Dissecting the importance of sex steroids balance for metabolic and reproductive health in men with Klinefelter syndrome: a randomized controlled study

SHORT TITLE – ACRONYM: THE KLIN-HEALTH study

Study Type:	Clinical trial with Investigational Medicinal Product (IMP)
Study Categorisation:	Category B
Study Registration:	Name of study registry (if not yet registered name the intended registry) Registration number (from the Swiss National Clinical trial Portal: SNCTP), other registries and numbers if applicable
Study Identifier:	Klin-Health
Sponsor, Sponsor-Investigator or Principal Investigator:	PD MERc Georgios Papadakis, MD Chief Resident, Service of Endocrinology, Diabetology and Metabolism CHUV Avenue de la Sallaz 8, CH-1011 Lausanne Georgios.Papadakis@chuv.ch; +41(0)79 556 03 08
Investigational Product:	Design for patients seeking fertility and negative mTESE: Group A: anastrozole - Anastrozole® Teva oral Group B: anastrozole - Anastrozole® Teva + gonadotrophine chorionique - Choriomon® subcutaneous Design for patients with high metabolic risk: Group A: control (standard care) – testosterone gel (Tostran®) Group B: anastrozole - Anastrozole® Teva oral Group C: semaglutide - Ozempic® subcutaneous
Design Version and Date:	Version 1 of 22.09.2022

Signature Page(s)

Note: Add more lines, functions and pages if relevant, e.g. for trial statistician, if relevant or design contributors

Study number	Study registry and registration number
Study Title	Dissecting the importance of sex steroids balance for metabolic and reproductive health in men with Klinefelter syndrome : a randomized controlled study

The Sponsor-Investigator and trial statistician have approved the design version 1 of DD.MM.YYYY, and confirm hereby to conduct the study according to the design, current version of the World Medical Association Declaration of Helsinki, the ICH-GCP guidelines and the local legally applicable requirements.

Sponsor-Investigator: Georgios Papadakis
Service of Endocrinology, Diabetology and Metabolism, CHUV

Place/Date	Signature

Local Principal Investigator at study site*:

I have read and understood this trial design and agree to conduct the trial as set out in this study design, the current version of the World Medical Association Declaration of Helsinki, ICH-GCP guidelines and the local legally applicable requirements.

Site	Service of Endocrinology, Diabetology and Metabolism, CHUV
Principal investigator	Georgios Papadakis

Place/Date	Signature

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STUDY SYNOPSIS

Sponsor / Sponsor-Investigator	PD MERc Georgios Papadakis
Study Title:	Dissecting the importance of sex steroids balance for metabolic and reproductive health in men with Klinefelter syndrome : a randomized controlled study
Short Title / Study ID:	KlinHealth study
Design Version and Date:	Version 1 of XXXX
Trial registration:	This study is registered on the international portal Clinicaltrials.gov (NCTXXX) and on kofam.ch (SNCTPXXX)
Study category and Rationale	Category B (according to the Clinical Trials Ordinance, art.19): the study product is authorised in Switzerland but it is not used in accordance with the prescribing information.
Clinical Phase:	Not applicable
Background and Rationale:	Klinefelter syndrome (KFS) is the most common chromosomal aberration in males with an estimated prevalence of 1 in 500 births. KFS is suspected in the setting of small testes, azoospermia, and high gonadotropins. The diagnosis is confirmed by a karyotype showing the presence of a supernumerary X chromosome (47, XXY). The majority of affected males have variable degrees of testosterone (T) deficiency due to testicular failure, as well as altered testosterone-to-estradiol (T/E) ratio due to an increased aromatase activity. The major co-morbidities in KFS include metabolic defects and infertility. Despite several benefits of testosterone replacement therapy (TRT), this is not effective in reversing the high metabolic risk in KFS men and does not treat their infertility. The advent of microdissection testicular sperm extraction (mTESE) biopsy has offered fertility potential in some KFS subjects, with successful sperm retrieval in approximately 30% of cases. The current study seeks to optimize the management of KFS focusing on hypogonadism, metabolic defects, and infertility by a randomized controlled trial. In men with high metabolic risk, based on several lines of evidence and preliminary data from our historic EDM KFS cohort, we will investigate (i) whether normalizing the T/E ratio by an aromatase inhibitor (anastrozole) will confer metabolic benefits as compared to classic TRT; and (ii) whether the use of glucagon-like peptide 1 (GLP1) receptor agonists achieves a more effective improvement of metabolic profile while also partly reversing T deficiency. In men who pursue fertility, we will assess two strategies of hormonal stimulation if there is no sperm retrieval after a first mTESE biopsy. Given the promising results on retrospective series, we will examine the efficacy of aromatase inhibitor alone or combined with human chorionic gonadotropin (hCG) in enhancing residual spermatogenesis. Underlying mechanisms including a shift in the intratesticular T/E ratio and changes in gene expression at the level of the testes will be explored.

Objective(s):	The study seeks primarily to determine whether modulation of systemic and testicular sex steroids balance by aromatase inhibitors will positively affect the metabolic health and spermatogenesis of men with Klinefelter syndrome as compared to the current state of the art for each issue. Secondary objectives of this study are (i) to unravel the heterogeneity of the reproductive and metabolic phenotype of men with KFS by performing a multi-omic analysis in a large cohort at baseline; (ii) to evaluate the efficacy of semaglutide-induced weight loss to achieve metabolic and reproductive benefit in men with Klinefelter syndrome as compared to standard testosterone replacement; (ii) to assess whether addition of hCG to aromatase inhibitors further increases intratesticular testosterone and promotes spermatogenesis in men with KFS.
Outcome(s):	The primary outcome for this study will be: • Design 1 (patients interested in fertility preservation): the **sperm retrieval rate (SSR) at second mTESE biopsy after 26 weeks of hormonal pre-treatment** (group A and B separately) in patients with a sperm-negative first mTESE biopsy**,** as compared to the expected rate without intervention • Design 2 (patients at high metabolic risk): the **change in insulin resistance**, **as assessed by the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) score** [1], **from baseline to end of treatment (week 26)** The main secondary outcomes will be: Design 1: - SSR at second mTESE biopsy in group A vs group B - Changes in markers of serum inhibin B, AMH and INSL3 from baseline to week 26 and correlation with the mTESE biopsy results - Change in testicular histology with particular focus to the fibrosis-hyalinization from baseline to week 26 - Change in transcriptomic status at the testes from baseline to week 26 - Change in intratesticular levels of sex steroids (T, E2) from baseline to week 26 Design 1: - Change in HOMA-IR score from baseline to week 4 and week 13 - Change in other insulin sensitivity indexes derived from oral glucose tolerance test from baseline to week 4, 13 and 26 - Change in total and visceral adiposity as assessed by DXA from baseline to week 13 and 26 - Change in inflammation serum markers (hs-CRP, TNF-a, IL-6, S100A8-9) from baseline to week 4, 13 and 26 - Change in serum T levers from baseline to week 13 and 26 in group 3 (semaglutide)
Study design:	Design 1: randomized controlled trial Design 2: randomized controlled trial Register study (only baseline): crossectional and at a later stage case-control study

Inclusion / Exclusion criteria:	**Design 1:**
	Key inclusion criteria are:
	• Diagnosis of KFS (47, XXY or mosaicism)
	• Age range: 16-40 years old
	• Intention to become parent or interest in fertility preservation
	• Confirmed azoospermia (lack of spermatozoids) after centrifugation of 2 semen samples
	Key exclusion criteria are:
	• Higher level of aneuploidy such as supra-Klinefelter (48, XXXY)
	• Prior exposure to aromatase inhibitors or hCG
	• Contraindications to testosterone-rising therapies, e.g., prostate or breast cancer, PSA > 4 µg/l, active liver disease, symptomatic heart disease
	• Other causes of infertility such as exposure to chemo- or radiotherapy, reducing the chance of successful mTESE
	• Decreased life expectancy due to advanced neoplasia or other terminal disease
	• Known or suspected non-compliance, drug or alcohol abuse
	• Inability to follow the procedures of the study, e.g. language problems, psychological or mental disorders, dementia
	• Any ascertained or suspected loss of ability to discern
	• Social precariousness contraindicating medically-assisted procreation
	
	Design 2:
	Key inclusion criteria are:
	• Diagnosis of KFS (47, XXY or mosaicism)
	• Age range: 18-60 years old
	• Moderate hypogonadism at diagnosis defined as serum T levels 4-14 nmol/l
	  ○ If TRT, the T level should be confirmed after wash-out
	• High metabolic risk: severe overweight with BMI > 27 kg/m^2 or a milder overweight (BMI 25-27 kg/m2) but with insulin resistance (fasting HOMA-IR > 2.6)
	  ○ If TRT, the BMI/HOMA-IR should be confirmed after wash-out
	Key exclusion criteria are:
	• Intention to become a parent during the course of the study or immediate interest in fertility preservation (if yes, directed to design 2)
	• Contraindications to TRT, e.g. known hypersensitivity or allergy to testosterone preparations, prostate or breast cancer, PSA > 4 µg/l, active liver disease, symptomatic heart disease
	• Contraindications to GLP1 receptor agonists, notably history of pancreatitis

	• Untreated endocrine disorders or medications with impact on metabolism • Decreased life expectancy due to advanced neoplasia or other terminal disease • History of fragility fractures at classic osteoporotic sites • Known or suspected non-compliance, drug or alcohol abuse • Inability to follow the procedures of the study, e.g. language problems, psychological or mental disorders, dementia <u>KFS patients for register study (only baseline)</u> Key <u>inclusion</u> criteria are: • KFS diagnosis (47 XXY or mosaic form) • Age 14-60 years • TRT naïve • Informed Consent as documented by signature • Non-compliance to the inclusion criteria of designs 1 and 2. <u>Reproductive controls (recruitment at a later stage):</u> Healthy volunteers fulfilling all of the following <u>inclusion</u> criteria are eligible • Age 18-60 years • Strictly normal serum T levels at morning fasting sample (> 14 nmol/l), plasma LH (2-9 U/L) and FSH (2-12 U/L) levels • Normal testicular volume at Prader (≥ 12 ml) and signs of virilization (pubic and axillary hair)

Measurements and procedures:	Screening visit: Informed consent, Medical history, Physical exam (BMI), Inclusion and exclusion criterion, Screening blood test for Design 2 Baseline: Clinical visit (physical exam, anthropometrics, sexual function questionnaire), Fasting blood sample (reproductive and metabolic profile + biobank + PaxGene for RNA studies), Oral Glucose Tolerance Test (OGTT), Dual X-ray absorptiometry (Bone and Body composition), Fibroscan of the liver, Testicular ultrasound, Whole Body calorimetry, Semen analysis, HCG stimulation test (Choriomon® 5000 U SC + T levels measurement after 72h), Randomization, Education regarding assigned study drug, Drug provision. For Design 1, mTESE biopsy n°1: sperm retrieval rate, testicular samples for gene expression studies and steroid measurements (T, E2) using liquid-chromatography – mass spectrometry (LC-MS) Week 4: Clinical visit (physical exam, anthropometrics, sexual function questionnaire), Fasting blood sample (reproductive and metabolic profile + biobank), Adverse events check, Adjustment of study drug's dose, Drug provision. Week 13: Clinical visit (physical exam, anthropometrics, sexual function questionnaire), Fasting blood sample (reproductive and metabolic profile + biobank), Adverse events check, Adjustment of study drug's dose, Drug provision. For Design 2, OGTT and body composition. Week 24: For Design 1, semen analysis. Week 26: Clinical visit (physical exam, anthropometrics, sexual function questionnaire), Fasting blood sample (reproductive and metabolic profile + biobank + PaxGene for RNA studies), Oral Glucose Tolerance Test (OGTT), Dual X-ray absorptiometry (Bone and Body composition), Fibroscan of the liver, Testicular ultrasound, Whole Body calorimetry, Semen analysis. For Design 2: HCG stimulation test (Choriomon® 5000 U SC + T levels measurement after 72h). For Design 1, mTESE biopsy n°2: sperm retrieval rate, testicular samples for gene expression studies and steroid measurements (T, E2) using liquid-chromatography – mass spectrometry (LC-MS). Adverse events check. Week 30: Follow-up study. Check for late adverse events. End of study. Reimbursement.

Study Product / Intervention:	**<u>Design 1 (see Study schedule for details):</u>** **Group A: anastrozole (Anastrozole Teva®)** Starting dose 1 tablet = 1 mg per day for 26 weeks • No scheduled adjustment of dosing regimen **Group B: anastrozole (Anastrozole Teva®) and hCG (Choriomon®)** For anastrozole: starting dose 1 tablet = 1 mg per day for 26 weeks • No scheduled adjustment of dosing regimen For hCG: starting dose according to the post-mTESE n°1 T levels • 1000 U every 48h if T < 8 nmol/l, 500 U every 48h if T > 8 nmol/l • The dose will be adjusted according to T levels at Week 4 of study, targeting T trough levels (48h post injection) between 10 and 15 nmol/l. Increments or reductions of 500 U to a maximum dose of 2000 U every 48h will be performed. In case of a dose adjustment, an additional blood test to measure T levels will be performed after 4 weeks to verify if the target was achieved or if additional dose adjustment is indicated. Total duration of treatment of 26 weeks. **<u>Design 2 (see Study schedule for details):</u>** **Group B: anastrozole (Anastrozole Teva®)** Starting daily dose 1 tablet = 1 mg per day for 26 weeks • No scheduled adjustment of dosing regimen **Group C: semaglutide (Ozempic®)** Starting weekly dose 1 subcutaneous injection of 0.25 mg for 4 weeks then 0.5 mg for 4 weeks, then 1 mg for 4 weeks, then 2 mg weekly up to the end of study (26 weeks) • If gastrointestinal symptoms grade 1 at the end of each dosing phase, the dose will not be further increased until resolution of side effects. If gastrointestinal symptoms grade 2 at the end or during each dosing phase, the dose will be decreased to the immediately lower available dose (for instance, decrease to 0.5 mg per week if side effects grade 2 on the 1 mg per week dosage). In case of grade 3 or 4 side effects, the medication will be immediately withheld.

Control Intervention (if applicable):	**<u>Design 1:</u>** At inclusion, all patients will undergo the control intervention which is a first mTESE biopsy without any hormonal pre-treatment (current state of the art). Subsequently, those without sperm retrieval – anticipated to be approximately 70% of total participants – will be randomly assigned to the two 2 experimental intervention. **<u>Design 2:</u>** **Group A: testosterone gel (Tostran®) for 26 weeks** Starting daily dose (1 push = 10 mg) according to T levels at baseline: • 20 mg if T 10-14 nmol/l, 30 mg if T 6-10 nmol/l, 40 mg if T < 6 nmol/l • Subsequent titration by increments or reductions of 1 push (10 mg) to target T levels 15-25 nmol/l, then reassess T levels after 4 weeks • If erythrocytosis (haematocrit > 52%) occurs during the study, target T levels 10-15 nmol/l
Number of Participants with Rationale:	<u>Design 1:</u> 40 patients (20 per group) with a negative (no sperm retrieval) first mTESE biopsy. Given that approximately 28% of patients have sperm retrieval, we will have to recruit 55-60 patients overall. This was calculated based on the positive sperm retrieval in retrospective series of men with KFS undergoing mTESE after anastrozole and also in the literature-derived expected positivity of a second mTESE biopsy is there was no hormonal pre-treatment at all (see Chapter 11). Design 2: 60 patients (20 per group), TRT-naïve or after TRT wash-out. This was calculated on the basis of expected efficacy of anastrozole and semaglutide for HOMA-IR reduction (see Chapter 11). <u>Register study:</u> all participants at Design 1 and 2 (baseline visit), as well as other novel KFS cases either diagnosed at EDM service or referred by providers on the condition that they are TRT-naïve.
Study Duration:	Each participant will be followed in one of the study's designs for 30 weeks (26 weeks of intervention and 4 weeks of follow-up)
Study Schedule:	Start of recruitment: November 2022 End of intervention: November 2025
Investigator(s):	PD MERc Georgios Papadakis, MD Chief Resident, Service of Endocrinology, Diabetology and Metabolism CHUV Avenue de la Sallaz 8, CH-1011 Lausanne Georgios.Papadakis@chuv.ch

Study Centre(s):	Single-center (CHUV, Lausanne) Visits, blood samples and dynamic tests will be performed at the service of Endocrinology, Diabetology and Metabolism (EDM) at CHUV. Testicular biopsies will be performed at the Unit of Medicine for Fertility (UMF) by Dr Vaucher Laurent or at specialized centers for reproductive urology (see list of investigators)
Statistical Considerations:	Design 1: The sample size was calculated based on the positive sperm retrieval in retrospective series of men with KFS undergoing mTESE after anastrozole and also in the literature-derived expected positivity of a second mTESE biopsy is there was no hormonal pre-treatment at all (see Chapter 11). The sperm retrieval rate in both interventional groups and every one separately will be compared to the expected rate without any pre-treatment (10%) using a Fischer exact test. Changes before-after intervention will be assessed by applying paired two-sample t-tests. Design 2: The sample size was calculated on the basis of expected efficacy of anastrozole and semaglutide for HOMA-IR reduction (see Chapter 11). The change score in primary and secondary outcomes will be compared between groups using a regression analysis adjusted for the baseline group.
GCP Statement:	This study will be conducted in compliance with the design, the current version of the Declaration of Helsinki, ICH-GCP, as well as all national legal and regulatory requirements.

ABBREVIATIONS

ACTH	Adrenocorticotrophic Hormone
ADAM	Androgen Deficiency in Aging Males
AE	Adverse Event
AI	Aromatase Inhibitor
AMH	Antimüllerian Hormone
AR	Androgen Receptor
ASR	Annual Safety Report
BASEC	Business Administration System for Ethical Committees, (https://submissions.swissethics.ch/en/)
BMD	Bone Mineral Density
BMI	Body Mass Index
BRB-seq	Bulk RNA Barcoding and Sequencing
CA	Competent Authorities (e.g. Swissmedic)
CEC	Competent Ethics Committee
CER-VD	Commission Cantonale d'Ethique de la Recherche sur l'Être Humain
CHH	Congenital Hypogonatropic Hypogonadism
ClinO	Ordinance on Clinical Trials in Human Research *(in German : KlinV, in French: OClin, in Italian : OSRUm)*
CRF	Case Report Form
CTCAE	Common Terminology Criteria for Adverse Events
DCN	Decorin
DEG	Differentially Expressed Genes
DISF-M-II	Derogatis Interview for Sexual Functioning in Men-II
DSMC	Data Safety Monitoring Committee
DSUR	Development Safety Update Report
DXA	Dual X-Ray Absorptiometry
E2	Estradiol
ECM	Extracellular Matrix
EDM	Endocrinology, Diabetes and Metabolism
eCRF	Electronic Case Report Form
ERa & ERβ	Estrogen Receptor Type A and β
FGF19	Fibroblast Growth Factor-19
FGF21	Fibroblast Growth Factor-21
FSH	Follicle-Stimulating Hormone
GC	Germ cells
GCP	Good Clinical Practice
GLP1	Glucagon-Like Peptide 1
hCG	Human Chorionic Gonadotropin
Ho	Null Hypothesis

HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
HRA	Federal Act on Research Involving Human Beings *(in German : HFG, in French: LRH, in Italian : LRUm)*
hs-CRP	High-sensitivity C-Reactive Protein (serum marker)
IB	Investigator's Brochure
ICD	International Classification of Diseases
ICTRP	International Clinical Trials Registry Platform
IIEF	International Index of Erectile Function
IIT	Investigator-Initiated Trial
IL-6	Interleukin 6 (serum marker)
IMP	Investigational Medicinal Product
INSL3	Insulin-like 3 Factor
ITT	Intention To Treat
KFS	Klinefelter Syndrome
LC	Leydig Cell
LC-MS	Liquid Chromatography – Mass Spectrometry
LLC	CHUV chemistry laboratory
LH	Luteinizing Hormone
MS	Metabolic Syndrome
mTESE	Microdissection Testicular Sperm Extraction
OGTT	Oral Glucose Tolerance Test
PI	Principal Investigator
R	Randomization
RCT	Randomized Controlled Trial
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
S100A8-9	Calprotectin
SC	Subcutaneous
SDV	Source Data Verification
SNCTP	Swiss National Clinical Trial Portal
SOP	Standard Operating Procedure
SPC	Summary of Product Characteristics
SRR	Sperm Retrieval Rate
SUSAR	Suspected Unexpected Serious Adverse Reaction
T	Testosterone
T/E	Testosterone/Estradiol
TMF	Trial Master File
TNF-a	Tumor Necrosis Factor-alpha
TRT	Testosterone Replacement Therapy
UCCR-CRC	Unité de Conseil et de Coordination de la Recherche Clinique

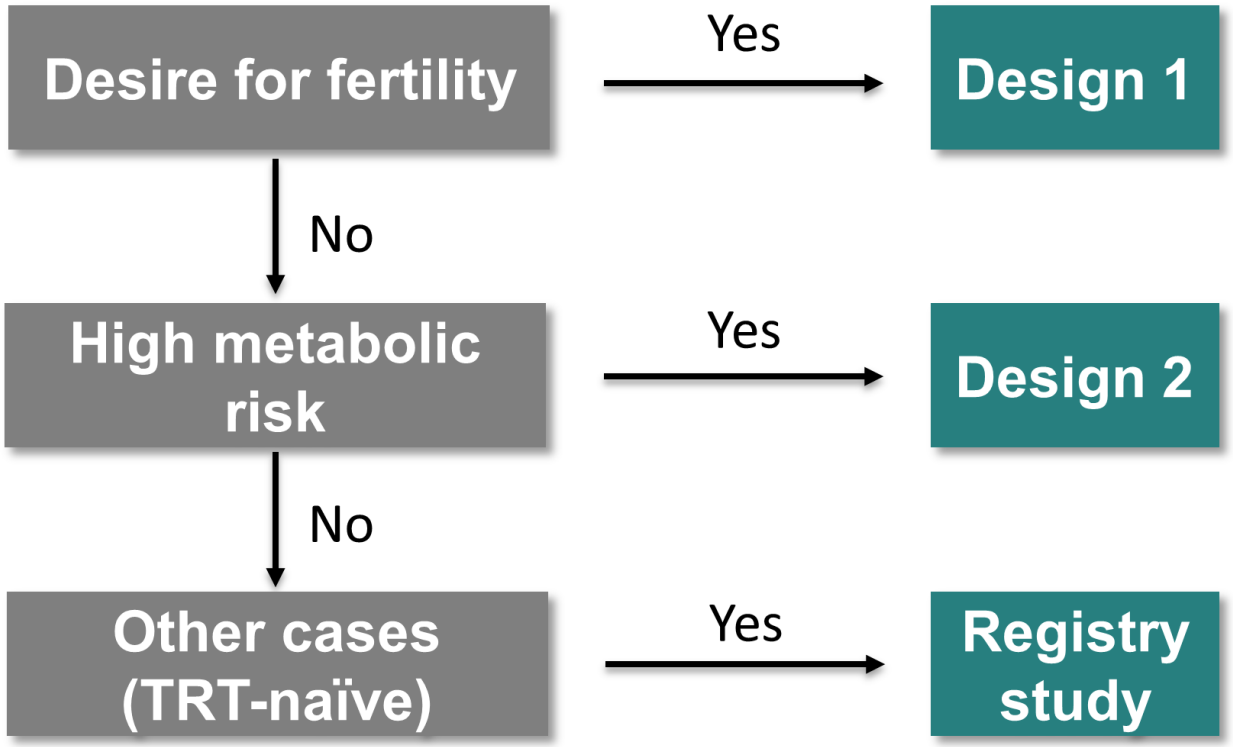
V1-8	Visits 1-8
VAT	Visceral Adipose Tissue

