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"Prevalence of Klinefelter Syndrome, clinical and biochemical evaluation: a multicentric, prospective, observational study".

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"*It always seems impossible until it's done*"

- Nelson Mandela –

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1. Introduction

1.1 Overview of Klinefelter Syndrome

Klinefelter syndrome (KS) is the most frequent aneuploidy of sex chromosomes in humans, characterized by the presence of a supernumerary X chromosome, leading to a 47,XXY karyotype or, in a minority of cases, to a 46,XY/47,XXY mosaicism [1, 2]. The syndrome was first described by Klinefelter et al. in 1942, as an endocrine disorder characterized by hypogonadism with high follicle stimulating hormone (FSH) plasma levels, small firm testes, azoospermia and gynecomastia [2-4].

Since then, the knowledge of KS has further improved, highlighting the complexity of this condition. In fact, the clinical burden of KS is not limited to the impairment of spermatogenesis and steroidogenesis, which is a quite constant clinical finding, but may lead to an increased prevalence of cardiovascular and metabolic diseases (e.g., impaired glucose and lipids metabolism), endocrinological comorbidities (e.g., osteoporosis and impaired thyroid function), oncological problems as well as neuropsychiatric alterations and development, behavioral and psychological issues [1]. Current literature data also report a shortened life expectancy (up to 2 years) of KS people when compared to general population [1].

Because of the wide heterogeneity in clinical phenotype, even in the presence of the same karyotype, the KS still continues to pose substantial diagnostic challenges, resulting in a significant number of patients misdiagnosed or undiagnosed [2, 5, 6]. Accordingly, it is common for the majority of Klinefelter subjects to remain undiagnosed or to have a late diagnosis during medical care for hypogonadism, sexual dysfunction or infertility workup [7, 8], well passed the optimal time for the appropriate management of many clinical manifestations and comorbidity related to KS.

Due to the variety of symptoms KS males tend to be considered as affected by a rare or chronic but, in any case, disabling condition. Although this aspect may help patients to receive adequate health assistance, on the other hand it could be considered as a handicap and lead to a social discrimination. Therefore, each patient should be individually investigated to get a correct and tailored judgement of possible problems. In view of these consideration a broadening of knowledge on KS and its manifestations is advisable, prompting to a multidisciplinary approach aimed at guaranteeing both physical and psychological wellbeing through the early diagnosis and adequate treatment and follow-up, from childhood to senescence.

1.2 Epidemiology of an underdiagnosed syndrome

The diagnosis of a KS male is based on the clinical appearance coupled with a karyotype finding of 47,XXY or mosaics thereof (usually with a 46,XY/47,XXY karyotype). People with additional sex chromosomes (48,XXXY, 48,XXYY and other polysomies) should not be considered KS males because they usually have a much more affected phenotype [9]. To date, there is no universal agreement on the main clinical

signs or stigmata that should lead to karyotyping [2, 5, 10, 11]. The clinical heterogeneity of the syndrome, especially in the absence of overt clinical signs, makes it difficult to distinguish males with KS from 46,XY males and represents one of the main determinants for the underdiagnosis of the disease [4, 12]. These aspects are even more significant in subjects with mosaicism (up to 20% of KS cases) since they may present larger testicular volume, more frequently sperm in the ejaculate, and higher androgen levels than their non-mosaic counterpart [13]. Moreover, chromosomal mosaicism can be present only in the testes, leading to a normal result on karyotype analysis of peripheral leukocytes.

Several studies indicate that only a minority of KS males are ever diagnosed (25-40% of cases) and only about 10% of them are diagnosed during childhood and adolescence [2, 3, 8, 14]. However, the current possibility of diagnosing aneuploidies even prenatally or in early pregnancy by amniocentesis, chorionic villus sampling, or cell-free DNA testing, resulted in an increased number of prenatal KS diagnosis [6, 15].

The possibility of early diagnoses brings with it ethical issues, including those related to abortion. Up to 85% of mothers carrying a 47,XXY fetus decide for legal abortion, potentially limiting the estimation of the real miscarriage rate of 47,XXY implanted embryos and reducing post-natal prevalence of KS, as confirmed by data from the Danish national register [5, 8, 16, 17]. Although the abortion rate seems to be only marginally affected by the low level of detection of prenatal KS, an optimization in diagnostic techniques (especially cell-free DNA testing) could allow an early detection of the syndrome and appropriate parental counselling in a wider number of cases.

To date, a population-based neonatal genetic screening may represent the most accurate method to clarify several questions concerning prevalence and phenotypic heterogeneity of KS even if the real impact of early diagnoses in reducing long-term morbidity and mortality is still being debated [6, 18-20].

Indeed, only 10% of KS males are detected prenatally, 3% are identified before the age of 20 due to developmental delays or behavioral problems, and only 2% are diagnosed due to delayed puberty or gynecomastia. The corresponding proportion of cases diagnosed in adulthood due to hypogonadism or infertility is reported to be 17% [14]. As previously mentioned, this leaves the majority of KS undiagnosed.

At the current state of knowledge, no precise data are available regarding the real prevalence of KS. The prenatal prevalence of KS is estimated to varies between 0.2%, when evaluated through amniocentesis and chorionic villi sampling [8], and 0.9%, when considering human blastocysts analyzed with preimplantation genetic testing for aneuploidies during IVF cycles [21]. Current data are scanty and do not allow the causes of this difference to be defined with certainty, despite some studies hypothesized lower implantation and higher miscarriage rate in 47 XXY blastocysts [2]. A higher prevalence of KS in human blastocysts may also be attributable to advanced maternal age (especially $\geq$ 35 years), as frequently observed in infertile couples undergoing IVF [21, 22]. Based on these considerations, some authors hypothesized that the prevalence of KS may be increasing. In fact, the progressive rise in maternal and paternal age at the time of conception is associated with a higher risk of meiotic non-disjunctions during gamete formation and thus 47,XXY karyotype [23].

Postnatal prevalence of KS is estimated to range from 85 to 223 per 100.000 males [16, 24-32]. In particular, in Denmark, where data from solid registries of newborns are available, authors estimated a prenatal prevalence of KS ranging from 153 to 173 per 100.000 males [8]. Interestingly, on a deeper analysis, it was found that only 25% of KS males were diagnosed in adulthood, leading to an estimated prevalence ranging from 12 to 40 per 100.000 males, far fewer than expected [5, 8, 16, 33].

Finally, it should be noticed that fewer prevalence data on KS mosaics are available. Although their scarcity, it is thought that mosaicism may occur in about 10-20% of all KS diagnoses, both at prenatal and postnatal evaluation [5, 13]. Although historically considered a rare condition, current data on prenatal or post-natal prevalence show that KS is not so uncommon as once thought. Actual discrepancies between prenatal and postnatal prevalence confirm that most patients are not correctly diagnosed, with potential detrimental long-term consequences in terms of morbidity and mortality due to lack of optimal management [6, 34, 35].

1.3 Genetic basis of Klinefelter Syndrome

KS males typically present a 47,XXY karyotype, accounting for the approximately 80-90% of the cases [2]. The presence of mosaicisms (mainly 46,XY/47,XXY) is observed in 10-20% of patients, while higher-grade aneuploidies (48,XXXY, 49,XXXXY or 48,XXYY) and structurally abnormal X chromosomes (e.g., 47,iXq,Y) are rarely observed in small minority of cases [2].

Most of the chromosomal disorders leading to aneuploidy in the offspring are due to impaired meiosis of the parental gametes [36]. The supernumerary X chromosome in KS comes from the father in more than half of cases, while in the remaining 40% of cases has maternal origin [2, 36]. In both cases, the XXY arrangement results from error occurring predominantly during the first meiotic division. During this phase, chromosomes pair with their corresponding chromosomes (X/Y chromosomes pair in men and X/X chromosomes pair in women) and exchange parts of the genetic material, favoring genetic variation in the gametes. Some author hypothesized mitotic errors during gametocyte replications (prior to meiosis), when the potential for error exists at each cell division [36, 37]. Alterations in the gametogenesis, regardless of the stage at which they occurs (either early embryogenesis, mitosis or meiosis), determines the development of gametes carrying a supernumerary X chromosome (i.e., 22,XX eggs or 22,XY sperm). Conversely, the presence of mosaicism (usually with 46,XY/47,XXY karyotype, accounting for 10-20% of KS cases) is thought to be due to post-meiotic event (i.e., errors in the very earliest days after conception such as non-disjunction in mitotic division of the developing 46,XY zygote, or loss of one of the X chromosome of a 47,XXY conception due to anaphase lagging) [22, 38].

The advanced maternal, and possibly paternal, age is a well-known risk factor for KS. Indeed, it has been reported that an advanced maternal age (> 40 years) correlates with higher the prevalence of KS, up to four times higher than in younger women. Moreover, advanced maternal age may also negatively impact on the

mitotic non-disjunction in the zygote, increasing the chance of a post-zygotic X chromosome non-disjunction as well [36].

Despite recent insights concerning genetic and biology of KS, our knowledge of the underlying pathophysiological mechanisms of this syndrome is still limited. Indeed, several mechanisms seems to be play a pivotal role in modulating the phenotype variability observed in KS, including the derivation of supernumerary X chromosome (maternal or paternal), the expression/inactivation status of the X chromosome genes, the dosage effect, the presence of mosaicism, the expression/activity of the genes located in the pseudoautosomal regions (PAR) of the sex chromosomes and the number of X-linked copy number variation, as well as epigenetic mechanisms [36].

Although some authors hypothesized that a parental origin of X chromosome could impact on clinical phenotype of KS (e.g., later onset and slower puberty development in case of paternal origin, impaired motor function and language/speech development) [22, 39, 40], the majority of current studies found no association at all between the parental origin of the supernumerary X chromosome and the clinical phenotype [5].

The random *inactivation of one X chromosome* (known as the "*dosage compensation*" phenomenon) occurring in female somatic cell, aimed at guaranteeing a gene dosage for X-linked genes comparable to male cells, also plays an important role in determining the phenotype of KS [36]. Moreover, a skewed X-inactivation (i.e., the preferential inactivation of one of the two X chromosomes occurring in females) has been observed also in a significant proportion of KS males (~ 40%), and this mechanism might further contribute in determining the wide clinical features of this syndrome [5, 22]. The responsible for silencing the extra X chromosome in somatic human cells is the *Xist gene* (X-inactive-specific transcript), located on the long arm of the inactivated X chromosome, whose expression suggest the presence of a supernumerary X chromosome in the somatic cell. The expression of *Xist* has been observed in blood cells of KS patients, and the Barr body (expression of X-silenced chromosome) was identified in both Sertoli and Leydig cells of KS patients, suggesting that one of the two X chromosomes is inactivated, as in 46,XX females. In woman, about 15% of X-linked genes is known to escape the X-inactivation process; however female metabolism is able to bare such doubled gene [36]. Likewise, also in KS somatic cells a group of X-linked genes escape the silencing process mediated by *Xist*, leading to a doubled gene dosage for these genes. Notwithstanding, male metabolism may be unable to adequately tolerate such a "female gene dosage" for these X-linked genes, and the X-inactivation escape of these genes may play a central role in determining KS phenotype [36]. An example of genes located on X chromosome and escaping X-inactivation process are *TXLNG* and *EIF2S3*. These genes are embroiled in regulation of the bone mass density and lipid metabolism, obesity, hypogonadism and mental retardation and are likely to be involved in several of the comorbidities of KS [41, 42]. Such mechanism may in part explain the milder phenotype (larger testicular volume, lower levels of luteinizing hormone and estradiol, and higher mean total sperm count) and the lower prevalence of comorbidities of KS men with 46,XY/47,XXY karyotype when compared with 47,XXY patients [13]. The inactivation of X chromosome mediated by *Xist* gene also includes male germ cells; however, a significant number of genes escape X-silencing process and are still expressed in these cells. Among them are some "*testis-specific genes*" (about 99 genes - 10% of all X-

linked genes), whose expression is essential for the survival of the germ cell within the mature testis. Therefore, in germ cells of KS patients, the doubled gene dosage of these genes may significantly alter both steroidogenesis and spermatogenesis, thus favoring the genesis of infertility [36].

The role of the *androgen receptor* (AR, mapped on X chromosome), in determining clinical phenotype in KS is debated. The N-terminal domain of the exon 1 of the AR gene is highly polymorphic and contains a sequence of CAG repeats, whose length negatively correlates with the function of the AR itself [2, 36]. Similar to other genes encoded on X chromosome, the AR gene undergo a random inactivation process as well. However, different studies suggest that the length of its AR allele (depending on the number of CAG repeats) may influence which of the two X chromosome to inactivate, but data are still controverse [5, 22, 43, 44]. Even when considering the impact of CAG repeats on clinical phenotype of KS, data are conflicting. Some author proved that longer AR allele (with lower receptor activity) was associated to a more severe clinical phenotype, with positive correlation with the final height and arm span and negative one with the levels of cholesterol and hematocrit [2, 5, 41, 45]. On the other hand, for other clinical elements (testicular volume, gynecomastia, and bone-related parameters), evidence is more inconsistent: in fact, some studies found a negative correlation between these measurements and CAG repeat length, whereas others found no correlation at all [5, 40].

Recent studies have focused their attention of the possible role of the *pseudoautosomal regions* PAR1 and PAR2; these are short, homologous regions between chromosomes X and Y, undergoing crossing over during meiosis (just like two real autosomes) and containing genes regulating the pairing of sex chromosomes during meiosis, which is an essential step for spermatogenesis [36]. All genes belonging to PAR1/2 normally escape X-silencing in both women and KS subjects, thus possibly influencing the final clinical phenotype. Among genes encoded in the PAR1/2 region, the *SHOX gene* (short-stature-homeobox-containing gene) is the only one that was proved to influence the KS phenotype. Indeed, the overexpression of *SHOX* is though be one of the determinants of the tall stature and long extremities in KS males, which is evident long before pubertal development and despite normal levels of IGF1 and IGF-BP3 [5, 36].

Another possible mechanism underlying the clinical KS phenotype was recently identified in the *X-linked CNVs* (copy number variations). It was recently demonstrated that KS is associated with a higher recurrence of CNVs on the X chromosome, including genes located in PAR1 region that usually escape the X-inactivation process [22, 46].

Lastly, some author proposed that epigenetic modifications of DNA (e.g., histone variants, acetylation, methylation and covalent modifications of DNA bases) might impact on the variability of the KS phenotype by modulating immune adaptive response, glucose, lipid and bone metabolism, endothelial and cardiac function, synaptic activity and plasticity [41, 47, 48].

1.4 General features of Klinefelter syndrome in adulthood

In addition to the traditional presentation described by Klinefelter et al. in 1942, probably reflecting a more severe degree of symptoms in a small cohort of patients seeking for medical consultation, KS may present a wide heterogeneity in clinical phenotype [3, 22]. Indeed, alternative and less severe phenotypes with mild, non-specific features have been described and because of this poor symptomatic manifestations KS often remained undiagnosed [22, 49]. Severity of signs and symptoms of KS result of from the combination of several factors including age, androgen deficiency, androgen receptor sensitivity and severity of expression of genetic defect [22, 43].

The "classical" adult man with Klinefelter's syndrome traditionally present an association of peculiar clinical findings as small and firm testes (resulting in androgen deficiency and azoospermia), gynecomastia, tall stature, narrow shoulders, and broad hips [3]. Among them, the significant reduction in testicular size (typically < 5 ml for each testicle) is the most characteristic clinical findings and is easily assessable by palpation (by means of a Prader orchidometer) or with ultrasonography [4, 22, 50]. The lack of testicular enlargement starts to be evident at puberty, while sexual secondary characteristics and penile growth usually progress towards the adult phenotype [22].

The degree of virilization is highly heterogeneous among KS patients, being more evident in those suffering from overt hypogonadism (reduced body and facial hair), further worsening with ageing [3, 4]. Up to 70% of KS subjects aged ≥ 25 years complain of symptoms of overt hypogonadism (including asthenia, decreased libido and erectile dysfunction), but this percentage varies according to different studies [51].

