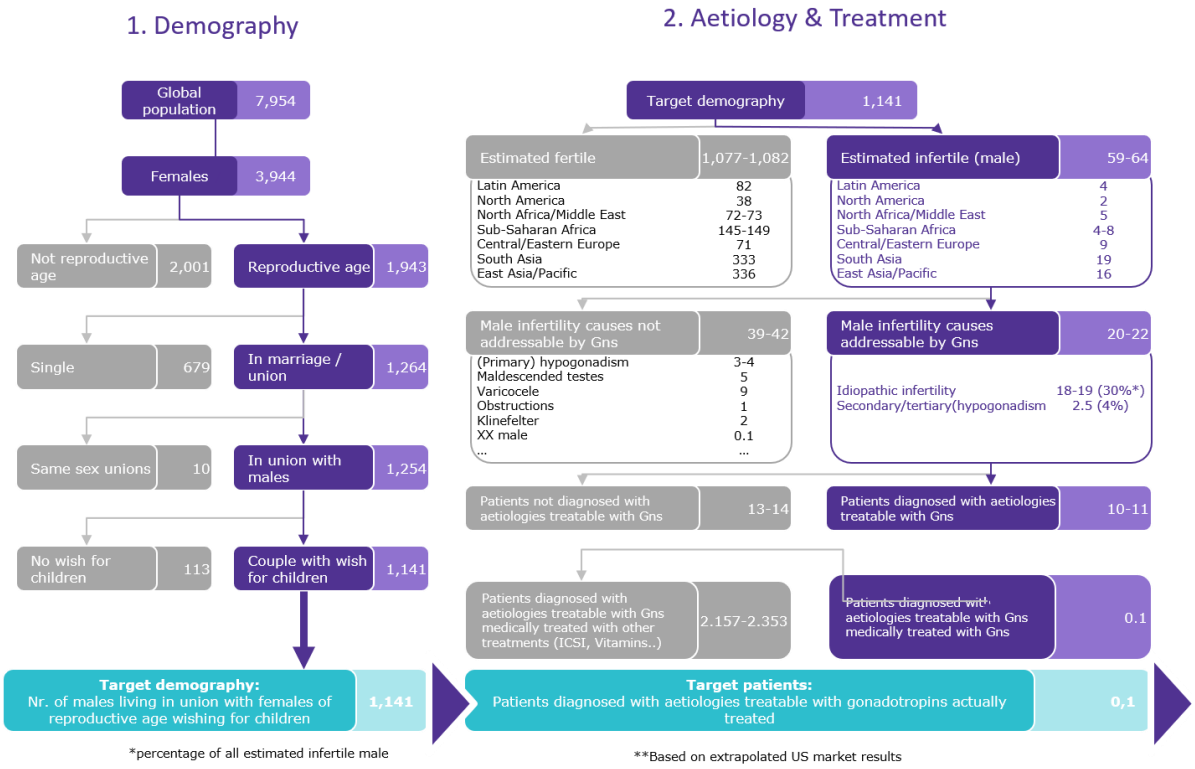

Figure 6. Initial projection of potential target Male Infertility population that could be suitable for gonadotropin treatment based on prevalence rates, diagnostic criteria, and treatment guidelines (Jungwirth et al., 2012; Agarwal et al., 2020).

Recently, The APHRODITE (Addressing male Patients with Hypogonadism and/or infeRtility Owing to altereD, Idiopathic TEsticular function) –system was proposed by a group of endocrinologists, andrologists, and MAR) specialists. APRHODITE is a novel method of classifying infertile patients with testicular dysfunction. This system aims to improve the fertility and pregnancy prospects of these patients through hormonal therapy. It is specifically designed for individuals with testicular dysfunction and may not benefit those with other confirmed infertility diagnoses, such as infection or obstruction.

Under the APHRODITE system, patients are classified based on their clinical descriptions (including history and physical examination) and the results of routine laboratory tests. The system defines five different groups, each with specific criteria, incidence rates of the condition, suggested therapeutic management, and relevant endpoints (**Figure 7**). The key objectives of this system are to enhance semen parameters for natural conception and to improve semen quantity and/or quality for couples undergoing ART treatments.

It's important to note that the APHRODITE system is independent of the specific cause of testicular dysfunction and provides recommendations for hormonal therapy tailored to each patient group. The scientific evidence supporting the classification and treatment proposed is reported in the original paper (Esteves *et al.*, 2023b).

Classification	Definition*	Prevalence	Suggested gonadotropin regimen	Endpoints†
Group 1 Hypogonadotropic Hypogonadism (acquired and congenital)	• Gonadal failure associated with reduced gametogenesis and reduced gonadal steroid production due to reduced gonadotropin production or action (Zegers-Hochschild et al. 2017) • FSH levels below reference range and reduced LH levels, reduced total testosterone levels (below the lower limit of the normal range of the assessing laboratory) and lowered semen analysis parameters (e.g., OAT or azoospermia)	1.9% of azoospermia cases (Chiba et al. 2016) and 1.6% of male infertility cases (Jungwirth et al. 2012) overall	hCG (+/-) FSH¶	Semen parameters/sperm retrieval rates, total testosterone levels, QoL, pregnancy rates
Group 2 Lowered semen analysis parameters, normal serum FSH and normal serum total testosterone	• Functional hypogonadism with reduced gonadotropin action • Lowered semen analysis parameters, including NOA • FSH levels within reference range1 and total testosterone levels within the normal range of the assessing laboratory	Idiopathic male infertility: Up to 44% of male infertility cases (Agarwal et al. 2021)	FSH alone	Semen parameters /sperm retrieval rates, SDF rates, QoL, pregnancy rates
Group 3 Lowered semen analysis parameters, normal FSH and reduced total testosterone levels	• Functional hypogonadism with reduced gonadotropin action and reduced testosterone production • Lowered semen analysis parameters, including NOA • FSH levels within reference range and reduced total testosterone (below the lower limit of the normal range of the assessing laboratory)	~20% of the total idiopathic male infertility population treated with hormonal therapy) (Romeo et al. 2023)	FSH¶ (+/-**) hCG	Semen parameters/sperm retrieval rates, total testosterone levels, SDF rates, QoL, pregnancy rates
Group 4 Lowered semen analysis parameters, elevated FSH levels and normal or reduced total testosterone levels	• Functional hypergonadotropic hypogonadism • Lowered semen analysis parameters, mainly NOA • FSH levels above the upper limit of the reference range and normal or reduced total testosterone levels (excluding genetic causes)	Up to 10% (Cocuzza et al. 2013)	hCG (+/- FSH*)	Semen parameters/sperm retrieval rates, total testosterone levels, SDF rates, QoL, pregnancy rates
Group 5 Unexplained male infertility in the context of unexplained couple infertility	FSH levels within reference range, testosterone levels within normal range, and normal semen analysis parameters	15% of couples presenting with unexplained infertility, and unexplained male infertility from 6–27% (Esteves et al. 2015)	FSH alone**	SDF rates, pregnancy rates