SUMMARY OF THE REVISION HISTORY IN CASE OF AMENDMENTS

Version Nr, Version Date	Chapter	Description of change	Reason for the change

KFS patients

EDM consultation & collaborators



Design 1 (men seeking fertility or interested in fertility preservation):

Study period	Recruitment	Screening	Baseline				Treatment					Follow-up
Visit	0	1	2	3		4	5	6	7	8		9
Time (days, week)	Day -180 to Day -3 before screening	Up to Month -6	Week -6	Week -6 + Day 3	Week -2 to Week -4	Day 1	Week 4	Week 13	Week 24	Week 26	Week 26 + 1-3 days	Week 30
Recruiting (study presentation, set-up)	X											
Patient Document Information	X											
Semen analysis	X		X						X			
Informed consent		X										
Medical history, Physical exam		X										
Inclusion and exclusion criterion		X										
TRT wash-out (if present)		X										
mTESE biopsy					X						X	
RandomizationN (if first mTESE negative)					X							
Drug provision (once a month)						X	X	X		X		
Physical exam												
clinical visit			X			X	X	X		X		X
weight, height, BMI			X			X	X	X		X		X
waist and hip circumference			X			X	X	X		X		X
testicular volume			X			X		X		X		
blood pressure, heart rate			X			X	X	X		X		X
ADAM questionary, DISF-M-II & IIEF scores			X				X	X		X		X
gynecomastia			X				X	X		X		X
check for adverse effects			X				X	X		X		X
Laboratory tests (fasting)												
Reproductive hormones			X			X	X	X		X		X
Metabolic and inflammatory status			X				X	X		X		X
Oral glucose tolerance test (2h)			X							X		
HCG stimulation test (5000 U unique dose)			X (inj)	X (lab)						X		
Blood samples (fasting)												
Serum (9.0 ml)			X			X	X	X		X		X
EDTA (4.9 ml) twice			X			X	X	X		X		X
Heparin (7.5 ml)			X			X	X	X		X		X
PaxGene (2.5 ml)			X			X				X		
Testicular ultrasound				X						X		
Testes samples for gene expression + LC-MS					X						X	
DXA (bone density)			X							X		
DXA (body composition)			X							X		
Whole body calorimetry				X						X		
Fibroscan			X	X						X		

Design 2 (men with high metabolic risk):

Study period	Recruitment	Screening 1	Screening 2 (if TRT)	Baseline		Treatment				Follow-up
Visit	0	1	2	3	4	5	6	7	8	9
Time (days, week)		up to Day -180	up to Day -14	Day -3	Day 1	Week 4	Week 13	Week 26	Week 26 + Day 3	Week 30
Recruiting (study presentation, set-up, TRT wash-out if present, screening info)	X									
Patient Document Information	X									
Informed consent		X								
Medical history, Physical exam (BMI)		X	X							
Inclusion and exclusion criterion		X								
Screening blood test (T, glucose, insulin)		X	X							
Randomization				X						
Drug provision (once a month)					X	X	X	X		
Physical exam										
clinical visit				X		X	X	X		X
weight, height, BMI				X		X	X	X		X
waist and hip circumference				X		X	X	X		X
testicular volume				X			X	X		
blood pressure, heart rate				X		X	X	X		X
ADAM questionary, DISF-M-II & IIEF scores				X		X	X	X		X
gynecomastia				X		X	X	X		X
check for adverse effects				X		X	X	X		X
Laboratory tests (fasting)										
Reproductive hormones				X		X	X	X		X
Metabolic and inflammatory status				X		X	X	X		X
Oral glucose tolerance test (2h)				X			X	X		
HCG stimulation test (5000 U unique dose)				X (inj)	X (lab)			X (inj)	X (lab)	
Blood samples (fasting)										
Serum (9.0 ml)				X		X	X	X		X
EDTA (4.9 ml) twice				X		X	X	X		X
Heparin (7.5 ml)				X		X	X	X		X
PaxGene (2.5 ml)				X				X		
DXA (bone density)				X				X		
DXA (body composition)				X			X	X		
Whole body calorimetry					X				X	
Fibroscan					X				X	
Testicular ultrasound					X				X	
Semen analysis (if not previously assessed)				X				X		

1. STUDY ADMINISTRATIVE STRUCTURE

1.1 Sponsor, Sponsor-Investigator

PD MERc Georgios Papadakis, MD

Chief Resident, Service of Endocrinology, Diabetology and Metabolism

CHUV Avenue de la Sallaz 8, CH-1011 Lausanne

Georgios.Papadakis@chuv.ch; +41(0)79 556 03 08

Role: Responsible for the scientific integrity of the study and overall supervision of the financial/administrative aspects of the project.

1.2 Principal Investigator(s)

PD MERc Dr Georgios Papadakis, MD

Chief Resident, Service of Endocrinology, Diabetology and Metabolism

CHUV Avenue de la Sallaz 8, CH-1011 Lausanne

Georgios.Papadakis@chuv.ch; +41(0)79 556 03 08

Study design and coordination, recruitment of participants, interpretation of results, and redaction of scientific articles

Co-investigators and collaborators:

Prof Nelly Pitteloud, MD

Head of the Service of Endocrinology, Diabetology and Metabolism, CHUV

Avenue de la Sallaz 8, CH-1011 Lausanne

nelly.pitteloud@chuv.ch; +41(0)79 556 27 36

Study design, infrastructure, recruitment of participants, and interpretation of results

PD MERc Dr Michael Hauschild, MD

Head of the unit of Paediatric Endocrinology, Department of paediatrics, CHUV

michael.hauschild@chuv.ch; +41(0)79 556 27 41

Study design, recruitment of adolescent participants, and interpretation of results

Dr Nathalie Vionnet, MD, PhD

Research Doctor, Service of Endocrinology, Diabetology and Metabolism, CHUV

Avenue de la Sallaz 8, CH-1011 Lausanne

nathalie.vionnet@chuv.ch; +41(0)79 556 52 15

Study design, database management, and participation in the interpretation of results

Rikiatou Francioli

Research Medical Assistant, Service of Endocrinology, Diabetology and Metabolism, CHUV

Avenue de la Sallaz 8, CH-1011 Lausanne

rikiatou.francioli@chuv.ch; +41(0)79 556 XX XX

Participation in the recruitment of participants and collection of data

Dr Laurent Vaucher, MD

Reproductive Urologist, mTESE biopsy specialist

Fertility Medicine and Gynaecologic Endocrinology, Department of Gynecologie, CHUV

VaucherL@genolier.net; +41(0)21 314 32 76

Study design, recruitment of participants, mTESE biopsies and interpretation of results

Dr Andrea Messina, PhD

Research Biologist, Service of Endocrinology, Diabetology and Metabolism, CHUV

Rue du Bugnon 7, CH-1011 Lausanne

Andrea.Messina@chuv.ch; +41(0)79 556 88 95

Study design, management of gene expression studies (blood, testes) and interpretation of results

Dr Pierre-Alain Binz, PhD, FAMH

Chemist, Chief of sector of mass spectrometry in clinical chemistry laboratory (LLC), CHUV

Rue du Bugnon 46, CH-1011 Lausanne

Pierre-Alain.Binz@chuv.ch; +41(0)79 556 67 23

Management of blood and testicular steroid profile by mass spectrometry, and interpretation of results

Dr Stephen Bruce, PhD, FAMH

Chemist, Sector of mass spectrometry in clinical chemistry laboratory (LLC), CHUV

Rue du Bugnon 46, CH-1011 Lausanne

steve.bruce@chuv.ch; +41(0)79 556 89 11

Management of blood and testicular steroid profile by mass spectrometry, and interpretation of results

Prof Andrew Dwyer, PhD, FNP-BC, FNAP, FAAN

Boston College, William F. Connell School of Nursing

140 Commonwealth Avenue, Chestnut Hill, MA 02467

andrew.dwyer@bc.edu; +1(0)(617) 552-1711

Study design, scientific consulting, contact with patients' associations

Dr Maria Mavromati, MD

Endocrinologist, Faculty doctor of unit of endocrinology at Geneva University Hospital

Service of endocrinology, diabetes, nutrition and therapeutic education of the patient

Hôpitaux Universitaire Genève (HUG)

Rue Gabrielle Perret-Gentil 4, CH-1205 Genève

Maria.Mavromati@hcuge.ch; +41(0)22 372 33 11

Recruitment of patients from Geneva and surrounding areas

Dr Stefan Fischli, MD

Endocrinologist, Co-head chief of endocrinology-diabetology at Lucerne Cantonal Hospital

Luzerner Kantonsspital

Spitalstrasse 6000, Luzern 16

stefan.fischli@luks.ch; +41 (0)41 205 51 03

Recruitment of patients from Lucerne and surrounding areas

Dr Julien Dagher, MD, PhD

Expert pathologist on testicular histology

University Institute of Pathology

Rue du Bugnon 25, CH-1011 Lausanne

Julien.Dagher@chuv.ch; +41(0)79 556 90 81

Systematic analysis of histologic samples (design 2) with particular focus on evolution of fibrosis

Dr Vietti Violi Naik, MD

Expert radiologist on urogenital imaging and ultrasonography

Service of radiodiagnostics and interventional radiology

Rue du Bugnon 46, CH-1011 Lausanne

Naik.Vietti-Violi@chuv.ch; +41(0)79 556 02 40

Coordination of testicular histology including exploration avec Doppler post injectable microbubbles

Dr Mohammed Barigou

Dr Elena Gonzalez-Rodriguez

Dr Montserrat Fraga

1.3 Statistician ("Biostatistician")

Dr Mohamed Faouzi, MD

Chief of Unit of Consultation Biostatistique (UCB)

Center for Primary Care and Public Health - Division of Biostatistics

Mohamed.Faouzi@unisante.ch; +41(0)21 314 72 87

1.4 Laboratory

Clinical Chemistry Laboratory, CHUV

Rue du Bugnon 46, CH-1011 Lausanne

Prof. Pitteloud's research laboratory, CHUV

Rue du Bugnon 7, CH-1011 Lausanne

Department of Pharmacy, CHUV

Rue du Bugnon 46, CH-1011 Lausanne

pha.etudes@chuv.ch; +41 21 314 4304

1.5 Monitoring institution

(ICH/E6 6.1.2; SPIRIT 5a-d)

ICH: Name and address of the …. monitor (if other than the sponsor).

Provide the name of the institution, place and country that monitors the study, if other than the Sponsor (may be referred to different document, e.g. separate agreement).

1.6 Data Safety Monitoring Committee

Not applicable.

1.7 Any other relevant Committee, Person, Organisation, Institution

Centre de Procreation Medicalement Assistée (CPMA) – Lausanne
Dr Nicolas Vulliemoz – Dr Lionel Micol
Rue de la Vigie 5, CH-1003 Lausanne

2. ETHICAL AND REGULATORY ASPECTS

The decision of the CED-VD and Swissmedic/foreign competent authority concerning the conduct of the study will be made in writing to the Sponsor-Investigator before commencement of this study. The clinical study can only begin once approval from all required authorities has been received. Any additional requirements imposed by the authorities shall be implemented.

2.1 Study registration

This study is registered on the Swiss National Clinical Trials Portal (kofam.ch) via BASEC (SNCTPXXX) and on clinicaltrials.gov (NCT0XXX).

2.2 Categorisation of study

This clinical trial of medicinal products goes under Category B according to OClin art. 19. The products in study (Anastrozole Teva®, Ozempic®, Choriomon®) are authorized in Switzerland, but not in accordance with the prescribing information.

2.3 Competent Ethics Committee (CEC)

The Principal Investigator ensures that the clinical study will not start before the approval of CER-VD. No change will be made to the design without prior Sponsor and CER-VD approval, except where necessary to eliminate immediate hazards to study participants. Premature study end or interruption of the study is reported within 15 days. The regular end of the study is reported to the CER-VD within 90 days, the final study report shall be submitted within one year after study end. Amendments are reported according to chapter 2.10.

2.4 Competent Authorities (CA)

The Principal Investigator ensures that the clinical study will not start before the approval of Swissmedic.

No change will be made to the design without prior Sponsor and Swissmedic approval, except where necessary to eliminate apparent immediate hazards to study participants.

Premature study end or interruption of the study is reported within 15 days. The regular end of the study is reported to the Swissmedic within 90 days, the final study report shall be submitted within one year after study end. Amendments are reported according to chapter 2.10.

2.5 Ethical Conduct of the Study

The study will be carried out in accordance to the design and with principles enunciated in the current version of the Declaration of Helsinki, the guidelines of Good Clinical Practice (GCP) issued by ICH, the Swiss Law and Swissmedic's requirements. The CER-VD and Swissmedic will receive annual safety and interim reports and be informed about study stop/end in agreement with local requirements.

2.6 Declaration of interest

No conflict of interest to declare.

2.7 Patient Information and Informed Consent

The principal investigator (or a co-investigator) will explain to each subject the nature of the study, its purpose, the procedures involved, the expected duration, the potential risks and benefits and any discomfort it may entail. Each subject is informed that the participation in the study is voluntary and that he/she may withdraw from the study at any time and that withdrawal of consent will not affect his/her subsequent medical assistance and treatment. The subjects are informed that he/she can ask any question, and consult with family members, friends, their treating physicians or other experts before deciding about their participation in the study. Enough time is given to the subjects.

The subjects are informed that authorised individuals other than their treating physician may examine his/her medical records. All subjects are given a subject information sheet and a consent form describing the study and providing sufficient information for the subjects to make an informed decision about their participation in the study. They will have at least 3 days in order to decide whether to participate or not.

The formal consent of a subject, using the approved consent form, is obtained before the subject is submitted to any investigation procedure. The subject should read, understand, and voluntarily agree before signing and dating the informed consent form, and is given a copy of the signed document. The consent form is signed and dated by the subject and the principal investigator (or her/his designee). The signed consent form it is retained as part of the investigation records.

The study will recruit adolescent patients aged 14 to 18 years. These participants will be seen by the PI along with their legal representative. They will receive a verbal briefing and written information with the same content as that given to their legal representative. Both the adolescent participant and his/her legal representative sign the consent form. Subjects lacking capacity of judgment (for example, intellectual deficit) will not be included in the interventional study (Design 1 and 2). Any signs and symptoms showing that the subject is unwilling to participate in the study will result in the subject being excluded from participation

2.8 Participant privacy and confidentiality

The investigator affirms and upholds the principle of the participant's right to privacy and that they shall comply with applicable privacy laws. Especially, anonymity of the participants shall be guaranteed when presenting the data at scientific meetings or publishing them in scientific journals.

Individual subject medical information obtained as a result of this study is considered confidential and disclosure to third parties is prohibited. Subject confidentiality will be further ensured by the assignment

to each subject of a unique subject identification number. This unique identification number that will appear on study's samples will be generated by the EDM research study database (SLIMS, Genhom – Lausanne, Switzerland).

For data verification purposes, authorised representatives of the Sponsor (-Investigator), a competent authority (e.g. Swissmedic), or an ethics committee may require direct access to parts of the medical records relevant to the study, including participants' medical history.

2.9 Early termination of the study

The Sponsor-Investigator (and any competent authority) may terminate the study prematurely according to certain circumstances, for example:

- ethical concerns,
- insufficient participant recruitment,
- when the safety of the participants is doubtful or at risk, respectively,
- alterations in accepted clinical practice that make the continuation of a clinical trial unwise,
- early evidence of benefit or harm of the experimental intervention

2.10 Design amendments

Substantial amendments are only implemented after approval of the CER-VD and Swissmedic respectively. Under emergency circumstances, deviations from the design to protect the rights, safety and well-being of human subjects may proceed without prior approval of the sponsor and the CER-VD/Swissmedic. Such deviations shall be documented and reported to the sponsor and the CER-VD/Swissmedic as soon as possible.

All non-substantial amendments are communicated to the Swissmedic as soon as possible if applicable and to the CER-VD within the Annual Safety Report (ASR).

3. BACKGROUND AND RATIONALE

3.1 Background and Rationale

Klinefelter syndrome (KFS) is the most common chromosomal aberration in men with an estimated incidence of 1 in 500-600 live births [2], and is caused by the presence of an extra X chromosome (47, XXY) [3]. KFS is a heterogeneous disease with a complex natural history [4]. Small testes with testicular malfunction are present in all patients, while other less invariable clinical signs include gynecomastia, learning difficulties and increased height [5, 6]. The majority of affected males have moderately low or low-normal testosterone (T) levels, while serum gonadotropins (Luteinizing hormone [LH] and Follicle-stimulation hormone [FSH] are invariably increased attesting the underlying testicular failure [7]. Another hormonal hallmark of the disease is the increased aromatase activity at the testes [8, 9]. **Enhanced aromatization of T to estradiol (E2) leads to high-normal serum E2 levels and decreased testosterone/estradiol (T/E) ratio** as compared to controls [10] and men with hypogonadism (=testosterone deficiency) of central origin [11]. Currently, testosterone replacement therapy (TRT) is the recommended therapy for KFS men with low or borderline T levels and/or clinical manifestations of hypogonadism. **This approach does not normalize the T/E ratio and does not correct two prominent comorbidities of the disease: (i) increased cardiometabolic risk; and (ii) infertility.**

Indeed, KFS men often tend to have excess adiposity and present with higher levels of visceral adipose tissue (VAT) as compared to age- and BMI-matched controls [12, 13], as well as with a **4-fold higher incidence of metabolic syndrome** (MS) [14-16]. Unsurprisingly, this results in an increased all-cause and cardiovascular mortality with a hazard ratio of 1.6-2.0 [17]. It has been hypothesized that testosterone deficiency is the cause of metabolic alterations in KFS men in a unidirectional way [14]. This was recently questioned by the finding of increased fat percent even in prepubertal KFS boys (T levels are physiologically low during childhood) [18]. Moreover, the efficacy of TRT, current state of art for men with KFS, to enhance metabolic health is limited. Three cross-sectional studies did not detect lower VAT or better insulin sensitivity in KFS men on TRT as compared to untreated patients [14, 19, 20]. A small crossover randomized controlled trial (RCT) of testosterone vs placebo for 6 months showed a slight decrease in total body fat but no effect on VAT or insulin resistance [13]. A retrospective Chinese study on 39 KFS men found no benefit of TRT on metabolic outcomes [21]. In this study, MS incidence was significantly higher in KFS than in men with central hypogonadism without the relative E2 excess. A difference of similar magnitude between KFS and patients with central hypogonadism due to pituitary failure was found in an Italian study [19]. Alternative explications for the high metabolic risk of KFS are:

(i) The propensity to MS is an intrinsic feature of KFS linked to the genetic defect. The link with reproduction is bi-directional and the occurrence of metabolic abnormalities aggravates the reproductive defects [20]. Notably, insulin resistance can affect the reproductive axis both centrally [22, 23] and peripherally by reducing the secretory capacity of Leydig cells [24].

(ii) Other hormonal changes such as the abnormal T/E ratio contribute to the metabolic phenotype. Several lines of evidence suggest that the enhanced aromatization in KFS could underlie the higher

MS incidence compared to other forms of hypogonadism, as well as the reduced efficacy of TRT. Higher serum E2 levels are associated with elevated risk of diabetes in men, even after adjustment for BMI [25]. One-week administration of letrozole, an aromatase inhibitor (AI) that blocks conversion of T to E2, increased insulin sensitivity in lean controls [26]. Oxandrolone, a non-aromatized androgen, reduced body fat in a randomized study on 93 prepubertal KFS boys [27]. Further, in an elegant study that identified 36 differentially expressed genes (DEG) in the peripheral blood of 132 KFS men, an ingenious pathway analysis highlighted elevated E2 as a clinical marker linked with several DEGs linked to waist circumference and inflammation [20].

Infertility is a major issue for KFS patients. Testicular failure in KFS is thought to result from a 'dosage effect' of genes that escape X-chromosome inactivation in the setting of an XXY karyotype [5]. The use of microdissection testicular sperm extraction (mTESE) has been a breakthrough, allowing the detection of focal areas of spermatogenesis in 30-40% of cases [28]. Nevertheless, this technique results in genetic fatherhood in only 10-20% of patients [28, 29], highlighting the need for improved strategies. Currently, there are no known predictors of mTESE success with the exception of worse outcomes in men aged > 35-40 years [30]. Recent findings from testicular biopsies in patients of different ages elucidated the natural history of testicular degeneration in KFS [31]. Principal alterations include (i) a progressive depletion of GC that accelerates during puberty [32]; (ii) a pronounced hyalinization of seminiferous tubules with altered expression of extracellular matrix (ECM) proteins; and (iii) a marked Leydig cell (LC) hyperplasia. The cause of hyalinization is unknown but it has been linked to an increased number of peritubular macrophages and mast cells [33]. Hormonal changes during puberty may also play a role as hyalinization is absent in prepubertal samples [31]. Increased aromatase activity could generate excessive conversion of intratesticular T to E2 as puberty initiates. In favor of such a link, transgenic mice overexpressing aromatase exhibit LC hyperplasia and macrophage activation [34]. Estrogen therapy in male-to-female transsexuals causes a marked increase of peritubular ECM proteins [35]. Based on prior use in men with idiopathic infertility and low serum T/E ratio [36], three retrospective series reported on aromatase inhibitors (AI) in KFS, alone [37, 38] or in combination with transdermal TRT [39]. Despite high sperm retrieval rates (50-70%), the absence of a control group precluded definitive conclusions and AI treatment is not systematically offered before mTESE [5]. Taking into account that sex steroids are potent regulators of gene expression [40], it is possible that the altered balance of testosterone and estradiol is linked to the extensive changes in expression of both X-linked and autosomal genes in the testes of KFS men [41-44]. D'Aurora *et al.* found that the majority of down-regulated genes were involved in spermiogenesis failure, whereas up-regulated genes were linked to increased apoptosis [42].

Based on the aforementioned gaps in literature and the currently suboptimal therapeutic choices, **this study's primary purpose is to assess whether optimization of sex steroids balance with aromatase inhibitors could prove more effective than testosterone alone for metabolic health and fertility** in KFS. The secondary purposes of the projects are to (i) to explore alternative therapeutic approaches to alleviate metabolic and reproductive defects such as weight-loss strategies using GLP1

receptor agonists; (ii) to dissect the heterogeneity of the KFS through an extensive phenotyping at baseline and using a multi-omic approach (transcriptomics, metabolomics, steroid profile).