Primary (hypergonadotropic) hypogonadism is one of the main characteristics of KS. At the beginning, the occurrence of reduced serum testosterone was considered as constantly associated with this syndrome [3]. However, testosterone concentration within normal range, although significantly lower when compared to age-matched healthy men, is reported in a minority of cases both at puberty and in adulthood [52, 53]. To date, the real prevalence of hypogonadism in KS patients widely varies among studies and has not been defined so far. Hyperestrogenism was another feature traditionally connected to KS, mainly because of the presence of gynecomastia and some other female features (e.g., female pubic escutcheon, sparse facial and body hair) [3, 22, 50, 52, 53]. Despite healthy men and KS patients have similar serum estrogens concentrations, the latter present higher estrogen-to-testosterone ratio and an increased aromatase activity [54].

Most patients suffer from azoospermia, due to impaired testicular spermatogenesis, making *infertility* to be the main symptom that induces KS patient to seek for medical consultation. KS testis displays various histological patterns, ranging from the classical and most severe (germ cell aplasia, tubular atrophy or hyalinizing fibrosis and relative hyperplasia of Leydig cells) to a less severe pattern in which residual foci of spermatogenesis can be detected [55].

Against this background, current guidelines recommend karyotype analysis for detecting KS in all males with primary hypogonadism combined with small testicular volume (< 5 ml per testis) and/or non-

obstructive azoospermia or severe azoospermia (i.e., sperm concentration < 10 million/ejaculate or < 5 million/ml) [2].

Bilateral *gynecomastia* is another common finding and can be present in nearly half of KS patients [3, 56]. The relative androgen deficiency and the imbalance in estrogen-to-testosterone ratio are known to favor the development of breast tissue; however, the role of hyper-estrogen status in inducing gynecomastia is still controversial [53, 56 - 58]. Testosterone replacement therapy can induce a partial regression of gynecomastia; otherwise, a surgical resolution by means of mastectomy and cosmetic breast surgery should be suggested, especially if gynecomastia troubles the patient [56, 59].

Considering *anthropometric parameters*, Klinefelter patients are usually taller than unaffected men (+ 5-7 cm), with eunuchoid proportions [5]. Leg length often exceeds that of the other segments of the skeleton, while the arm span seldom exceeds the body height, differently from other forms of hypogonadism [4, 22]. The high stature, still present before pubertal onset, is likely to be caused by the supernumerary copy of the SHOX gene [4, 22]. Waist circumference, body mass index (BMI) and waist-to-hip ratio are also significantly greater in KS males than in controls; however, the physical trait may range from slim to obese phenotype depending on individual differences, ethnicity and age [12, 60, 61].

The severity of clinical phenotype may also significantly impact on quality of life (QoL) of patients with KS. The awareness of being affected by a genetic condition, as well as body appearance (reduced androgenization, gynecomastia, eunuchoid habitus) and composition (overweight/obesity), disabilities and behavioral issues, may negatively impact on patient's body image and social interaction, resulting in psychological, self-confidence, self-esteem and social complications, finally reducing the QoL [62]. Furthermore, the presence of infertility may result in a significant linked to the poor probability of fatherhood and related problems in the relationship with the partner.

1.4.1 Hypogonadism

KS is considered one of the most frequent causes of male organic hypogonadism [22]. During childhood, pituitary–gonadal axis function is relatively preserved in KS patients. In fact, in the early puberty an increase in FSH, LH, inhibin B, INSL-3, and testosterone levels occurs, leading to initial increase in testicular size (up to 6-8 ml) and development of secondary sexual characteristic, with a minority of patients showing delayed puberty [2, 63, 64].

However, from mid-stage of puberty the situation is reversed: testosterone, INSL-3 and inhibin B progressively decrease, as well as testicular volume, suggesting an impaired testicular function, and the resulting increase in LH and FSH concentration outlines a condition of overt hypergonadotropic hypogonadism [2]. Simultaneously, an increase in estrogens and sex hormone-binding globulin (SHBG) concentration occurs,

while AMH declines to pathologically low levels, probably reflecting the hyalinization of seminiferous tubules [65].

Although Leydig cells may retain to a lesser extent the responsivity to exogenous GnRH, adult KS males usually display a variable degree of testosterone deficiency, ranging from clinically overt hypogonadism to normal androgenic status [66]. Also in KS males, similar to what happens in healthy men, testosterone levels decline with age but this reduction occurs at a younger age than in the general population [63] Accordingly, serum testosterone is often in the low-mid normal range in early adulthood and then progressively reaches values below the normal range in 65–85% of patients with KS after the age of 25 [56, 51]. Thus, periodical evaluation of serum testosterone is advisable, in order to promptly diagnose and treat the hypogonadal state.

It has been suggested that hypogonadism should play a causal role in many comorbidities frequently observed in KS patients, such as obesity and metabolic alterations, reduced bone mineral density (BMD) and increased cardiovascular risk [67]. Against this background testosterone replacement therapy (TRT) may positively impact on symptoms of hypogonadism and improve metabolic and cardiovascular outcome; notwithstanding, the real contribution of TRT to several outcomes in KS has not been completely elucidated yet.

Several observational studies have evaluated the effect of TRT in patients with KS [12, 60, 68 - 79]. Conversely, only few RCTs are available [80]. In the vast majority of studies, TRT had a limited effect on several metabolic and body composition parameters [81]. Similar to those observed in patients with non-organic or functional hypogonadism, TRT may improve both lean and fat mass, without however modifying the body weight. Moreover, a neutral effect on lipid profile was observed, although a possible improvement in fasting glucose and Homeostatic Model Assessment (HOMA) index was detected [82, 83]. Accordingly, current guidelines do not support the use of TRT with the sole aim of improving body composition and metabolic profile [84].

Some case reports or case series have documented a possible improvement in psychopathological symptoms after TRT in patients with KS. However, data on the effects of TRT on mood, and depressive symptoms are controversial and no data from RCTs are currently available [85].

1.4.2 Infertility

Despite being characterized by a highly variable phenotype, infertility is almost a constant clinical finding in KS males, and currently represent the main reason for these patients to seek for medical consult. Most subjects suffer from non-obstructive azoospermia and for many decades the use of donor semen represented the only viable option to become father for KS men. However, motile sperms retrieval in ejaculate and spontaneous pregnancies have been reported, especially when 46,XY/47,XXY mosaicism is present, leading to less severe clinical manifestation of the syndrome and to some germ cell preservation [4, 86-88].

To date, the pathophysiological mechanism underlying testicular damage and infertility in these patients have not been completely understood and is still matter of debate whether the germ cell failure (a hallmark of KS) lies in their inherent primarily genetic defect or if their maturation is compromised by an impaired gonadal microenvironment. Inside testes of KS males, a progressive degeneration including tubular atrophy, hyalinization and sclerosis occurs, leading to maturation arrest and testicular failure [2].

While pre-pubertal testicular development in KS is generally described as similar to the one of unaffected boys, some evidence suggests that germ cell hypoplasia can be present already during fetal development, between 18 and 22 weeks of gestation [89]. Histopathological pictures detected by testicular biopsies confirmed a reduced number of germ cell in pre-pubertal phase, along with maintenance of a normal morphology of seminiferous tubules, Sertoli and Leydig cells. After puberty onset, a progressive hyaline fibrosis of the seminiferous tubules occurs and continues to extend into adulthood [90, 91].

Although the majority of KS men are azoospermic, a less severe impairment of spermatogenesis has been reported and vital spermatozoa were retrieved in the ejaculate in up to 8% of patients. Moreover, residual single foci with preserved spermatogenesis in the testis of KS males have been described and a successful sperm recovery by conventional or microsurgical testicular sperm extraction (TESE and micro-TESE, respectively) could be achieved in up to 50% of patients [4, 92-94].

Hence, KS men should not be considered anymore infertile and appropriate management is advisable to maximize their fertility potential. Indeed, because of recent advances in TESE and assisted reproductive techniques (ART), a significant proportion of men with KS will have sperm detected with TESE/micro-TESE, of which a 50% pregnancy and live birth rate can be expected [94]. Reported sperm retrieval rates of about 40% resulted to be independent of several clinical and biochemical parameters, including age, testis volume, and hormonal status at baseline. In addition, the use of retrieved sperm allowed live children to be born in ~40% of ICSI cycles meaning a final live birth rate of 16% [94, 95].

Currently, there are no established guidelines for appropriate timing and/or harvesting technique choices, and only the sperm cryopreservation is considered acceptable for standard cares. Therefore, when patients are able and willing to provide an ejaculated specimen, the more invasive surgical interventions should be avoided. Cryopreservation of ejaculated samples or testicular tissue samples should be offered to all young, post-puberal KS men before starting testosterone replacement therapy [2, 91].

Although data on specific predictors of underlying success in surgical sperm retrieval are still conflicting, some authors emphasized that younger age (below 35 years) may ensures a better outcome [96 - 98]. The presence of germ cells has been reported in the testes of 50% of peripubertal KS boys [99], fostering the hypothesis that testicular spermatozoa might be retrieved in pubertal KS boys before the occurrence of progressive germ cell degeneration. However, success rates of TESE in patients younger than 15 years was similar to that observed in older subjects (15-19 years) [100]. Furthermore, some author postulated that the focal spermatogenesis could arise from XXY spermatogonia undergoing an erroneous self-renewal (with loss of one X chromosome self-correcting to XY spermatogonia); therefore, early sperm retrieval at adolescent age may even be contra-productive [101, 102].

According to some author, hormonal pattern should also be taken into account when considering potential predictors of surgical sperm retrieval [87]. In patients already receiving androgen replacement therapy, it has been suggested to discontinue this treatment for at least 6 months prior to TESE [103]. Furthermore, medical treatment (i.e., aromatase inhibitors, clomiphene citrate, or human chorionic gonadotropin) aimed at improving intratesticular testosterone levels has been proposed, but data supporting the routinary use of these regimens are scanty [98].

In contrast to this view, a recent meta-analysis pointed out that neither age nor testicular volume and hormonal pattern were predictors of sperm recover in KS males, suggesting that the focal spermatogenesis in KS testes is not involved in the progressive seminiferous tubule hyalinization, although the origin of a scattered preserved spermatogenesis in KS is still hypothetical [94].

Finally, although higher success rates of sperm retrieval using a microTESE approach when compared to classical TESE have been described in general population, no difference by comparing these techniques emerged in KS males, probably due to the low testes volume in these patients [94]. Moreover, when considering the whole testes volume, the amount of testicular tissue removed during TESE should impair testicular function and worsen hypogonadism.

Nevertheless, the risk of transmission of chromosomal aneuploidies to the offspring is a major concern for KS males and should be taken into account. Although conception from KS men is apparently safe and KS patients should be counseled that the majority of them will probably father a normally genetic child, preimplantation genetic diagnosis (PGD) should be offered to couples with KS who undergo successful TESE and ART [86, 104].

1.4.3 Gynecomastia

Gynecomastia is defined as the benign enlargement of the male breast caused by proliferation of glandular tissue; it is the most common breast alteration in males, occurring more frequently during infancy, puberty, and old age [58, 57]. Gynecomastia represents one of the hallmarks of clinical manifestation of KS, with higher prevalence in these patients (ranging from 40 to 75%) when compared to unaffected males [2]. Even when considering pubertal age, gynecomastia of various degrees is present in the majority of KS boys (up to 88%) and it is more likely to persist through adulthood [105].

Despite being a benign proliferation with no remarkable clinical consequences, the presence and the degree of gynecomastia may negatively impact on patient's self-perception and body image, possibly resulting in psychological, self-confidence, self-esteem and social complications.

Gynecomastia is usually bilateral, but may be asymmetric in size. It usually develops in clinical situations in which the levels or activity of estrogens is relatively high when compared to androgens (i.e., high estrogen-to-androgen ratio): this hormonal milieu may result either from high estrogen or low androgen

concentrations or activity [57, 58]. KS patients often display both androgen deficiency and increased estrogen concentration, the latter probably reflecting an increased peripheral conversion or an enhanced testicular secretion, owing to excessive FSH stimulation of gonadal aromatase activity [106].
Moreover, elevation in estrogens concentration might induce an increased synthesis of the sex hormone-binding globulin (SHBG), whose binding affinity for estrogens is lower than for androgens, further reducing the amount of circulating free-testosterone.

Although this hormonal pattern may account for the genesis and persistence of gynecomastia, the real etiology of this phenomenon still persists to be unknown. In fact, gynecomastia of KS patients is characterized by unique histologic changes, such the hyperplasia of the inter-ductal tissue, which is not a typical feature of hyperestrogenism [107]. This might partially explain the higher risk of developing breast cancer in these subjects, despite gynecomastia is not considered as a risk factor for male breast cancer. Hence, periodical physical examination including palpation of breast tissue, as well as breast US if required, should be performed in these patients [108]

Testosterone replacement therapy can partially induce the regression of gynecomastia; otherwise, a surgical resolution (mastectomy and cosmetic breast surgery) should be suggested, especially if gynecomastia troubles the patient [57, 58].

1.5 Metabolic alterations in Klinefelter syndrome

Several studies pointed out that KS males are at higher risk of hospitalization (> 70%) and occurrence of metabolic and cardiovascular diseases than age-matched non-KS males. These comorbidities negatively impact on quality of life and life expectancy, possibly explaining the higher mortality rate (up to 50%) and the lower expected lifespan (median -2.1 years) observed in many KS patients [2, 31, 67, 109, 110].

Overweight and obesity are common clinical finding among subjects suffering from KS, although the underlying pathophysiological mechanism have not been fully elucidated yet. In particular, KS patients often show a reduced lean body mass and increased truncal fat deposition, the latter representing a well-established risk factor for insulin resistance and metabolic syndrome, regardless of testosterone concentrations [5, 60, 67]. Hypogonadism and the supernumerary X chromosome are likely to play a causative effect by reducing insulin sensitivity and favoring deposition of abdominal fat, thus increasing the peripheral aromatization of testosterone and reducing the serum androgen concentrations [22, 50].
In particular, the genetic background could directly affect the risk of obesity, regardless of testosterone concentrations. This hypothesis is supported by the increased body fat and decreased lean body mass in young KS males when compared to healthy people (at an age when hypogonadism is relatively less pronounced). Moreover, KS males maintain an increased fat deposition compared with controls even after reaching adequate serum testosterone concentration on replacement therapy [5, 111].

In addition to obesity, subjects with KS also present an increased prevalence of several metabolic abnormalities, including type 2 diabetes (T2DM), dyslipidemia, and metabolic syndrome, when compared to 46,XY males [2, 5, 67, 109, 112-113]. The higher prevalence of metabolic disorders contributes to the higher mortality observed in KS [56, 74, 114, 115].

Similarly to other clinical manifestation of KS, the causal factors underlying the increased prevalence of metabolic disorders have not been completely identified.

Hypogonadism is well established risk factor for metabolic diseases, causing unfavorable change in body composition by increasing truncal adiposity and reducing lean mass, leading to an unfavorable muscle/fat ratio as frequently evidenced in KS subjects [111, 116]. In particular, the increase in visceral adiposity is associated with increased cardio-metabolic risk [117]. Moreover, androgens regulate several biological functions related to adipose tissue remodeling and energy storage (i.e., lipid uptake, lipogenesis/lipolysis, adipogenesis, mitochondrial function and lipoprotein lipase activity) [118, 119].

Notwithstanding, metabolic abnormalities observed in KS are only in part the direct consequence of hypogonadism *per se*. Some of the changes in body composition, visceral obesity and metabolic syndrome may be present early before puberty, suggesting a role of non-hormonal factors [2, 111]. In healthy men high SHBG levels have been reported to correlate with lower risk of T2DM [120, 121], while low estrogen concentration (ad reported in hypogonadal men) may promote the development of T2DM by triggering metabolic tissues, particularly the adipose tissue [122]. However, KS patients display higher levels of both SHBG and estrogens compared to their peers, further supporting the hypothesis that the higher incidence of metabolic disease does not derive exclusively from hypogonadism [4, 123].

Whether the association of KS with T2D and the metabolic syndrome could be explained, at least partially, by genetic abnormalities is unclear. The majority of deregulated genes in men with KS are not located on the X chromosome, suggesting that changes in the epigenome and transcriptome may be involved rather than alterations of single genes on the supernumerary X chromosome [48, 112]. Furthermore, also the CAG repeats of AR may play a causal rose, since the correlations between length of CAG repeats, HOMA-IR and cardiovascular risk factors (e.g., high LDL and low HDL levels) were reported [124-126].