Figure 7. Characteristics of 5 APHRODITE groups. The Aphrodite concept classifies Male Infertility patients benefitting from hormonal treatment. FSH, follicle stimulating hormone. hCG, human chorionic gonadotropin. LH, luteinizing hormone. NOA, non-obstructive azoospermia. OAT, oligoasthenoteratospermia. QoL, quality of life. SDF, Sperm DNA fragmentation. *FSH treatment could improve DNA fragmentation and sperm quality; however, indiscriminate use of FSH will reset the parameters. †Sperm parameters are the primary outcome as they are the primary outcome of hormonal treatment. ¶Regimen can be tailored according to the congenital or acquired forms of HH **The suggestion for FSH alone is based on empirical evidence and the opinion of the authors, and will need to be updated as more data become available. If FSH levels are low, treatment with exogenous FSH can be considered. ¶Groups 1 and 3 (Italy only) would meet the label for FSH treatment. Groups 2–5 would be off-label treatment.

Upon analyzing **Figures 6 and 7**, it was determined that APHRODITE Group 1, 2, 3, and 4 have potential business implications. APHRODITE Group 1 aligns with the current r-hFSH

pharmaceutical indications for congenital and acquired HH males when combined with hCG. In 2017, the International Glossary on Infertility and Fertility Care (Zegers-Hochschild *et al.*, 2017) defined HH as a condition characterized by "gonadal failure associated with reduced gametogenesis and reduced gonadal steroid production due to reduced gonadotropin production or action". APHRODITE Group 3 is defined by altered gametes and reduced testosterone, and it falls within the HH ICMART definition and the existing indication for r-hFSH. However, the most promising group from a business perspective is APHRODITE Group 2 due to its high incidence rate (50% of the male infertile population) and the existing scientific literature supporting the efficacy and safety of FSH treatment. Group 4 could be mainly treated with hCG associated with FSH only if FSH downregulation is reached after that hCG administration started, this population presents low incidence and few available clinical data on efficacy and safety of hormonal treatments. Group 5 represents an interesting target population. These individuals have normal FSH and total testosterone levels, as well as normal semen analysis parameters. They have unexplained infertility, as they show no history of fertility-affecting diseases and have normal findings in physical examinations and laboratory tests, including genetic analysis. Currently, these patients are not individually treated because no abnormalities are detected after analysis of semen and sex hormone profile.

However, this group of male patients could potentially benefit from FSH treatment, if a "stimulatory" therapeutic approach is taken rather than a "substitutive" one, as suggested by Simoni and Santi in 2019. Physiologically, it has been observed that spermatogenesis does not reach its maximum capacity, and additional FSH stimulation may further enhance sperm quantity and quality. This could potentially lead to higher pregnancy rates and reduced time to live birth for couples undergoing fertility treatments.

In summary, Group 5 consists of males with unexplained infertility who currently receive treatment aimed at their female partners. However, by applying FSH stimulation to this group, a potential improvement in sperm quality and quantity may be achieved, offering new possibilities for enhancing pregnancy outcomes. Unfortunately, no clinical data are available on the use of gonadotropins in this population and ART is the main treatment available today to overcome such condition.

In summary, based on the analysis performed, APHRODITE Group 1 and 3 resulted parts of the r-hFSH approved indication.

Meanwhile APHRODITE Group 2, even considering that ART approach is the golden standard treatment used for this population, has been considered of business interest to elaborate a full business case on r-hFSH as treatment. In parallel, r-hCG indication expansion for APHRODITE

Group 1, 3 and 4 was also assessed in an independent model that is not included in this thesis but briefly examined in the business case results and in the discussion session.

Drivers	**Barriers**
• MI increasingly considered as important global scale problem: rising comorbidities and reduced sperm quality • MI will increase due to rising comorbidity factors • Arising belief in importance to treat the male partner (public & key opinion leaders) • Increasing interest of men to participate in the treatment journey • Improving male factors could be an important lever to improve IVF/ICSI success rates and offspring wellbeing • Research efforts to prove effectiveness of Hormones in MI • Increasing offering of sperm test at home (detecting potential MI)	• ICSI as gold standard treatment without other considerations - source of income of MAR center • Lacking Health Care professionals (HCP) awareness for necessary diagnoses & treatment • Male patients are underdiagnosed and under treated • Impatience on HCP and couple side • Different level of scientific evidence • Lack of treatment options and high costs • Options as lifestyle changes help and are less expensive • Cultural barriers

Figure 8. Business Drivers & Barriers. Male infertility is a huge unmet Medical Need and as such a very important area for the future, but definitively hard to uncover and to dig. Figure 8 summarizes drivers and barriers considered as relevant for the business model. MI: Male Infertility

Other Assumptions - This thesis cannot provide, for confidentiality reasons, Company information contributing, as assumptions, to the model proposed such as:

- Clinical development and Health Authority costs and timing
- Estimating the volume of sales based on the target population, ART cycles and barriers
- Estimation of the net selling price of the products once the new indication becomes available
- Considering the average cost of goods, which includes the production costs of each product
- Estimation of costs associated with product launch and marketing

3.4.2. Business case model

APHRODITE Group 2- The model used in this exercise consists of a bottom-up approach and a top-down approach (**Figure 9**).