3.2 Investigational Product (treatment) and Indication

For Design 1:

Group A: Anastrozole Teva® is a medication in oral form (film-coated tablets) contained in packages of 30 pills of 1 mg per blister. The active principle is anastrozole, a non-steroid inhibitor of aromatase, blocking the conversion of testosterone to estradiol and of androstenedione to estrone. The excipients of the product are: lactose, povidone K 29-32, carboxyméthylamidon sodique (type A) (corresponding to 0.21 mg of sodium), magnesium stearate, hypromellose, titanium dioxide (E171) and macrogol. Anastrozole is used as an adjunt treatment in menopausal women with hormonosensible form of breast cancer (presence of estrogen or progesterone receptors), as well as to treat women with advanced breast cancer. In men, aromatase inhibitors have been used in adolescent and young adults as part of clinical trials to treat idiopathic short stature [45] and delayed puberty [46] with proved efficacy and good tolerability. In particular, anastrozole has been used as an off-label treatment in men with idiopathic infertility [36], but also specifically in KFS men to induce spermatogenesis [37-39]. For this study, Anastrozole Teva® is considered within the dispensing category B and its authorization number is 65922 (Swissmedic). The authorized enterprise producing is Teva AG, 6330 Cham.

Group B: Anastrozole Teva® - see Group A. Choriomon® is a medication in form of a powder contained in a pierceable vial. The medication kit contains also a prefilled syringe containing 1 ml of NaCL 0.9% to use as a solvent. The active principle is human chorionic gonadotropin (HCG) which is extracted from human urine originated from China Republic and/or Netherlands). The excipient of the powder is lactose. The medication is reconstituted by dissolving the powder in the solvent, then slowly injecting the solution subcutaneously. HCG is acting as a long-acting form of LH, stimulating the Leydig cells of the testes to produce testosterone. It is authorized to treat men with cryptorchidism, hypogonadotropic hypogonadism and delayed puberty, as well as women with lack of ovulation. HCG has been used off-label to promote spermatogenesis in men with Klinefelter. The latest published report was a retrospective series in large group of KFS men originated from China and no safety issues were reported [47]. Choriomon® is in the dispensing category B and its authorization number is 33524 (Swissmedic). The authorized enterprise producing is IBSA Institut Biochimique SA, 6915 Pambio-Noranco.

For Design 2:

Group B: Anastrozole Teva® - see Design 1, Group A.

Group C: Ozempic® is a medication for subcutaneous auto-injection in a pre-filled pen. The active principle is semaglutide, an analogue of GLP-1 whose sequence has 94% homology with the sequence of human GLP-1. Semaglutide acts as a GLP-1 receptor agonist, which selectively binds and activates the GLP-1 receptor, the target of native GLP-1.The principal excipients of the product are: sodium phosphate dibasic dihydrate, Propylene glycol, hydrochloric acid and sodium hydroxide (for pH adjustment). Semaglutide is used to treat patients with type 2 diabetes that have not achieved sufficient

glycemic controls despite lifestyle changes. In recent clinical trials, it was shown to be very effective for weight loss induction also in non-diabetic subjects [48]. An authorization request by Novonordisk is ongoing to commercialize semaglutide 2.4 mg weekly for obesity as the main indication (commercial name Wegovy® expected to launch in Swiss market for March 2023). Ozempic® is in the dispensing category B and its authorization number is 66604 (Swissmedic). The authorized enterprise producing is Novo Nordisk Pharma AG, 8302 Kloten.

3.3 Preclinical Evidence

For Design 1 and 2:

Anastrozole Teva®

Acute toxicity:

In acute toxicity studies, the mean lethal dose in rodents was greater than 100 mg/kg/day orally, and greater than 50 mg/kg/day intraperitoneally. In dogs, the mean oral lethal dose was greater than 45 mg/kg/day in an acute oral toxicity study.

Long-term toxicity (or repeated dose toxicity):

Chronic toxicity studies were performed in rats and dogs by repeated administration. During these toxicity studies, the critical dose threshold at which toxicity occurs was not established for anastrozole. Effects observed at low (1 mg/kg/day) and mid (dog: 3 mg/kg/day; rat: 5 mg/kg/day) doses were attributable either to pharmacological properties or to induction of anastrozole, and were not associated with any toxic or degenerative changes.

Reproductive toxicity:

Oral administration of doses ≤1.0 mg/kg/day to pregnant rats and doses ≤0.2 mg/kg/day to pregnant rabbits was not teratogenic. Only effects of pharmacological origin were observed, such as an increase in placental volume in rats or abortions in rabbits.

For Design 1:

Choriomon®

There are no relevant preclinical data for this preparation.

For Design 2:

Ozempic®

Preclinical data from conventional studies of safety pharmacology, repeated dose toxicity or genotoxicity reveal no special risk for humans.

Carcinogenicity

Non-lethal thyroid C-cell tumors seen in rodents are a class-specific effect of GLP-1 receptor agonists. In 2-year carcinogenicity studies in rats and mice, semaglutide induced thyroid C-cell tumors at clinically

relevant exposures. C-cell tumors in rodents are due to a non-genotoxic, specific, GLP-1 receptor-mediated mechanism to which rodents are particularly susceptible. The relevance of these results for humans is probably low.

Reproductive toxicity

In fertility studies in rats, semaglutide did not affect mating performance or male fertility. In female rats, cycle prolongation and a slight decrease in corpus luteum (ovulation) were observed at doses associated with reduced maternal weight. In embryo-fetal development studies in rats, semaglutide caused embryotoxicity at exposures below clinically significant levels. Semaglutide caused a marked reduction in maternal weight and a decrease in embryonic growth and survival. In foetuses, major visceral and skeletal malformations have been observed, including effects on the long bones, ribs, vertebrae, tail, blood vessels and cerebral ventricles. Mechanistic evaluations indicated that embryotoxicity involved a GLP-1 receptor-mediated abnormality in nutrient delivery to the embryo via the rat yolk sac. Due to differences in yolk sac anatomy and function between species, and due to the lack of expression of GLP-1 receptors in the yolk sac of non-human primates, this mechanism is probably not relevant for the man. In developmental toxicity studies in rabbits and monkeys, an increase in miscarriages and a slightly increased incidence of fetal abnormalities were observed at clinically significant exposures. These results coincided with a marked reduction in maternal weight of up to 16%. It is unclear whether these effects are related to reduced maternal food intake as a direct effect of GLP-1. Postnatal growth and development were assessed in monkeys. Infants were slightly smaller at parturition, but recovered during lactation. In young male and female rats, semaglutide delayed sexual maturation. These delays had no impact on the fertility and reproductive capacity of either sex.

3.4 Clinical Evidence to Date

Information concerning different medications according to the professional information is available on **www.swissmedicinfo.ch**.

For Design 1 and 2:

Anastrozole Teva®

Pharmacodynamics:

Highly sensitive assay methods have shown that administration of anastrozole to postmenopausal women at a daily dose of 1 mg resulted in a >80% decrease in estradiol concentration. Anastrozole Teva is devoid of any progestin, androgenic or estrogenic activity. Doses up to 10 mg/day of anastrozole do not affect cortisol or aldosterone secretion, measured before and during an ACTH challenge. Consequently, simultaneous administration of corticosteroids is not necessary.

Efficacy and safety:

Several studies have confirmed the efficacy of anastrozole for its authorized indication showing superiority compared to tamoxifen for reduction of breast cancer recurrence [49]. In the setting of KFS,

anastrozole at daily dose of 1 mg for 3-6 months was used in three small retrospective studies [37-39]. Sperm retrieval rates during mTESE ranged between 50-100% which is higher than the mean reported rate in the literature. In these studies and for this duration of treatment (similar to the current design), the authors did not report any serious side effects.

Pharmacokinetics:

The pharmacology of anastrozole is dose-linear. The pharmacokinetics of anastrozole in postmenopausal women is independent of age. Clearance in volunteers with stabilized liver cirrhosis or renal insufficiency is within the normal limits observed in healthy volunteers.

Absorption

Anastrozole is rapidly absorbed; peak plasma concentrations are reached within 2 hours after dosing on an empty stomach. Oral bioavailability is 100-104%. Ingestion of food causes a slight decrease in the rate at which the maximum plasma concentration is reached (Cmax = 36 ng/ml after the intake of 1 mg of anastrozole), without however modifying the extent of the resorption. At a dose of 1 mg daily, 7 days are required to reach approximately 90-95% of steady-state plasma concentration.

Distribution

Protein binding of anastrozole is approximately 40%. The volume of distribution Vdss/F amounts to approximately 90 litres.

Metabolism

Anastrozole is extensively metabolized in the liver. The metabolic transformation takes place by N-dealkylation, hydroxylation and glucuronidation. Triazole, one of the main metabolites in plasma and urine, has no inhibitory effect on aromatase. Studies performed on human liver microsomes lead to the conclusion that CYP3A4 is mainly responsible for the oxidative metabolism and UGT1A4 for the glucuronidation of anastrozole.

Elimination

Anastrozole is eliminated primarily in the urine as metabolites. Less than 10% of a dose is recovered in the urine unchanged within 72 hours of administration. The elimination half-life of anastrozole amounts to 40-50 hours.

For Design 1:

Choriomon®

In men, Choriomon stimulates the interstitial Leydig cells and therefore the secretion of androgen hormones.

Pharmacodynamics and mechanism of action

Human chorionic gonadotropin (hCG), the active ingredient of Choriomon, is secreted by the placenta. It is extracted from the urine of pregnant women. Its biological activity largely matches that of LH produced by the anterior lobe of the pituitary gland, but its considerably longer half-life gives it a stronger

activity.

Pharmacokinetics

Absorption

When administering Choriomon, peak plasma levels of hCG are reached after about 2-6 hours (depending on the dose).

Distribution

No data.

Metabolism/Elimination

Chorionic gonadotropin is eliminated in two phases: the biological half-life of the first phase is 8-12 hours, while that of the second phase is 23-37 hours. 80-90% of hCG is metabolized in the kidneys. Due to the slow elimination of hCG, repeated administrations at short intervals (e.g. daily) may induce an accumulation.

For Design 2:

Ozempic®

Efficacy and safety

Compared to native GLP-1, semaglutide has an extended half-life of approximately 1 week, making it suitable for once-weekly subcutaneous injection. The primary mechanism of prolongation is albumin binding, which results in decreased renal clearance and protection against metabolic breakdown. Additionally, semaglutide is stabilized against degradation by the DPP-4 enzyme.

The clinical effects of semaglutide has been shown in the setting of multiple large randomized controlled trials in diabetic and obese patients [48, 50, 51]. Semaglutide lowers blood glucose in a glucose-dependent manner by stimulating insulin secretion and reducing glucagon secretion. When blood sugar is high, insulin secretion is stimulated and glucagon secretion is inhibited. The blood glucose lowering mechanism also causes a slight delay in gastric emptying in the early postprandial phase. During hypoglycaemia, semaglutide decreases insulin secretion without altering glucagon secretion. Semaglutide reduces body weight and fat mass by decreasing energy intake. The mechanism includes a general reduction in appetite, including an increase in satiety and a decrease in hunger. Insulin resistance is reduced. This is probably due to the reduction in body weight. In addition, semaglutide reduces the incidence of cardiovascular events and the overall mortality [50]. In 8 clinical trials, 4,792 patients were exposed to Ozempic as monotherapy or in combination with other antidiabetic medicines. The duration of treatment ranged from 30 weeks to 2 years. The most frequently reported adverse reactions during clinical trials were gastrointestinal disorders, including nausea, diarrhea and vomiting. In general, these reactions were mild or moderate in intensity and of short duration.

Pharmacokinetics

Absorption

The maximum concentration was reached 1 to 3 days after dosing. Steady-state exposure was achieved after 4-5 weeks of once-weekly dosing. In type 2 diabetic patients, mean steady-state concentrations after subcutaneous administration of 0.5 mg and 1 mg semaglutide were approximately 16 nmol/l and 30 nmol/l, respectively. In a trial in which the doses of semaglutide 1 mg and 2 mg were compared, the mean concentrations at steady state were 27 nmol/l and 54 nmol/l respectively. Semaglutide exposure increased in a dose-proportional manner for doses of 0.5 mg, 1 mg and 2 mg. Similar exposure was obtained with subcutaneous administration of semaglutide in the abdomen, thigh or upper arm. The absolute bioavailability of semaglutide by the subcutaneous route was 89%.

Distribution

The mean volume of distribution of semaglutide after intravenous administration in type 2 diabetic patients was approximately 12.5 l. Semaglutide was highly bound to plasma albumin (>99%).

Metabolism

Semaglutide is metabolized by proteolytic cleavage of the peptide chain and sequential beta-oxidation of the fatty acid side chain. The most abundant plasma metabolite accounted for less than 8% of total exposure and was identified as semaglutide with truncation of the first 13 amino acids from the N-terminus.

Elimination

Elimination of semaglutide-associated material was primarily via urine and faeces. Approximately 3% of the dose was excreted as intact semaglutide in the urine. In type 2 diabetic patients, the clearance of semaglutide was approximately 0.05 l/h. With an elimination half-life of approximately 1 week, semaglutide will remain in circulation for approximately 5 weeks after the last dose.

Specific groups of patients

Liver function disorders

Hepatic impairment had no impact on semaglutide exposure. The pharmacokinetics of semaglutide have been evaluated in patients with varying degrees of hepatic impairment (mild, moderate, severe) compared to subjects with normal hepatic function in a single dose trial of 0.5 mg semaglutide

Renal function disorders

Renal impairment did not affect the pharmacokinetics of semaglutide in a clinically meaningful way, although Cmax decreased and manifested later in patients with progressive renal dysfunction. This has been observed with a single 0.5 mg dose of semaglutide in patients with varying degrees of renal impairment (mild, moderate, severe or dialysis patients) compared to subjects with normal renal function. This has also been observed in type 2 diabetic patients with renal impairment based on phase 3a trial data (population pharmacokinetic analysis).

Elderly patients

In patients 20 to 86 years of age, age has no effect on the pharmacokinetics of semaglutide.

3.5 Rationale for the dosage, route, regimen

Design 1 and 2:

Anastrozole Teva®

Dose 1 mg per day, oral for 6 months. Dose unchanged during the study.

The choice of dosage and route is in line with the current authorization of the medication. According to professional information available on www.swissmedicinfo.ch, postmenopausal women at a daily dose of 1 mg resulted in a >80% decrease in estradiol concentration. Men with KFS receiving anastrozole 1 mg daily for 6 months had approximately 60% lower E2 levels that those without any treatment or receiving HCG in a small retrospective study [38]. We, thus expect, this dosage and duration to effectively normalize the T/E ratio and allow to test the hypothesis of the study for both designs.

Design 1:

Choriomon®

For Group B of design 2, the addition of hCG will aim to further increase intratesticular testosterone levels as compared to the group A (anastrozole alone). The dose of hCG will be adjusted to target serum T levels at the lower normal range (10-14 nmol/l) when measured at 48h post injection. Details on initial dose and subsequent adjustment are available in Chapter 8 – Study Intervention. The maximal dose will be 2000 U every 48h in line with the current clinical practice for central hypogonadism and the pre-existing retrospective studies on men with KFS.

Design 2:

Ozempic®

Based on the current recommendations in the treatment of diabetes, which were reproduced in the randomized trial that proved the efficacy of semaglutide for weight loss in non-diabetic population [48]), the medication will be initiated then gradually titrated according to the following schematic: 0.25 mg per week for 4 weeks, then 0.5 mg per week for 4 weeks, then 1 mg for 4 weeks, and then 2 mg per week as final dose. In case of gastro-intestinal adverse effects, the maximum tolerated dose will be maintained. Based on the aforementioned study of New England Journal of Medicine, a substantial weight loss of 12% was present in the treatment group already at 28 weeks of study, suggesting that the 6-month period is sufficient to achieve weight loss in Group C of Design 2 and verify the effect on reproductive function. The choice of semaglutide over liraglutide which is the currently authorized GLP1 receptor agonist for treatment of obesity was motivated by (i) the ease of applications (semaglutide, once weekly; liraglutide, once daily) and (ii) larger efficacy of semaglutide for weight loss in a head-to-head randomized controlled study [52].

3.6 Explanation for choice of comparator (or placebo)

In design 1, all patient will undergo the current state of art intervention, which is mTESE biopsy. Subsequent, only patient with a negative result (no sperm) and thus a clinically unmet need will be included to the exploratory group and be randomly assigned to two potential interventions (see study schedule). In this setting, the use of placebo does not convey any benefit (the primary outcome being the appearance of spermatozoids, a placebo effect is highly unlikely). In addition, a placebo group for 6 months before a minimally but still invasive procedure (second mTESE biopsy) is not ethically justified.

The comparator in Design 2 will be TRT (Group A), the current state of art. Among the different formulations for testosterone replacement, we opted for a T gel to apply daily because the obtained plasmatic levels of testosterone are more stable than with intramuscular injections of testosterone [53]. **Tostran gel®** is an approved drug for treatment of male hypogonadism. It is classified in the dispensing category B and its authorization number is 57959 (Swissmedic). The authorized enterprise producing is Cederberg GmbH, 4102 Binningen. The use of the placebo arm in design 1 would be ethically unacceptable as it would expose patients to untreated hypogonadism for 6 months, as opposed to the current design in which a certain rise of serum T levels is expected with all three arms.

3.7 Risks / Benefits

The main expected benefit of the study is the identification of novel treatments for men with KFS. This will be increasingly relevant as KFS is not a rare disease and the gap in diagnostic of the disease is currently improving both ante- and post-natally. The study aims to improve the management of two key comorbidities: metabolic health and fertility, which have direct impact to patients' long-term survival but also quality of life and mental health. In addition to these immediate benefits, this study will allow a better understanding of the disease and will elucidate at least in part the underlying mechanisms (hormonal, transcriptomic) that lead to the very large and heterogeneous clinical spectrum of KFS.

The risks of the study are the possible adverse effects of study medications. There is sufficient experience in a clinical setting with all prescribed medications and eventual side effects are expected to be of minor or moderate intensity.

- **Tostran® gel** is very well tolerated according to the PI's large clinical experience on treatment of male hypogonadism. According to the professional information available on www.swissmedicinfo.ch, the most commonly reported adverse reactions in a controlled clinical study with doses up to 4 g (80 mg) of Tostran® were application site reactions (26%), including: paraesthesia, dry skin, pruritus and rash. The majority of these reactions were of mild to moderate severity and diminished or disappeared despite continued applications. The rest of reported risks of testosterone, notably erythrocytosis (increased red blood cell number), are dose-dependent and occur mostly in case of excessive dosing. The plasmatic levels of testosterone will be regularly monitored during the study and the dosing of Tostran® will be adjusted to target T levels in the mid-normal range (15-25 nmol/l) and thus avoid side effects. In case of erythrocytosis

(haematocrit > 52%), lower T levels between 10-15 nmol/l will be targeted.

- Data on the safety of **aromatase inhibitors** (**anastrozole** – Design 1 and 2) in men are derived mostly from their off-label and empirical use for decades to treat male infertility [54]. In this setting, various side-effects have been reported but most are mild and well tolerated [54]. In a RCT comparing letrozole, another aromatase inhibitor, to testosterone for 1 year in boys aged 14-15 years with delayed puberty, the most common adverse events in the letrozole group were (i) musculoskeletal symptoms such as transient back pain and joint pain (20%); (ii) gastrointestinal symptoms such as abdominal pain, diarrhea and nausea (13%) and neurological symptoms such as dry eyes and migraine (13%) [46]. Only two boys had short treatment pauses, one because of transiently elevated liver enzymes and the other because of myocarditis caused by parainfluenza virus infection (not treatment-related).
- **hCG - Choriomon®** is also very well tolerated based on the PI's large prior use for fertility induction in men with central hypogonadism. Few undesirable effects are reported in the professional information available on www.swissmedicinfo.ch, including systemic hypersensitivity reaction such as erythema, rash, angioedema (rare incidence ≥1/10,000 but <1/1000), injection site reactions such as redness, swelling, itching, bruising or pain (rare incidence ≥1/10,000 but <1/1000), fluid retention (occasional incidence ≥1/1000 but <1/100). The PI has observed the rare occurrence of gynecomastia (breast tissue development) in men receiving high doses of hCG due to stimulated aromatization at the testes but this risk seems low in this particular study because men who will receive hCG (Design 2, Group B) will also be on anastrozole that has an antiestrogenic effect.
- The tolerance of **semaglutide** (Design 2, Group C) is good. In the largest clinical trial to date using dosing for obesity (up to 2.4 mg weekly for 68 weeks), 94% of participants completed the study [48]. At 6 months of the study, 90% of patients assigned to semaglutide exhibited good adherence to the medication. Nausea and diarrhea were the most common adverse events with semaglutide; they were typically transient and mild-to-moderate in severity and subsided with time. Serious adverse events were reported in 9.8% and 6.4% of semaglutide and placebo participants, respectively, with the difference due primarily to the incidence of serious gastrointestinal disorders (1.4% of participants in the semaglutide group and 0% in the placebo group) and hepatobiliary disorders (1.3% with semaglutide and 0.2% with placebo). One death was reported in each group, with neither considered by the independent external event adjudication committee to be related to receipt of semaglutide or placebo.

Currently, there is no ongoing or registered competing study on KFS and the use of anastrozole or other aromatase inhibitors (search on clinicaltrials.gov in July 2022).

3.8 Justification of choice of study population

Klinefelter syndrome is an excellent disease model to assess the effect of sex steroid imbalance on metabolic homeostasis and spermatogenesis in men. In particular, the choice of this particular study population is justified by the following arguments: (i) relatively common entity with potential large impact;

(ii) high burden of comorbidities (metabolic syndrome, infertility) that are not sufficiently treated by current therapeutic options; (iii) dissociation between the two principal sex steroids (low-normal testosterone, high-normal estradiol) allowing mechanistic studies to assess individual contribution to the phenotype. For design 1, the choice of investigation population reflects the decision to focus on KFS men with high metabolic risk, which are approximately 40% of the whole KFS population according to published reports and the retrospective review of our historic cohort at EDM. For design 2, the choice of investigation population is fully representative of KFS patients. The age limit is justified by literature data suggesting lower sperm retrieval rates in men > 40 years (see chapter 3.1).

The design will not recruit vulnerable subjects. Given the delicate character of fertility issues, all patients with an intellectual deficit or severe psychiatric conditions potentially impairing the capacity of discernment. General intelligence is not affected in KFS [55], thus we do not expect that there will be a lot of patients excluded for this reason. For adolescent patients between age 16 and 18 years in Design 1 and between age 14 and 18 for the register study, the PI will present the study to both the candidates and their legal representatives. Both will need to give their consent so that the patient can participate.

4. STUDY OBJECTIVES

4.1 Overall Objective

The purpose of this study is to improve the health of men with Klinefelter syndrome focusing on metabolic health and fertility.

4.2 Primary Objective

The study seeks primarily to determine whether modulation of systemic and testicular sex steroids balance by aromatase inhibitors will positively affect the metabolic health and spermatogenesis of men with Klinefelter syndrome as compared to the current state of the art for each issue.