The effects of TRT on the risk of developing T2DM and improvement of glycemic control in men with KS remains poorly understood due to the lack of randomized controlled trials. To date, TRT in hypogonadal KS male proved to be effective in ameliorating body composition, but its favorable impact on glycosylated hemoglobin and lipid levels is still debated [71, 77, 127, 128]. TRT increases lipid oxidation and a decreases glucose oxidation, promoting a shift in metabolic substrate to favor lipid utilization; moreover, TRT promote changes in body composition and physical fitness that may help in improving glucose and lipid metabolism [129]. However, the persistence of a worse metabolic profile in KS subjects after TRT does not support the use of TRT with the only aim of reverting insulin resistance or improving glucose metabolism [2, 49].

To date, insufficient data are available to identify the most appropriate drugs for the treatment of diabetes or its complications in KS patients, but drugs promoting weight/fat loss (considering the body composition and the visceral fat accumulation of KS subjects), should be preferred [112, 130].

1.6 Cardiovascular disease in Klinefelter syndrome

Cardiovascular diseases significantly contribute in determining the aforementioned increase in mortality observed in KS [31, 110]. Ischemic heart disease accounts for the higher risk of hospitalization in these patients, together with thrombophlebitis, venous thrombosis and pulmonary embolism [2, 109]. Along with acquired cardiovascular disease, congenital heart malformations have an important role in increasing the overall cardiovascular risk of KS patients [31, 109].

Congenital heart malformation includes transposition of the great arteries, patent arteriosus ductus, partial atrioventricular canal defect and tetralogy of Fallot [131]. Some author also reported the presence of left ventricular structural abnormalities an increased prevalence of mitral valve prolapse, resulting in an increased risk of mitral regurgitation, chordal rupture and sudden death [5, 131].

When considering acquired disorders, both structural and functional left ventricular abnormalities have been described in KS males. Echocardiographic studies pointed out systolic velocities and strain rate significantly lower in KS patients, and a precocious diastolic impairment was also reported. Alterations in cardiac kinesis were found to correlate with important metabolic parameters: diastolic dysfunction was found to negatively correlate to truncal body fat, while and systolic dysfunction was negatively associated with both truncal body fat and fasting triglyceride levels, underlining the strong relation between metabolic syndrome and left ventricular performance in these patients [132].

In view of these considerations, echocardiography is strongly recommended in KS patients (both children and adults) in order to detect cardiac congenital abnormalities and acquired disorders.

Besides left ventricular morpho-functional alterations, increased cardiovascular risk in KS is also associated to worse exercise performance and subclinical atherosclerosis. KS males have shown to have reduced maximal oxygen consumption, increased intima-media thickness, and chronotropic incompetence in response to exercise, thus suggesting multiple preclinical cardiovascular alterations that may precede or at least be part of the poor cardiovascular clinical outcome [5, 74]. Furthermore, anatomic alteration in arterial structure, including a reduced artery diameter, have been reported in KS adults [133]. Remarkably, testosterone exerts a potent effect on endothelial cells, acting primarily as a vasodilator, thus possibly explaining the reduced artery diameter observed in some study [5, 133]. Notwithstanding, clinical effectiveness of TRT in increasing artery diameter, as well as its anti-thrombotic effects (mediated by anti-inflammatory action) are lacking [5].

Endothelial dysfunction also plays a role in increasing cardiovascular risk in KS. In fact, an absolute lower number of endothelial progenitor cells, an independent predictor of atherosclerosis progression, was found in KS patients when compared to unaffected men, independently from the presence of any other cardiovascular risk factor and testosterone levels [74, 134].

Finally, an increase risk of rhythm abnormalities (e.g., atrial fibrillation and short QTc, the latter eventually leading to sudden cardiac arrest) have also been reported [2]. The underlying mechanism could rely on genetic aspects of the syndrome: for example, the *SLC25A6 gene*, a pseudoautosomal gene highly expressed in KS involved in calcium signaling pathway and metabolism, seems to be associated with the short QTc reported in KS patients [41].

1.7 Bone alterations and Klinefelter syndrome

Metabolic comorbidities of KS also include quantitative and qualitative alteration in bone architecture and density (i.e., osteopenia and osteoporosis), leading to a higher fracturative risk [109, 111]. Reduced bone mineral density (BMD) is reported in up to half of the patients (from 25 to 48% of KD adult males), whereas overt osteoporosis accounts in 6–15% of cases [135, 136].

In young KS boys and adolescents normal lumbar BMD and whole-body bone mineral content (evaluated by Dual X-Ray Absorption technique) have been reported, indicating that the risk of osteopenia/osteoporosis might start after puberty [2, 111]. Since then, a progressive reduction in bone mass rate occurs, estimated in ~ -1.2% per year at the lumbar level and ~ -1.0% per year at the femoral neck level [137]. Together with quantitative evaluation of bone density, recent advanced in bone imaging techniques, (e.g., high resolution computed tomography and biomechanical computation model) allowed to evaluate bone strength, cortical and trabecular micro-architecture, providing qualitative data on bone health without the need for an invasive approach (i.e., bone biopsy) [138]. Interestingly, the few studies who assessed these latter aspects reported divergent findings on BMD, but all reported an impaired bone strength, suggesting that the higher risk of fragility fractures observed in KS patients might not entirely depend from BMD values [138, 139].

Although the exact fracture rates in KS population still remains unknown, a correlation between fractures and increased morbidity and mortality in KS was reported [31, 109, 140].

Both reduced bone formation and increased bone resorption contribute to reduced BMD observed in KS, but the underlying causes still have to be fully understood [56, 141, 142].

Hypogonadism is a well-acknowledged cause of overall bone loss and probably represents the most important risk factor for reduced BMD and osteoporosis, especially in adolescence, when it might compromise the reaching the peak bone mass that normally occurs soon after puberty [142]. Notwithstanding, the precise role of testosterone in the development and maintenance of bone health, and conversely the contribution of low testosterone, to the genesis of osteoporosis is still debated.

The AR has been found to be expressed in several bone cells, including osteoblasts (bone deposition), osteoclasts (bone resorption), osteocytes (bone homeostasis), and pluripotent mesenchymal bone marrow stromal cells. The AR pathway is particularly effective in osteoblast and into trabecular bone, where testosterone increases and preserves trabecular numbers, suppress trabecular resorption and reduces trabecular spaces, therefore increasing the overall trabecular bone volume [143]. The net effect of testosterone is to promote bone formation (by stimulating both longitudinal and radial growth) during puberty and to reduce bone resorption during adult life [142].

Notably, testosterone activity on bone tissue strictly depends from the function and sensitivity of the AR [144]. Reduced AR activity on genetic basis (i.e., number of CAG repeats) has been reported in KS, along with a decreased activity of bone 5α-reductase [135, 145]. Nevertheless, the AR expression in bone has never been studied in KS.

Albeit these evidences, to date no clear relation between testosterone serum levels and BMD has been found in patients with KS, and osteopenia/osteoporosis might also be present despite testosterone concentrations within the normal range [76, 146, 147]. Moreover, the prevalence of low BMD is similar when comparing hypogonadal KS men and KS patients with normal testosterone levels, suggesting the presence of other possible causal factors besides hypogonadism.

Current data evaluating the impact of TRT on bone health in KS patient are conflicting. Some author reported that TRT in hypogonadal KS men with low BMD does not fully reverse the decreased bone mass, especially when started after puberty; conversely, TRT in young age (i.e., before reaching the peak bone mass) was reported to normalize BMD, according to other authors [72, 136, 142, 148]. As reported in general population, the effect of TRT was more pronounced at lumbar level rather than femoral neck [84, 81, 149]. However, when interpreting these results, the reduced AR expression and/or activity in bone might should be taken into account, since it might contribute to explain the heterogeneity of available data in KS patients. Moreover, no studies have been performed in osteoporotic KS subjects with fractures as primary outcome on treatment intervention and further studies are required.

In addition to directly regulating bone metabolism through the androgen receptor, testosterone also exerts indirect effects on bone tissue through being aromatization into estrogens. *Estradiol* plays a pivotal role in regulating bone turnover and estrogen levels are more closely correlated with BMD and bone turnover markers than serum testosterone ones [150]. Although the estradiol levels are generally within or slightly above normal range in KS subjects, low estrogen levels were reported to be associated with reduced BMD and with the rate of bone loss in these patients [137, 151]. However, these data have not been replicated in further studies and conclusions on this possible etiological mechanism cannot be drawn.

FSH might also be involved in the regulation of bone metabolism, primarily through stimulation of aromatase activity. Interestingly, FSH function may not exerts only in the testes, but also on several extra-gonadal sites, including the bone [152]. In fact, FSH seems to directly modulate the bone metabolism by influencing pro-inflammatory and pro-osteoclastogenic cytokine expression, such as the *Receptor Activator of NF-κB* (RANK) located on osteoclasts, therefore inducing osteoclastogenesis [153, 154]. More recently, a possible link

between FSH and RANKL in men with KS has been proposed, leading to hypothesize that elevated FSH levels with concomitant declining sex steroids could contribute to decrease BMD and increase the fracturative risk [155].

When considering the complex mechanisms underlying bone alteration in KS, other hormone besides sexual steroids and FSH should be taken into account. *Insulin-like factor 3* (INSL-3), a peptide hormone produced by Leydig cells under stimulation of LH, whose concentration progressively increase throughout puberty and then decline over time, is deeply involved in bone turnover [15 -158]. Indeed, INSL-3 maintains the osteoblast/osteoclast balance by exerting both an anabolic effect (acting directly on osteoblasts and osteocytes) and stimulating osteoclastogenesis [159-161]. In adult KS subjects with reduced testosterone levels, also very low levels of INSL-3 were reported, reflecting the testicular atrophy and decreased Leydig cell function. However, discordant results when correlating INSL-3 concentrations and BMD were observed [156, 162].

Vitamin D might be another possible modulator of bone health in KS, because of its regulatory activity on calcium homeostasis and bone metabolism [2, 163]. The testis expresses an LH-dependent isoform of CYP2R1 enzyme, with a pivotal role in vitamin D activation through its 25-hydroxylase activity [164]. An impaired testicular function, as occurs in KS, contributes in decreasing circulating 25-hydroxyvitamin D levels, thus reducing the BMD [2, 165]. Accordingly, lower 25-hydroxyvitamin D levels in KS compared to healthy controls were reported [76, 160].

Hence, vitamin D supplementation is fundamental in maintaining an adequate BMD in KS males [2]. When considering vitamin D supplementation, lumbar and femoral BMD in KS were positively associated with 25-hydroxyvitamin D, and both lumbar and femoral BMD were significantly reduced in KS patient with vitamin D deficiency respect to KS subject with 25-hydroxyvitamin D $\geq$ 50 nmol/L [76]. Moreover, KS subjects treated with calcifediol and TRT showed a significant increase in lumbar BMD when compared to those treated with T alone [76].

Lastly, another possible mechanism involved in the development of bone loss in KS might be related to sarcopenia and the unfavorable fat/muscle ratio caused by hypogonadism, hypovitaminosis D, increased visceral adiposity and reduced lean mass [109, 166].

1.8 Thyroid and Autoimmune disease in Klinefelter syndrome

The prevalence and the etiopathogenesis of thyroid dysfunction in KS are still unclear, and available data are mostly contradictory and derived from small cohort of patients. Adult KS patients tend to have normal fT3 concentration but lower serum T4 levels, clustering around the lower limits of the reference range, without evidence of compensatory increase in TSH concentration nor in TSH/fT4 ratio [167]. This might reflect an inadequate pituitary secretion of TSH in response to TRH stimulus, possibly increasing the risk of secondary thyroid insufficiency [167-170].

A recent multicenter study focused on evaluating thyroid function in KS reported a prevalence of Hashimoto's thyroiditis of 7% in KS compared to 4% in unaffected males. FT4 confirmed to be was significantly lower in KS than in controls, without any difference in TSH concentrations, supporting the hypothesis of a hypothalamic–pituitary–thyroid axis dysregulation. In particular, KS patients may suffer from an impaired production of T4, with an adaptive increased response type II deiodinase activity, allowing to maintain normal peripheral circulating amount of fT3 and TSH levels [171]. Even when evaluating the TSH index, that provides an accurate estimate of the severity of pituitary dysfunction in hypopituitary patients, both TSH index and fT3/fT4 ratio did not differ between KS and non-KS males, living unexplained the origin of the lower FT4 levels in KS [172]. Notably, the fT3/fT4 ratio was found to be a reliable predictor for the risk of developing metabolic syndrome and insulin resistance in both KS and non-KS adults [172].

While only few case-reports describing hyperthyroidism or goiter in KS patients are available, large data from Danish register confirmed higher prevalence of hypothyroidism in KS men than controls (0.7 vs. 0.005%) but with no differences in prevalence of Hashimoto's thyroiditis [109]. Moreover, frequency of elevation in the anti-TPO antibody in KS males was similar to that found in control group [167, 173].

Current evidences further suggests that KS patients may be at increased risk developing some autoimmune diseases, but data are not substantial [5]. Interestingly, a higher prevalence of type 1 diabetes mellitus was observed in KS subjects compared to controls, with significantly higher positivity of the related auto-antibodies (i.e., GAD-65 and IA2) [173, 174]. Furthermore, data from a national dataset recording all hospital day cases and inpatient admissions in England (from 1999 to 2011) aimed at identifying the occurrence of autoimmune diseases, pointed out that KS subjects had a significantly increased risks of Addison's disease (RR 11.7) and multiple sclerosis (RR 4.3), as well as type 1 diabetes mellitus (RR 6.1) and acquired hypothyroidism (RR 2.7) [174].

The reasons accounting for the increase prevalence of autoimmune diseases are thought to rely on genetic background, since X chromosome contains several genes involved in the regulation of the immune response (e.g., CD40 ligand and Toll-like receptor 7) [175, 176].

1.9 Neuro-Psychological features in Klinefelter patients

The neurocognitive phenotype in KS is associated with several deficits, although it displays a high variability. Several data suggest that supernumerary X chromosome may significantly impact on brain anatomy and morphology, possibly explaining some aspects of KS behavioral and psychological phenotype. Neuroanatomical and functional studies depicted significant differences in KS males as reduced total brain volume (especially in smaller caudate, cerebellar, temporal and frontal lobe) and enlarged ventricles [5, 177, 178]. Furthermore, significant grey matter volume reduction in insular cortex and the left orbitofrontal cortex (OFC), amygdala and hippocampus were also reported. Particularly, OFC handle neuronal processes involving the evaluation of sensory stimuli and social decision making, while the amygdala reductions may determine atypical temperament, passivity and reduced sexual desire [179]. Moreover, altered structure and functionality of brain areas in the frontal lobe have been observed in KS; these areas are involved in the "executive functions", essential for flexible adaptation in complex situations (i.e., goal-directed behavior and flexibility in problem-solving and processing thoughts) [180]. Cognitive flexibility, a specific ability strictly related to social adaptation, especially in disengaging attention away from one source and then reengaging it to another, may also be impaired [181].

Intellectual abilities are not impaired and Intelligence Quotient (IQ) of KS subjects almost overlap to those of unaffected males, as well as visual and spatial cognitive abilities, while verbal and nonverbal memory, arithmetic abilities and executive functions are mildly impaired [182, 183].
Concerning the *verbal area*, verbal deficits are among the most characteristic functional features of KS, identified in the majority of KS males (70-80%) [5, 29]. Learning disabilities in reading and spelling, delayed in early language development, impairments in syntax, word retrieval and both language production and perception have been reported, determining a verbal IQ slightly lower than performance IQ [5, 184]. These alterations can be observed in both children and adolescents with KS and currently represent one of the most distinctive traits in the neuropsychological functioning of these individuals [185-187]. In fact, many children with KS are "late-talking children", displaying a small vocabulary and compensating vocabulary difficulties using communicative gestures, probably reflecting a difficulty in verbal production rather than a general communicative deficit [188]. However, language problems (e.g., understanding complex grammatical constructions, syntactic productions and word retrieval abilities) and phonological difficulties (e.g., errors in word pronunciation or accentuation) usually persist during childhood and adolescence and could be associated to distractibility and inattentive symptoms [189, 190]. Indeed, as language skills advance and become more complex, higher-level language deficits are common (e.g., poor grasp of verbal concepts, decreased verbal fluency, and social communication difficulties) [5]. Hence, a specialized help at school and specific speech and language therapy are advisable, especially in young patients, since language-based learning difficulties and social cognitive impairments may trigger derision from peers and poor socialization, resulting in the impossibility of achieving satisfactory, anxiety and mood disorders [184, 191].