The bottom-up approach utilizes market research and extrapolation based on the number of healthcare professionals (HCPs). It aims to estimate market size by considering the insights gathered from research and projecting them based on the HCP population. To perform the market research HCPs were provided with a virtual r-hFSH Target Product Profile (TPP), including the idiopathic male infertility indication, to explore their willingness to use the treatment in this population (market research questionnaire not provided for confidentiality reasons).

On the other hand, the top-down approach involves an epidemiological model (EPI-model) that estimates treatment numbers. These estimates are then adjusted and modeled according to the local country experts' input. After comparing both approaches, it was determined that the top-down EPI-model was the most accurate. This final model considers multiple countries and adjusts for specific market characteristics. It is further refined by considering various drivers and factors and aligning it with available real market data (e.g. in market-sales and prescription data).

It is important to note that modeling accuracy is never 100%, but the chosen approach is considered sufficiently reliable for estimating the current and future market for the intended purpose. A pilot study will be conducted to provide additional details and to validate the business case assumptions and opportunity.

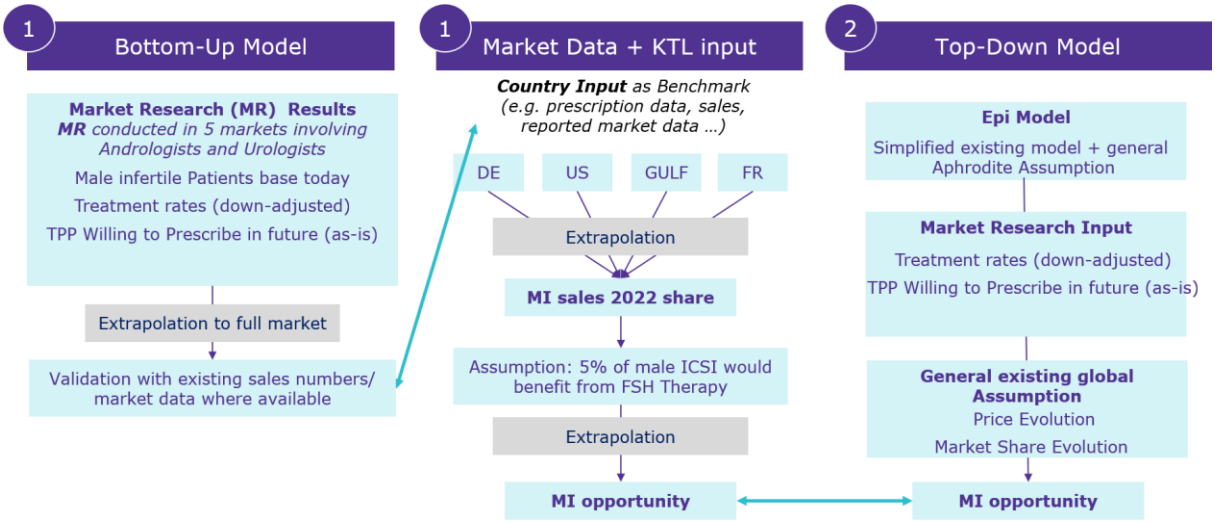

Figure 9. Bottom-up and top-down models to assess business case of r-hFSH as treatment for APHRODITE Group 2. Legend - TPP: Target product profile; DE: Germany; US: United State; FR: France; GULF; Gulf Countries

3.4.3. Business case Results

As explained above, Company confidential information was used in the application of the described model to obtain a robust and objective assessment of the business case. Considering such limitation, **Figure 10** provides a simulation of the estimation of market share and revenue using the model described in session 3.4.2.

In particular, simulation is based on German epidemiology data. Extremely interesting is the provision of the business evolution up to 2030 (**Figure 11**).