4.3 Secondary Objectives

Secondary objectives of this study are (i) to unravel the heterogeneity of the reproductive and metabolic phenotype of men with KFS by performing a multi-omic analysis in a large cohort at baseline; (ii) to evaluate the efficacy of semaglutide-induced weight loss to achieve metabolic and reproductive benefit in men with Klinefelter syndrome as compared to standard testosterone replacement (design 2, Group C); (ii) to assess whether addition of hCG to aromatase inhibitors further increases intratesticular testosterone and promotes spermatogenesis in men with KFS (design 1, Group B).

4.4 Safety Objectives

The study aims to assess the medium-term (6 month) safety of all studied drugs with particular focus on

the risk of bone density loss under aromatase inhibitors (anastrozole), as well as their tolerability in terms of gastrointestinal side effects (especially for semaglutide).

5. STUDY OUTCOMES

5.1 Primary Outcome

The primary outcome will be:

- Design 1 (patients interested in fertility preservation); the **sperm retrieval rate at second mTESE biopsy after 26 weeks of hormonal pre-treatment** (group A and B separately) in patients with a sperm-negative first mTESE biopsy**,** as compared to the expected rate without intervention (10%)
- Design 2 (patients at high metabolic risk and not interested in fertility preservation): the **change in insulin resistance**, **as assessed by the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) score** [1], **from baseline to end of treatment (week 26)**

5.2 Secondary Outcomes

Secondary outcomes will be:

Design 1:

- **Sperm retrieval rate (qualitative) at second mTESE biopsy in group A vs group B**

  Even though there are no reports clearly indicating the efficacy of hCG in improving sperm retrieval rate in men with KFS, achieving higher levels of serum T levels pre-intervention (which are probably a surrogate marker for intratesticular T levels) was found to be a positive predictive markers in several retrospective series [30, 47, 56]. We will thus compare the efficacy of two approaches to promote spermatogenesis: a primarily anti-estrogen approach (group A, anastrozole) versus a primarily pro-testosterone (intra-testicular) approach (group B, anastrozole + hCG).

- **Changes in markers of serum inhibin B, AMH and INSL3 from baseline to week 26 and correlation with the mTESE biopsy results**

  Serum inhibin B and AMH are reliable markers of the Sertoli cell numbers and correlate well with the presence and intensity of seminiferous tube defects in KFS and other pathologies [57-60]. Retrospective studies were unable to reveal a reliable predictive value of these easily accessible serum markers but it is possible that this was due to selection bias or incomplete data. We will assess study these hormones both crossectionally (as compared to the first mTESE biopsy) and longitudinally in a prospective study.

- **Change in testicular histology with particular focus to the fibrosis-hyalinization from baseline to week 26**

  Although the germ cell loss is early in KFS, the fibrosis and eventual hyalinization of the seminiferous tubes and testicular parenchyma is absent in prepubertal samples [60]. Several indirect lines of evidence suggest that the pubertal presumably excessive rise of intratesticular E2 could contribute to the pathogenesis or at least the acceleration of this process. We will thus quantify hyalinization as previously detailed [31] to assess the potential of reversibility after a 6-month

treatment with anastrozole +/- hCG. The expression of decorin (DCN), a surrogate marker for hyalinization in the KFS testis [33], will also be assessed.

- **Change in transcriptomic status at the testes from baseline to week 26**

  Few testes transcriptomic studies in a very limited number of KFS men have shown relevant changes in pathways linked to spermatogenesis (downregulation) and apoptosis (upregulation) [41, 42]. The significance of these results (cause or consequence of the testicular degeneration) remains uncertain. The overlap in the between-studies results is limited, highlighting the large heterogeneity of KFS. In this unique model, we will be able to compare the gene expression status before and after a considerable hormonal shift, allowing to dissect the link between sex steroids status and gene expression. We will assess the significance of transcriptomic changes both crossectionally (association with first mTESE biopsy results) and longitudinally (association with group assignment and second mTESE biopsy results). Initially, the focus will be on the transcriptional signatures of spermatogonia and other evolutionary forms of the spermatogenesis cascade based on genes identified from a recent single cell RNA-seq study [61]. Further, we will study downstream pathways of androgen receptor (AR) and the two principal estrogen receptors (ERα, ERβ) by conducting gene set enrichment analysis before and after AI. Lastly, an exploratory analysis on the whole transcriptome will be performed to identify novel target genes that are differentially expressed. In selected samples with the largest effect after AI exposure, single-cell RNA-seq will be applied to illustrate the transcriptional impact of reduced intratesticular E2 at single cell resolution. We will compare our results with the only available study of single cell RNA-seq on KFS [62] and other published datasets at that time. The comparison of testicular samples from the same subjects before and after intervention will allow to limit the intrinsic variability of the disease. Gene expression in testis and blood samples will be assessed in close collaboration with Dr Messina in Pr Pitteloud's lab and with the team of Pr. Deplancke at Ecole Polytechnique Fédérale de Lausanne, who recently developed a novel technique for high-throughput transcriptomics, Bulk RNA Barcoding and Sequencing (BRB-seq) [63]. BRB-seq was shown to perform greater than the standard approach (TruSeq) for low-input/quality RNA samples, while being significantly cheaper, making this approach ideal for screening a large number of samples. Differential gene expression analysis will be performed using edgeR [64] and DESeq2 [65] methods.

- **Change in intratesticular levels of sex steroids (T, E2) from baseline to week 26**

  In healthy men, intratesticular testosterone levels are at least 1000-fold higher than in serum [66, 67], an element that is considered crucial for fertility. There are very scarce data on intratesticular T and none on intratesticular E2 in KFS. An Italian study measured testosterone in testicular extracts using radioimmunoassay (RIA) and reported surprisingly elevated levels as compared to controls, postulating the presence of a vascular defect of the KFS testes preventing adequate transfer of T to the systemic circulation [68]. In the current study, we will assess for the first time the T/E ratio at the level of the KFS testes using liquid chromatography - mass spectrometry (LC-MS) in collaboration with CHUV experts (see list of investigators). The levels of sex steroids in the testes will be assessed both crossectionally (in relation with the first mTESE biopsy result) and

longitudinally (in relation with the group assignment and the second mTESE biopsy result).

Design 2:

- **Change in HOMA-IR score from baseline to week 4 and week 13**

  It will be important to assess the rapidity of the expected favourable impact on glucose homeostasis and correlate with the specific hormonal changes in group 1 and group 2

- **Change in other insulin sensitivity indexes (area under the curve for glucose and insulin) derived from oral glucose tolerance test from baseline to week 4, 13 and 26**

  These are additional markers of insulin sensitivity and their assessment will be critical to reinforce the conclusions from the expected results in the primary outcome.

- **Change in total and visceral adiposity as assessed by DXA from baseline to week 13 and 26**

  Sex steroids impact on metabolic health are mediated at least in part by positive changes in body composition with decrease of adiposity and expansion of lean mass.

- **Change in inflammation serum markers (hs-CRP, TNF-a, IL-6, S100A8-9) from baseline to week 4, 13 and 26**

  Low-grade inflammation with excess of cytokines mostly from visceral adipose tissue is a key component of metabolic syndrome and associated cardiovascular morbidity. Greater anti-inflammatory efficacy of group B or C could indicate potential long-term additional clinical benefits.

- **Change in serum T levers from baseline to week 13 and 26 in group C (semaglutide)**

  Based on the preliminary results of the PI, weight loss in KFS improves hypogonadism, presumably by removing the obesity-associated central inhibition of gonadal function or by alleviating the negative effect of insulin resistance on the secretory capacity of Leydig cells. It is thus reasonable to evaluate how testosterone and other reproductive hormones will change in group 3.

5.3 Other Outcomes of Interest

Both designs:

- As part of the baseline evaluation of both designs, KFS men (either TRT-naïve or after TRT washout) will undergo a complete metabolic and reproductive phenotyping at baseline using a multi-omic approach (transcriptomics, metabolomics). They will subsequently be matched for age- and BMI with reproductive controls (healthy volunteers with strictly normal reproductive status) and compared crossectionally in order to refine our understanding of the metabolic phenotype of KFS and identify disease-specific markers.

Design 1:

- Change in metabolic health such as glucose metabolism (HOMA-IR) and body composition (fat mass percent, VAT) from baseline to end of treatment (Week 26)

- Change in testicular volume and vascular pattern from baseline to end of treatment (Week 26)

  Both anastrozole and hCG can have a stimulatory effect on the testes. Based on a recently published report [69], we will attempt to specifically investigate the testicular microvascular flow before and after intervention.

- Change in blood RNA expression from baseline of end of treatment (Week 26).

  The weight loss as well as the change in sex steroids ratio may influence the expression of key genes in the blood.

Design 2:

- Change in other metabolic parameters from baseline to end of treatment (Week 26), such as lipid profile (cholesterol, triglycerides, LDL cholesterol, HDL cholesterol), Hb1Ac, hepatic function (ASAT, ALAT, NAFLD score) and other key metabolic hormones (leptin, FGF21, FGF19, adiponectin).
- Change in blood RNA expression from baseline of end of treatment (Week 26).

  The weight loss as well as the change in sex steroids ratio may influence the expression of key genes in the blood.

- Change in Leydig cell secretory capacity as assessed by the hCG stimulation test, from baseline to the end of treatment (Week 26).

  The latter consists of intramuscular injection of 5000 IU of hCG and measurement of T levels at the time of injection and 72 hours after injection as previously reported [70].

- Change in lipidomics and metabolomics from baseline to end of treatment (Week 26)

  In an exploratory analysis, we will assess changes in key pathways for metabolic health such as sphingolipids-ceramides and branched-chain amino-acids. This will be done in collaboration with the Metabolomics UNIL Lab (Dr Ivanisevic).

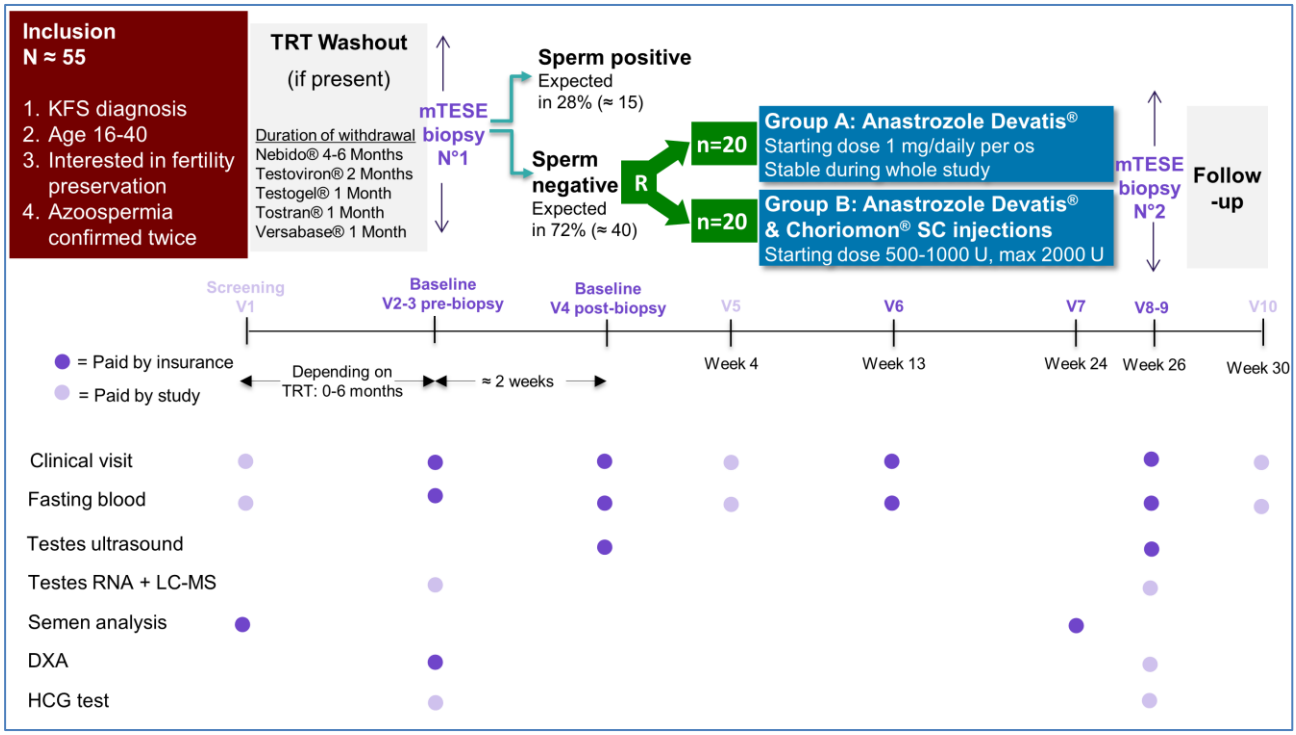
5.4 Safety Outcomes

- Incidence of erythrocytosis, defined as a haematocrit > 52%, related to the testosterone-rising treatments for both designs
- Incidence and severity of bone mineral density (BMD) loss related to the antiestrogenic properties of anastrozole for both designs. This will be monitored with serial DXA for measurement of BMD at relevant sites (lumbar spine, total hip, femoral neck) at baseline and end of treatment (Week 26).
- Incidence and severity of gynecomastia related to study drug of group B (hCG) of design 1.
- Incidence and severity of gastrointestinal side effects related to study drug of group C (semaglutide) of design 2.

6. STUDY DESIGN

Figure 1: Sex steroid role in fertility optimization of men with Klinefelter syndrome

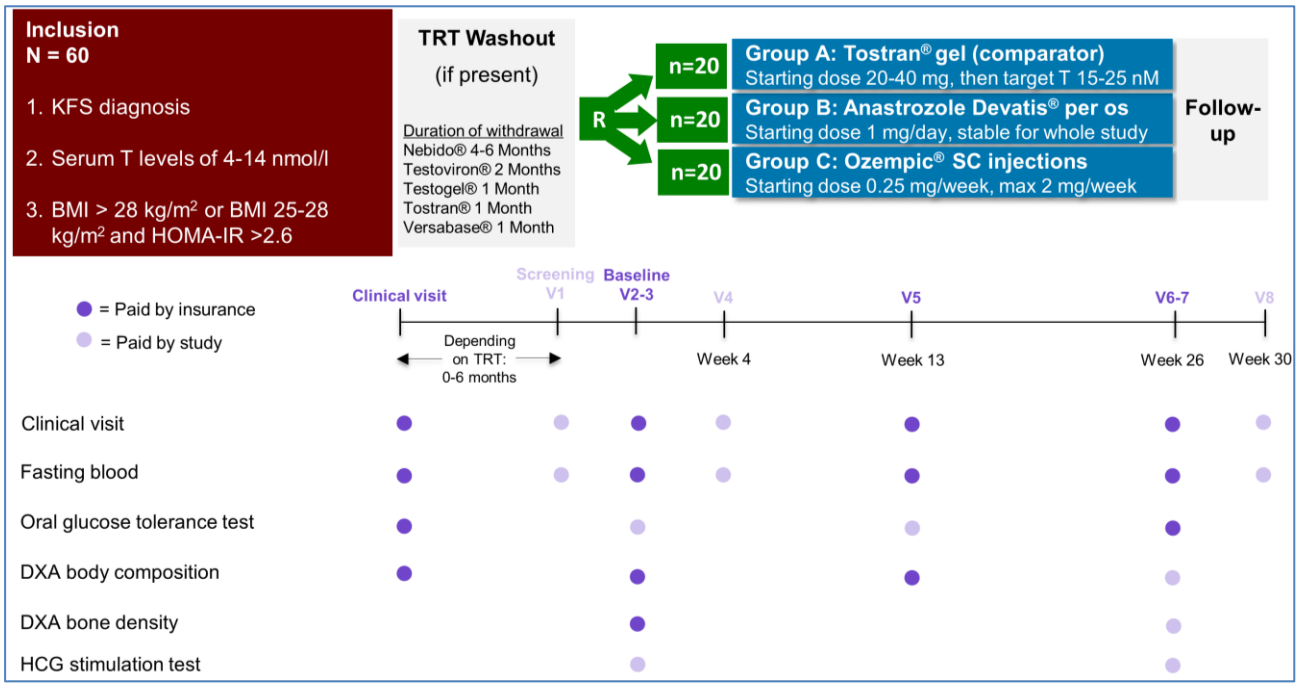
Design 1 / Randomized controlled study, non-blinded, allocation 1:1



KFS, Klinefelter syndrome; TRT, testosterone replacement therapy; LC-MS, Liquid chromatography – mass spectrometry; DXA, Dual X-ray absorptiometry; HCG, Human Chorionic Gonadotropin; ,mTESE, microdissection testicular sperm extraction; R, Randomization; SC, subcutaneous; V1-4, Visits 1-4.

Figure 2: Sex steroid role in metabolic health of men with Klinefelter syndrome

Design 2 / Randomized controlled study, non-blinded, allocation 1:1



KFS, Klinefelter syndrome; T, testosterone; TRT, testosterone replacement therapy; BMI, body mass index; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; DXA, Dual X-ray absorptiometry; HCG, Human

Chorionic Gonadotropin; R, Randomization; SC, subcutaneous; V1-4, Visits 1-4

6.1 General study design and justification of design

A graphic summary of the two designs is shown above.

Baseline cohort – registry:

In order to dissect the phenotypic heterogeneity of KFS, we plan to recruit a large cohort of men with KFS and perform a detailed phenotyping in agreement with published recommendations [5] and our recent publication of a patient-oriented medical passport [71]. We will include (i) all newly diagnosed KFS cases (in our centre or referred by co-investigators or other providers) aged > 16 years; (ii) selected patients aged 14-16 years for whom a TRT needs to be started to induce puberty; and (iii) the baseline assessment of all eligible patients for designs 1 and 2 (see below) after they consented to and went through a TRT wash-out in order to participate to the respective intervention. Depending on the number of KFS cases included in the registry study during 3 years, healthy male volunteers with strictly normal reproductive status (testicular volume > 12 ml with Prader, normal timing of puberty, serum total T > 14 nmol/l, serum LH and FSH 2-9 IU/l) will be enrolled via public advertisements and matched to the patient group. The ratio and the specific age- and BMI characteristics will be specified in a future amendment, once the recruitment of KFS patients is near completion.

Regarding the wash-out of TRT in previously treated KFS cases, due to the very long and unpredictable half-time life of intramuscular T injections such as Nebido®, at least 4 months of withdrawal will be required in most patients to achieve adequate wash-out (attested by return of serum T and plasma LH and FSH to the pre-treatment levels). To ensure that the patients are not exposed to hypogonadal symptoms for a prolonged period, transient covering by a T gel will be offered to those with low T levels but persistently low LH and FSH levels suggesting a residual drug effect. The baseline visit will be performed after 4 weeks after the discontinuation of the T gel.

All patients will undergo a baseline visit [71] consisting of (i) a targeted clinical evaluation (anthropometrics, testicular volume, Tanner staging); (ii) an assessment of hypogonadal symptoms (ADAM questionnaire [72]), as well as specific scores to estimate sexual desire (Derogatis Interview for Sexual Functioning in Men–II [DISF-M-II]) [73] and erectile function (International Index of Erectile Function [IIEF]) [74]; (iii) a fasting blood sample to measure reproductive hormones (LH, FSH, E2, T, SHBG, inhibin B, AMH, INSL3), adipokines (leptin, adiponectin, FGF21), inflammation markers (hs-CRP, cytokine panel); (iv) an oral glucose tolerance test (OGTT) to evaluate glucose metabolism and insulin sensitivity by several calculated indexes including HOMA-IR [1], insulin sensitivity index [75] and corrected insulin response [76]; (v) a DXA scan to assess body composition (VAT measurement) and quantify bone mineral density (BMD) and indexes of microarchitecture [77]; (vi) a testicular ultrasound to assess testicular volume and vascularisation using a contrast enhancement ultrasound (CEUS) approach with microbubble injection,

as performed in standard clinical practice. This is wildly available, easy to perform that is not nephrotoxic and can be used regardless renal function and without radiation exposure [78]. The side effects of sulfur hexafluoride microbubbles (Sonovue®) are mild and limited in time. The most encountered side effects are headache, nausea and cutaneous reaction in injection site; (vii) a semen analysis if the patient is able to ejaculate and if this test was not performed in the past as part of a fertility pursue management. Serum and a PaxGene tube will be collected and stored for metabolomics and gene expression studies. According to their metabolic status and fertility desire, eligible KFS men will then be included in design 1 (fertility preservation) or design 2 (high metabolic risk). KFS patients that are not eligible for any of the designs will complete their participation to the study after the baseline assessment.

Design 1:

KFS patients who seek fertility or are interested in fertility preservation will be offered participation to **design 1**. Currently, there are no recommendations regarding hormonal management before mTESE biopsy for sperm extraction, although most experts recommend against initiation of TRT in case of active fertility pursue [55]. We will thus withdraw prior TRT if present based on half-time life of specific preparation (Figure 1). Because of reduced retrieval of mature spermatozoids in young adolescents (< 15 years) according to a recent systematic review [79], only patients aged 16-40 years will be included to this design. Azoospermia will be confirmed in two centrifuged semen specimens before inclusion. Subsequently, the study will take place in two phases:

(i) **a first unilateral mTESE biopsy without any hormonal pre-treatment (control intervention)** with an expected success rate of 28% according to the largest published series to date [80]. In addition to the aforementioned baseline assessment, a peripheral blood and a testicular sample will be drawn for gene expression studies. Lastly, a testicular sample will be homogenised as previously reported [68], then the extract will be used to measure intratesticular T and E2 by LC-MS (collaboration with Dr Binz and Dr Bruce).

(ii) **a second contralateral mTESE biopsy in patients with no sperm retrieval at the first biopsy**. This practice has a low chance of success (5-10%) in the absence of any hormonal stimulation [28, 81]. Therefore, all participants will receive anastrozole 1 mg daily given that this treatment showed promising results in retrospective series (see background in Chapter 3.1). In addition, they will be randomly assigned to receive no additional treatment (group A) or concomitant hCG injections every 2 days (starting dose 500 U if pre-biopsy T levels > 8 nmol/l, otherwise 1000 U). The dose of hCG will be adjusted once every 4 weeks by increments of 500 U to a maximum dose of 2000 U every 2 days to target T trough levels (48h post injection) of around 10-14 nmol/l. A blood sample to measure hormone levels will be done at week 13. At the end of the 26 week-intervention, a complete evaluation and a new semen analysis will be repeated before the second mTESE biopsy. **The primary outcome will be the sperm retrieval rate at the second mTESE biopsy for group A or B or the two groups combined if comparable SSR**.

The choice of this particular design for design 2 was justified by ethic issues and more specifically by our willingness to not expose KFS patients that would have a positive mTESE result anyway to an

experimental hormonal pre-treatment. The absence of placebo is also due to ethical concerns and the fact that a placebo group before the second mTESE biopsy would not be acceptable given the very slight chance of success after a first negative biopsy controlaterally.