When considering *behavioral* issue, the main problems in KS patients affect social domain, with higher prevalence of social withdrawal and anxiety, impulsivity, shyness, and inappropriate or antisocial behavior than in general population. Particularly, anxiety, depression and low self-esteem are the most prevalent and debilitating affective symptoms among KS patients, including adolescents [5, 185]. As a consequence, significant difficulties in coping with social situations, especially the high levels of distress during social interactions, are frequently observed [5, 192]. All these aspects have a detrimental impact on the quality of life, which proved to be are considerably inferior in KS subjects compared to general population. Some author suggested a potential association between mood disorders (especially depression) and concomitant hypogonadism, although available data are still controverse [193]. Finally, few studies also reported associations between KS, bipolar disorders and schizophrenia-spectrum pathology [187, 194, 195].

In view of these considerations, an early diagnosis of KS may guarantee as optimal and early intervention aimed at adequately manage the neurocognitive features of the syndrome; at once, a comprehensive psychological or psychiatric assessment to manage mood and behavioral disorders, as well as psychosis, is advisable and should be offered to all KS patients [2].

1.10 Klinefelter syndrome and sexuality

Testosterone is known to play a central role in regulating sexual behavior and sexual function, and it is deeply involved in neurovascular events underlying penile erection and ejaculation [196]. Actually, sexual behavior is regulated by a highly complex neuroanatomical and neurohormonal network. Among them, the amygdala is essential in integrating information and processes with their emotional significance, leading to the conscious awareness of feelings and drives, including sexual desire. Furthermore, amygdala contributes to gender dimorphism in both dimension and function, along with testosterone: in fact, amygdala largely expresses the AR and higher exposure to androgen (even in the intrauterine life) is associated with masculinized brain and behavior [197]. In view of these considerations, the androgen deficiency occurring in KS patients has the potential to alter the normal development of brain structures involved in emotional processing and sexual behavior. Moreover, in KS patients a reduction in the volume of the amygdala and other regions involved in emotional processing and sexuality has been observed, leading to potentially impaired sexuality [196, 198]

Metabolic derangements can also have negative consequences on sexual function. Obesity, visceral adiposity and insulin resistance, frequently reported in KS adults, may directly worsen gonadal steroidogenesis and reduce the amount of circulating androgens, also enhancing arterial atherogenesis and erectile dysfunction [199].

Evidences on sexual function in KS men are scanty, despite these patients often present several risk factors for sexual dysfunctions, including hypogonadism, metabolic disorders, impaired behavioral and social problems.

The first available studies, dating back in 1980, reported a significant increase in sexual thoughts and sexual excitement in KS males after the initiation of testosterone replacement therapy. Recent findings of a large cohort of 1386 men consulting for sexual dysfunction (of which 23 affected by non-mosaic 47,XXY KS), reported a higher prevalence of low sexual desire and guilt with masturbation, reduced ejaculate volume and later andrological referral than unaffected men. Notably, when comparing these KS men with 92 controls matched for serum testosterone concentrations, age and smoking habits, the aforementioned differences were not confirmed, thus suggesting a predominant role of androgen deficiency in the genesis of sexual alterations rather than KS itself [5]. Further data from small cohort, comparing 15 non-mosaic 47,XXY KS to 20 acquired hypergonadotropic hypogonadal men with a prepubertal onset, no differences at baseline in erectile function and penile blood flow were observed, as well as similar improvement after TRT [73].

However, data on the impact of TRT on sexual function in this setting are lacking. Evidences from small pilot observational studies showed that testosterone treatment effectively improves sexual function, libido, and decreased fatigue in adult KS males [5].

When compared to 46,XY males, reduced sexual desire and lower prevalence of premature ejaculation in men with KS were also reported [200]. Finally, when evaluating sexual function through validated questionnaire (i.e., IIEF-15), lower scores in erectile function, sexual desire and satisfaction domains were observed in KS males [201]. Besides having a worse metabolic profile, almost half KS patients reported a history of difficulties in relationships and sexuality, as well as body uneasiness or embarrassment [201]. Leaving aside the role of hypogonadism in the genesis of sexual dysfunction, these data pointed out that erectile deficiency and impaired sexuality has a more complex pathogenesis, probably involving metabolic factors and psycho-relational issues.

1.11 Oncological disease in Klinefelter syndrome

Oncological problems in KS currently represent an open issue, with lack of evidences on the correct clinical approach and management in these subjects. Although KS is associated to an increase of all-cause mortality, oncological diseases do not seem to contribute to this increase and their overall incidence in KS subjects is closer to that of the general male population.

Nonetheless, evidence is compelling about a higher prevalence in KS patients of specific malignancies, including extragonadal germ cell tumors, breast cancer and hematological malignancies [2, 202, 203]. Since the overall incidence of these neoplasms is low, a routinary screening in asymptomatic KS patients is not recommended yet. The underlying pathophysiological mechanism are largely unexplained, but according to recent hypothesis an aberrant hormonal milieu together with the supernumerary X-chromosome and its

consequences on DNA methylation and gene regulation (e.g., gene dosage effects and X-silencing process) might exert a predominant role [202]. On the other hand, hormonal profile in KS (i.e., either hypogonadism or suboptimal TRT) might exert a protective effect against the occurrence of hormone-dependent neoplasm as prostate cancer, partially explaining its low prevalence among KS males [5].

Germ cell tumors (GCTs) are a group of histologically heterogeneous neoplasms deemed to originate from the aberrant proliferation of primordial germ cells during intrauterine life. These tumors typically affect the gonads but in a minority of cases they may develop in extra-gonadal sites, typically along midline structures (e.g., retroperitoneum and mediastinum), with a peak age in adolescence. The majority of these lesions are classified as *non-seminomatous*, with Yolk sac tumor and teratoma being the most common [202]. Clinical presentation varies according to the localization, size and stage of the neoplasm; hormonal secretion (i.e., human chorionic gonadotropin) can be present.

Data from large Danish cohort estimates a 67-fold increase of risk in developing of mediastinum GCTs and significant higher risk of hospital admission because of mediastinal cancer (HR 14.2) in KS patients compared to unaffected controls [109, 204]. On the other hand, a remarkable prevalence of KS (about 3%) was reported in a cohort of children diagnosed with a GCT, predominantly with extra-gonadal localization (70% mediastinum), leading to a significant risk of developing GCT (RR 18.8) among KS males compared to unaffected men [205].

Male breast cancer (MBC) is a rare disease. Although it is now recognized that KS patients are at risk for MBC, the extent of this relationship still remains uncertain due to scarcity of epidemiological data [108]. The risk of MBC in KS males ranges from 19 to 50%, according to different sources, with significantly higher standardized mortality ratio [206, 207]. Gynecomastia and increased levels of estrogens should not be considered as risk factors for MBC [56-58]. Similarly to what discussed for gynecomastia, the etiological mechanism are still obscure. However, both hormonal and metabolic alterations (i.e., relative hyperestrogenism, increased estrogen-to-testosterone ratio and obesity) and genetic susceptibility might be involved [56].

Nowadays, the increase risk of *hematological cancer* in KS patients is still debated, but seems to affect predominantly patient with more than three sex chromosomes rather than non-mosaic 47,XXY KS patients [208, 209]. Indeed, current data are scares and controverse. The underlying mechanisms may account of the higher frequency of gene fusion and/or translocation during cell division due to extra chromosome in the cellular lines [34].

More broadly aneuploidy might play an important role in tumorigenesis thus explaining, at least in part, the higher occurrence of malignancies in KS patients.

Several studies found positive correlations between the degree of aneuploidy, proliferation, and cell cycle transcriptional signatures that are believed to be indicative of promoting tumorigenesis [57, 210]. Some

evidence reported that aneuploidy positively correlates with cell proliferation, possibly resulting in a selective advantage and increases the tumorigenic behavior of human cancer cells. However, according to other data, it would seem that aneuploidy might suppresses rather than promotes tumorigenesis, exerting a detrimental effect on cell proliferation and survival [36].

Several elements (e.g., tumor stage, cytotype, tumor microenvironment, and immune system interactions) concur in determining the circumstances under which aneuploidy can drive tumorigenesis. In fact, the same chromosome might be gained some tumor type, but lost in another, demonstrating that no single chromosome gain or loss is universally able to promotes tumorigenesis [208].

2. Methods

2.1 Aim of the study

In view of these considerations, we are currently cooperating with the *Klinefelter Italian National Group* (K.I.N.G.) in a multicenter, prospective, observational trial aimed at evaluating the prevalence of KS in the Italian population and evaluating clinical and biochemical aspect of the syndrome, as well as the quality of life of KS males through the administration of self-administered questionnaires (TIB, SCL-90, TCR-R and a structured andrological interview).
However, the block of the outpatient activity in 2020 and part of 2021 due to the Sars-Cov2 pandemic determined a significant delay on the scheduled steps, so that the process of recruitment and data collection is still ongoing and results of this study will be available only in the next years.

In the lights of these limitations, since only data from our center were currently available for analysis, we conducted an observational, cross-sectional, single center study with the primary endpoint of evaluating the biochemical and clinical characteristics of adult males with KS. The secondary outcome of the study was a focus on specific aspect of cognitive skills (i.e., Intelligence Quotient - IQ) and behavior (i.e., experience, expression and control of rage) of KS patients, comparing data to those deriving from cohort of unaffected males.
All the procedures used in the present study adhere to the tenets of the Declaration of Helsinki. Approval was granted by the Ethics Committee of our Institution.
Study quality was assessed using the ‘‘STROBE’’ (STrengthening the Reporting of OBservational studies in Epidemiology).

2.2 Patients

Patients with KS referring to the Andrology Unit of the "*S.C.U. Endocrinology, Diabetology and Metabolism – Department of Medical Sciences, University of Turin*" were consecutively enrolled from 1st November 2018 until 1st September 2021.
All adult males (age ≥ 18 years) with new or previous diagnosis of KS (confirmed by karyotype analysis) on outpatient follow-up were considered eligible for the study.
We excluded from the study patients: a) who did not sign or withdraw informed consent; b) who were on outpatient follow-up at another Hospital and were referring to our unit only for bureaucratic issues (i.e., prescription of testosterone replacement therapy).

All patients underwent a full clinical and andrological anamnesis, physical examination, hormonal and laboratory evaluation.

Data concerning andrological features (i.e., bi-testicular volume, presence of varicocele, gynecomastia) were collected during the routine outpatient visits.

Clinical data on past medical history regarding andrological (age at diagnosis, efficacy and safety of TRT, sperm alterations and fertility rate), metabolic (prevalence of cardiovascular events, alterations in glucose and lipid metabolism, thyroid disorders and bone metabolism), neurological, oncological and psychological/psychiatric aspects of the syndrome were collected.

Efficacy of TRT was defined in presence of serum testosterone in the low-mid normal range [211, 212]

When considering alterations in glucose and lipid metabolism, target levels of LDL-c cholesterol and HbA_1c were determined in accordance to current guidelines [213, 214].

Validated questionnaires (i.e., TIB and STAXI-2) were also administered and fulfilled in part during the outpatient visit and partly at home. The TIB test was then administered to a control group of adult males, matched for age and years of study. Scores in STAXI-2, instead, were compared to those obtained in the validation cohort of test.

2.3 Questionnaires

2.3.1 TIB

The "Brief Intelligence test" (*Test d'Intelligenza Breve - TIB*) is a validated test for assessing the Intelligence Quotient (IQ). The TIB is based on the correlation between global intelligence and reading skills and leads to explore the areas of language and learning. It consists of a list of 54 words (validated in Italian language): 34 main words with different accentuation (irregular/regular) alternating to 20 words commonly used in everyday life [215].

The patient must read aloud all the 54 words: both errors in pronunciation and accentuation only in the 34 main words will determine the final *TIB score* while the remaining 20 words will work as "control words", and mistakes in their pronunciation/accentuation will not influence the final *TIB score* of the test. A higher number of mistakes will lead to a higher final *TIB score* and a lower IQ, estimated by using the following equations:

- $IQ_{verbal} = 93.551 + (0.133 \times age) + 3.3825 + (0.772 \times years\ of\ study) - (0.943 \times TIB_{score})$
- $IQ_{performance} = 102.938 + (0.198 \times age) + 3.316 - (1.319 \times TIB_{score})$
- $IQ_{total} = 98.471 + (0.168 \times age) + 3.605 + (0.388 \times years\ of\ study) - (1.196 \times TIB_{score})$

Verbal IQ is designed to provide a measure of an individual's overall verbal intellectual abilities. It measures acquired knowledge, verbal reasoning, and attention to verbal materials.

Performance IQ provides a measure of an individual's overall visuospatial intellectual abilities by evaluating fluid reasoning, spatial processing, attentiveness to details, and visual-motor integration.
IQ scores in the examined cohort were than compared with a. cohort of healthy men matched by age and years of study.

2.3.2 STAXI-2

The STAXI-2™ questionnaire is an easily self-administered tool for assessment of the experience, expression, and control of anger for adult individuals ≥ 16 years. The STAXI-2 was developed to assess components of anger and anger expression for a detailed evaluation of normal and abnormal personality and to measure the way these anger components contribute to medical conditions such as hypertension and coronary heart disease.
The 54-item STAXI-2, validated in Italian language, consists of 6 scales (*State Anger*, *Trait Anger*, *Anger Expression-Out*, *Anger Expression-In*, *Anger Control-Out* and *Anger Control-In*), 5 subscales (*Feeling Angry*, *Feel Like Expressing Anger Verbally*, *Feel Like Expressing Anger Physically*, *Angry Temperament* and *Angry Reaction*), and an *Anger Expression Index* that provides an overall measure of total anger expression [216].

State Anger (**R/S**) refers to the intensity of anger as an emotional state at a particular time (either at the time of testing, or a time and situation specified by the test administrator). The **R/S** presents subscales: *Feeling Angry* (**RS/S**), *Feel Like Expressing Anger Verbally* (**RS/V**), and *Feel Like Expressing Anger Physically* (**RS/F**). People with high scores in this scale and subscales experience moderate to intense anger which may be manifested as a desire to scream or break things. Mild to moderate activation of the sympathetic nervous system with increased heart rate and blood pressure are possible. It is likely that the high R/S score reflects a momentary rather than a chronic state of being.

Trait Anger (**R/T**) analyze an individual's general predisposition to become angry and plays a fundamental role in understanding how often a person becomes angry in a variety of situations. The *Angry Temperament* subscale (**RT/T**) allows measuring whether a person has an overall angry temperament and is predisposed to experience anger quickly and with little provocation. On the other hand, the *Angry Reaction* subscale (**RT/R**) evaluates the tendency of a person to respond with anger or to become agitated when perceiving to be treated unfairly, being criticized or receiving negative feedback from others.

The *Anger Expression-Out* (**ER/out**) scale describes the extent to which a person expresses his/her emotional experience of anger (both verbally and physically) in a poorly controlled manner towards other people or objects if they are seen as somehow related to the source of rage or are simply nearby when the angry outburst occurs.

The *Anger Expression-In* (**ER/in**) scale measures the extent to which ad individual suppresses anger and holds things in when feeling angry or furious. Sometimes, the person may replace the repressed anger with feelings of guilt and depression, blaming himself/herself for the surrounding problems.

The *Anger Control-Out* (**CR/out**) scale describes the extent of energy spent to monitor, prevent and control the physical or verbal expressions of anger toward other persons or objects in the environment, while the *Anger Control-In* (**CR/in**) scale measures how often a person attempts to relax, calm down, and suppress anger before losing control.

The *Anger Expression Index* (**ER/Index**) provides an overall estimate of the person's tendencies to express anger either inwardly toward himself/herself or outwardly toward other people. It can be derived with the following equation: ***ER/Index*** *= (ER/out + ER/in) – (CR/out + CR/in) + 48*.