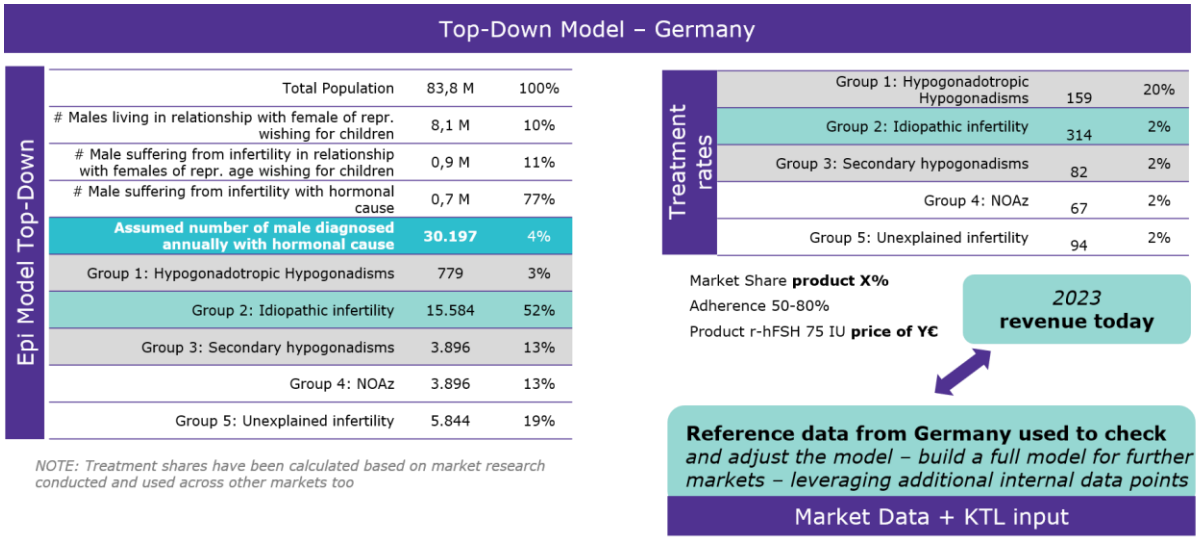

Figure 10. Business model applied to Germany.

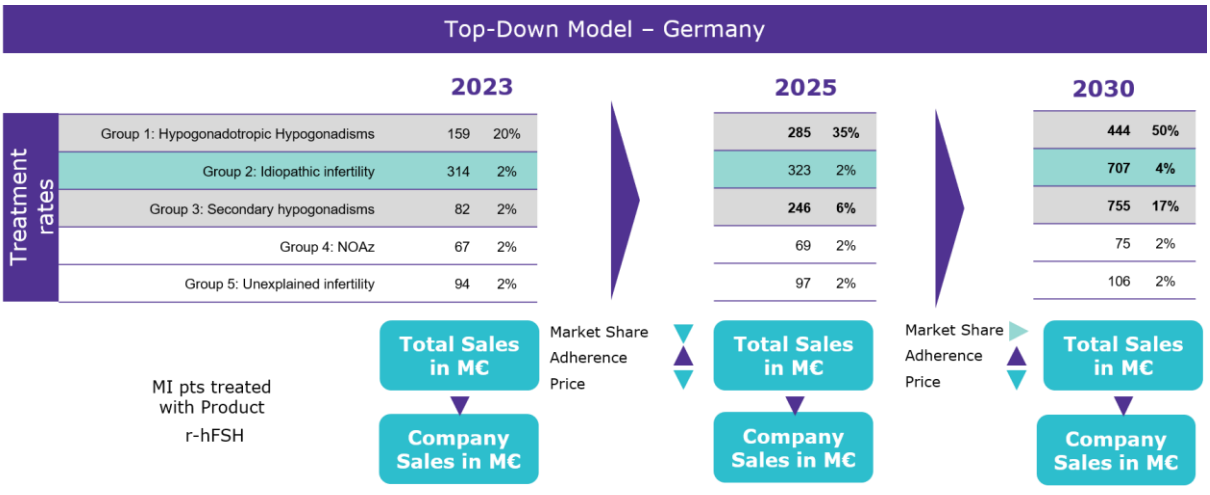

Figure 11. Business model applied to Germany with provision up to 2030. Market share should decrease in 2025 due to competition, meanwhile adherence should increase driven by patients and clinicians' education. Finally, products should suffer of a price decrease due to Health Authorities reconsideration.

The difference in revenue between today and the future and deducting the required investment is generating the NPV. A positive NPV and Return Ratio indicate that the investment is

expected to generate more cash inflows than outflows, potentially creating value for the Company.

The Company is currently reviewing the Business Case results to make an informed decision regarding whether Mele infertility represents an unmet medical need that justifies further exploration. This evaluation includes assessing the potential expansion of indications for the existing hormonal products portfolio. The exercise brought to some recommendations for Company consideration (**Figure 12**)

Parameter	Gr 1&3 r-hFSH	Gr 1&3 r-hCG	Gr 2 r-hFSH
Clinical Development strategy (studies required)	No regulatory study required	Formulation development and Bioequivalence (BE) Study	Ph2 1-2 Ph3
Study investment	None	Medium (avg internal cost for BE study and formulation dev.)	Double digit Mio €
Probability of success	n/a	Benchmark	Benchmark
Time-to-market	immediately	4-5 years	5-6 years
Incremental Topline per annum	2-3 x baseline	Marginal only	xx Mio €
Financial KPIs	Positive eNPV & Return Ratio	Negative financial KPIs > Not viable case	Positive eNPV & Return Ratio
Recommendation for discussion	*Execute*	*Stop assessment*	*Enter next phase (explore development strategy)*

Figure 12. Evaluation of NPV for male infertility opportunity. Legend - NPV is a financial metric used to evaluate the profitability of an investment or project. NPV calculates the difference between the present value of cash inflows and the present value of cash outflows over a specified time-period. Return Ratio is defined as return or profit generated from an investment relative to its cost. It is expressed as a multiple (factor) and is calculated by dividing the net profit from the investment by the initial investment cost.

4. Discussion

This PhD project has provided some evidence that could serve as a starting point to better understand the landscape of male infertility. However, many aspects still require exploration and the development of solid solutions.

Exploring LH and hCG differential induced steroid secretion profile of target Leydig cells (mouse cell line) & pharmacodynamics and safety of r-hLH (Investigational treatment) and u-hCG (standard treatment) in HH men

LH and FSH are glycoproteins supporting steroidogenesis, gametogenesis, and reproduction. hCG acts during pregnancy via the same receptor for LH, the LHCGR, to stimulate progesterone production by the corpus luteum and maintain pregnancy. In addition, gonadotropins are growth and differentiation factors, modulating cell proliferation, survival, and apoptosis. In the male, only FSH and LH are physiologically produced. Indeed, the regulation of spermatogenesis involves a complex interplay of endocrine, paracrine, and metabolic interactions among Sertoli, Leydig, peritubular, and germ cells. These interactions are crucial to support proliferation and differentiation of spermatogenic cells. LH regulates the production of testosterone by Leydig cells, which are endocrine cells located in the testicular interstitium. Testosterone is essential for male virilization and, in combination with FSH, it initiates and sustains spermatogenesis. The combined action of testosterone and FSH occurs within Sertoli cells, which form the wall of the seminiferous tubules and provide support for germ cells as they undergo progressive development into mature sperm (Oduwole *et al.*, 2021). hCG is produced by corpus luteum, and even though it is not naturally produced in male, hCG acts through the membrane G protein-coupled LH receptor (LHCGR) and induces testosterone synthesis (Fanelli *et al.*, 2023). In the clinical setting, hCG is routinely used to mimic LH physiological activity.