Design 2:

Sixty KFS patients with (i) moderate or borderline hypogonadism (serum T levels 4-14 nmol/l at diagnosis; for patients on TRT also confirmed after the wash-out) and (ii) high metabolic risk suggested by the presence of a BMI > 27 kg/m^2 or a milder overweight (BMI 25-27 kg/m^2) but complicated by insulin resistance (fasting HOMA-IR > 2.6). For patients on TRT, the high metabolic risk criterion should be met at initial evaluation (on TRT) and persist at the screening visit after TRT wash-out. Based on our historic KFS cohort at EDM, approximately 40% of KFS cases correspond to these criteria. **This will be a randomized controlled study for 26 weeks** and the comparator will be a T gel (Tostran®, group A). Based on previous trials in congenital hypogonadotropic hypogonadism and obesity-associated hypogonadism, the study duration of approximately 6 months should be sufficient to reveal a relevant shift (if present) in glucose homeostasis and body composition. Following a baseline visit as described above (with the addition of an hCG stimulation test [70]), all participants will receive lifestyle counselling [82] and will be randomly assigned to receive the comparator (group A, Tostran®), an aromatase inhibitor (group B, Anastrozole Teva®) or a GLP1 receptor agonist (group C, Ozempic®). Follow-up visits at 4 and 13 weeks will study the metabolic changes and measure fasting T levels to perform dose adjustments for group A. At the end of 26-week period, a complete metabolic evaluation will be performed. **The primary outcome will be the changes in HOMA-IR levels from baseline to end of treatment.** Secondary, safety and other outcomes are summarized in Chapters 5.2-5.4. Given the presence of three different route of administrations (Tostran® transdermal, Anastrozole Teva® oral, Ozempic® subcutaneous), a double-blinded study was considered extremely challenging and we opted for an unblinded design with TRT (current state of art) as the control arm.

6.2 Methods of minimising bias

6.2.1 Randomisation

The allocation of participants to different groups will be randomized with a ratio of 1:1 in both designs. The randomization list (2 for design 1, 3 for design 2) will be established by the CHUV pharmacy and the two persons in charge of clinical trials: Dr Isabelle SOMMER and Dr Aline VOIDEY according to their standard process. Only men will be included in this study, thus there will be no need to stratify the randomization according to sex. Participants who will not participate to the final visit (Week 26) will be considered as drop-out. The shipment of different medications will be done to the CHUV pharmacy (Tostran® provided by Cederberg, Anastrozole® provided by Teva, Ozempic® provided by Novonordisk and Choriomon® provided by IBSA) (see Chapter 8).

6.2.2 Blinding procedures

There will be no blinding of participants nor investigators in this study. In Design 1, the primary outcome is the development of sperm in the setting of an altered testes, a development that is highly unlikely to be prone to a placebo-effect. Therefore, we estimated that a blinding process does not add to the scientific value of this design. In Design 2, the presence of three different route of administrations (Tostran® transdermal, Anastrozole Teva® oral, Ozempic® subcutaneous), the use of placebo as well as a double-blinded study were considered extremely challenging and we opted for an unblinded design. This study is still largely exploratory. In case of positive findings, a larger multi-centric randomized and ideally double-blinded trial will be performed to validate the change in the state of art of KFS.

6.2.3 Other methods of minimising bias

All the questionnaire used for the study are validated. These will include ADAM questionnaire for hypogonadal symptoms [72], Derogatis Interview for Sexual Functioning in Men–II (DISF-M-II) to estimate sexual desire [73] and International Index of Erectile Function (IIEF) to assess erectile function [74].

6.3 Unblinding Procedures (Code break)

Not applicable. There will be no blinding in this study.

7. STUDY POPULATION

7.1 Eligibility criteria

General context prior to eligibility assessment:

The study's purpose is to improve the metabolic and reproductive health of men with KFS by focusing on the potential role of sex steroids balance. Infertility is a frequent reason to seek medical advice in this disease. Moreover, even when affected men are not immediately seeking fertility (for instance, adolescent or young adults that are diagnosed for other symptoms such as signs of testosterone deficiency or gynecomastia), it is recommended to inform them regarding the possibility to preserve fertility by performing a sperm count to search for spermatozoids (approximately 5% of patients) or undergo a mTESE biopsy in those with azoospermia (approximately 95% of patients) [55]. This is part of the standard clinical practice. We recommend that patients undergo mTESE before the age of 35-40 years because of some data showing reduced success beyond this age limit. In the end, it is the patient's decision whether he wants to immediately seek fertility preservation (in order to freeze spermatozoids for future use if the biopsy is successful) or if he prefers to defer this intervention to a later stage or to renounce to this possibility for personal reasons [83]. When assessing candidates for inclusion to this clinical trial, their preference for fertility management will be the crucial element to guide their evaluation

for design 1 (fertility desire or active interest for fertility preservation) or design 2 (no fertility desire or already managed in the past). In addition, TRT-naïve KFS men could undergo the baseline assessment and participate to the KFS registry even if they do not meet the criteria for design 1 or design 2. This will assist in the secondary purpose of the study which is to fully phenotype KFS men in order to dissect the large clinical heterogeneity of the disease. The eligibility criteria will be verified by the PI in collaboration with the medical doctors of EDM at CHUV or external providers (endocrinologists and urologists) that regularly refer KFS cases to the PI.

Design 1:

Participants fulfilling all of the following inclusion criteria are eligible for the study:

- Informed Consent as documented by signature
- Diagnosis of KFS (47, XXY or mosaicism)
- Age range: 16-40 years old
- Intention to become parent or interest in fertility preservation
- Confirmed azoospermia (lack of spermatozoids) after centrifugation of 2 semen samples
- Consent to a wash-out of TRT (if present) prior to fertility management
- Consent to undergo 1 or 2 mTESE biopsy based on the study's design

The presence of any one of the following exclusion criteria will lead to exclusion of the participant:

- Higher level of aneuploidy such as supra-Klinefelter (48, XXXY)
- Prior exposure to aromatase inhibitors or hCG
- Contraindications to testosterone-rising therapies, e.g., prostate or breast cancer, PSA > 4 µg/l, active liver disease, symptomatic heart disease
- Known hypersensitivity or allergy to aromatase inhibitors or hCG
- Other causes of infertility such as exposure to chemo- or radiotherapy, reducing the chance of successful mTESE response
- Decreased life expectancy due to advanced neoplasia or other terminal disease
- History of fragility fractures at classic osteoporotic sites (vertebrae, hip, distal radius, proximal humerus, pelvis) occurring after the age of 16 years
- Known or suspected non-compliance, drug or alcohol abuse
- Inability to follow the procedures of the study, e.g. due to language problems, psychological or mental disorders, dementia, etc. of the participant
- Any ascertained or suspected loss of ability to discern
- Social precariousness contraindicating medically-assisted procreation

- Participation in another clinical study
- Previous enrolment into the current study
- Enrolment of the investigator, his/her family members, employees and other dependent persons

If the recruitment for this Design is slower than expected, it is possible that the interventional study will be offered to patients that have had previous negative mTESE biopsies prior to the initiation of the study, provided that they still meet the other inclusion criteria (notably the age limit). For this patients the primary outcome will still be assessable but we will not be able to evaluate the longitudinal change of gene expression status and intratesticular steroids given that the first mTESE biopsy took place before their inclusion to the study.

Design 2:

Participants fulfilling all of the following inclusion criteria are eligible for the study:

- Informed Consent as documented by signature
- Diagnosis of KFS (47, XXY or mosaicism)
- Age range: 18-60 years old
- Consent to a wash-out of TRT (if present) prior to randomization
- Moderate hypogonadism defined as serum T levels 4-14 nmol/l at diagnosis
    - For patients on TRT, this level of T should also be confirmed after the wash-out
- High metabolic risk: severe overweight with BMI > 27 kg/m2 or a milder overweight (BMI 25-27 kg/m2) but complicated by insulin resistance (fasting HOMA-IR > 2.6)
    - For patients on TRT, HOMA-IR & BMI should also be confirmed after the wash-out

The presence of any one of the following exclusion criteria will lead to exclusion of the participant:

- Higher level of aneuploidy such as supra-Klinefelter (48, XXXY)
- Intention to become a parent during the course of the study (if yes, directed to design 1)
- Immediate interest in fertility preservation (if yes, directed to design 1)
- Contraindications to TRT, e.g. known hypersensitivity or allergy to testosterone preparations, prostate or breast cancer, PSA > 4 µg/l, active liver disease, symptomatic heart disease
- Contraindications to GLP1 receptor agonists, notably history of pancreatitis
- Untreated endocrine disorders with impact on metabolism (i.e. active Cushing or acromegaly, untreated thyroid disease)
- Medications with impact on metabolism (i.e. glucocorticoids, anabolic steroids, insulin)
- Previous exposure to aromatase inhibitors or GLP1 receptor agonists
- Severe renal insufficiency

- Decreased life expectancy due to advanced neoplasia or other terminal disease
- History of fragility fractures at classic osteoporotic sites (vertebrae, hip, distal radius, proximal humerus, pelvis) occurring after the age of 16 years
- Known or suspected non-compliance, drug or alcohol abuse
- Inability to follow the procedures of the study, e.g. due to language problems, psychological or mental disorders, dementia, etc. of the participant
- Participation in another clinical study
- Previous enrolment into the current study
- Enrolment of the investigator, his/her family members, employees and other dependent persons

KFS patients for register study (only baseline assessment)

KFS men not fulfilling the inclusion criteria for designs 1 and 2 will be offered to undergo the baseline phenotyping (one visit). Submitting patients to TRT wash-out without any subsequent trial inclusion would be excessive. Thus, only TRT-naïve patients will be recruited for this arm.

Patients fulfilling all of the following inclusion criteria are eligible

- KFS diagnosis (47 XXY or mosaic form)
- Age 16-60 years
- TRT naïve
- Informed Consent as documented by signature
- Non-compliance to the inclusion criteria of designs 1 and 2.

Reproductive controls will be enrolled at a later stage (after a protocol amendment):

Healthy volunteers fulfilling all of the following inclusion criteria are eligible

- Age 18-60 years
- Strictly normal serum T levels at morning fasting sample (> 14 nmol/l), plasma LH (2-9 U/L) and FSH (2-12 U/L) levels
- Normal testicular volume at Prader (≥ 12 ml) and signs of virilization (pubic and axillary hair)
- No TRT, anabolic steroids or other testosterone-raising medications
- No signs of endocrine disorders (Cushing, acromegaly, hyper- and hypothyroidism) at targeted physical examination

The age and BMI of healthy volunteers will be closely matched to KFS men of the baseline registry.

7.2 Recruitment and screening

The recruitment of the participants will be performed by the PI and the study's medical assistant. Potential candidates will be preselected upon review of medical records from the following sources:

- Patients followed at the EDM consultation, CHUV, Lausanne by the PI or other endocrinologists
- Patients currently being followed at other hospitals of French speaking Switzerland (Geneva University Hospital, Fribourg regional hospital, Bienne cantonal hospital) with whom the PI has formed collaborations
- Patients referred to the PI by private endocrinologists and paediatric endocrinologists across French speaking Switzerland
- Adolescents and young adults that are transitioning by the paediatric endocrinology consultation (Dr Hauschild, co-investigator) provided that they are > 16 years old
- Patients referred by reproductive urologists collaborating with the PI (Dr Vaucher, UMF-CHUV, co-investigator; Dr Micol, CPMA)
- Patients referred *via* the Klinefelter syndrome associations (Klinefelter.CH, VALENTIN A.P.A.C. - *Association de Porteurs d'Anomalies Chromosomiques* in France)
- Patients referred by endocrine network of Swiss German centers

The PI plans to put in motion additional means to increase recruitment sources while aiming at reducing the diagnostic gap (expected rate 1:600 men) which is a crucial issue for KFS and: (i) launch a public awareness campaign by contacting family doctors of Vaud canton (and later on the rest of French-speaking Switzerland) to screen patients for KFS by performing testicular palpation, a simple and inexpensive examination; (ii) contact all urologists and fertility gynecologists to increase their awareness for KFS in case of unexplained azoospermia; (iii) via CHUV Datawarehouse, extract a list of CHUV patients' complying with the diagnosis of KFS (in the list of diagnostics) and the inclusion criteria of this study (age, BMI). This list will include the following info: first and last name, birthdate, CHUV coded number (IPP), all available physical addresses, emails and contact numbers. The coded number (CHUV IPP) will allow access to the CHUV medical folder to confirm, before contact, that they really comply with our study criteria; (iv) a similar extraction will be requested by the service of medical genetics at CHUV based on their database of karyotypes.

All potentially eligible KFS patients that are not currently being followed at EDM consultation will be receive written information of the study (e-mail, post) as well as a telephone contact to respond their first questions. Those possibly interested by the study will be invited for a research consultation in person (or via zoom in case of residence away from canton of Vaud) with the PI in order to assess eligibility. They will subsequently have at least 3 days to think about the study before reaching a decision about whether they want to participate. For these patients, there will be no charge for the research consultation nor the screening laboratory test (for design 2: fasting testosterone if no current TRT, glucose and insulin

to calculate HOMA-IR if BMI < 25 kg/m^2). Some of the study's tests that are part of the clinical follow-up of men with KFS will be charged to the patient's insurance (Figures 1 & 2 – dark purple spots).

For the patients followed at EDM, the majority of research visits for both designs will be grouped with clinical consultations (**Figure 1-2**). Indeed, these patients are regularly followed every 3 months in our service to adjust hormonotherapy and monitor clinical and metabolic response. The visit at Week 4 for both designs will be an exclusively research visit and patients will be reimbursed for the transport expenses. The same will be done for additional short visits to perform blood checks (to adjust Tostran® or Choriomon® dose) or to measure T levels 72h after the HCG stimulation test. Based on our estimation, the added presence time for each individual participant in designs 1 or 2 will be 5-10 hours.

For patients followed by external providers, all visits will be considered as exclusively research visits and patients will be reimbursed for transport fees. For these patients, there will be no medical consultation charged to the insurance given that they are already being followed by an external endocrinologist. However, laboratory tests and other examinations that are part of the standard clinical practice [71] and are reimbursed will be covered by the patient's insurance and the PI will coordinate with the external provider to communicate these results and ensure that there will be no unnecessary repetition of the same tests at a clinical setting.

Taken into account the aforementioned data, participants in designs 1 and 2 will receive a compensation of 150 CHF (in addition to the transport fees as detailed above). KFS that undergo only the baseline assessment will receive 50 CHF per person.

7.3 Assignment to study groups

The allocation of participants to different groups will be randomized with a ratio of 1:1 in both designs. The randomization list (2 for design 1, 3 for design 2) will be established by the CHUV pharmacy and the two persons in charge of clinical trials: Dr Isabelle SOMMER and Dr Aline VOIDEY according to their standard process. Only men will be included in this study, thus there will be no need to stratify the randomization according to sex.

7.4 Criteria for withdrawal / discontinuation of participants

Each participant is aware of the possibility to withdraw his/her consent at any moment for any reason and without having to justify it.

Participants will be excluded if any of the following situation occurs:

- Severe side effects attributed to the study drugs
- Insufficient compliance to the study drugs and trial procedures

- Justified decision of the principal investigator

Data, including biological samples, obtained from participants who withdrew from the clinical trial will not be destroyed. They will be kept coded and may still be analysed. Participants who will not undergo the final visit at Week 26 (+/- 2 weeks in case of emergent reason to postpone) will be considered as drop-out. Please refer to Section 9.2.5 for description of follow-up procedures.

8. STUDY INTERVENTION

8.1 Identity of Investigational Products

8.1.1 Experimental Intervention

Design 1 + 2, Anastrozole Teva® (generic)

Brand name: Arimidex

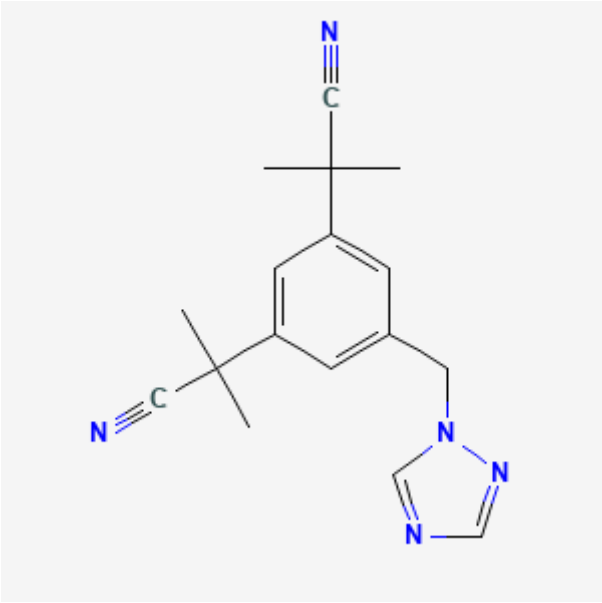
Active principle: anastrozole

Chemical name: α,α,α',α'-Tetrametyl-5-(1H-1,2,4-triazol-1-ylmetyl)-m-benzenediacetonitrile

Chemical Formula: C17-H19-N5

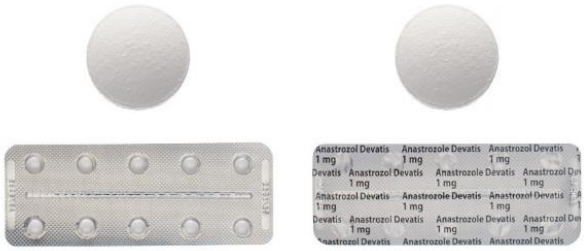
Molecular Weight: 293

Structural form:



Colour: off-white powder

Source: Anastrozole Teva® is a medication in oral form (film-coated tablets) contained in packages of 30 pills of 1 mg per blister. The active principle is anastrozole. The excipients of the product are: lactose, povidone K 29-32, carboxyméthylamidon sodique (type A) (corresponding to 0.21 mg of sodium), magnesium stearate, hypromellose, titanium dioxide (E171) and macrogol. Each tablet has a diameter of 6 mm, a round form and a grey-white colour.



Physicochemical properties: Anastrozole has moderate aqueous solubility (0.5 mg/mL at 25°C); solubility is independent of pH in the physiological range. Anastrozole is freely soluble in methanol, acetone, ethanol, and tetrahydrofuran, and very soluble in acetonitrile.

Physiological properties: anastrozole is a non-steroid inhibitor of aromatase, blocking the conversion of testosterone to estradiol and of androstenedione to estrone. Anastrozole is used as an antineoplastic drug and in particular as an adjunt treatment in menopausal women with hormonosensible form of breast cancer (presence of estrogen or progesterone receptors), as well as in women with advanced breast cancer. In men, aromatase inhibitors have been used in adolescent and young adults as part of clinical trials to treat idiopathic short stature [45] and delayed puberty [46] with proved efficacy and good tolerability. In particular, anastrozole has been used as an off-label treatment in men with idiopathic infertility [36], but also specifically in KFS men to induce spermatogenesis [37-39]. Anastrozole Teva® is considered within the dispensing category B and its authorization number is 65922 (Swissmedic).

Design 1, Group B: Choriomon®

Brand name: Choriomon

Active principle: human chorionic gonadotropin

Chemical Formula: -

Molecular Weight: -

Structural form: hCG consists of two non-covalently linked subunits, alpha and beta. Within a species, the alpha subunit is virtually identical to the alpha subunits of the three pituitary glycoprotein hormones (TSH, LH, and FSH), but the beta subunit is unique and confers its biological specificity.

Source: Choriomon® is a medication in form of a powder contained in a pierceable vial. The medication kit contains also a prefilled syringe containing 1 ml of NaCL 0.9% to use as a solvent. The active principle is human chorionic gonadotrophine (hCG), which is extracted from human urine originated from China Republic and/or Netherlands). The excipient of the powder is lactose. The medication is reconstituted by dissolving the powder in the solvent, then slowly injecting the solution subcutaneously.

Physiological properties: hCG is acting as a long-acting form of LH, stimulating the Leydig cells of the testes to produce testosterone. It is authorized to treat men with cryptorchidism, hypogonadotropic (central) hypogonadism and delayed puberty, as well as women with infertility due to lack of ovulation. HCG has been used off-label to promote spermatogenesis in men with Klinefelter. The latest published report was a retrospective series in large group of KFS men originated from China and no safety issues were reported [47]. Choriomon® is in the dispensing category B and its authorization number is 33524 (Swissmedic).

Design 2, Group C: Ozempic®

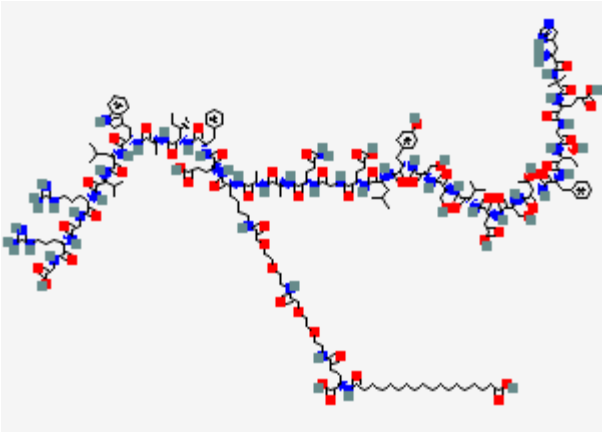
Brand name: Ozempic

Active principle: semaglutide

Chemical Formula: $C_{187}H_{291}N_{45}O_{59}$

Molecular Weight: 4114

Structural form:



Source: Ozempic® is a medication for subcutaneous auto-injection in a pre-filled pen. The active principle is semaglutide. The principal excipients of the product are: sodium phosphate dibasic dihydrate, Propylene glycol, hydrochloric acid and sodium hydroxide (for pH adjustment).

Physiological properties: Semaglutide, an analogue of GLP-1 whose sequence has 94% homology with the sequence of human GLP-1. Semaglutide acts as a GLP-1 receptor agonist, which selectively binds and activates the GLP-1 receptor, the target of native GLP-1. Semaglutide is used to treat patients with type 2 diabetes that have not achieved sufficient glycemic controls despite lifestyle changes. In recent clinical trials, it was shown to be very effective for weight loss induction also in non-diabetic subjects [48]. Ozempic® is in the dispensing category B and its authorization number is 66604 (Swissmedic).

8.1.2 Control Intervention (standard/routine/comparator treatment)

In Design 1, all patient will undergo the current state of art intervention, which is mTESE biopsy. Subsequent, only patients with a negative result (no sperm) and thus a clinically unmet need will be randomly assigned to two exploratory interventions. The primary outcome being the appearance of spermatozoids, a placebo effect is highly unlikely.

In Design 2, we will use the standard therapy (control intervention), which is TRT, in group A. The use of the placebo arm in design 1 would be ethically unacceptable as it would expose patients to untreated hypogonadism for 6 months, as opposed to the current design in which a certain rise of serum T levels is expected with all three arms. Among the different formulations for testosterone replacement, we opted for a testosterone gel to apply daily because the obtained plasmatic levels of testosterone are more stable than with intramuscular injections of testosterone [53]. **Tostran gel®** is an approved drug for treatment of male hypogonadism. It is classified in the dispensing category B and its authorization number is 57959 (Swissmedic). The authorized enterprise producing is Cederberg GmbH, 4102 Binningen. There will be no deviation from the commercial product.

Brand name: Tostran 20 mg/g

Active principle: testosterone

Pharmaceutical form: gel

Colour: Clear, colourless to slightly yellow.