Patients rate themselves on 4-point scales aimed at assessing both the intensity of their anger in a particular moment and the frequency that anger is experienced, expressed, and controlled. The final score of the test can be interpreted through normative tables providing raw score-to-percentile and raw score-to-normalized T point conversions for each scale and subscales, according to individual's age groups: 16-19 years, 20-29 years, and 30 years and older.

Patients whose score exceeds the 75th centile (equal to T normalized score $\geq$ 56.75 points) are considered to be more prone in experiencing or suppressing feelings of anger. Higher scores in ER/index, ER/out or ER/in positively correlate with incidence of arterial hypertension, cardiovascular disease (including myocardial infarction) and increased risk of all-cause hospitalization [217-220].

2.4 Statistical analyses

Data are expressed as mean and standard deviation (SD), median and interquartile range (IQR) or absolute number and percentage.

Distributions of continuous variables were examined by the Shapiro-Wilk and were logarithmically transformed as appropriate.

Data were evaluated by Chi^2 test, parametric (Student T-test for independent samples) or non-parametric (Mann-Whitney) tests, as appropriate.

Correlation analysis calculating Spearman coefficient was performed to assess the strength of the association between the different variables. Partial correlation on variables of clinical interest was conducted to analyze the potential interfering effects of covariates.

Statistical significance was assumed at $p < 0.05$.

All analyses were performed with SPSS (version 27.0.1.0, SPSS Inc, Chicago, IL, USA).

3. Results

3.1 Overview

Among the initial cohort of 98 patients, 72 met the inclusion criteria and were enrolled in the study. The majority of them (66 subjects, 91.7%) agreed in filling all the proposed questionnaires and results from almost half of them (34 subjects, 47.2%) were currently available. The whole selection process is described in *Figure 1* [*Fig. 1*].

Enrolled patients had a median age of 27.5 (17.0 – 39.0) at diagnosis and 45.0 (32.5 – 53.0) years at the time of evaluation [*Tab. 1*]. Notably diagnosis of KS was performed prenatally in 5 cases (6.9%) and within the first year of life in 1 case, due do the presence of urological malformations and cryptorchidism. Moreover, diagnosis was performed during late-childhood or adolescence in 9 cases (12.5%), mainly because of pubertal delay and clinical features suggesting KS (e.g., undescended testis, tall stature, gynecomastia) or, less frequently (3 cases, 4.2%), because of learning difficulties. Finally, 3 patients (4.2%) were diagnosed over the course of medical evaluation for military service. All other patients (54, 75.0%) were diagnosed in adulthood, mainly during clinical workup for hypogonadism and/or infertility.

Almost the entire cohort exhibited classical 47,XXY karyotype (69 patients, 95.8%), while 46,XY/47,XXY mosaicism was found in the remaining 3 patients (4.2%) [*Tab. 1*].

3.1.1 Andrological features, testicular function and fertility

When considering andrological characteristics, the majority of patients (70, 97.2%) were presenting a significant reduction in *bilateral testicular volume*, with a median volume of 5.3 (3.0 – 7.0) ml [*Tab. 1*]. Interestingly, the remaining patients with preserved bi-testicular volume (35 and 36 ml, respectively) were affected by a 46,XY/47,XXY mosaicism.

Hypogonadism requiring TRT was reported in a high number of patients (65 subjects, 90.3%), while few of them (7 subjects, 9.7%) showed persistence of adequate testicular steroidogenesis, guaranteeing serum testosterone levels within the normal range [*Tab. 1*]. Almost two thirds of patients on TRT (41 cases, 56.9% of the whole cohort) were using long-acting injectable preparations (i.e., undecanoate testosterone), while 22 of them (30.6%) were on transdermal formulations, allowing to reach an adequate serum testosterone concentration in 49 cases (67.1%) [*Tab. 1*]. No correlation was observed between the achievement of adequate plasmatic testosterone levels and the route of administration of TRT (i.e., transdermal vs intramuscular). Conversely, a negative correlation between BMI and the attainment of testosterone level within normal range was reported (ρ -0.337, $p < 0.05$) [*Tab. 2*].

In 2 cases (2.8%) TRT had been temporarily withdrawn at the time of evaluation due to the occurrence of side effects (i.e., erythrocytosis) [*Tab. 1*]. Particularly, erythrocytosis was the most common adverse events occurred on TRT, reported in 26 patients (36.1%) and requiring phlebotomy in a significant number of cases (8 subjects, 11.1% of the whole cohort and 30.1% of patients with erythrocytosis) [*Tab. 1*]. When considering this subgroup, a significant prevalence of smoking habits (11 cases, 42.3%), overweight/obesity (10 cases, 38.5%) and Obstructive Sleep Apnea Syndrome (3 cases, 11.5%) was observed.
No correlations emerged between this side effect and the achievement of adequate serum testosterone concentrations [*Tab. 2*]. Nevertheless, the route of administration of TRT was found to correlate with the occurrence of erythrocytosis, in particular when using long-acting intramuscular testosterone (ρ 0.482, $p < 0.001$). No further correlation between this side effect and other variables of clinical interest emerged [*Tab. 2*].

Varicocele of various clinical degrees, already diagnosed or further confirmed by testicular ultrasound, was observed in 11 subjects (15.3%) [*Tab. 1*]. Various degrees of *gynecomastia* were reported in a remarkable proportion of KS males (28 cases, 38.9%) and surgically treated in a small number of them (6 cases, 21.4% of all cases of gynecomastia) [*Tab. 1*]. No correlations between variables of clinical interest and varicocele nor gynecomastia were observed [*Tab. 2*].

A minority of patients, especially aged ≥ 40 years, were suffering from Erectile Disfunction (ED) or Benign Prostate Hyperplasia (BPH) (17 and 12 cases, 23.3% and 16.4%, respectively) [*Tab. 1*]. The occurrence of ED proved to be associated with ageing (ρ 0.466, $p < 0.0001$) and arterial hypertension (ρ -0.509, $p < 0.0001$) [*Tab. 2*]. Both these variables were also positively associated with occurrence of BPH (ρ 0.559, $p < 0.0001$ and ρ 0.474, $p < 0.0001$, respectively). Correlation analysis also pointed out a peculiar negative correlation between BPH and smoking habits which, however, was not confirmed after correcting for possible confounders (i.e., patients age at evaluation).

In relation to *fertility*, 59 subjects underwent at least one semen analysis during their lifetime and almost all of them were found to be azoospermic (57 cases, 96.6%) [*Tab. 1 bis*]. Surgical sperm retrieval using TESE/microTESE was performed in 19 of them (32.2%) with low percentages of sperm retrieval rate (10.5% - 2 cases, both followed by unsuccessful ART) [*Tab. 1 bis*]. The remaining subjects declined surgery, primarily because non interested in fatherhood due to their advanced age (29 cases) while 9 patients, mostly young aged and thus potentially involved in future fatherhood, refused anyway the procedure after being exhaustively informed about potential clinical consequences of their choice [*Tab. 1 bis*].
Anyhow, 2 cases of spontaneous pregnancies were reported, both involving a 46,XY/47,XXY adult male with preserved testicular volume and normal semen parameters. Five other cases of fatherhood have been reported, by means of heterologous ART (4 cases) or adoption (1 case) [*Tab. 1*].

Lower testicular volume and ongoing TRT were found to significantly impact on the occurrence of azoospermia (ρ -0.380, $p < 0.05$ and ρ 0.330, $p < 0.05$, respectively) [*Tab. 2*], especially after adjusting for possible confounders (i.e., age and adequate serum testosterone levels: ρ -0.935, $p < 0.001$ and ρ 0.421, $p < 0.05$, respectively). Although median age of patients who underwent TESE/micro TESE did not differ significantly from subjects who were not submitted to this procedure (24.9 vs 28.4 years, $p = 0.427$), a negative correlation emerged between the patients' age at diagnosis of KS and the execution of sperm retrieval surgery (ρ -0.336, $p < 0.001$) [*Tab. 2*].

3.1.2 Metabolic and Cardiovascular disease

Median *BMI* in overweight range emerged in our KS males: 26.5 (23.1 – 29.5) Kg/m^2 [*Tab. 3*]. In fact, overweight and obesity were observed to be highly prevalent in the examined cohort, affecting 27 (37.5%) and 15 (20.8%) patients, respectively [*Tab. 3*]. Higher BMI scores were found to positively correlate with age (ρ 0.257, $p < 0.05$) and the occurrence of cardio-metabolic diseases such hypercholesterolemia (ρ 0.376, $p < 0.001$) and arterial hypertension (ρ 0.320, $p < 0.05$) [*Tab. 4*]. Conversely a negative correlation between BMI and adequate serum testosterone concentration (ρ -0.337, $p < 0.05$) emerged [*Tab. 4*], being further confirmed after controlling for possible confounders (i.e., age; ρ -0.335, $p < 0.05$).

A significant number of subjects were suffering from *hypertriglyceridemia* (17 cases, 23.6%) and/or *hypercholesterolemia* (31 cases, 42.5%), with adequate LDL cholesterol levels for cardiovascular-risk reported in only a minority of them (7 cases, 21.6%) [*Tab. 3*]. Both these alterations in lipid metabolism positively correlated with age and almost all cardio-metabolic risk factors, with the sole exception of smoking habits [*Tab. 4*]. Achievement of normal plasmatic testosterone concentration was associated with a lower occurrence of lipid metabolism alterations, despite not significant.

Likewise, an elevated percentage of subjects with impaired *glucose metabolism* was also reported: 17 men (23.6%) were suffering from Impaired Fasting plasma Glucose (IFG) or Impaired Glucose Tolerance (IGT), while 11 of them (15.3%) had overt type 2 diabetes mellitus, adequately controlled in the majority of cases (81.8%) [*Tab. 3*].
Similar to what observed for lipid alterations, also the occurrence of impaired glucose metabolism was observed to positively correlate with age and other cardio-metabolic diseases, but not with the achievement of target values of serum testosterone [*Tab. 4*].

Only 1 patient (1.4%) had a positive anamnesis for a *major cardiovascular event* (i.e., myocardial ischemia). However, several cardiovascular risk factors were observed to be highly prevalent in the examined

cohort, in addition to the aforementioned hypercholesterolemia and diabetes mellitus, as *arterial hypertension* (24 patients, 32.9%) and *smoking habits* (22 patients, 30.1%) [*Tab. 3*].
Notably, delayed diagnosis was significantly associated to a higher occurrence of all cardio-metabolic diseases, including hypertriglyceridemia (ρ 0.263, $p < 0.05$), hypercholesterolemia (ρ 0.394, $p < 0.001$), impaired glucose metabolism (ρ 0.336, $p < 0.05$) and arterial hypertension (ρ 0.395, $p < 0.001$) [*Tab. 4*], even when adjusted for BMI and occurrence of normal serum testosterone. However, none of these correlations were confirmed when correcting for the age at evaluation.

A minority of patients (21 subjects, 30.1%) already underwent cardiac ultrasound at time of evaluation, which allowed diagnosing valvular abnormalities in 6 of them (28.6% of examined patients; 8.2% of the whole cohort) [*Tab. 3*]. Mild tricuspid insufficiency was the most prevalent alteration, either alone (3 cases) or associated with mild mitral insufficiency (2 cases), while in 1 case mild aortic insufficiency was observed. In the remaining patients, cardiac ultrasonography was requested or already scheduled [*Tab. 3*].

The occurrence of *arrhythmic diseases* was also observed in the examined cohort: sinus bradycardia and atrial fibrillation/flutter were each reported in 3 subjects (4.2%), in one case requiring the implantation of a pace-maker; isolated cases of supraventricular extrasystoles and right bundle brunch block were also observed [*Tab. 3*].
None of the examined clinical variable showed any correlation with the occurrence of valvular alterations nor arrhythmic disease [*Tab. 4*].

Finally, a not negligible number of venous thrombosis was pointed out: 6 cases (8.3%) [*Tab. 3*], without any evident correlation with age, smoking habits, ongoing TRT or BMI.

3.1.3 Bone parameters, endocrine dysfunction and autoimmunity

In relation to *bone health* the majority of KS males (57 patients, 79.1%) already underwent lumbar and/or femoral morphometry with Dual X-Ray Absorption technique, while the remaining 15 subjects are scheduled to be tested in the next few months. BMD was found to be normal in half the of examined patients (37 men, 51.4% of the whole cohort), while reduced values consistent with osteopenia or osteoporosis were reported in 14 (19.4% of the whole cohort, 25.0% of examined cases) and 6 (8.2% of the whole cohort, 10.7% of examined cases) subjects, respectively [*Tab. 3*]. No cases of fragility fractures were recorded in the examined cohort.
Not surprisingly, vitamin D deficiency revealed to be highly prevalent among KS males (60 cases, 82.2%); all of them were on replacement therapy at time of evaluation (either with cholecalciferol or calcifediol) or were advised to initiate it [*Tab. 3*]. In 1 patient a hormonal profile consistent with primary normocalcemic hyperparathyroidism was observed.

Patients' age demonstrated to be significantly related to the occurrence of vitamin D deficiency and (ρ 0.289, *p < 0.05*) reduced BMD (ρ 0.386, *p < 0.05*), while no correlation emerged between BMD, Hypovitaminosis D and BMI, testosterone levels on target and smoking habits [*Tab. 5*]. The age at diagnosis showed an initial positive correlation with both reduced BMD and occurrence of thyroid disease (ρ 0.415, *p < 0.05 and* ρ 0.285, *p < 0.05*, respectively) [*Tab. 5*]; however, these data were not confirmed after adjusting for potential confounder (i.e., age at evaluation and BMI).

Thyroid function was preserved in almost all patients (67 subjects, 93.1%), with median TSH levels of 1.9 (1.2 – 2.8) μU/ml [*Tab. 3*]. Thyroid goiter and Hashimoto's thyroiditis were reported in 2 and 3 patients respectively; in 1 case concomitant hypothyroidism occurred, requiring replacement therapy [*Tab. 3*]. No correlations between variables of clinical interest and occurrence of thyroid disease occurred [*Tab. 5*].

Interestingly, a few cases of autoimmune disease were recorded, including psoriasis (1 case), multiple sclerosis (1 case) and Chron's disease (1 case). Finally, 1 subject was suffering from both type 1 diabetes mellitus and celiac disease, without concomitant alteration in thyroid or adrenal function.

3.1.4 Neuro-psychological features and other comorbidities

A high prevalence of *neurological diseases* was recorded among KS males (17 cases, 23.6%) including peripheral neuropathy (3 cases), headache (2 cases), memory deficit (1 case), syncope or drop attack (4 cases) [*Tab. 3*].
Notably a significant number of patients (8 cases, 11.0%), most of which aged ≤ 60 years, were suffering from *essential tremor*, requiring medical therapy in almost all of them [*Tab. 3*]. None of the examined clinical variables showed any correlation with the occurrence of neurological disease nor essential tremor [*Tab. 5*].

In the examined cohort, 17 cases (23.6%) of psychiatric/psychological disease were described [*Tab. 3*]. Anxiety and/or depression were accounting for the major part of these diseases (13 cases, 18.1%), but also cases of psychosis (1 subject) and "not otherwise specified" personality disorders (2 cases) were reported. Interestingly, the achievement of adequate levels of testosterone was found to negatively correlate with the occurrence of psychiatric/psychological disease (ρ -0.250, *p < 0.05*) [*Tab. 5*], even when adjusted for age at time of evaluation [*Tab. 5*].

Nine cases (12.3%) of both benign and malignant *neoplasms* were reported, including 1 case of testicular cancer and 1 of Leydig cell hyperplasia, 2 cases of testicular formation whose diagnostic workup is still ongoing, 1 case of mediastinum germ cell tumor, 2 benign breast lesions [*Tab. 3*]. No correlations between variables of clinical interest and occurrence of cancer emerged [*Tab. 5*].

Furthermore, in the examined cohort occurrence of hyperuricemia/gout (2 cases), obstructive sleep apnea syndrome (4 cases), non-alcoholic fatty liver disease (2 cases), aortic dilatation (either ectasia or aneurism, 2 cases), pericarditis (1 case) and congenital malformations including bifidum scrotum with urethral stenosis, diaphragmatic hernia and solitary kidney were recorded [*Tab. 3*].