Despite LH and hCG sharing approximately 80% homology and binding to the same receptors, they serve distinct biological purposes in females. LH is involved in folliculogenesis, while hCG plays a role during pregnancy. In clinical practice, hCG is used in Ovarian Stimulation (OS) protocols during ART to replace LH's physiological activity. Studies by Casarini et al. (2012) have demonstrated that LH and hCG activate different biological pathways in granulosa cells. These findings, observed in stabilized and primary cell line models, are now considered crucial to take clinical decisions about the use of LH in OS protocols. Considering this strong evidence generated in a female setting, it becomes relevant to question whether LH and hCG also activate different pathways in Leydig cells, potentially leading to a reevaluation of the clinical use of hCG.

Recently, a specific pathway called the "backdoor" pathway has been identified for DHT production. Unlike the conventional pathway, that involves testosterone intermediacy, the backdoor pathway leads to DHT synthesis via neurosteroid production from progesterone and 17OH-progesterone (O'Shaughnessy *et al.*, 2019). This pathway has been observed in the testes of tammar wallabies and immature mice, and it is suggested to be present in humans as well. Pathologic mutations in genes involved in the backdoor pathway have been linked to undermasculinized external genitalia in genetic males, and urine steroid profile analysis supports the relevance of the activated backdoor pathway to abnormal virilization in genetic females with cytochrome P450 oxidoreductase deficiency and 21-hydroxylase deficiency (O'Shaughnessy *et al.*, 2019). It is likely that the backdoor pathway primarily operates in the fetal testes, producing sufficient DHT for male sex development in physiological conditions. In pathological conditions with increased 17-hydroxyprogesterone levels, there would be an interplay between fetal adrenal and liver, and placental steroidogenesis, driving the backdoor pathway. These findings provide new insights into androgen biosynthesis in both physiological and pathological conditions and raise the intriguing hypothesis that hCG and LH may differentially activate the two pathways for DHT production (Fukami *et al.*, 2013). Since hCG is responsible for fetal virilization during pregnancy, and LH is involved in development from childhood to adulthood, further research is needed to explore this theory. If such a theory is confirmed, it could potentially impact clinical practice by replacing hCG treatment with LH in testosterone-deficient patients.

The results obtained during this PhD fellowship brought some evidence regarding the differential pathway activated by LH and hCG. First, we developed a LC-MS/MS method to accurately measure the levels of 20 steroids, including those belonging to the canonical and backdoor androgen pathways, as well as P4 metabolites. The validity of the method was confirmed using the mLTC1 cell line, which are expressing the murine LH receptor. This receptor shares structural similarity with the human receptor and binds both human LH and hCG. It's important to note that human ligands primarily stimulate the synthesis of Δ4 hormones, although Δ5 steroid production has also been observed. However, when comparing mLTC1 to human Leydig steroidogenesis, variations in their enzymatic environments should be considered.The LC-MS/MS method exhibited good recovery, precision, trueness, and stability. The impact of matrix effects was minimal thanks to the careful separation of compounds by LC and the selection of specific fragment ions. Additionally, to ensure the reliability of the steroid results in this study and in future *in vitro* and *in vivo* investigations, we

verified the absence of interferences occurring from steroids not included in the panel and from phosphodiesterase 5 inhibitors.

We extracted steroids from supernatants of mLTC1 cells that were either unstimulated or exposed to hCG. The extracted steroids were then subjected to analysis using positive electrospray ionization and multiple reaction monitoring. We successfully measured the levels of 16OH-progesterone, 11-deoxycortisol, androstenedione, 11-deoxycorticosterone, testosterone, 17OH-progesterone, androstenedione, epitestosterone, dihydrotestosterone, progesterone, androsterone, and 17OH-allopregnanolone. Traces of 17OH-dihydroprogesterone, androstanediol, and dihydroprogesterone were also detected, while no peaks were observed for androstenediol, 17OH-pregnenolone, dehydroepiandrosterone, pregnenolone, and allopregnanolone.

Upon hCG stimulation, we observed a significant increase of 80.2-102.5 folds in 16OH-progesterone, androstenedione, and testosterone levels. Dihydrotestosterone showed a 16.6-fold increase, while epitestosterone, progesterone, and their metabolites exhibited increases ranging from 12.2 to 27.5 folds. The levels of 17OH-allopregnanolone increased by 8.1 folds, and the levels of 5α and 5α,3α steroids increased by 3.3 folds or less. These findings demonstrate that the method developed in this study is suitable for assessing steroid production in mLTC1 cells with sufficient sensitivity.