Source: Tostran® is a medication in form of a tube containing the gel which is delivered by pressing on the canister piston as showed in the adjacent photo. One gram of gel contains 20 mg testosterone. One depression of the canister piston delivers 0.5 g of gel containing 10 mg testosterone. The excipients are: Propylene glycol (E1520), Butylhydroxytoluene (E321).

Physiological properties: Tostran is a hydroalcoholic formulation that dries quickly when rubbed into the skin. The dose can be applied to the abdomen or to both inner thighs. Daily rotation between the abdomen and inner thighs is recommended to minimize application site reactions. The gel should be applied to clean, dry, intact skin. It should be rubbed in gently with one finger until dry. Hands should then be washed with soap and water.

Precautions testosterone gel can be transferred to other persons by close skin to skin contact. This transfer is avoided by wearing clothes covering the application area or bathing or showering prior to contact. As a result, the following precautions are recommended in standard clinical care:

- wash hands with soap and water after applying the gel,
- cover the application area with clothing once the gel has dried
- bathe or shower before any situation in which this type of contact is foreseen.

To guarantee partner safety the patient should be advised for example to observe a minimum of four hours between Tostran application and sexual intercourse, to wear clothing covering the application site, during contact period or to bathe or shower before sexual intercourse. Furthermore, it is recommended to wear clothing covering the application site during contact periods with children, in order to avoid a risk of contamination to children's skin. Absorption studies of testosterone conducted in patients treated with Tostran indicate that patients should wait at least two hours between gel application and bathing or showering. The PI has large experience with prescription of this product in the clinical setting as well as providing the necessary advice to avoid contact to partners and children.

8.1.3 Packaging, Labelling and Supply (re-supply)

For the purpose of the study, the CHUV pharmacy will receive all the medications in batches and then redistribute them to the PI and EDM service for storage. The expected duration of both designs is 3 years as it is the expiration date for all product. The PI has taken contact to individual commercial providers to inform them on this study and request price reductions that will be documented in the contract between the drug providers and the CHUV pharmacy. Each batch will include the quantity necessary to complete the study for 5 patients on Tostran®, Ozempic® and Choriomon®, as well as 15 patients on Anastrozole Teva®. Given that this is not a double blinded study, there will be no re-labelling or re-packaging of the medications. Once the randomization will be performed by the CHUV pharmacy, we will reserve the necessary quantity of the chosen medication for the whole study duration, ideally from the same lot. These packages will be labelled with the subject's research number. All medications

will then be stored in a secure room at the EDM, at a stable temperature of 25°C±5°C (ensured by a digital thermometer).

8.1.4 Storage Conditions

You may find below the recommended storage conditions according to compendium for the comparator and the investigational products.

Tostran®

Store at room temperature (15-25°C). Do not store in the refrigerator. Do not freeze. Keep container upright.

Anastrozole Teva®

Store at 15-30°C in the original packaging.

Choriomon®

Store at room temperature (15–25°C). Keep out of reach of children. Store contents in outer carton to protect from light.

Ozempic®

Before first use: store in the refrigerator (between 2°C and 8°C), do not freeze, protect from light. After the first use: store at a temperature below 30°C or in the refrigerator (between 2°C and 8°C), do not freeze. Keep the cap on the pen when the Ozempic pen is not in use to protect it from light. Ozempic should be protected from excessive heat and light. Always remove the needle after each injection and store the Ozempic pen without the needle attached. This prevents the risk of clogged needles, contamination, infection, solution leakage and incorrect dose.

Tostran®, Anastrozole Teva® and Choriomon® will be stored in a secure room at the EDM, at a stable temperature of 15-25°C, as continuously monitored by a digital thermometer. In case of temperatures between 25-30°C for a short period time (< 1 week), this is considered still ambient temperature by pharmaceutical convention and is acceptable based on standard clinical practices. In case of higher ambient temperatures or prolonged periods of environmental hit, the storage temperature will be cooled by application of cold packs (commercially available) or external ventilation of the specific storage unit. Only the PI and the research medical assistant will have access to this storage space.

Ozempic® will be stored in one of the EDM service fridge at a mean temperature of 5°C. The shelf with the medication of the study will be clearly separated and labelled differently to ensure that there is no confusion with the medications kept in the clinic. A limited number of persons have access to the fridge notably the EDM pharmacist and EDM nurses that will be informed on this storage space specifically destined for this study.

8.2 Administration of experimental and control interventions

8.2.1 Experimental Intervention

Design 1 (see Figure 1 for details):

Group A: anastrozole (Anastrozole Teva®) – experimental intervention

Starting daily dose 1 tablet = 1 mg per day

- No scheduled adjustment of dosing regimen

Group B: anastrozole (Anastrozole Teva®) and hCG (Choriomon®) – experimental intervention

For anastrozole: starting daily dose 1 tablet = 1 mg per day

- No scheduled adjustment of dosing regimen

For hCG: starting dose according to the post-mTESE n°1 T levels

- 1000 U every 48h if T levels < 8 nmol/l, 500 U every 48h if T levels > 8 nmol/l
- The dose will be adjusted according to T levels at Week 4 of study, targeting T trough levels (48h post injection) between 10 and 15 nmol/l. Increments or reductions of 500 U to a maximum dose of 2000 U every 48h will be performed. In case of a dose adjustment, an additional blood test to measure T levels will be performed after 4 weeks to verify if the target was achieved or if additional dose adjustment is indicated.

A follow up visit will be performed at Week 4 post completion of the study (last mTESE biopsy) in all groups to assess clinical status and eventual late adverse events. The patient will then be referred back to clinical follow-up (EDM or external provider).

Design 2 (see Figure 2):

Group B: anastrozole (Anastrozole Teva®) – experimental intervention

Starting daily dose 1 tablet = 1 mg per day

- No scheduled adjustment of dosing regimen

Group C: semaglutide (Ozempic®) – experimental intervention

Starting weekly dose 1 subcutaneous injection of 0.25 mg for 4 weeks then

- 0.5 mg for 4 weeks, then 1 mg for 4 weeks, then 2 mg weekly up to the end of study
- If gastrointestinal symptoms grade 1 at the end of each dosing phase, the dose will not be further increased until resolution of side effects. If gastrointestinal symptoms grade 2 at the end or during each dosing phase, the dose will be decreased to the immediately lower available dose (for instance, decrease to 0.5 mg per week if side effects grade 2 on the 1 mg per week dosage). In case of grade 3 or 4 side effects, the medication will be immediately withheld.

A follow up visit will be performed at Week 4 post completion of the study in all groups to assess clinical

status and eventual late adverse events. The patient will then be referred back to clinical follow-up (EDM or external provider).

8.2.2 Control Intervention

Design 2 (see Figure 2):

Group A: testosterone gel (Tostran®) – control intervention

Starting daily dose (1 push = 10 mg) according to T levels at baseline visit (V1):

- 20 mg if T 10-14 nmol/l, 30 mg if T 6-10 nmol/l, 40 mg if T levels < 6 nmol/l
- Subsequent titration by increments or reductions of 1 push (10 mg) to target T levels 15-25 nmol/l, then reassess T levels after 4 weeks
- If erythrocytosis (haematocrit > 52%) occurs during the study, target T levels 10-15 nmol/l

A follow up visit will be performed at Week 4 post completion of the study in all groups to assess clinical status. The patient will then be referred back to clinical follow-up (EDM or external provider).

8.3 Dose modifications

There are three planned drug dose changes as described in Chapters 8.2.1 (Design 1, group B, Choriomon®; Design 2, group C, Ozempic®) and 8.2.2 (Design 2, group A, Tostran®). These regimens are in line with the recommended use of these drugs in clinical practice. Anticipating potential harms, we included additional dose adjustment rules in case of erythrocytosis (design 1, group A, Tostran®) or gastrointestinal side effects (design 1, group C, Ozempic®). It is possible that in case of unexpected adverse events or participant's request, the PI can decide dose adjustments based on his large clinical experience. These will be reported as note to files.

8.4 Compliance with study intervention

A diary will be provided to participants to monitor the compliance to the study (including injection dates for Ozempic® and Choriomon®). This diary will have to be brought to every visit at EDM together with the used and unused medications. Monitoring laboratory tests of reproductive hormones will allow an indirect assessment of compliance for most groups. During the visit at week 4, the compliance to the drug medication will be estimated with the participant based on the diary information and unused medication. In case of an estimated > 10% of missed doses (or a single missed injection in the Ozempic® group), the participant will have a closer monitoring by the research medical assistant with a weekly motivational phone interview up to a renewed assessment of the compliance after 4 weeks. If the compliance issues persist at this stage, the participants will be warned for a risk of premature withdrawal from the study. Indeed, participants with persistently low compliance (> 30% missed doses) at the Week 13 visit will be considered as drop outs and be replaced. For this potential risk, the randomization list will contain an additional 20% of number participants.

8.5 Data Collection and Follow-up for withdrawn participants

Participants who interrupt the study will be requested to return unused medication and have a clinical visit (vital signs, physical exam) for safety reasons. If participants were in the interventional study for at least 12 weeks, they will be asked to perform a standard follow-up visit 4 weeks after withdrawal. Refer to paragraph 9.3 for details on the follow-up visit.

8.6 Trial specific preventive measures

The CRF will include all the treatments usually taken by the participant. Participants will have to inform the co-investigator of any treatment change due to any acute or chronic event. A needle procedure in occasion of blood tests may be harmful for adolescent participants. In this particular group and in accordance with the standard practices of pediatric endocrinologists, a topical anesthetic product, such as Emla cream 5% or Emla patch, will be proposed to help numb the skin. To minimize any pain to participants, they will be offered to put the anesthetic product 60-120 minutes before any needle procedures. A mild reaction (paleness or redness of the skin, slight puffiness, initial burning or itching) may occur on the area on which Emla Cream is used. These are normal reactions to the cream and will disappear in a short while without any measures being needed. Emla Cream contains *macrogolglycerol hydroxystearate*, which may cause skin reactions in some people.

8.7 Concomitant Interventions (treatments)

If the participant starts taking a new treatment (including vaccine) during the clinical trial, he/she will have to inform the co-investigator and the treatment will be reported in the CRF. In this occasion, any contraindication will be verified. Relevant changes in reproductive hormones, notably testosterone and prolactin, have been observed in men following a treatment with these medications: **spironolactone, ketoconazole, corticosteroids, antiemetics, opioids, and psychotropic drugs (antidepressants, antipsychotics, and anticonvulsants).** If a participant is under one of the previously cited treatments at the screening visit, the eventual impact on the reproductive axis will be verified and the patient will not be included in case of drug-induced hypogonadism (hypogonadotropic hypogonadism) or significant elevation of plasma prolactin levels (> 30 µg/l).

8.8 Study Drug Accountability

Medications will be ordered once the study is authorized by the CER-VD and Swissmedic. The pharmaceutic companies will provide the CHUV pharmacy in batches (once every 6 months) that correspond to approximately 25% of total study needs (15 patients for 6 months of Anastrozole Teva®, and 5 patients for 6 months of each of Tostran®, Ozempic® and Choriomon®. The lot/batch number and the expiration date of each vial used during this study will be registered. The medications will be stored at the EDM service (Hotel des patients) in a dry, clean, well-ventilated area, at room temperatures for

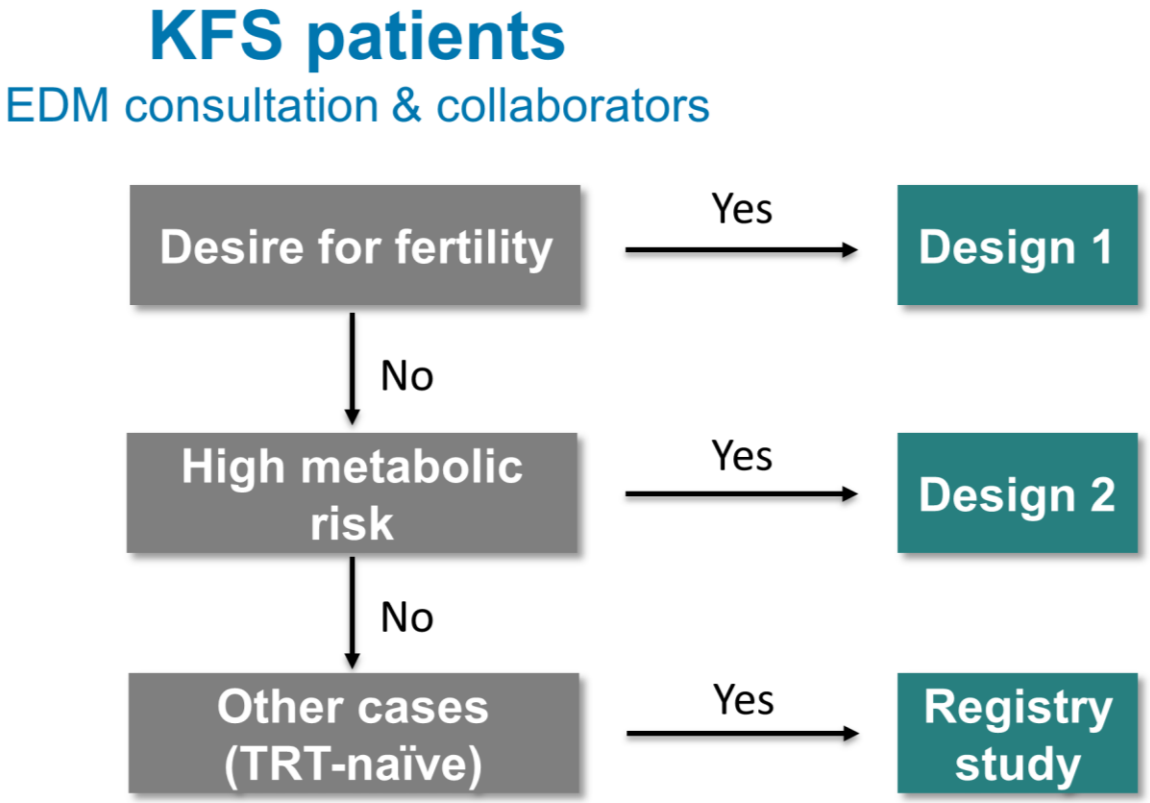
Anastrozole Teva®, Tostran® and Choriomon®, whereas Ozempic® stocks will be refrigerated (2-6°C). Following randomization of each participant, the appropriate quantity of the selected medication will be labelled with the subject's research number. Study drugs will be handled and allocated to the participants by the research medical assistant under the supervision of the PI. The necessary quantity until the next research visit will be provided with an extra additional 2 weeks in case of rescheduling of the research visit due to emergency. Participants will have to bring all the medications (used and unused) at each visit for verification of correct use. Participants will also be required to complete a diary to identify dates and sites of injections for Choriomon® and Ozempic®, any concomitant medications and adverse events.

8.9 Return or Destruction of Study Drug

We will retrieve the used material of Choriomon® and Ozempic® and will ensure adequate recycling in collaboration with the CHUV pharmacy. Drug packages that have been opened but not finished will be destroyed. Drug packages that have not been opened will be returned to the respective drug company.

9. STUDY ASSESSMENTS

9.1 Study flow chart(s) / table of study procedures and assessments



Design 1 (men seeking fertility or interested in fertility preservation):

Study period	Recruitment	Screening	Baseline				Treatment					Follow-up
Visit	0	1	2	3		4	5	6	7	8		9
Time (days, week)	Day -180 to Day -3 before screening	Up to Month -6	Week -6	Week -6 + Day 3	Week -2 to Week -4	Day 1	Week 4	Week 13	Week 24	Week 26	Week 26 + 1-3 days	Week 30
Recruiting (study presentation, set-up)	X											
Patient Document Information	X											
Semen analysis	X		X						X			
Informed consent		X										
Medical history, Physical exam		X										
Inclusion and exclusion criterion		X										
TRT wash-out (if present)		X										
mTESE biopsy					X						X	
RandomizationN (if first mTESE negative)					X							
Drug provision (once a month)						X	X	X		X		
Physical exam												
clinical visit			X			X	X	X		X		X
weight, height, BMI			X			X	X	X		X		X
waist and hip circumference			X			X	X	X		X		X
testicular volume			X			X		X		X		
blood pressure, heart rate			X			X	X	X		X		X
ADAM questionary, DISF-M-II & IIEF scores			X				X	X		X		X
gynecomastia			X				X	X		X		X
check for adverse effects			X				X	X		X		X
Laboratory tests (fasting)												
Reproductive hormones			X			X	X	X		X		X
Metabolic and inflammatory status			X				X	X		X		X
Oral glucose tolerance test (2h)			X							X		
HCG stimulation test (5000 U unique dose)			X (inj)	X (lab)						X		
Blood samples (fasting)												
Serum (9.0 ml)			X			X	X	X		X		X
EDTA (4.9 ml) twice			X			X	X	X		X		X
Heparin (7.5 ml)			X			X	X	X		X		X
PaxGene (2.5 ml)			X			X				X		
Testicular ultrasound				X						X		
Testes samples for gene expression + LC-MS					X						X	
DXA (bone density)			X							X		
DXA (body composition)			X							X		
Whole body calorimetry				X						X		
Fibroscan			X	X						X		

Design 2 (men with high metabolic risk):

Study period	Recruitment	Screening 1	Screening 2 (if TRT)	Baseline		Treatment				Follow-up
Visit	0	1	2	3	4	5	6	7	8	9
Time (days, week)		up to Day -180	up to Day -14	Day -3	Day 1	Week 4	Week 13	Week 26	Week 26 + Day 3	Week 30
Recruiting (study presentation, set-up, TRT wash-out if present, screening info)	X									
Patient Document Information	X									
Informed consent		X								
Medical history, Physical exam (BMI)		X	X							
Inclusion and exclusion criterion		X								
Screening blood test (T, glucose, insulin)		X	X							
Randomization				X						
Drug provision (once a month)					X	X	X	X		
Physical exam										
clinical visit				X		X	X	X		X
weight, height, BMI				X		X	X	X		X
waist and hip circumference				X		X	X	X		X
testicular volume				X			X	X		
blood pressure, heart rate				X		X	X	X		X
ADAM questionary, DISF-M-II & IIEF scores				X		X	X	X		X
gynecomastia				X		X	X	X		X
check for adverse effects				X		X	X	X		X
Laboratory tests (fasting)										
Reproductive hormones				X		X	X	X		X
Metabolic and inflammatory status				X		X	X	X		X
Oral glucose tolerance test (2h)				X			X	X		
HCG stimulation test (5000 U unique dose)				X (inj)	X (lab)			X (inj)	X (lab)	
Blood samples (fasting)										
Serum (9.0 ml)				X		X	X	X		X
EDTA (4.9 ml) twice				X		X	X	X		X
Heparin (7.5 ml)				X		X	X	X		X
PaxGene (2.5 ml)				X				X		
DXA (bone density)				X				X		
DXA (body composition)				X			X	X		
Whole body calorimetry					X				X	
Fibroscan					X				X	
Testicular ultrasound					X				X	
Semen analysis (if not previously assessed)				X				X		

9.2 Assessments of outcomes

9.2.1 Assessment of primary outcome

The primary outcomes will be:

Design 1 (patients interested in fertility preservation); the **sperm retrieval rate (SSR) at second mTESE biopsy after 26 weeks of hormonal pre-treatment** (group A and B separately) in patients with a sperm-negative first mTESE biopsy**,** as compared to the expected rate without intervention

During the mTESE biopsy, several samples of the testes are performed as guided by macroscopic and microscope real-time assessment. These samples are rapidly assessed by the andrology laboratory in search of viable spermatozoids. A second sample is send in the CHUV pathology lab. This outcome is qualitative and will be either presence of spermatozoids (SSR positive) or absence of spermatozoids (SSR negative).

Design 2: the **change in insulin resistance**, **as assessed by the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) score** [1], **from baseline to end of treatment (week 26)**

HOMA-IR is calculated based on fasting plasma glucose and insulin levels according to according the formula (glucose in mmol/l × insulin in mU/L)/22.5. All blood samples of the study will be performed after an overnight fasting (no meal or drink other than water after 22 pm the day before). Given that insulin is very sensitive, particular attention will be driven to perform a gentle blood sampling to limit risk of hemolysis and samples will be immediately conveyed to the LLC CHUV laboratory for analysis.

9.2.2 Assessment of secondary outcomes

Please refer to Chapter 5.2 for clinical importance of selected secondary outcomes.

Secondary outcomes will be:

Design 1:

- **Sperm retrieval rate (qualitative) at second mTESE biopsy in group A vs group B**

  See primary outcome – Chapter 9.2.1.

- **Changes in markers of serum inhibin B, AMH and INSL3 from baseline to week 26 and correlation with the mTESE biopsy results**

  Testicular peptides will be measured at fasting serum samples. These hormones are not currently measured at CHUV. We will directly centrifuge and freeze the serum/plasma at -80°C (the EDM service disposed of two -80°C freezers). The measurement will be performed for the samples altogether at the end of the study, which could allow better pricing offers and reduce the variability due to different batches. The PI is aware of 2-3 laboratories in France which perform these analysis.

- **Change in testicular histology with particular focus to the fibrosis-hyalinization from**

baseline to week 26

This will be performed in the histologic samples that are routinely taken during the mTESE biopsies. The PI will collaborate for this with an experienced pathologist in testicular histology (see list of co-investigators).

- **Change in transcriptomic status at the testes from baseline to week 26**

  Detailed methods are provided in Chapter 5.2.

- **Change in intratesticular levels of sex steroids (T, E2) from baseline to week 26**

  Detailed methods are provided in Chapter 5.2. Briefly, intratesticular sex steroids levels will be measured in testicular extracts sampled during the mTESE biopsies. Fresh testicular tissue will be homogenised using an ultrasound at the LLC CHUV (Dr Bruce) based on detailed techniques previously reported [68]. Subsequently, LC-MS will be applied in the liquefied phase of the sampled material.

Design 2:

- **Change in HOMA-IR score from baseline to week 4 and week 13**

  See primary outcome – Chapter 9.2.1

- **Change in other insulin sensitivity indexes (area under the curve for glucose and insulin) derived from oral glucose tolerance test from baseline to week 4, 13 and 26**

  These will be calculated based on the oral glucose tolerance test. Briefly 75 gr of a glucose solution will be ingested by the patient with subsequent plasma glucose and insulin measurements every 30 minutes for 2 hours (5 samples in total). Based on these values, area under the curve for glucose and insulin can be calculated.

- **Change in total and visceral adiposity as assessed by DXA from baseline to week 13 and 26**

  This is a routine clinical application of Dual X-ray absorptiometry.

- **Change in inflammation serum markers (hs-CRP, TNF-a, IL-6, S100A8-9) from baseline to week 4, 13 and 26**

  These markers will be measured in standard fasting blood samples (serum or plasma) without need for additional preanalytic or analytic precautions.

- **Change in serum T levers from baseline to week 13 and 26 in group 3 (semaglutide)**

  Testosterone levels in the serum will be measured by LC-MS via the steroid profile of LLC – CHUV.

9.2.3 Assessment of other outcomes of interest

- Change in blood RNA expression from baseline of end of treatment (Design 1-2, Week 26)

  Blood transcriptomics will be assessed in collaboration with Dr Messina, biologist expert of Nelly Pitteloud's lab. The BRB-seq platform of EPFL will be implemented for sequencing.