3.2 Verbal abilities

In the examined cohort, a subgroup of 34 patients underwent TIB test in order to evaluate language skills in the area of reading and learning, thus estimating verbal, performance and overall IQ.
These subjects had a median age of 46.5 (45.75 – 54.75) years and an average length of studies of 15.0 (8.0 – 15.0) years [*Tab. 6*]. Data from this subgroup where then compared to a cohort of non-KS adult males, matched for both age ($p = 0.934$) and education level ($p = 0.275$) [*Tab. 6*].
KS patient were more prone to make mistakes in accentuation ($p < 0.001$), but not in pronunciation ($p = 0.119$) than controls [*Tab. 6*]. As a consequence, higher TBI scores were observed in KS subjects ($p < 0.001$), leading to inferior verbal IQ, performance IQ and overall IQ when compared to unaffected men ($p < 0.001$) [*Tab. 6*].
Younger age at time of diagnosis, as well as the achievement of adequate serum testosterone levels, did not show any correlation with the performance in TIB test and IQ [*Tab. 7*].
Not surprisingly, a positive correlation between verbal IQ and education level was observed (ρ 0.468, $p < 0.05$) [*Tab. 7*]. Furthermore, peculiar associations between older age and both performance and overall IQ (ρ 0.464, $p < 0.05$ and ρ 0.377, $p < 0.05$, respectively) were recorded [*Tab. 7*].

3.3 Experience, expression and control of rage

Among 34 patients who underwent STAXI-2 test, 26 of them returned a complete filled questionnaire, while in the remaining 8 cases incomplete or unclear responses were provided, thus invalidating the whole test. The main anamnestic and clinical characteristics of this subgroup are described in *Table. 8* [*Tab. 8*].
When considering each scale or subscale of the questionnaire, the majority of patients reached scores within 25^{th} and 75^{th} centile, which is currently considered the normalcy. However, up to one fourth of the KS males obtained scores > 75^{th} centile in few specific domain (i.e., ER/out, CR/in and ER/Index) [*Tab. 8*].
Interestingly, in young KS males (aged 20-29 years) lower scores in R/S scale ($p < 0.05$) and its subscales (RS/V and RS/F, $p < 0.0001$ for both) were reported when comparing to the age-matched validation cohort of STAXI-2 questionnaire [*Tab. 9*]. Lower scores in RS/V and RT/R subscales were observed in KS subjects (aged > 30 years) when compared to age-matched controls [*Tab. 9*].

Positive correlation between age at evaluation and normalized T-scores for R/S and RS/V emerged and were confirmed after correcting for potential confounders (ρ 0.418, $p < 0.05$ and ρ 0.435, $p < 0.05$, respectively) [*Tab. 10, 11*]. Interestingly, significant associations were observed between higher score in CR/in (ρ 0.418, $p < 0.05$), lower scores in ER/Index (ρ 0.435, $p < 0.05$, respectively) and earlier diagnosis of KS, after adjusting for covariates [*Tab. 10, 11*].

BMI has been shown to positively correlates with several items regarding both the predisposition to feel rage (i.e., R/T and RT/R, $p < 0.05$ for both) and its expression (i.e., ER/out and ER/in, $p < 0.05$ for both); correlations were maintained after adjusting for possible confounders [*Tab. 10, 11*].

Notably, KS males with adequate serum testosterone concentrations were reported to have higher scores in ER/Index (ρ 0.450, $p < 0.05$), as well as in scales/subscales involved in feeling rage (i.e., R/T and RT/R, $p < 0.05$ for both), similarly to that observed for BMI [*Tab. 10, 11*]. Moreover, these subjects also were characterized by higher scores in scales involved in expression of rage towards other people or objects (ER/out; ρ 0.408, $p < 0.05$) and lower scores in scales involved in its control (CR/out; ρ -0.513, $p < 0.05$), even after adjusting for covariates [*Tab. 10, 11*].

No correlation emerged between scores (both absolute number and normalized T-score) in STAXI-2 scales/subscales and other variables of clinical interest (i.e., arterial hypertension, occurrence of psychiatric/psychological disease, TRT and ED) [*Tab. 10, 11*]. In particular, no associations were observed between the occurrence of arterial hypertension and scores above 75th centile in all scales and subscales of STAXI-2 questionnaire.

4. Discussion

4.1 Prevalence and diagnosis

The cross-sectional design of the study does not allow to estimate a reliable prevalence of KS among male population, and multi-center studies, as well as the creation of a national registry, are advisable in order to have more detailed information about this topic.
With these limitations in mind, a cohort of 98 adult males with KS is currently referring to our Unit for periodic evaluation and follow-up, leading to an estimated prevalence of 8.9 cases per 100000 males. On the basis of the overall male population of our city and its district (~ 1.4 millions of people) and the number of hospitals providing andrological units (ranging from 8 to 12), it is possible to roughly estimate a current prevalence of KS ranging from 71 to 109 cases per 100.000 males, similar to what reported in literature [8, 16, 24-32].
However, the uneven distribution of the population and the unequal proportion of KS males referring to each hospital makes it very difficult to estimate the real prevalence of the syndrome and no definitive conclusion can be driven based on actual data.

As expected, the majority of KS males in the examined cohort had a classical 47,XXY karyotype, while only in 3 cases a 46,XY/47,XXY mosaicism was observed, representing the 4.2% of the diagnosis. This percentage is significantly lower than expected, since mosaicism is known to account for 10-20% of KS cases [5, 13]. We suppose that such discrepancy mainly relies on the limited sample size examined, which may not adequately reflect the characteristic of the KS male population of our city and its district. Furthermore, it should be considered that several mosaic KS males could still be underdiagnosed, since the milder phenotype that characterizes this karyotype (e.g., larger testicular size and less severe degree of androgen deficiency and impaired spermatogenesis) prompt less frequently patients to seek for medical consult [2, 4]. In accordance, two out of three 46,XY/47,XXY mosaic patients of the examined cohort were displaying a preserved bi-testicular volume, adequate serum testosterone levels and a normal concentration of vital sperm at semen analysis.

According to literature data, we observed that the majority of enrolled subjects were diagnosed in young adulthood (75% of cases, mainly ranging from 17 to 39 ages), often well passed the optimal time for the appropriate management of many clinical manifestations of KS, mainly regarding fertility and neuro-psychological issues [6-8].
Notably, a significant percentage of cases were diagnosed either prenatally or within the first year of life (8.3 of cases), confirming the efficacy and the usefulness of current screening techniques (i.e., amniocentesis, chorionic villus sampling, cell-free DNA testing and preimplantation genetic tests on blastocytes) in posing early diagnostic suspicion of KS, further confirmed by post-natal karyotype test in all cases [2, 6, 8, 14]. This

approach can be extremely helpful, since an early diagnosis can guarantee both adequate medical/psychological counselling to parents (thus reducing the number of aborting KS fetuses) and an adequate, specialized support to KS patients since childhood, particularly in areas on behavior and learning.
If the proportion of cases diagnosed prenatally was similar to what reported in literature, a remarkable percentage of KS diagnoses in the examined cohort was performed during childhood, leading to an overall rate of diagnosis in this age range higher than expected [2, 6, 8, 14]. While emphasizing once again the importance of early diagnosis, also during childhood, it is important to point out that the clinical features which lead to karyotype analysis in these subjects were mainly related to their physical appearance (e.g., tall stature, undescended testis) rather than learning difficulties or behavioral aspects (only 3 cases). Nevertheless, it is exactly during school age that several distinctive characteristics of KS become evident, especially learning disabilities in learning and reading, impaired word retrieval and syntax [5, 184]. Furthermore, the social setting in school attendance might also exacerbate behavioral problems frequently associated with KS, including shyness, impulsivity and withdrawal anxiety [5, 185]. All these attitudes, along with impaired verbal skills, are commonly observed among children in school age and are not pathognomonic of KS. Notwithstanding, such symptoms must not be underestimated, since they might represent a "*red-flag*" allowing early diagnosis of KS in a large number of patients. Therefore, a greater awareness and information on KS is recommended not only among physicians and pediatricians but also among teachers and school staff.
Lastly, a minority of patients received the diagnosis of KS over the course of medical evaluation for military service. Because military service is no longer compulsory in our country from 2003, young males undergo much more rarely a full andrological examination, contributing to increase the number of missed diagnoses.

4.2 Andrological features, testicular function and fertility

As expected, *testicular atrophy* confirmed to be a hallmark of KS, especially in presence of the classical 47,XXY phenotype [2-4]. The presence of firm and small testis (< 5 ml) is a peculiar characteristic of KS and it is easily assessable over the course of the andrological evaluation, thus representing e reliable ad effective tool for suspecting this syndrome. Testicular atrophy reflects the depletion of germ cells, the hyalinization of the tubules, the degeneration of the Sertoli cells and the hyperplasia of the Leydig cells observed in these patients [92]. Progressive degeneration of testicular structure is supposed to begin after pubertal onset; however, available data are still conflicting. The reasons underlying this clinical feature are largely obscure, but genetic background is thought to play a predominant role.

A remarkable number of examined patients (about 90%) was on TRT because of *primary hypogonadism*, confirming existing data on prevalence of androgen deficiency in KS males. Serum testosterone is known to settle in low-mid normal range in early adulthood and then progressively reducing below the normal range by the age of 25 years in a significant number of patients, ranging from 65% to 85%

[51, 56]. From this perspective the slightly higher prevalence of hypogonadal patients in our cohort is likely to be attributable to the median age of examined males (median 45 years).

Patients' age might also have influenced the therapeutic choice concerning the route of administration of testosterone, with a predominant number of patients using long-acting formulation, while a minority of them were on transdermal preparations. The latter might probably represent a better option in naïve and older people since it may offer advantages in terms of flexible dosing, self-administration and, in case of side-effects, immediate decrease in serum testosterone levels after cessation.

Notably, adequate testosterone serum concentrations were observed in about 70% of patients, regardless of the route of administration of TRT. Several factors may contribute in explaining the non-negligible number of subjects with inadequate testosterone levels, starting with the high prevalence of overweight and obesity in our cohort. In fact, the excess of adipose tissue may determine an increase in aromatase activity, thus reducing circulating testosterone and making it harder to reach adequate plasmatic concentrations. In addition, increased aromatization leads to a the relative hyperestrogenism and subsequent increase in hepatic SHBG synthesis, further contributing to reduce the serum androgen levels. Along with these hormonal mechanisms, the occurrence of pandemic Sars-Cov2 outbreaks significantly contributed the worsening hormonal profile of KS patients. Indeed, many patients did not attend their scheduled visit, resulting in a non-renewal of the prescription for TRT, which was then temporarily withdrawn. Finally, it should be noticed that some patients may also have temporarily stopped or reduced the dose of TRT because of concomitant occurrence of side effects, mainly represented by erythrocytosis.

Erythrocytosis was the main adverse event reported in our cohort during TRT. Erythrocytosis is defined as a hematocrit concentration > 53% or hemoglobin levels Hb > 18.5 g/dL in men, although this definition may vary according to different sources [211, 221, 222].

Exogenous testosterone administration is known to increases both hemoglobin and hematocrit up to 3.15 times, especially in older men, depending on testosterone dose, route of administration (higher risk was reported when using short-acting rather than long-acting injectable and transdermal formulations) and circulating concentrations [211, 221-223]. Potential mechanisms explaining the relationship between TRT and erythrocytosis include the direct stimulation of erythroid progenitor cells, the increased production of erythropoietin (EPO) by the kidneys and the suppression of hepcidin, leading to an augmented iron absorption, systemic transport and enhanced erythropoiesis. Moreover, recent advances hypothesized that estradiol could contribute to the genesis of erythropoiesis by increasing hematopoietic stem cells proliferation and survival.

According to our data, erythrocytosis was more likely to occur in patients on TRT with long-acting intramuscular formulations rather than transdermal testosterone, leading to hypothesize a more pronounced stimulus on bone marrow exerted by depot formulations. From this perspective the pharmacokinetic of transdermal preparations could better reproduce the circadian rhythm of testosterone, avoiding a sustained stimulation of bone marrow, and guarantee a rapid decrease in serum testosterone levels after discontinuation in case of side-effects.

Notably no correlation emerged between erythrocytosis and the achievement of adequate serum testosterone levels. Several factors may contribute in explaining this aspect. In particular, the rise of hematocrit can firstly be due to preanalytical errors (i.e., excessive time elapsed between blood sample collection and analysis) or patients' hydration status, the latter being negatively influenced from both reduced liquid intake (e.g., decreased thirst perception in elderly) or increased water loss (e.g., concomitant diuretic therapy) [221, 223]. In addition, smoking habits and metabolic comorbidities (e.g., obesity and OSAS) are known to stimulate red blood cell synthesis, and all these risk factors were found to be highly prevalent in the examined cohort, despite non being significantly related to the occurrence of erythrocytosis, mainly because of the sample size [211, 221-223]. It is also reasonable to assume that the heterogeneity in individuals' bone marrow response to exogenous testosterone might rely on different threshold, thus explaining why some patients are more prone than others to develop erythrocytosis even in presence of similar risk factors and serum testosterone below or in the low normal range. Finally, the choice of considering the achievement of adequate serum testosterone levels might have hindered a correlation between erythrocytosis and plasmatic testosterone, since the latter should be more correctly interpreted as a *continuum* rather that a dichotomic variable.

Erythrocytosis enhances blood viscosity and platelet adhesiveness, suggesting an increased cardiovascular risk [221]. This aspect is even more important in KS, because these subjects already present an increased cardiovascular and thrombotic risk. However, to date no randomized or prospective studies have observed a direct relation between TRT-induced erythrocytosis and thromboembolic events [221, 222]. Furthermore, it is still unknown the hematocrit level at which the risk of neuro-occlusive or cardiovascular events increases and the frequency of neuro-occlusive events in men with hypogonadism enrolled in RCTs of T who developed erythrocytosis has been very low [211].

Data from our study further confirmed *infertility* to be, along with hypogonadism, a hallmark of KS. Azoospermia was observed in almost all patients who underwent semen analyses, thus confirming current literature data [2, 4, 94].

Azoospermia directly relies on the maturation arrest and progressive testicular tubular atrophy, hyalinization and sclerosis, but whether these processes lie on primarily genetic defects or are a direct consequence of the impaired gonadal microenvironment is still debated [2]. Likewise, the exact timing at which these alterations arise is also unclear. On the one hand, some author hypothesized that testicular degeneration and germ cells hypoplasia might be present already during intrauterine development, in contrast with evidences reporting the presence of testicular germ cells (despite reduced in number) in pre-pubertal KS boys, along with a preserved tubular morphology [89, 91]. These latter aspects suggest that testicular progressive degeneration actually arises at puberty, and is supported by the mild testicular enlargement and serum elevation of testicular synthetized hormones as testosterone, Inhibin B and INSL-3 observed in the early pubertal phase [2, 63, 64]. AR is thought to significantly contribute to these processes. In fact, in healthy males, the AR is located in the cytoplasm in absence of androgens, while it was proved to appear in immature Sertoli cell nuclei soon before the onset of puberty, together with the rising in FSH and testosterone concentrations [40]. Interestingly, KS

boys display a constant AR expression in their Sertoli cell cytoplasm instead of in Sertoli cell nuclei when compared to age-matched controls [40]. Such differences are likely to rely on the "female gene dosage" caused by the supernumerary X chromosome. In particular, a large number of belonging to the "*testis-specific-genes*" and mapped on X chromosome, escape X-silencing process. These genes are currently expressed in male germ cells and play a crucial role in guaranteeing both their survival and differentiation. Thus, it is not surprising that molecular and genetic mechanisms that alter the proper dosage of X-encoded genes in testicular cells may, during puberty, initiate the degeneration process in the testes of boys with KS [224].

This hypothesis is in accordance with the progressive decrease in testicular volume and hormonal alterations (i.e., progressive increase in gonadotropin, estrogens and SHBG along with reduction in testosterone, inhibin B, INSL-3 and AMH concentrations) observed from mid-puberty [2, 65].