As second phase of this research, mLTC1 cells were independently exposed to LH or hCG and the steroidogenesis pathways assessed by the newly developed and validated LC-MS/MS method. Comparison between untreated vs LH- and hCG-induced steroid production demonstrated indeed a differential activation of the steroidogenesis pathway and highlighted the hCG higher potency in activating DHT synthesis in Leydig cells (session 3.1.2.7). In particular, hCG upregulated also backdoor neurosteroids, i.e. allopreganolone, androsterone and androstanediol, whose production is unexpected in the Leydig cell, while they are overall inhibited by LH. Finally, hCG is also more potent than LH in inducing the production of deoxicorticosterone and 11-deoxicortisol, which are metabolites of progesterone and 17OH-progesterone. These data suggest that, in male target reproductive cells, LH and HCG activate different signals and that hCG action could be exerted preferentially through the “backdoor” pathway. Such preliminary data corroborate the hypothesis that hCG could have a primary role during fetal period in activating fetus virilization using the “backdoor”, while LH main role is exerted during childhood and adult life via the classical steroidogenic pathway, in collaboration with FSH. These *in vitro* studies must to be considered as a proof of concept and must be confirmed using human Leydig cells. However, the collection of human Leydig cells is

challenging as they are not commercially available and difficult to generate from primary cell lines derived from human testis. As a continuation of this PhD project, the Company, in collaboration with Modena and Reggio Emilia University, is initiating a project to develop a stabilized human Leydig cell line using stem cells. These experiments are currently ongoing.

Another very important observation comes from the RHYTM trial, a pharmacodynamics study ongoing on HH patients (3.2.), comparing the LH- and hCG-induced testosterone production in HH patients. This study consists in an important assessment of validity of the *in vitro* study described above. The clinical study is still ongoing but, meanwhile, the preliminary data are indicative of relevant findings. Up to date, one patient received hCG in the control group and 4 patients were enrolled in the study group receiving LH. As expected, hCG significantly increased testosterone levels after the first administered dose. Instead, the four patients treated with LH did not show any significant increase of testosterone production, even at the highest dose. These preliminary findings suggest that hCG is more potent in inducing testosterone production than LH, in HH patients. Unfortunately, The LC-MS/MS method is not suitable for testing steroids and their metabolites in human serum due to high background signal and interference with other proteins. Therefore, a specific method needs to be developed and validated for accurate testing. As a result, it is currently not possible to confirm if LH and hCG differently activate the two pathways for DHT synthesis *in vivo*. Further research and development of appropriate testing methods are required to explore this aspect. However, we could speculate about a LH and hCG differential action based on analysis of past published evidence. Santi et al. (2017b) published a clinical case involving a patient with an atypical giant pituitary adenoma secreting FSH. This patient had elevated FSH serum levels in the presence of central hypogonadism. However, there was no observed testicular enlargement or increased sperm count, indicating a possible reduction of FSH biological activity. Despite undergoing neurosurgery, the patient's hypogonadism persisted, and semen analyses demonstrated azoospermia. In any case, the reduction of serum FSH levels suggested that the gonadotropin should have, at least in part, biological activity impacting the feedback regulation of the hypothalamus-pituitary-gonadal axis. In further investigations, the patient underwent trials consisting of short-term treatment with low doses of either LH or hCG, in three consecutive treatment schemes. Remarkably, LH and hCG demonstrated similar efficacy in stimulating testosterone levels. These findings shed light on the potential effectiveness of both molecules in triggering testosterone synthesis (Santi *et al.*, 2017b). Considering the conflicting results of studies mentioned above, one could propose that LH may require FSH for efficient activation of steroidogenesis and spermatogenesis, while hCG could have a higher sterodogenic potential

displayed even when FSH has relatively weak activity. This hypothesis suggests that FSH might facilitate communication or interactions between Sertoli and Leydig cells, fundamental to support LH steroidogenic action. Instead, I may speculate that hCG activity may rely on the backdoor pathway.

Both *in vitro* and *in vivo* experimental settings conducted as part of this PhD project hold significant potential value for clinical practice. However, additional data are needed before drawing conclusive insights. These findings could also contribute to the evaluation of product development strategies, although it is still too early to assess specific business opportunities.

To further validate results, confirmatory experiments could consist in the optimization of a reliable method to assess steroidogenic pathways in serum supernatant. This would help to determine which pathway is activated *in vivo* preferentially by hCG and LH. Additionally, the RHYTM trial could be amended or modified by adding a study arm including FSH and LH co-treatment. Outcomes obtained from this modified trial arm would either confirm or refute the hypothesis proposed above.

If confirmed, hCG could still be considered a viable therapy for individuals with profound testosterone deficiency in APHRODITE Group 1 and 4. Similarly, FSH and LH co-treatment could be explored as a potential solution for cases of idiopathic male infertility (Group 2), as well as APHRODITE Group 3, where testosterone deficiency is borderline and associated with normal FSH levels. These further investigations could provide essential insights for clinical decision-making and development of new treatment strategies in the future.

Biological/clinical correlation between sperm DNA fragmentation, steroidogenesis (Testosterone serum production) and semen parameters in patients treated with FSH. Testosterone Serum Levels Are Related to reduced Sperm DNA Fragmentation Index, after FSH Administration, in Males with Idiopathic Infertility

In the attempt to identify new treatments for male infertility, it is crucial to consider the lack of robust clinical endpoints to assess treatment efficacy. Currently, there is no clear consensus on which endpoint should be used. In the field of female infertility treatment, live birth (LB) or clinical pregnancy (CP) are commonly used as natural endpoints to assess the efficacy of treatments. However, in controlled ovarian stimulation, the primary endpoint is typically the number of oocytes retrieved. Several studies have established a correlation between the number of oocytes retrieved and clinical outcomes such as CP or LB. This correlation supports the notion that the number of oocytes retrieved may serve as a reliable surrogate for assessing the likelihood of achieving CP, or LB, in infertility treatment. These endpoints have been

extensively studied and validated over many years, allowing for more confident conclusions in clinical trials. However, in the context of male infertility, the identification of appropriate clinical endpoints is challenging, and CP or LB could suffer of significant limitations due to impact of multiple variables unrelated to the condition. The use of LB or CP would require large sample sizes and prolonged trial durations.