- Change in testicular volume and vascular pattern from baseline to end of treatment (Design 1, Week 26)

  Based on a recently published report [69], we will perform a testicular ultrasound with Doppler to investigate the testicular microvascular flow before and after intervention. This will be done in collaboration with Dr Vietti Violi, referent radiologist for urogenital imaging at CHUV.

- Change in other metabolic parameters from baseline to end of treatment (Design 2 - Week 26)

  Most of these parameters (cholesterol, triglycerides, LDL cholesterol, HDL cholesterol, ASAT, ALAT) will be measured using Cobas/Roche platform of LLC. FGF21 and adipokines will be measured by commercially available ELISA kits. Hb1Ac is also measured at LLC using a chromatography assay (D-100 / Bio-Rad Laboratories AG).

- Change in Leydig cell secretory capacity as assessed by the hCG stimulation test, from baseline to the end of treatment (Design 2, Week 26).

  For this test specifically, testosterone will be assessed using the currently used method of LLC – CHUV (CMIA, Architect I / Abbott).

- Change in lipidomics and metabolomics from baseline to end of treatment (Design 2, Week 26)

  This will be done in collaboration with the Metabolomics UNIL Lab (Dr Ivanisevic). The current methods for sphingolipids and amino acid are summarized in the following articles [84, 85].

9.2.4 Assessment of safety outcomes

Participants will report any event considered abnormal either during the planned visits or by contacting the investigator. A supplementary blood test will be performed in case of any relevant clinical event.

9.2.4.1 Adverse events

Participants will receive medical care throughout the study and will be supervised in case of side effects. During each interventional design, they will be asked at each visit to report any adverse event (participants will be provided with a diary) as well as details regarding the time of onset, duration, intensity and eventual resolution. All adverse events, considered to be related to the treatment, will be recorded in the CRF. If a participant presents serious side effects (grade 3) that are thought to be related to one of the study's drugs, the treatment will be stopped immediately and the participant will be followed until healing or stabilization of his/her condition. A clinical exam and a blood draw to verify the hormonal concentrations will be performed in case of mild or moderate side effects and the treatment's dose (Tostran®, Ozempic®, Choriomon®) will be adjusted based on the results and the overall clinical reasoning. Refer to Section 10 for adverse events definition and procedures.

9.2.4.2 Laboratory parameters

The following laboratory parameters will be considered as adverse events:

- **Haematocrit elevation > 52%**, a known potential adverse event of TRT and all testosterone-raising therapies such as Anastrozole® and Choriomon®. The effect of testosterone on blood cells is clearly dose-dependent. A reduction of the dose of the medications will be done and the haematocrit will be reassessed after 4 weeks or before according to the PI's clinical judgment.
- **PSA absolute elevation at Week 13 > 1.0 µg/l or relative elevation of more than > 1.4 µg/l compared to baseline** values. The risk of prostate cancer under TRT is very low and a causal link is not proven. Nevertheless, a monitoring of PSA is recommenced in men > 50 years [8]. For safety reasons, we will follow this parameter in all participants. In case of an elevation meeting the aforementioned criteria, the participant will be referred for a urologic consultation. Given the use of short-acting drugs (Tostran®, Anastrozole®, Choriomon®), the study's medication will not be withdrawn awaiting the urologic verdict, in agreement with what is done in routine clinical practice.

9.2.4.3 Vital signs

Blood pressure will be measured at each visit to detect any high blood pressure onset. In case of high value (> 140/90 mmHg), the measurement will be repeated twice in supine position after 5 minutes resting. Given that a weight loss is expected in some study's groups, the blood pressure will also be measured at upright position once to ensure that there is no orthostatic hypotension (defined as a drop in systolic blood pressure > 20 mmHg and/or a drop in diastolic blood pressure > 10 mmHg) and need to recommend a reduction of anti-hypertensive treatment if present. Blood pressure and heart rate will be reported in the CRF.

9.2.5 Assessments in participants who prematurely stop the study

If a participant has serious side effects that are thought to be related to the study's medication, the treatment will be stopped immediately. The participant will be followed until healing or stabilization of

his/her condition. Refer to Section 10 for adverse events definition and procedures.

9.3 Procedures at each visit

9.3.1 Recruiting and screening visit

Recruiting (Visit 0)

The patient eligibility (inclusion criteria) will be pre-checked during the clinical follow-up of KFS patients at the EDM consultation or the medical follow-up by collaborators. When contacted by external endocrinologists, surrounding hospitals or other physicians, the PI will perform a first check-up of the eligibility. This will be done anonymously for external patients (via the provider-collaborator). The first question will be whether the patient a fertility pursue is wanted (Design 1) and if yes whether an azoospermia was present at previous semen analysis. If the patient does not seek fertility or fertility preservation, the PI will check the eligibility for Design 2, in particular (i) whether a TRT is present or serum T levels without any TRT are at the inclusion range – see Figure 2; AND (ii) whether the patient's BMI is > 28 kg/m^2 or 25-28 kg/m^2 but with an elevated HOMA-IR score (> 2.6) based on fasting glucose and insulin levels. These are standard measurements – laboratory tests that are easily retrievable in the medical file of KFS men. If the patient is potentially eligible, the PI will:

- For patients followed at EDM clinic, briefly meet them before or after a clinical appointment
- For external patients and after the referring physician has obtained the patient's consent, contact them by phone or visioconference (Webex) to present the study

By this contact, the PI will present the study to potential participants who will receive the written information of the study (design 1 or 2 based on their characteristics) by post, e-mail or in hand for those followed in the EDM clinic. A follow-up phone contact to have their feedback and organize the screening visit in case of a positive response, will be scheduled at the earliest 3 days after.

<u>For Design 1 (patients seeking fertility)</u>

Screening (Visit 1): any time of the day

T0-T30': The participant will received at the EDM clinic by the PI and the research medical assistant (if a blood sample is necessary). During the visit, a detailed presentation of the study design will be performed with explanations on the purpose of the study, the randomized design, the expected beneficial effects of potential study drugs and the eventual adverse events. The need to withdraw TRT before the mTESE biopsy as also recommended in clinical practice will be underlined. The formal **consent** of the participant (and/or his/her legal representative), using the approved consent form, will be obtained before the participant is submitted to any study procedure.

T30'-T45': Checklist inclusion and exclusion criteria and signature of consent form

T45'-T60': Physical exam (weight, height, blood pressure, pulsation, waist and hip circumference, arm

span, testicular volume, gynecomastia)

If patient is on TRT, the duration of the wash-out will be defined according to the TRT type (Figure 1). The baseline visit will be scheduled according to the indicated duration of the wash-out.

For Design 1 (patients with high metabolic risk)

Screening I (Visit 1): any time of the day if TRT is present OR 08h00 AM fasting if TRT-naive

T0-T30': The participant will received at the EDM clinic by the PI and the research medical assistant (if a blood sample is necessary). During the visit, a detailed presentation of the study design will be performed with explanations on the purpose of the study, the randomized design, the expected beneficial effects of potential study drugs and the eventual adverse events. The formal **consent** of the participant (and/or his/her legal representative), using the approved consent form, will be obtained before the participant is submitted to any study procedure.

T30'-T45': Checklist inclusion and exclusion criteria and signature of consent form

T45'-T60': Physical exam (weight, height, blood pressure, pulsation, waist and hip circumference, arm span, testicular volume, gynecomastia)

T60' (only TRT-naïve patients): screening blood sample (testosterone, glucose, insulin)

If patient is on TRT, the duration of the wash-out will be defined according to the TRT type (Figure 2).

Screening II (Visit 2): after adequate wash-out only for patients on TRT at Visit 1

08h00-08h30: The patient will have a brief physical exam (weight and height to calculate BMI) and a fasting blood test to measure (i) testosterone (eligible if T levels 3-14 nmol/l) and (ii) glucose and insulin levels to calculate HOMA-IR only if the BMI is between 25-28 kg/m^2 (eligible if HOMA-IR > 2.6). If the BMI is > 28 kg/m^2 the patient is considered at high metabolic risk and there is no need to calculate HOMA-IR for eligibility. Once the eligibility of the participant is confirmed, the baseline visit will be planned and the randomization of the patient will be requested to the CHUV pharmacy.

9.3.2 Baseline visit

Design 1 (patients seeking fertility)

The baseline visit will be conducted on two half-days, at least 2 weeks before the first mTESE biopsy in order to ensure that the semen analysis results will be available before the biopsy. If the biopsy fails to detect viable spermatozoids, the patient will be eligible for the interventional study. A complementary short visit to verify reproductive hormones will be done 2-4 weeks after the biopsy given the mild risk of a new-onset surgery-induced hypogonadism. The patient will receive and start the allocated study medication at the same day.

Baseline I – Visit 2 (at least 2 weeks before the mTESE biopsy):

Identical to Design 2 (see below). If the semen analysis was already performed by the referent reproductive urologist, it will not be repeated, provided that azoospermia was confirmed by at least two different semen samples.

Baseline II – Visit 3 (3 days post Visit 1):

Identical to Design 2. The research medical assistant will not perform the education regarding the study drug nor request the randomization of the patient, given that his eligibility for the interventional trial depends on the mTESE biopsy outcome. Based on the scheduled date of the biopsy, the baseline III – Visit 4 will be organized.

mTESE testicular biopsy n°1:

This will be performed according to the usual clinical procedure either at CHUV (Dr Vaucher, EDM patients), at CPMA (Dr Micol) or at HUG (Dr Tran). This open biopsy is typically performed under general anaesthesia but in an ambulatory setting. A unilateral approach will be followed with multiple samples guided by microscope as at standard mTESE design. Two research samples will be performed during the study:

(i) a testicular biopsy at the beginning of the process using dedicated material (scalpet) that is sterile and not exposed to RNA-ase. The biopsy material will be rinsed with physiologic serum (NaCL 0.9%) then will added in a dedicated Eppendorf tube filled with RNA later (Quiagen) then stored at 4°C until transfer to EDM service (either manually for CHUV and EPFL or by a dedicated express transport for HUG samples)

(ii) a second testicular sample, taken toward the end of the procedure just before closing the scrotum when very frequently some testicular tissue remains exposed. This will be homogenised in order to measure using LC-MS intratesticular steroids (mainly T and E2) in the tissue extracts. For biopsies conducted at CHUV, the small tissue sample will be inserted in a classic conic tube and be transferred directly to the LLC laboratory. For biopsies performed elsewhere, the tissue will be frozen locally and transported to LLC in batches.

The urologist will communicate the result of the biopsy to the PI. In case of failed sperm retrieval, the PI will request the randomization of the patient by the CHUV pharmacy.

Baseline III - Visit 4 (2-4 weeks after the mTESE biopsy) = Day 1 of interventional study:

For patients with positive sperm retrieval at mTESE biopsy, this will be the follow-up and last visit.

For patients with negative sperm retrieval at mTESE biopsy, this will be the first day of the interventional study.

08:00-09:00: Consultation with research medical assistant and PI to (i) perform anthropometric

measurements; ii) to measure basic reproductive hormones in order to exclude a possible reproductive impairment owing to biopsy-induced trauma; (iii) inform the patient on the randomly assigned study group and educate him for subcutaneous auto-injections if he is assigned to group B (Choriomon®); (iii) provide adequate drug supply for at least 4 weeks and inform the patient on handling and storage of the medication; (iv) to plan the dates of all the follow-up visits up to the end of the follow-up.

Design 2 (patients with high metabolic risk)

The baseline visit will be conducted on two half-days.

Baseline I - Visit 3:

07:15-07:45: Semen analysis if the patient is able to ejaculate. This will be performed at the andrology laboratory of CHUV (Maternité). Abstinence from all masturbation and sexual intercourse will be requested at least 2-7 days before the semen sample in line with published recommendations [86].

08:00-11:00: The patients will be welcomed by the research medical assistant. Anthropometric measurements will be performed (weight, height, waist and hip circumference). A blood sample will be performed fasting and a venous catheter will be placed in the forearm to facilitate blood sampling during the oral glucose tolerance test (OGTT). The blood sample will be divided in two parts: one that will be brought to CHUV LLC for immediate measurement (basic reproductive and metabolic parameters) and a second for exploratory analysis (adipokines, cytokines, transcriptomics, metabolomics) that will be rapidly centrifuged, then frozen at -80°C (EDM service freezers). This will also include a biobank. After ingestion of 75 gr of glucose, a blood sample for glucose and insulin will be done every 30 minutes for 2 hours. At the end of the test, a breakfast will be offered to the participant. During the breaks between the blood samples, the PI will undertake the physical status (testicular volume, gynecomastia) and will also open the randomization envelop with the patient and inform him regarding the attributed group. The research medical assistant will complete the reproductive axis medical questionnaire (ADAM questionary, DISF-M-II & IIEF scores) and at the end of the OGTT test will administer a subcutaneous injection of HCG 5000 U (Choriomon®) to launch the HCG stimulation test.

11:00-12:00: Dual X-ray absorptiometry scan at Center of Bone Diseases, CHUV (Hôpital Orthopédique) for bone density and body composition assessment.

Baseline II - Visit 4 (3 days post Visit 1) = Day 1 of the interventional study:

07:15-07:45: Testicular ultrasound at CHUV radiology service under the supervision of Dr Vietti Violi.

08:00-08:30: A whole body calorimetry will take place in the service of clinical nutrition of EDM service (Dr Barigou) to assess the basic metabolic rate (resting energy expenditure).

09:00-10:00: Consultation with research medical test to (i) perform a blood test for testosterone measurement (72h post HCG to complete the HCG stimulation test); (ii) to educate the patient for the assigned study drug, in particular the subcutaneous auto-injections of Ozempic®; (iii) to provide

adequate drug supply for at least 4 weeks and inform the patient on handling and storage of the medication; (iv) to plan the dates of all the follow-up visits up to the end of the follow-up.

10:30-11:00: Liver fibroscan at service of gastroenterology at CHUV (Dr Fraga) to quantify hepatic steatosis and grade of accompanying fibrosis.

9.3.3 Interventional study visits

Design 1 (patients seeking fertility)

Interventional study I – Visit 5 (Week 4 post baseline)

08:00-09:00: Consultation with research medical assistant. The patient comes to EDM clinic after an overnight fasting. He will be asked to bring with him all drug provision (in order to assess the compliance) as well as the study diary where he was asked to note all new symptoms since the beginning of the trial. The following actions will be undertaken during this visit:

- Anthropometric measurements as at baseline visit (except for testicular volume)
- Review of potential adverse events list including revision of injection sites to search for abnormal local reactions if any
- ADAM questionary and other sexual function scores as at baseline visit
- A fasting blood sample will be taken to measure reproductive and metabolic parameters. Additional plasma and serum will be rapidly centrifuged and then aliquoted and frozen at -80°C for future analysis in batches.
- Provision of drug for at least 10 more weeks

Interventional study II – Visit 6 (Week 13 post baseline)

08:00-09:00: Consultation with research medical assistant and PI. In addition to the procedures described in the Interventional study I, this visit will include:

- A physical examination and testicular palpation to estimate their volume by the PI
- Provision of drug for at least 12 more weeks

Interventional study III – Visit 7 (Week 24 post baseline)

07:15-07:45: Semen analysis. This will be performed at the andrology laboratory of CHUV (Maternité). Abstinence from all masturbation and sexual intercourse will be requested at least 2-7 days. This test will be done at least 2 weeks before the second mTESE biopsy to ensure that azoospermia persisted and that the second biopsy is necessary.

Interventional study IV – Visit 8 (Week 26 post baseline)

The last visit of the study for this design will take place in single day (the patient will be at CHUV for

different exams from 07h00 to 15h00). The patient will arrive after an overnight fasting. He will be able to drink water.

07:15-07:45: Testicular ultrasound at CHUV radiology service under the supervision of Dr Vietti Violi.

08:00-11:00: The patients will be welcomed by the research medical assistant. Anthropometric measurements will be performed (weight, height, waist and hip circumference). A blood sample will be performed fasting and a venous catheter will be placed in the forearm to facilitate blood sampling during the oral glucose tolerance test (OGTT). The blood sample will be divided in two parts: one that will be brought to CHUV LLC for immediate measurement (basic reproductive and metabolic parameters) and a second for exploratory analysis (adipokines, cytokines, transcriptomics, metabolomics) that will be rapidly centrifuged, aliquoted then frozen at -80°C (EDM service freezers). This will also include a biobank. After ingestion of 75 gr of glucose, a blood sample for glucose and insulin will be done every 30 minutes for 2 hours. At the end of the test, a breakfast will be offered to the participant. During the breaks between the blood samples, the PI will undertake the physical status (testicular volume, gynecomastia). The research medical assistant will complete the reproductive axis medical questionnaire (ADAM questionary, DISF-M-II & IIEF scores). We will ensure that the patient has enough of study medication until the date of the second mTESE biopsy.

11:00-12:00: Dual X-ray absorptiometry scan at Center of Bone Diseases, CHUV (Hôpital Orthopédique) for bone density and body composition assessment.

12:00-13:30: Lunch at CHUV restaurant offered by the study

13:30-14:00: A whole body calorimetry will take place in the service of clinical nutrition of EDM service (Dr Barigou) to assess the basic metabolic rate (resting energy expenditure).

14:30-15:00: Liver fibroscan at service of gastroenterology at CHUV (Dr Fraga) to quantify hepatic steatosis and grade of accompanying fibrosis.

mTESE testicular biopsy n°2:

This will be identical at design as the first mTESE biopsy and will performed by the same urologist surgeon (Dr Vaucher, CHUV + EDM patients; Dr Micol, CPMA, Dr Tran, HUG). The contralateral testes will be chosen for the biopsy unless there is a clear asymmetry between the two testes or ultrasonographic findings that indicate a better chance of sperm retrieval if the biopsy is repeated in the same testes as in the first biopsy. Similar to baseline, two research samples will be performed during the biopsy for RNA extraction and LC-MS intratesticular steroid measurement. A strictly identical method will be followed as at the baseline visit.

Design 2 (patients with high metabolic risk)

Interventional study I – Visit 5 (Week 4 post baseline)

08:00-09:00: Consultation with research medical assistant. The patient comes to EDM clinic after an

overnight fasting. He will be asked to bring with him all drug provision (in order to assess the compliance) as well as the study diary where he was asked to note all new symptoms since the beginning of the trial. The following actions will be undertaken during this visit:

- Anthropometric measurements as at baseline visit (except for testicular volume)
- Review of potential adverse events list including revision of injection sites to search for abnormal local reactions if any
- ADAM questionary and other sexual function scores as at baseline visit
- A fasting blood sample will be taken to measure reproductive and metabolic parameters. Additional blood samples will be rapidly centrifuged and then aliquoted and frozen at -80°C for future analysis in batches.
- Provision of drug for at least 10 more weeks

Interventional study II – Visit 6 (Week 13 post baseline)

08:00-11:00: Consultation with research medical assistant and PI. In addition to the procedures described in the Interventional study I, this visit will include:

- a physical examination and testicular palpation to estimate their volume by the PI
- an oral glucose tolerance test similar to the detailed description in Baseline I (Visit 3)
- Provision of drug for at least 12 more weeks

11:00-11:30: Dual X-ray absorptiometry scan at Center of Bone Diseases, CHUV (Hôpital Orthopédique) for body composition assessment.

Interventional study III – Visit 7 (Week 26 post baseline)

This will be identical to Baseline I – Visit 3. Additionally, the patient will be questioned regarding adverse events.

Interventional study IV – Visit 8 (Day 3 post Visit 7)

This will be identical to Baseline II – Visit 4. The study medication will be stopped and the patient will be requested to return remaining stocks in any. The participant will be also reminded that he should bring us a copy of all the transport fees at the follow-up study.

9.3.4 Follow-up visits

Design 1 & 2

The follow-up visit for both designs will take place approximately 4 weeks after the discontinuation of the study medication. This visit will include:

- Anthropometric measurements
- Screening for late and/or persistent adverse events
- A fasting blood test to measure reproductive and metabolic parameters as well as stored serum and plasma for exploratory measurements in batches at the end of the study
- Return of all unused study medication (if any) + study diary
- Estimation of the whole reimbursement owed to the participant (study remuneration + transport fees) and clarification of the bank coordinates for the money transfer
- Resumption of the standard clinical care (TRT) at EDM or with the referring physician depending on the biochemical results of the follow-up study and the patient's preferences and needs.

10. SAFETY

This study will require several venepunctures (6-8 depending on the design) to perform blood tests by insertion of a small needle into a forearm vein. Each blood test may involve a risk of pain, hematoma at the puncture site or superficial phlebitis. All medications used in the present study are already commercialized and their safety has been evaluated. Furthermore, clinical studies have been done in hypogonadal men even with the medication for which an official SwissMedic indication is accepted only for women (i.e. anastrozole). To avoid recruiting subjects who may be more prone to adverse events of these medications, the exclusion criteria will be scrupulously followed.

10.1 Drug studies

During the entire duration of the study, all adverse events (AE) and all serious adverse events (SAEs) are collected, fully investigated and documented in source documents and case report forms (CRF). Study duration encompassed the time from when the participant signs the informed consent until the last design-specific procedure has been completed, including a safety follow-up period.

10.1.1 Definition and assessment of (serious) adverse events and other safety related events

An **Adverse Event (AE)** is any untoward medical occurrence in a patient or a clinical investigation participant administered a pharmaceutical product and which does not necessarily have a causal relationship with the study procedure. An AE can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal (investigational) product, whether or not related to the medicinal (investigational) product.

A **Serious Adverse Event (SAE)** is classified as any untoward medical occurrence that:

- results in death,
- is life-threatening,
- requires in-patient hospitalization or prolongation of existing hospitalisation,
- results in persistent or significant disability/incapacity, or
- is a congenital anomaly/birth defect.

In addition, important medical events that may not be immediately life-threatening or result in death, or require hospitalisation, but may jeopardise the patient or may require intervention to prevent one of the other outcomes listed above should also usually be considered serious. Examples of such events are intensive treatment in an emergency room or at home for allergic bronchospasm, blood dyscrasias or convulsions that do not result in hospitalisation, or development of drug dependency or drug abuse.

All SAEs, occurring during the study, will be registered and followed until resolution and stabilization of the participant. Participants with ongoing SAEs at study termination will be further followed up until

recovery or until stabilization of the disease after termination.

Assessment of Causality

The Sponsor-investigator will make a causality assessment of the event to the study drug, based on the criteria listed in the ICH E2A guidelines:

Relationship	Description
Definitely	Temporal relationship Improvement after dechallenge* Recurrence after rechallenge (or other proof of drug cause)
Probably	Temporal relationship Improvement after dechallenge No other cause evident
Possibly	Temporal relationship Other cause possible
Unlikely	Any assessable reaction that does not fulfil the above conditions
Not related	Causal relationship can be ruled out
*Improvement after dechallenge only taken into consideration, if applicable to reaction	

Unexpected Adverse Drug Reaction

An “unexpected” adverse drug reaction is an adverse reaction, the nature or severity of which is not consistent with the applicable products information:

https://compendium.ch/product/1108837-tostran-gel-20-mg-g

https://compendium.ch/product/1116422-anastrozol-teva-cpr-pell-1-mg

https://compendium.ch/product/1425521-ozempic-fixdose-4-mg-3ml-1-mg-dose

https://compendium.ch/product/1211587-choriomon-2000-ui-c-solv-seringue

Suspected Unexpected Serious Adverse Reactions (SUSARs)

The Sponsor-Investigator evaluates any SAE that has been reported regarding seriousness, causality and expectedness. If the event is related to the investigational product and is both serious and unexpected, it is classified as a SUSAR.