Albeit being historically considered infertile because of the very high prevalence of azoospermia, recent studies reported the existence within KS testis of foci with preserved spermatogenesis, leading to vital sperm retrieval through mini-invasive surgery (i.e., TESE and microTESE) or, much less frequently, directly from ejaculate [4, 92-94].

In our cohort one out of three patients with azoospermia underwent surgical approach, especially those who were early diagnosed. Notably, among patients who declined the procedure there were not only older people, no longer interested in fatherhood, but also younger patients (aged < 25 years), who preferred to reconsider surgical intervention in the next future or unwilling to discuss about the fertility issue. In particular, this latter aspect should prompt clinicians to always exhaustively inform patients about the potential consequences of their choice, eventually reviving the discussion over time.

This issue acquires even more importance in lights of the percentages of sperm retrieval reported in literature, accounting for up to 50% of the procedures and leading to successful ART fertilization and live birth rate in 40-50% and 16% of cases, respectively [94, 95]. From this point of view, the sperm retrieval rate observed in our study was even lower (~ 10%, 2 cases), primarily because of the small sample size. Unsuccessful ART was reported, and patients had to resort heterologous ART or adoption.

In order to maximize sperm retrieval rate, several predictive factors have been proposed, including younger age, larger testicular volume and hormonal pattern, thus emphasizing earlier surgical intervention and/or the use of medication aimed at improving intratesticular testosterone concentrations in order to ensure a better outcome [87, 96-98, 103]. In accordance to these evidences, data from our cohort confirmed that a lower testicular volume (likely reflecting a more severe testicular degeneration) and exogenous administration of testosterone (leading to reduced pituitary secretion of FSH/LH, further decreasing intratesticular testosterone levels), were positively associated with the occurrence of azoospermia. At present we are not able to evaluate whether these variables are also reliable in predicting the surgical outcome, since the very low number of successful sperm retrieval makes it difficult to exhaustively analyze this issue.

Notwithstanding, none of these clinical variables were found to predict the sperm recover rate in KS males, suggesting that the focal spermatogenesis in KS testes may survive the progressive seminiferous tubule hyalinization and that other factors, including individual's predisposition, should be involved [94].

Gynecomastia, of various degrees, was confirmed to be a common clinical finding in adult KS males, although the prevalence recorded in our cohort (~40%) is slightly lower than that reported in literature (up to 75%) [2]. This discrepancy might in part be due to the small size of the examined group but could also rely on an underdiagnosis of this clinical feature in our daily clinical practice. Although the presence of hypertrophic breast tissue can usually be confirmed by physical examination, in some cases the correct diagnosis of gynecomastia could be challenging, in particular when glandular enlargement is modest or when concomitant fat deposition is present (i.e., adipomastia and mixed gynecomastia), requiring ultrasound examination in order to confirm the diagnosis [57, 58].

Etiology of gynecomastia is still debated, both in KS and non-KS males. Androgen deficiency is known to favor the genesis of gynecomastia, but other factors should be taken into account, including increased aromatase activity (stimulated by both high FSH concentration and excess of adipose tissue) and increased serum SHBG concentration, leading to relative or absolute hyperestrogenism [57, 58, 106]. From this perspective, the cross-sectional design of the present study does not allow to draw any conclusion about possible causal factors of gynecomastia, since precise data on age of onset, concomitant hormonal profile and clinical features are not available. However, the lack of correlation between the occurrence of gynecomastia and clinical features possibly involved in its genesis (BMI, age at diagnosis, achievement of adequate serum testosterone concentrations), further support the hypothesis of a multifactorial etiology.

The absence of correlation with adequate plasma testosterone levels also confirms that TRT alone is not effective in inducing complete regression of the gynecomastia also in KS males. Despite being benign and not representing a risk factor for breast cancer, gynecomastia may alter patients' body image and, in some cases, reduce his self-confidence and self-esteem perception [62]. This latter aspect was clearly pointed out in our study, where 25% of patients underwent surgical approach for resolving gynecomastia.

Physical examination and testicular ultrasound allowed diagnosing *varicocele* in 12.5% of cases, in most cases monolateral and of mild entity. Current data on prevalence of varicocele in KS males are scanty. An Italian study in a cohort of 40 adult KS patients pointed out a similar prevalence of varicocele (11.5%), while slightly higher percentages (23.3%) were reported in an Argentinian cohort, possibly due to the young age of enrolled patients (44 out of 94 subjects were aged < 18 years) [63, 225]. Albeit KS males present a peculiar testicular anatomy and architecture, to date there are no evidences suggesting a higher occurrence of varicocele in these subjects, whose prevalence is substantially comparable to that observed in the general male population (5-20%) [226].

To date, data regarding the prevalence of *BPH* in KS males are really scanty and dates back in the early 80s. As far as we know, prostate volume and prostate-specific antigen concentration does not significantly differ between KS and non-KS males, both before and during TRT [227].

Interestingly, an Italian study enrolling 121 naïve non-mosaic KS patients and 60 age-matched healthy male controls, reported a positive correlation between prostate volume and growth during TRT and visceral obesity, insulin-resistance in KS, independently from androgen or estrogen levels [75]
Notably, a lower risk of prostate cancer was observed in a wide cohort of KS (HR 0.58) when compared to non-KS patients [206, 228]
The underlying causes may only partially depend from hypogonadal state, since the lower prevalence of prostate cancer is confirmed also in patients on TRT. From this perspective, estrogens are likely to play a pivotal role. In fact, KS males are known to have a slightly higher estrogen concentration, exerting an antiproliferative and anti-inflammatory actions on prostate gland by interacting with their ß receptor (ER-ß). On the other hand, obesity and smoking habits may promote a chronic inflammatory state, leading to a reduced expression of ER-ß in favor to its α isoform (ER-α, whose activation promotes cell growth and proliferation), thus theoretically increasing the risk of malignancies and explaining in part the occurrence of prostate cancer in this subgroup [229-231].

4.3 Metabolic and Cardiovascular disease

According to current data, KS is burdened with a higher mortality rate (almost doubled) and a lower expected lifespan (ranging from 2 up to 5.6 years) when compared to unaffected men. Metabolic and cardiovascular comorbidities are highly prevalent among adult KS males, and are thought to be one of the main determinants of the abovementioned increased mortality [2, 5, 31, 67, 109, 110, 115].

The present study pointed out a remarkable prevalence of both *overweight* and *obesity* among adult KS males, the latter almost doubled when compared to non-KS adult males (20.8% vs 10%) [232]. Likewise, also the prevalence of *IGT/IFG* (~ 23%) and overt *T2DM* (~ 15%) observed in the examined cohort were significantly higher than in the general population (10% and 6%, respectively). Finally, when compared to healthy non-KS males, increased prevalence of *hypercholesterolemia* (42.5% vs 20-30%) but not of *hypertriglyceridemia* (23% vs 30%) were reported [232].
Noteworthy, the multiple correlations emerged between weight excess, alterations in lipid and glucose metabolism, arterial hypertension and older age depict a subgroup of patients with several cardiovascular risk factors, making an appropriate cardiological investigation and follow-up advisable.
The mechanisms underlying these metabolic alterations are largely unknown. Hypogonadism is supposed to play a pivotal role by reducing insulin sensitivity and causing unfavorable changes in body composition (reduced lean mass along with abdominal fat deposition), further reducing the serum androgen concentrations because of enhanced aromatase activity [5, 22, 50, 60, 67]. Accordingly, the negative correlation emerged in the present study between BMI and achievement of adequate serum testosterone concentrations directly

support this hypothesis, even if others confounders (i.e., the reduced absorption of transdermal testosterone described in obese men) should be considered.
Furthermore, a possible causal role of estrogens and SHBG concentrations, especially in the genesis of T2DM, was hypothesized, but supporting evidences are conflicting [2, 4, 122, 123].
On the other hand, recent data suggested that insulin-resistance might also involve Leydig cells, reducing testicular response to both pituitary LH and exogenous human choriogonadotropin, further compromising the already hampered testosterone production in KS males [5].
Nevertheless, the genetic background itself might directly affect the risk of obesity and impaired glucose and lipid metabolism, regardless of testosterone concentrations. In fact, these features can be retrieved also in pubertal and young adult KS males, at an age when androgen deficiency could have not occurred yet or is relatively less pronounced. Moreover, this peculiar body composition partially persists even after reaching adequate serum testosterone concentrations with TRT, further suggesting that these alterations are, at least in part, independent from hypogonadism [5, 60, 67, 111].

When considering *cardiovascular disease* in the present study only 1 major cardiovascular event was reported. However, the high prevalence of well-established cardiovascular risk factors and ponderal excess, arterial hypertension and impaired lipids and glucose metabolism, allows to define these patients as having an increased cardiovascular risk, confirming current knowledge on KS [2, 31, 109, 110]. The cross-sectional design of the study and the small sample size with the occurrence of a single cardiovascular event did not allow to analyze the impact of TRT on cardiovascular outcomes. Therefore, further researches are needed to better describe the cardiovascular complications in KS, possibly elucidating the involved pathophysiological mechanisms and defining the contribution of TRT in restoring cardiovascular health in these subjects.

In view of these considerations, the occurrence of *ED* in this clinical setting should not be underestimated. About 1 out of 4 patients enrolled in the study was suffering from ED (23%), especially when aged > 40 years. This data confirms the prevalence of ED in non-KS matched-age Italian males, estimated in ~ 20% and then increasing over time [233]. Similarly to that observed in the general population, the correlation between ED, age and arterial hypertension strongly supports the hypothesis of a vasculogenic component of the disease [234].
Noteworthy, it should be highlighted that ED represents a well-established predictor of cardiovascular events [235-237]. In fact, the onset of ED may anticipate of a few years the occurrence of a major cardiovascular events, probably because of the smaller diameter of penile arteries when compared to coronary ones [235, 236]. Moreover, the presence of ED (especially when severe) was demonstrated to double the patients' cardiovascular risk, even in younger subjects, currently considered as a "low-risk" population [237].
Hence, moving from the knowledge that KS patients have an increased cardiovascular risk, the occurrence of ED in a KS males should not be underestimated, since it might represent an "alarm sign" allowing the early diagnosis of cardiovascular disease and subsequent appropriate diagnostic and therapeutic workup.

The aforementioned increase in cardiovascular risk and mortality does not rely only on metabolic comorbidities but it might be influenced also by alterations in cardiac morphology, kinesis and rhythm along with a thrombophilic state. Indeed, KS patients are characterized by subclinical abnormalities in left ventricular (LV) systolic and diastolic function and endothelial function, which, when associated with chronotropic incompetence, may lead to reduced cardiopulmonary performance [238].

Furthermore, alterations in valvular morphology and functionality (mainly affecting mitral valve) could contribute in worsening the cardiac function [5, 131].

Only one third of enrolled patients already underwent cardiac ultrasound at time of evaluation, thus limiting the possibility of depicting an exhaustive overview of cardiac alterations in the examined cohort. Mild tricuspid insufficiency was found to be the most prevalent alterations, but this condition is frequently observed in the general population (up to 70% of people) and, in presence of a structurally normal valve, it should not be considered as pathologic [239]. Likewise, all other valvopathy were mild and without any significant clinical and hemodynamic impact. Mild left ventricular hypertrophy was finally reported is few cases but, in our opinion, it should be more likely related to ageing and the presence of arterial hypertension rather than to KS itself.

Our study also highlighted the occurrence of few cases of *arrythmia*, predominantly atrial fibrillation or flutter, thus confirming the few available literature data [2, 5]. To date, the real prevalence of atrial fibrillation and, more in general, rhythm abnormalities in this setting is unknown. However, when evaluating a cohort of 851 KS males with no history on atrial fibrillation nor myocardial infarction, the KS itself was found to be an independent risk factor for occurrence of atrial fibrillation [240].

No cases of short QTc-intervals were reported in our cohort. Nevertheless, in a recent study KS males were observed to have a shorter QTc-interval than non-KS controls and, notably, a shorter QTc was described among patients on TRT [77]. In this same study, no mutations in genes related to short QT syndrome were found; however, the overexpression of genes encoded in the pseudoautosomal region of X chromosome (e.g., SLC25A6 gene, involved in calcium signaling pathway) might contribute in shortening the QTc of KS patients [2, 41, 77]

In accordance to literature data, the present study confirmed the high prevalence of *venous thrombosis* in KS patients [2, 67]. As far as we know, thrombotic risk in KS patients is comparable to that of an inherited thrombophilia, as factor V Leiden and prothrombin mutations.

The fact that venous thrombosis often occurs before diagnosis of KS, and consequently before the start of TRT, suggests that the underlying mechanism might rely, at least in part, on a genetic background. Moreover, the lack of correlation with known pro-thrombotic risk factors (smoking habits, older age, BMI) reported in this study seems to strengthen this hypothesis.

In accordance, increased levels and activity of factor VIII coagulant (whose gene is mapped on the X chromosome) were observed in a KS patient with venous thrombosis, as well as homozygous mutations in methylenetetrahydrofolate reductase (MTHFR) gene and elevated activity of plasminogen activator inhibitor-1 (PAI-1), the latter favoring platelets aggregation [67, 241]

4.4 Bone parameters, endocrine dysfunction and autoimmunity

At the time of evaluation, all enrolled patients already underwent regular and periodic assessment of serum vitamin D; in a large number of them the BMD evaluation had already been performed or scheduled in the following months, underlying a great awareness among clinicians about bone complications of KS.

With regard to bone metabolism, *vitamin D deficiency* confirmed to be highly prevalent among KS males [2, 76, 160].

Similar to vitamin D deficiency, *reduced BMD* consistent with osteopenia or osteoporosis was recorded in one out of three patients who underwent lumbar and/or femoral morphometry (~ 35%), thus confirming the high prevalence of bone alterations in KS. In particular the prevalence of both osteoporosis and osteopenia is in accordance with current literature evidences (10% vs 6-15% and 25% vs 25-48%, respectively) [5].

Similar to what observed in the general population, our study confirmed also in KS males the relationship between ageing and both hypovitaminosis D and reduced BMD. However, reduced BMD usually occurs in older people than what observed in our cohort (median age 45.0 years), suggesting the existence of other pathophysiological mechanisms involved in the genesis of bone alterations. From this perspective, the extent and duration of untreated hypogonadism, the reduction of LH-mediated testicular vitamin D hydroxylation and INSL-3 synthesis, as well as FSH-induced osteoclastogenesis might be indicated as potential causal factors [2, 76, 84, 146, 149, 153, 159, 160, 165].

Moreover, older people are known to have a higher prevalence of vitamin D deficiency, due to a reduced dermal capacity to activate vitamin D, impaired renal hydroxylation of 25(OH)D to $1,25(OH)_2D_3$ with declining renal function and resistance to the action of vitamin D metabolites on the bowel mucosa [242]. Vitamin D also stimulates the proliferation and differentiation of skeletal muscle fibers, maintaining and improving muscle strength and physical performance. Therefore, the age-dependent decline in vitamin D concentrations could induce sarcopenia, thus reducing the BMD and increasing the risk of falls and bone fractures [243].

Further researches are needed in this field, in order to better understand the real impact of early diagnosis of KS and TRT on bone outcomes. In addition, the advent of new diagnostic techniques able to evaluate both mineral density (BMD) and bone microarchitecture (e.g., *Trabecular Bone Score* - TBS) are advisable and could lead to further breakthroughs in understanding bone alterations and estimating fracturative risk in KS.

Concerning *thyroid function*, few cases of goiter and autoimmune thyroiditis were observed. In particular the prevalence of Hashimoto's thyroiditis was similar to that observed in non-KS males (~ 4%) [109, 167, 173]. The small number of enrolled patients, as well as the cross-sectional design of the study and the variability of collected data regarding thyroid function (e.g., hormonal evaluation not performed in centralized laboratory and frequent lack in fT4 and/or fT3 evaluation along with TSH dosage) did not allow us do draw any further conclusion about thyroid dysfunction in KS males. Alteration in pituitary response to TRH and reduced fT4 secretion followed by an adaptative response of type 2 deiodinase activity were hypothesized in

KS males, although further studies are needed to deeply investigate this issue [171].
Finally, few cases of *autoimmune disease* were reported, according to observational data from national registers. [5, 174]. However, the correlation between KS and autoimmunity is still debated and further research on a larger cohort are required.