Semen analysis, as the number of oocytes retrieved, is considered as a suitable surrogate endpoint for male infertility, although it has limitations in accurately differentiating between fertile and infertile males (Luján *et al.*, 2019). A recent study by Santi et al. (2023) attempted to address this challenge by proposing the percentage increase of sperm concentration after FSH administration, as a predictor of treatment efficacy and pregnancy outcomes. Mathematical analyses were used to develop a function that predicts the required increase in sperm concentration for achieving pregnancy based on the baseline sperm count.

Recently, Villani et al. (2022) published a study describing results from a retrospective descriptive analysis of MAR cycles, including both males and females' parameters. In this study 22,013 in vitro fertilization and ICSI cycles were considered and semen analysis of male partner assessed according to the WHO manual, used for the interpretation and the sperm alterations. In the context of in vitro fertilization (IVF), various parameters related to sperm motility and morphology have been found to be predictive of fertilization rate, pregnancy rate, and LB rate. Progressive motility and motility after capacitation have been identified as significant predictors of fertilization rate. Additionally, sperm motility has been found to predict pregnancy and LB rates.

In cycles involving ICSI, sperm morphology has been found to be a predictor of fertilization rate. Moreover, sperm morphology has been shown to significantly predict both pregnancy and LB rates. Researchers have identified a cut-off of 5.5% in sperm morphology as a threshold to predict CP. These findings highlight the importance of assessing sperm motility and morphology in determining the success of IVF and ICSI treatments.

In summary, sperm motility and morphology could be used as indicators of sperm quality. The findings of Villani et al. shed light on the clinical approach that should be considered when dealing with infertile couples, particularly those with a dominant male factor. In cases where patients present severe male factor infertility, performing ICSI or IVF with the sperm may not yield optimal results.

Other potential endpoints include endocrine biomarkers of spermatogenesis such as serum FSH, Inhibin B, Testosterone and sDF.

Sperm DNA integrity plays a crucial role in fertilization and the development of healthy offspring. During spermatogenesis, the sperm undergoes molecular remodelling occurring in the nucleus, protecting the genetic content by compaction. However, maturation defects, abortive apoptosis in the testes, and oxidative stress in post-testicular phases can lead to sDF. sDF can affect both natural conception and assisted reproduction (Agarwal *et al.,* 2020)

Various clinical and environmental factors have been identified as contributors to sperm DNA damage. The relationship between sperm DNA damage and male infertility is increasingly recognized by numerous studies (Agarwal 2020). However, scientific societies currently do not recommend routine assessment of sDF in clinical practice.

Several randomized controlled clinical trials have shown a reduction in sperm DNA damage in men treated with FSH (Santi *et al.,* 2018b). Studies have demonstrated that infertile men have higher sDF indexes compared to fertile men, and FSH administration can lead to a decrease in sDF by approximately 4.24% in cases of idiopathic infertility (Esteves *et al.,* 2020). However, it remains unclear whether the improvement in sDF after FSH administration is limited to men with altered baseline levels of DNA damage. Additionally, it is important to investigate whether sDF can serve as a marker of FSH efficacy, differentiating between "responders" and "non-responders" to FSH treatment.

In our study, which is part of this PhD project, we conducted a retrospective, post-hoc re-analysis using prospectively collected raw data from clinical trials involving idiopathic infertile men who received FSH treatment. The primary aim of your study was to explore the relationship between testosterone serum levels and changes in the sDF index following FSH administration. The secondary aim was to confirm the validity of the sDF index as a biomarker of FSH effectiveness in male idiopathic infertility.

The study reveals novel aspects of male infertility management, including the improvement of conventional semen quality and a 20% decrease of sDF baseline index after FSH treatment. FSH stimulation not only leads to improved semen parameters, but also increases testosterone, inhibin B, and anti-Müllerian hormone (AMH) serum levels. The rise in inhibin B and AMH levels is expected since they reflect the proliferative *status* of the testicular germinative epithelium, which is influenced by FSH. However, the unexpected finding is the increase in testosterone levels following FSH administration, suggesting a more complex role of FSH in testicular function than previously thought.

The study suggests that FSH may directly impact Sertoli cells, supporting spermatogenesis, and potentially exerting indirect effects on the testis interstitial compartment. This finding reflects previous results demonstrating that the stimulation with supraphysiological FSH doses can

sustain spermatogenesis even in the absence of LH action. Again, these data elegantly explain RHYTM preliminary results, which were discussed above, about the role of FSH to support LH steroidogenic action via classical pathway, in Leydig cell.

The correlation between testosterone increase and sDF decrease, occurring in men after FSH treatment, is a novel observation that may have implications for evaluating the response to treatment in male idiopathic infertility.

The study emphasizes the connection between the seminiferous and interstitial testicular compartments, which have typically been underestimated in the clinical practice due to the complexity of assessing intratesticular testosterone levels.

In summary, our analysis highlights the association between the rise of testosterone levels and improvement of sDF following exogenous FSH administration in men with idiopathic infertility. Testosterone levels and male age serve as markers of FSH effectiveness, although specific thresholds for clinical application were not identified in this study. These findings suggest that the evaluation of fertility *status* and treatment response should extend beyond traditional semen parameters.

Moreover, baseline sDF levels can predict the response to FSH treatment, in men having a basal sDF index greater than 16.75%, who have increased sensitivity to FSH-induced improvement of sDF. However, further investigations are needed to understand how these data may be translates into clinically relevant outcomes, such as pregnancy rates.