Assessment of Severity

The Principal Investigator evaluates any AE using the following severity grading scale, as described in the *Common Terminology Criteria for Adverse Events Version 5.0.*

Intensity	Description
Grade 1	Mild: asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.
Grade 2	Moderate: minimal, local or non-invasive intervention indicated, limiting age appropriate instrumental activities of daily living.

Grade 3	Severe or medically significant but not immediately life threatening: hospitalization or prolongation of hospitalization indicated; disabling; limiting self-care activity of daily living.
Grade 4	Life-threatening consequences: urgent intervention indicated.
Grade 5	Death related to AE.

10.1.2 Reporting of serious adverse events (SAE) and other safety related events

Reporting of SAEs

All SAEs must be reported immediately and within a maximum of 24 hours to the Sponsor-Investigator of the study. The Sponsor-Investigator will re-evaluate the SAE and return the form to the site. SAEs resulting in death are reported to the Ethics Committee via BASEC within 7 days. Non-life threatening SAEs will be reported to CER-VD and Swissmedic in the safety annual report. Reporting of SAEs or other safety relevant events to the Marketing Authorization Holder (MAH) of the drugs will be described in detail in the contract with each individual MAH.

Reporting of SUSARs

The Sponsor-Investigator will report SUSARs to CER-VD (via BASEC) and to Swissmedic within 7 days, if the event is fatal, or within 15 days (all other events).

Reporting of immediate safety and protective measures

All suspected new risks and relevant new aspects of known adverse reactions that require immediate safety-related measures, will be reported to the Sponsor-Investigator within 24 hours. The Sponsor-Investigator will report these measures within 7 days to CER-VD (via BASEC) and to Swissmedic.

Reporting and Handling of Pregnancies

Not applicable because only men will be recruited for this study (occurrence of KFS only in men).

Periodic reporting of safety

An annual safety report (ASR) will be submitted once a year to the CER-VD and Swissmedic by the Sponsor-Investigator. The ASR will be written in compliance to ICH Harmonised Guideline E2F for non-commercial sponsor. The start date for the ASR will be the date of the first authorisation by CER-VD and Swissmedic to initiate the clinical trial in Switzerland.

10.1.3 Follow up of (Serious) Adverse Events

All participants presenting AEs or SAEs will be followed by the PI or a co-investigator until complete recovery and stabilization of the participant. The PI will refer the patient to the appropriate specialist in

case of persistent adverse event requiring specialized care (for example, referral to an urologist in case of a persistent PSA elevation). In case of loss to follow-up, the PI will attempt to contact the participant via different means to restore monitoring. He will also inform the family doctor if the patient is not reachable despite the aforementioned efforts.

10.2 Assessment, notification and reporting on the use of radiation sources

This clinical trial will not involve therapeutic products capable of emitting ionising radiation. The only irradiation-emitting investigation of the study is the Dual X-ray absorptiometry. The irradiation level of this investigation is extremely low (approximately 0.001 mSv) and is completely in accordance with Article 45 of the Radiological Protection Ordinance of 26 April 2017.

11. STATISTICAL METHODS

Baseline variables between groups will be compared using Fischer exact test for categorical variables (e.g. presence or absence of diabetes) and Student's t-test for continuous variables (or non-parametric tests such as Mann-Whitney in case of markedly skewed distribution). The associations between metabolic outcomes (BMI, body fat, VAT, HOMA-IR) and sex steroids levels (T, SHBG, LH) will be explored using linear regression. Assessment of changes at month 6 versus baseline will be conducted with paired sample t-test. A p-value of less than 0.05 will be considered as statistically significant.

In Design 1, the primary outcome (presence of sperm in mTESE) will be compared between groups using a Fisher's exact test. In the interventional groups, the changes of intratesticular steroids between baseline and month 6 will be assessed with a paired sample Student’s t test. For gene expression studies, to address the issue of multiple testing, a Bonferroni correction will be applied when determining the level of statistical significance.

In Design 2, to compare the change score (month 12 versus baseline) for the primary outcome a regression analysis will be performed adjusted for the intervention (study group). Similar analysis will be performed for all the secondary outcomes. To examine the dynamic changes of this outcome, a secondary analysis including all timepoints (baseline, months 1, 3 and 6) will be done using a linear random effects model, to evaluate the interaction with other variables (for instance for the secondary outcome of change in endogenous T levels, interaction with metabolic parameters such as BMI and insulin resistance).

11.1 Hypothesis

For Design 1, the hypothesis is that pre-treatment with anastrozole (group A) or anastrozole and HCG (group B) will increase the rate of sperm retrieval in patients with a negative first mTESE biopsy and who currently have almost null chance of a sperm retrieval in contralateral biopsy (10% according to the

mean levels in the literature).

For Design 2, the hypothesis is that treatment with anastrozole (group B) will result in superior improvement in insulin resistance than standard care treatment with testosterone gel (group A).

11.2 Determination of Sample Size

Design 1:

The sample size for this design was estimated based on a one-sample comparison of proportion to the hypothesized value of expected SSR if a second mTESE biopsy is performed without any particular hormonal pre-treatment. This value was calculated on the basis of two publications: a systematic review and metanalysis that detected a 7% higher SSR in bilateral vs unilateral biopsy approach [28] and a small retrospective study with sequential biopsies detecting SSR in 3 out of 18 patients (success rate 16%). We estimated the expected added value of anastrozole (present in both groups of design 2) based on the two largest reports on mTESE outcomes with and without this treatment. Sabbaghian et al [80] (mean age 32.6 years) published the largest report on mTESE without hormonal pre-treatment reporting a SSR of 28.4% (mTESE+ in 38/134). This is the anticipated success rate of the first mTESE biopsy. Schiff et al [37] (mean age 32.8 years) pre-treated the majority of their participants showing a SSR of 79.2% in the AI only group (mTESE+ in 19/24). We thus expect that if the hypothesis is true, at least one of the two groups will have a SSR of approximately 51% (79-28) at the second mTESE. With a type I error probability (two-tailed α) of 0.05, a statistical power of 0.80, an expected SSR proportion in the general population of 10% (p=0.10) and a relatively moderate assumption for an expected SSR of 35% in at least one of the study groups (alternative p=0.35), inclusion of 16 subjects per group would be sufficient to detect a difference versus the expected SSR without any pre-treatment. Taking into account a potential 10-20% drop-out rate, 20 patients per group will be recruited

Design 2:

For group B, the sample size calculation was based on the assumption that correcting abnormal T/E ratio with anastrozole in KFS will correct the metabolic ‘resistance’ to TRT and will allow similar HOMA-IR reduction as TRT in men with congenital hypogonadotropic hypogonadism (CHH) who have a normal T/E ratio. The only longitudinal study assessing the effect of TRT on HOMA-IR in KFS men found a very mild decrease of 18% [87]. This is in contrast with two studies evaluating the effect of TRT on HOMA-IR in CHH men that reported relative decreases of 89% [88] and 54% [89], thus a mean reduction of 72%. In our retrospective EDM cohort of 44 men with KFS, seventeen met the criteria for inclusion in Design 1 (HOMA-IR > 2.6). Most of these patients were already on TRT and they had a mean HOMA-IR of 6.1 ± 3.1 which is assumed as the expected result in group A (TRT). Anticipating a relative difference for efficacy of 72-18=54% in favor of group B, we expect a mean HOMA-IR of 2.8 after intervention with anastrozole at this group. Assuming a similar standard deviation as group A, a type I error probability (two-tailed α) of 0.05, a statistical power of 0.80 and a 1:1 enrolment ratio for group B vs group A, 14

subjects per group would be sufficient to detect a higher efficacy in group B. Assuming a 20% of drop-out rate, we decided to recruit 20 men in each arm.

For group C, the sample size calculation was based on two objectives:

(i) The primary outcome (HOMA-IR reduction as for group B). Currently, there are no studies assessing the effect of semaglutide or other GLP1 receptor agonists in the metabolic health of men with KFS. Post-hoc analysis of the STEP RCT trials that evaluated semaglutide vs placebo (lifestyle intervention) for weight loss in obese populations reported a higher HOMA-IR reduction in semaglutide with at least 30% relative benefit compared to lifestyle alone [90]. The absolute HOMA-IR reduction for each group was not reported in the main manuscript nor the supplementary material of this article. Our KFS cohort is expected to be more severely affected than the STEP population which were only mildly insulin resistant (mean baseline HOMA-IR ≈ 3.2 versus 6.1 in our historic EDM KFS cohort). Taken together, these data support that we could expect at least a 45% relative more efficacy in group C compared to standard care (group A). Assuming a type I error probability (two-tailed α) of 0.05, a statistical power of 0.80 and a 1:1 enrolment ratio, 20 subjects would be sufficient to detect a higher efficacy in group C.

(ii) The secondary outcome of rise in endogenous T levels. Sample size was calculated based on a case report of EDM service on a KFS obese man that exhibited a 100% serum T increase following 18% of weight loss. With an anticipated weight loss of 8% with semaglutide by Week 26, a proportional 44% increase of serum T would be expected. Our historic KFS cohort had a mean serum T of 8.6 ± 4.7 nmol/l. Thus the expected mean T levels at Week 26 would be 12.4 nmol/l and a mean difference of 3.8 nmol/l. Assuming a standard deviation of differences similar to the baseline, with a type I error probability (two-tailed α) of 0.05 and a statistical power of 0.80, inclusion of 15 patients would be sufficient to detect a similar difference.

Given these results, inclusion of twenty patients per group was considered adequate for Design 2.

11.3 Statistical criteria of termination of trial

For Design 1, the primary outcome is the SRR at the second mTESE biopsy. This will be immediately communicated to the participants due to the clinical significance of the result. Thus, results will be also available to the PI as the study progresses. In case of a very low SSR (< 5-10%) at second mTESE biopsy after inclusion and treatment of twenty participants with a negative first mTESE biopsy (half of the targeted population), we will consider terminating this part of the study.

11.4 Planned Analyses

11.4.1 Datasets to be analysed, analysis populations

This randomised trial will be analysed following the intention-to-treat approach. Eligible participants, will be assigned to Design 1 or Design 2 and will subsequently be randomly assigned to a specific group as

previously described (refer to paragraph 6.1 and 6.2.1). All data will be included in the analyses. In addition to the interventional analysis, baseline data from both designs will be grouped to perform a crossectional analysis on the predictive factors of metabolic and reproductive phenotype as well as a more exploratory multi-omic approach to dissect the phenotypic variability. Patients that are not eligible for any of the study's designs but are TRT-naïve at screening will be offered only the baseline visit and could also participate to a registry study with a crossectional analysis. A comparison with age- and BMI-matched controls may be performed at a later stage but this will be specified via an amendment of the design (once the recruitment of the KFS cohort is near completion).

11.4.2 Primary Analysis

All analysis will be performed by the PI and will be verified by the referent statistician of the study or another member of the UCB at Unisanté. For Design 1, the primary analysis (presence of sperm in mTESE at group A or group B versus the expected rate of 10% without any treatment) will be performed using a Fisher's exact test. Subgroup analysis will compare the SSR in men who were TRT-naïve or had received prior TRT before the first mTESE biopsy. For Design 2, mean differences between the three groups regarding the change score of HOMA-IR (between week 26 and baseline) will be assessed by a regression analysis, adjusted for the baseline values.

11.4.3 Secondary Analyses

All analysis will be performed at the end of the study with an intention-to-treat approach.

For the register study (baseline only), association between metabolic and reproductive variables will be assessed using linear regression. Pearson r correlation indexes will be calculated. Based on the first results, it is possible that some multivariate analysis are performed. These will be conducted by the referent statistician.

For Design 1, the comparison of sperm retrieval rate between the two interventional groups will be conducted by a Fischer's exact test. The changes of intratesticular steroids between baseline and month 6 will be assessed with a paired sample Student's t test. A similar methodology will be utilized for gene expression changes. To address the issue of multiple testing for this analysis, a Bonferroni correction will be applied when determining the level of statistical significance.

For Design 2, secondary analysis will include between-group comparison of change scores in several secondary outcomes, adjusted for the intervention (group). For group C, the secondary outcome of change in endogenous T levels under semaglutide therapy will be analysed by a paired sample Student's t-test. To examine the dynamic changes of this outcome, a secondary analysis including all timepoints (baseline, months 1, 3 and 6) will be done using a linear random effects model, to evaluate the interaction with other variables such as BMI and insulin resistance (HOMA-IR).

11.4.4 Interim analyses

No interim analysis has been planned.

11.4.5 Safety analysis

Safety analysis regarding bone health will performed in all groups using paired sample Student's t-test for bone mineral density before and after intervention (Week 26).

11.4.6 Deviation(s) from the original statistical plan

The final report will include justifications for any deviation from the original statistical plan.

11.5 Handling of missing data and drop-outs

A drop-out rate of 20% was taken into account in the sample size calculation. Thus, if this limit is not exceeded (< 3 drop-outs per group), the drop-outs will not be replaced. In case of a larger than expected drop-out rate, we will amend the research design and modify the statistical plan to allow for drop-outs substitution. Missing data will be handled using multiple imputation or likelihood-based approaches based on the specific variables with missing data and after consultation with the referent statistician. Three sensitivity analysis will be performed: (i) a per protocol analysis; (ii) an analysis including only subjects without missing data for respective outcomes; (iii) a compliance analysis excluding subjects with reported suboptimal adherence to drug treatment defined as less than 90% intake of the study drug.

12. QUALITY ASSURANCE AND CONTROL

12.1 Data handling and record keeping / archiving

The competent authorities, i.e. the CER-VD and Swissmedic, will have access to all the data, including non-coded data, in case of audit, inspection, or monitoring procedures. All study related documents will be kept in the electronic case report from through the secured web application REDcap or the protected sample database (SLIMS). Source documents and consent forms will also be stored in paper format in a secured area at the EDM (accessible only to the medical staff of the study).

12.1.1 Case Report Forms

All study data will be recorded with electronic Case Report Forms (e-CRF) using the secure web application REDcap. Once included in the study, the participant will be entered in the EDM research study database supported in SLIMS (Genhom – Lausanne, Switzerland). The SLIMS database will

generate a specific code for the patient. All samples for each visit of the study will be created in SLIMS database as well, that will generate specific labels to identify different blood tubes, frozen aliquots of serum/plasma and tissue specimens. For each enrolled study participant, a specific e-CRP will be kept to record specific data during all the visits. Only the SLIMS-generated code and the year of birth of the patient will appear on the paper CRFs. Each person who will contribute to the collection of data, notably the research medical assistant and/or the PI will insert his/her initials to electronically sign the e-CRFs. Only the investigators authorized by the PI will be allowed to enter data on the CRF. Data derived from laboratory measurements performed at the LLC will be received in electronic form (via Molis) and will be imported to the e-CRF to avoid transcription errors. If a transcription of data is needed, a double data entry will be performed with one investigator inserting the data and the second one reading them loudly while checking the inserted values. Only the investigators authorized by the PI will be allowed to access the SLIMS database, including the participants' list with the codes.

12.1.2 Specification of source documents

The source documents will include (i) all data collected during the screening visit, i.e. the signed informed consent, the inclusion and exclusion checklist, the medical history and medical treatment sheets as well as results of laboratory screening test for candidates for Design 2; (ii) the randomization envelop received by the CHUV pharmacy; (iii) several documents completed during the study such as the ADAM questionnaire and other sexual function questionnaire, as well as medical reports of the exams that will be performed as part of the clinical practice and whose results will be used for the study (testicular ultrasound, bone and body composition DXA, calorimetry). Part of the results of this tests will be included coded in the eCRF while the whole results will be stored as a source document. The original informed consent forms will be stored in a "source documents" folder at the EDM in a secured area. All documents regarding tests done in a strictly research setting (coded) will be scanned to be attached to the individual SLIMS account, only accessible to the co-investigators of this study.

12.1.3 Record keeping / archiving

All study data will be archived for a minimum of 10 years after study termination or premature termination of the clinical trial in a secured area at the EDM and/or on the SLIMS database.

12.2 Data management

12.2.1 Data Management System

We will use two platforms for data management: (i) REDcap to store eCRF and all results of different study tests. This will be put in place in collaboration with the *Unité de Conseil et de Coordination de la Recherche Clinique* (UCCR-CRC) of CHUV-UNIL; The data structure will follow the data description in

Section 9.3 by visit. (ii) SLIMS database to reliably label and trace all study's samples including biobanks and tissue samples. All samples will be stored in the EDM freezers at Hotel des Patients (-80°C) which are connected to SLIMS. SLIMS is a platform that has been used for multiple clinical research studies of EDM service. It allows adequate quality processes for data entry, verification, storage and traceability.

12.2.2 Data security, access and back-up

Only the PI and the co-investigators will have access to the study's REDcap files and SLIMS database and, in case of entry error, they will have the right to modify the registered data. Any modification on the databases will be automatically saved and traceable.

12.2.3 Analysis and archiving

The REDcap (and/or SLIMS for screening) database will be exported and analysed once data will be validated. Both software supports tracking of the exported data by precisely indicating date and time of the export. Coded data will be stored, protected by passwords, for at least 10 years on the CHUV servers and/or the SLIMS database.

12.2.4 Electronic and central data validation

Validity, consistency and completeness of the data will be verified at different timepoints during the study by the PI and one of the co-investigators. Data will be filled directly in the eCRF (REDcap) which will serve to extract the majority of results data base. All laboratory data assessed at LLC as well as the DXA results will be available in electronic form and will be directly imported to the eCRF to avoid transcription errors. The source document data will be entered in the eCRF and double-checked after entry (one person reading the data, the other checking the CRF). In addition, the distribution of the data will be regularly verified and all outlier values will be re-checked in the source documents. At the end of the study, once all queries will be solved, the database will be exported on a standard file (e.g. *.csv), protected by a password and used by only one person who will be performing the statistical analysis.

12.3 Monitoring

A monitoring strategy will be organised by a mandated Monitor XXXX in accordance with the Good Clinical Practice (GCP).

The objectives of the monitoring are to verify that:

a) The rights and the well-being of the participants are preserved

b) Reported test data are accurate, complete and verifiable from source documents

c) The conduct of the test is in accordance with the approved design, current GCP, and applicable regulatory requirements.

The monitor will conduct a site initiation visit, intermediate follow-up visits and a closing visit, in accordance with a predefined monitoring plan and written standard operating procedures (SOPs) that will be provided before inclusion of the first patient. The PI agrees to be available for these visits, to provide direct access to all test data, documents and reports, and to respond to monitor's questions during follow-up visits. During the visits, the monitor will carry out a quality control of the progress of the study. Data will be evaluated for compliance with the design and accuracy to the source documents (verification of source data). The monitor will discuss of any problems with the PI and the study staff and, if appropriate, will define with them the actions to be undertaken and will write a report.

12.4 Audits and Inspections

In the case of an audit or inspection, all source data and relevant documents will be accessible to the auditing team, and we will answer to their questions. All the involved parties will keep the participant data strictly confidential.

12.5 Confidentiality, Data Protection

Data protection will be carefully enforced during the conduct of the study and the data analysis. The design will be available for all the co-investigators of the study. Personal data, collected during the clinical trial, will be coded and accessible only by the research team for scientific analysis. However, direct access to source documents will be permitted to the competent authorities for purposes of monitoring (refer to paragraph 12.3), audits and inspections (refer to paragraph 12.4). The dataset and the statistical code will be accessible to the co-investigators only at the end of the study. In case of publication of the results of this study, individual data will never be published nor disseminated.

12.6 Storage of biological material and related health data

All biological material will be coded and stored in a freezer at -80°C at the EDM service, Hotel des Patients, CHUV for at least 10 years after the end of the study; afterwards they will be destroyed together with the code break. The medical results will be stored on computer files on a protected server at CHUV. The patient will be requested to consent to the possible reutilization of the study's data for similar research purposes.

12.7 Use of biological samples and health data in related research projects

In addition to this project, the stored biological samples and related health data could be used for further related research projects during at least 10 years after the end of the study. The specific aims and objectives of these potential studies will arise depending on the initial results from the current project and other research teams involved in the field of Klinefelter syndrome. Their specific research questions will be defined in detail later and could involve study designs such as observational analyses (e.g.

studies on the reproductive and metabolic status of KFS versus age- and BMI-matched controls). These related future projects will be first submitted either as an amendment of the current design or a separate new designs to the CER-VD for approval. The biological samples or health related data will be transferred as coded data and participants will be free to withdraw at any time like for the current study (section 2.7).

13. PUBLICATION AND DISSEMINATION POLICY

At the end of this clinical trial, the results of the study will be orally transmitted to the participants by the PI or one of the providers-collaborators if the participant reside very far from Lausanne. All the results of the study tests will also be transferred to the provider at the end of the study and/or the endocrinology physician that will continue the clinical follow-up of the patient. The family doctor/general practitioner will also be contacted according to the patient's wish. Multiple scientific publications in peer-reviewed journals may result from the analysis of the results of this study. The PI retains full responsibility and decision over the publication and dissemination process, as well as further scientific collaborations. All co-investigators that are implicated to the study realization including specialists for different tests, as well as providers that have referred a significant number of eligible patients (> 5-10) will be included as co-authors to one or several scientific articles. There is no intended use of professional writers, the PI and collaborators have sufficient experience with scientific writing in English. Based on the requirements of the targeted journal, it is possible that the PI deposes in a publicly accessible repository the scientific design, anonymized data sets and/or statistical plan.

14. FUNDING AND SUPPORT

14.1 Funding

This clinical study will be funded by an SNF Ambizione grant attributed to the PI (grant n° PZ00P3_202151 / 1) between May 2022 and April 2026. This will cover protected research time 80% for this PI, the salary of a research medical assistant at 50% as well as the majority of project cost including medications fees. The PI has taken contact with several pharmaceutical firms and will sign a contract with Teva pharm (Anastrozole) and Cederberg (Tostran) for price reduction offers without any intervention to the design or the study realization. Administrative support and infrastructure is provided by the EDM, CHUV. A part of the investigations scheduled in the study such as transcriptomic studies and metabolomics were not part of the budget accepted by the SNF grant and the PI is currently searching for additional sources of funding. In the meantime, the necessary samples for these analysis will be stored.

15. INSURANCE

Regarding the possible risks and damages caused to the participants, the CHUV will act as the Sponsor and provide insurance according to the official legal dispositions (liability fund of the canton of Vaud).

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17. APPENDICES

Documents that do frequently change during the course of the study are provided separately and listed here.

1. IMP: IB or SPC
2. Monitoring Plan
3. Patients' Recruitment Plan
4. Participant information and informed consent
5. Case Report Form (e.g. CRF)
6. Design of biobank for endocrine diseases – EDM service

e.g. Other documents given to the patients