As introduced in this thesis, recent evidence suggests that the male factor may have an impact on the health of offspring when MAR is applied to overcome it. There is a correlation between male factors and health conditions of offspring born through ICSI. These considerations reflect the relevance of sperm quality and emphasizes that it is a parameter to be optimized prior to natural intercourses or any MAR technique. Increasing number of studies are suggesting that hormonal therapeutic interventions improve sperm quantity/quality and overall male health, ultimately resulting in better reproductive outcomes, even when ICSI is the only option (Faure *et al.*, 2014; Esteves *et al.*, 2016, 2020, 2023a; Kirby *et al.*, 2016; Salas-Huetos *et al.*, 2017; Samplaski *et al.*, 2017; Vanegas *et al.*, 2017; Ricci *et al.*, 2018; Santi *et al.*, 2018a; Omar *et al.*, 2019; Lira Neto *et al.*, 2021; Persad *et al.*, 2021; Bian *et al.*, 2022; Humaidan *et al.*, 2022).

By integrating existing evidence with new findings from this PhD project, it is possible to speculate on addressing one of the key gaps in male infertility treatment: the need for reliable biomarkers to assess the efficacy of hormonal treatments and transition towards evidence-based approaches. Semen analysis emerges as a primary biomarker for male fertility, and it should be

considered by evaluating sperm concentration along with sperm quality, indicated as motility and morphology.

The mathematical function identified in the study by Santi et al. (2023) may serve as a valuable primary endpoint in future clinical trials, enabling the assessment of gonadotropin treatment effectiveness in improving male fertility. Additionally, sDF can be considered as a co-biomarker to predict the response to hormonal treatment and as a co-endpoint in male infertility studies, providing insights into the clinical efficacy of interventions.

Testosterone is a known diagnostic and clinical biomarker for patients with hypogonadism or testosterone deficiency. In addition, this hormone could be utilized together with sperm analysis in idiopathic male infertility conditions to assess hormonal treatment efficacy. This combined approach may enhance the comprehensive evaluation of male fertility and provides useful information for treatment decision-making.

In summary, this project aimed to address the need for reliable biomarkers in male infertility treatment. The integration of sperm analysis, sDF, and testosterone assessment may contribute to an evidence-based approach in the evaluation and management of male infertility. Future research should focus on further experimental validation of these biomarkers and exploring their clinical utility in different treatment context.

Data describing different activity between LH and hCG in the activation steroidogenic pathways will be extremely helpful, if confirmed, to guide clinical study designs that could better address patient's needs.

The APHRODITE concept could serve as an initial platform by applying these concepts and generating data, and it could be also possible to refine and improve the classification system over time for patients benefit.

Business Case on male infertility deserving hormonal treatments and final considerations.

Missing knowledge on etiology of male infertility, diagnostic biomarkers, and clinically measurable endpoints, resulted in empirical treatment approaches for this condition. This has led to reduced interest from healthcare system actors and pharmaceutical companies to invest in research and development of specific therapies for male infertility.

In this Industrial PhD project, critical aspects related to male infertility, such as mode of action and efficacy of hormonal treatments on specific target populations, were addressed. Another goal was to identify potential reliable endpoints to be used in clinical trials. By leveraging data from this PhD project and available scientific knowledge, I aimed to build a solid business case

to evaluate the feasibility of developing appropriate therapies and determine their potential success in the market.

The business case evaluation is essential for making informed decisions related to the expansion of the Company portfolio. Pharmaceutical companies often use this standard approach to assess market opportunities before making critical decisions that involve significant investments.

In this specific business case, decision-making is based on the NPV obtained through the developed model. NPV is a financial metric used to assess the profitability of an investment or project. It calculates the difference between the present value of cash inflows and outflows over a specified time-period, by discounting future cash flows to their present value using an appropriate discount rate. A positive NPV indicates that the investment is expected to generate more cash inflows than outflows, potentially adding value to the Company or to the project. Instead, a negative NPV suggests that the investment may not generate sufficient returns to cover the initial investment and related costs.

According to **Figure 12**, a positive NPV is obtained for promoting the existing indication for r-hFSH in APHRODITE Group 1 and 3, as well as for developing a new r-hFSH indication for APHRODITE Group 3 focusing on idiopathic male infertility. Based on this information, the Company is currently developing a potential implementation plan that will be discussed in the early 2024 for further decision-making.

The evidence generated from this PhD research, not only holds value for the Company's business case, but also plays a crucial role in addressing the open questions surrounding male infertility management. By delving into this complex field, the research provides a first response to face the existing, various uncertainties. It emphasizes the importance of expanding data generation within the scientific community to better serve patients and meet their medical needs.

One significant aspect of this research is the implementation of the APHRODITE criteria. They enable the generation of data from homogeneous populations, ensuring that findings are reliable and applicable across different contexts. This approach helps to move beyond purely empirical practices and fosters the adoption of evidence-based strategies in male infertility management. Additionally, the use of the APHRODITE criteria allows for the validation of existing clinical endpoints or the identification of new, robust ones.

This PhD project emphasizes the importance of education in addressing the barriers associated with male infertility management. Lack of awareness and knowledge among patients, clinicians, health authorities, service providers, and society, poses a significant challenge in this field. By advocating for increased education and awareness, the research highlights the need

for concerted efforts to overcome these barriers. It emphasizes the responsibility of both scientific societies and pharmaceutical companies to collaborate and work synergistically to effectively reduce these obstacles.

It's worth noting that projects included in this PhD will not terminate at the end of the academic year. Instead, they will continue beyond this period to ensure the robustness of data and uphold the integrity of the scientific plan. This commitment to continuous research reflects the dedication to producing reliable and long-term insights in the field of male infertility management.

Overall, this PhD research aims to significantly advancing our understanding of male infertility and improving the management approaches currently available. By generating evidence, addressing open questions, implementing robust criteria, advocating for education, and ensuring project continuity, it seeks to make a positive impact on both the scientific community and patients in need.

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