4.5 Neuro-psychological features and other comorbidities

Current knowledge on *neurological feature* and comorbidities on KS are very scarce and mostly deriving from case-reports or small case series [5, 244, 245]. The first reports date back to the 70s, when the prevalence of epilepsy in KS patients was found to be higher than in healthy men, but not dissimilar to that observed in patients in mental hospitals. Therefore, authors hypothesized that the occurrence of neurological symptoms in KS had basically to be regarded as coincidental findings rather than being related to the chromosome abnormality [245]. In the next decades, only few studies reported cases of epilepsy in KS male; in accordance, no cases of epilepsy were recorded in our study.

Among neurological comorbidities of KS, the occurrence of *essential tremor* in up to 25% of KS males was reported in different case series, especially in young subjects [5, 244, 245]. Interestingly, out study confirmed the higher prevalence of this peculiar disease in 8 cases (11% of the whole cohort), currently representing one of the most numerous subgroups ever described in the literature. Notably, the tremor was confirmed to arise in young age in almost all patients and significantly reduced their quality of life, often requiring the start of medical therapy.
Essential tremor is basically characterized by an action tremor, with a less evident resting component, predominantly affecting bilateral upper extremities, with few patients having head and voice tremor. Mild resting tremor was previously reported in five KS patients.

Furthermore, KS males often suffer from nonspecific motor impairments encompassing reduced muscle strength, running speed, agility, and coordination as well as a large prevalence of essential tremor [5, 246, 247]. In particular, the lack of muscle buildup, leading to decreased lean body mass and muscular hypotonia was often observed in KS males, regardless of testosterone levels and with poor response to TRT [247].

According to our data, about one out of four patients was suffering from *psychiatric/psychological disorders*, predominantly anxiety and depression, followed by schizophrenia spectrum disorders. These findings further confirm current literature data, emphasizing the high prevalence of behavioral and emotional alterations in KS male which might negatively impact on patients' social domains, social interactions and quality of life [5, 190, 248, 249].
In particular, it has been questioned whether the occurrence of *depression* and *anxiety* relies on genetic alterations in KS or are consequences of physical and emotional traits due of the syndrome. Morphological

alterations in cerebral nuclei involved in the regulation of emotions, aggressive and impulsive behaviors (i.e., amygdala and other limbic structures) are likely to contribute to the etiology of psychiatric features on KS [248]. On the other hand, the causal role of acquired hormonal factors, such as androgen deficiency, should be taken into account. Indeed, testosterone plays a pivotal role in maintaining balance within the multi-dimensional psychological network of mood, behavior, self-perception and perceived quality of life in men of any age. Testosterone is known to positively and actively modulate various traits of mood, aggression and anxiety (encompassing phobic anxiousness, panic syndromes and unfocussed fear) [249]. Accordingly, androgen deficiency is often observed in patients complaining of low vigor and vitality, irritability, lack of assertiveness, anxiety and depression [249]. However, current data on beneficial impact of TRT on depression and mood alterations in KS patients are scanty and controverse [248].

When considering the schizophrenia spectrum disorders, KS has been found to show higher scores in all domains regarding schizotypal and schizophrenia symptoms. Conversely, a higher prevalence of KS when compared to general population was reported in cohorts of patients suffering from schizophrenia and other psychotic disorders [248].

Finally, it should be emphasized that late or misdiagnosis of all the aforementioned alterations in mood and behavior observed in KS males, regardless from their underlying pathophysiological mechanisms, may have the greatest negative impact on quality of life and social behavior oh these patients. Indeed, a greater awareness of the psychiatric and psychological features in KS is recommended, in order to guarantee early diagnosis and appropriate support to all needy patients.

Concerning the occurrence of *oncological diseases*, the reduced sample size and the cross-sectional design of the study does not allow to draw any exhaustive conclusion with regard to this topic. According to literature evidences, KS is associated with higher risk of specific malignancies, especially extragonadal germ cell tumors and breast cancer [2, 202, 203]. However, because of the low overall incidence of these neoplasms, a routinary screening in asymptomatic KS patients is not recommended yet [2, 202]. With these limitations in mind, one case on mediastinal germ cell tumor and 2 cases of benign breast lesions were reported, along with testicular cancer and Leydig cells hyperplasia. To date, few studies reported the occurrence of testicular neoplasms and Leydig cell hyperplasia in KS, allowing to speculate that alterations in gonadal architecture, along with increase in gonadotropin concentrations and a peculiar intratesticular hormonal milieu, might contribute to the oncogenesis of these complications [225].

4.6 Verbal abilities

Verbal deficits, especially in the areas of learning and reading, word retrieval and syntax, are often described to affect KS patients since their early childhood [5, 184]. The pathophysiology underlying language dysfunctions is still a matter of debate but a growing body of literature supports a predominant role of the genetic background. In fact, the X-chromosome inactivation (XCI) pattern and neurocognitive X-linked gene expression were tested and correlated with intelligence quotient (IQ) scores as well as morphological abnormalities of the temporal lobe [5, 250]. Although a small, but significant downward shift in mean overall IQ has been reported, many KS subjects are not significantly impacted by cognitive concerns and achieve success in academic, personal, and career endeavors [195, 251]. Nevertheless, according to recent data, less than 10% of KS males achieve a higher education, whereas retirement age on average is more than 15 years earlier (43.5 vs. 60.3 years) than in non-KS males [5].

Disabilities in reading and learning arise and become more evident during school age and, if correctly recognized, they might allow diagnosing KS in a larger number of young people and provide them adequate specialized support [5]. Earlier detection and intervention of reading, grammar and phonological errors may reduce the risk for later language and literacy challenges and optimize academic, and ultimately social and behavioral difficulties later in life. On the other hand, the underestimation and misdiagnosis of these features might exacerbate behavioral problems (e.g., shyness, impulsivity and withdrawal anxiety), further worsening the neuropsychological background of these patients [5, 185].

In lights of these considerations, the use of validated questionnaires (in particular when self-administered) could represent a very useful, quick and effective tool for screening verbal disabilities in a wide number of people. In accordance with this hypothesis, the administration of the validated TIB questionnaire proved to be effective in highlighting differences between adult KS males and age-matched unaffected controls. This test confirmed that both overall and verbal IQ tend to be slightly lower in KS males than non-KS males, without however reaching values within disability range [5, 195].

Our study also pointed out that KS males are much more prone to make mistakes in accentuation while reading than controls, regardless of patients' age at the time of diagnosis, thus confirming that it probably constitutes an intrinsic feature of KS. To date, few studies analyzed the performance of self-administered questionaries for estimating IQ or exploring verbal skills in KS, and there are no available reports in literature on the use of TIB test on KS patients. With these limitations in mind, the high number in accentuation mistakes, rather than the lower IQ, can be interpreted as a "red-flag" for early recognizing verbal disabilities. Because difficulties in learning and reading are quite common among children in school age, the TIB might represent a screening tool for suspecting impaired verbal skills (high sensibility) rather than a test aimed at confirming the diagnosis of KS (scarce specificity).

However, at present the TIB test was validated only in patients ages > 16 years and further studies are needed to verify its applicability at a younger age. Meanwhile, a greater awareness and information on KS is recommended not only among physicians and pediatricians but also among parents, teachers and school staff.

4.7 Experience, expression and control of rage

Behavioral alterations in KS, including antisocial or inappropriate behavior relying on increased *impulsivity* and *anger*, have been reported in KS. If misdiagnosed, these aspects may have a detrimental impact on patients' abilities of coping with social situations characterized by high levels of social distress and/or social interactions, thus exacerbating feelings of anxiety, low self-esteem and worsening their quality of life [5, 185].

When evaluating the experience, expression, and control of anger in KS males through the STAXI-2 questionnaire, the majority of patients reached scores similar to those observed in the general population and ranging between the 25th and the 75th centile, which is currently considered the normalcy [216]. Notably, KS patients were characterized by significantly lower scores than non-KS males in domains evaluating their emotional state of anger and its verbal expression at time of filling the questionnaire, both at young and older age. Furthermore, reduced scores in the RT/R domain, evaluating the tendency of a person to respond with rage or to become agitated when perceiving to be treated unfairly, being criticized or receiving negative feedback from others, were observed in KS adult patients when compared to the control group. The underlying mechanism of anger expression and control in KS have not been deeply studied and understood yet. Lower scores in patients' anger level and expression at time of filling questionnaire could rely on a *bias* (i.e., people might be calmer when filling the module in a hospital, in front of a physician) but we can hypothesize that they might be a peculiar feature of KS syndrome, potentially caused by both androgen deficiency and anatomical brain morphology in areas involved in controlling emotions owing to genetic predisposition [5, 177-179]. This latter hypothesis could be supported by the not-negligible number of KS males (up to 25%) reaching scores > 75th centile in specific domains evaluating the overall index estimating the person's tendencies to express anger, in particular outwardly toward other people observed in our study. These findings further allow to speculate that KS males could be aware of their tendence to feel and express anger more easily than other people, thus prompting them in spending several energies for controlling these feelings, as confirmed by the high number of subjects (one out of four patients) reaching scores > 75th in the CR/in domain, which evaluates persons' ability and efforts to relax, calm down, and suppress anger before losing control.

Patients' age is likely to significantly impact on the experience, expression and control of rage. Older KS patients tend to experiment higher intensity of anger at a particular time, possibly reflecting the negative impact of body image (e.g., testicular atrophy, gynecomastia, obesity), self-esteem, and clinical features of the syndrome (e.g., impaired fertility, hypogonadism) on their emotional status, rather than being caused by e genetic background. From this perspective, the positive correlation observed between BMI and predisposition to both feeling anger and expressing rage seems to further confirm this hypothesis.

On the other hand, early diagnosis may be crucial for ensuring adequate psychological support to these patients, thus improving their awareness and comprehension of the disease, clinical manifestation and comorbidities, and finally leading to an improved quality of life [2]. In accordance to this theory, an earlier diagnosis of KS was found to correlate with a reduced overall tendence to express anger along with an increase in efforts to control the rage before losing control.

Serum testosterone concentration could also be involved in modulating anger expression and control in KS

patients. Data from our study highlighted a positive correlation between the achievement of adequate testosterone levels and patients' predisposition in experimenting feelings of anger.

Testosterone levels are known to impact on both feelings and expression of anger, especially when administered at supraphysiological doses. On the other hand, the real impact of restoring the physiological serum hormone concentration obtained with TRT on determining the intensity of anger feelings and expression is still debated, and several factors might be involved (i.e., stress-induced cortisol secretion, individual's sensitivity to testosterone, mono-amino-oxidase A activity) rather than the absolute serum testosterone concentration [252, 253]. To date few studies evaluated the impact of TRT on the emotional sphere in KS males, with no specific focus on anger [254]. Hence, prospective and interventional studies are required to deeply investigate this issue.

Notwithstanding, neuroimaging techniques in adult males have shown that testosterone activates the amygdala enhancing its emotional activity and its resistance to prefrontal restraining control, thus suggesting a possible mechanism underlying TRT-induced rage expression in KS, whose amygdala is known to present a peculiar morphology [179-255].

Notably, some authors observed that higher scores (> 75th centile) in domains evaluating overall anger experience and expression (either inwardly toward himself/herself or outwardly toward other people) were positively correlate with the incidence of arterial hypertension, and cardiovascular disease (including myocardial infarction) along with an increased risk of all-cause hospitalization [217-220]. In relation to these issues, no correlations were recorded in our study, probably because of the small size and the young age of the examined subgroup, the latter representing a probable *selection bias* on the prevalence of arterial hypertension and cardiovascular diseases.

At present, no data about the use of STAXI-2 questionnaire for the evaluation of anger experience, expression, and control in KS males are reported and further studies are needed to confirm our findings in a larger number of patients.

Moving from the fact that KS patients present a higher cardiovascular risk and that scores > 75th in STAXI-2 questionnaire were associated to an increased risk of cardiovascular events, the application of this test in the daily clinical practice might represent an additional tool for the assessment and stratification of patients' cardiovascular risk, thus providing specific diagnostic and therapeutic workup [217-220]. In fact, an improved ability of anger-control was associated to a decreased risk of myocardial infarction (HR 0.75) and holistic approach based on mindfulness-based art therapy was proved to be effective in inducing both psychological/emotional stability and coronary relaxation in patients with established coronary artery disease [256]

4.8 Study analysis

This study allows to depict an exhaustive overview on KS characteristics in a cohort of adult patients in a "real world evidence", underlying the high complexity of this syndrome. In particular the availability of data regarding both clinical (i.e., hormonal, metabolic) and behavioural/cognitive parameters allowed to draw an initial evaluation about the complex interactions between these factors and their impact on patients' health and quality of life, even in hitherto unexplored areas. Moreover, the significant results obtained using validated questionnaires (TIB and STAXI-2) might pose the basis for their more extensive use in KS males, both with research and diagnostic purposes.

Nonetheless, these results should be interpreted in the light of some limitations. First of all the small size of the examined cohort, especially when considering specific subgroups, may have hindered to properly analyse the occurrence of some clinical features (e.g., erythrocytosis, sperm retrieval rate, etc.) as well as limiting the possibility of individuating their predictors or etiological factors.

Secondly, the cross-sectional design of the study did not allow to fully depict the baseline clinical and hormonal characteristics of the cohort, especially prior to the start of TRT. Similarly, an exhaustive evaluation of the diagnosed comorbidities over time, as well as the evaluation of TRT impact on the comorbidities themselves and other clinical outcomes of interest was foreclosed. Furthermore, the cross-sectional design provides only statistical associations between analysed variables but does not allow deriving a causal relationship. Hence, multicentre and prospective studies are needed to further confirm these findings and exhaustively investigate the pathophysiological mechanisms underlying physical and psychological alterations of the syndrome. Our Andrological Unit is currently participating to a national multicenter, prospective, observational trial on adult KS males promoted by the *Italian Society of Andrology and Sexual Medicine* (SIAMS) and the *Klinefelter Italian National Group* (K.I.N.G.). From this perspective, all data collected in the present study will be made available for a national analysis, thus contributing to estimate the real prevalence of KS in the Italian population and to evaluate clinical and biochemical features of the syndrome, as well as the quality of life of KS patients.

The last limitation concerns serum testosterone levels assessment, which was not standardised: analyses were not performed in a centralized laboratory, daily time of blood collection was widely variable among subjects as well as the time elapsed since the last testosterone administration (despite medical advices, both for intramuscular and transdermal preparations) and in some cases only free testosterone but not total serum testosterone was available. Therefore, testosterone was interpreted as a dichotomic variable (i.e., adequate or non-adequate serum testosterone levels) rather than a continuous one, possibly hampering an adequate analysis and correlation with outcomes of clinical interest.

5. Conclusions

Even after 80 years from being firstly described, Klinefelter syndrome confirmed to be highly complex, with a wide heterogeneity in clinical phenotype which still continues to pose substantial diagnostic challenges, resulting in a significant number of patients misdiagnosed or undiagnosed.

Diagnosis in often delayed, accounting in the majority of cases during medical care for hypogonadism, sexual dysfunction or infertility workup, well passed the optimal time for the appropriate management of many clinical manifestations and comorbidity related to KS.

Along with testicular atrophy, hypogonadism and infertility, which confirmed to be a hallmark of this syndrome, KS males present a higher prevalence of several hormonal (vitamin D deficiency, thyroid disorder and increase fracturative risk) and metabolic comorbidities (diabetes mellitus, dyslipidemia, and metabolic syndrome), thus increasing their cardiovascular risk and overall mortality.

A dedicated cardiovascular, hormonal and metabolic workup, aimed at reducing their cardiovascular risk through precocious diagnoses and adequate management of the aforementioned comorbidities, is advisable.

Moreover, KS males face a bewildering array of neurocognitive, behavioral and social problems, including a greater vulnerability to psychopathology, which are only beginning to become apparent in recent years but also require early diagnosis and a tailored approach to reduce their impact on patients' quality of life.

In view of these considerations a broadening of knowledge on KS and its manifestations is advisable, prompting to a multidisciplinary approach aimed at guaranteeing both physical and psychological wellbeing through the early diagnosis and adequate treatment and follow-up, from childhood to senescence.

Figure 1. Flow-chart of screening and enrolment process.

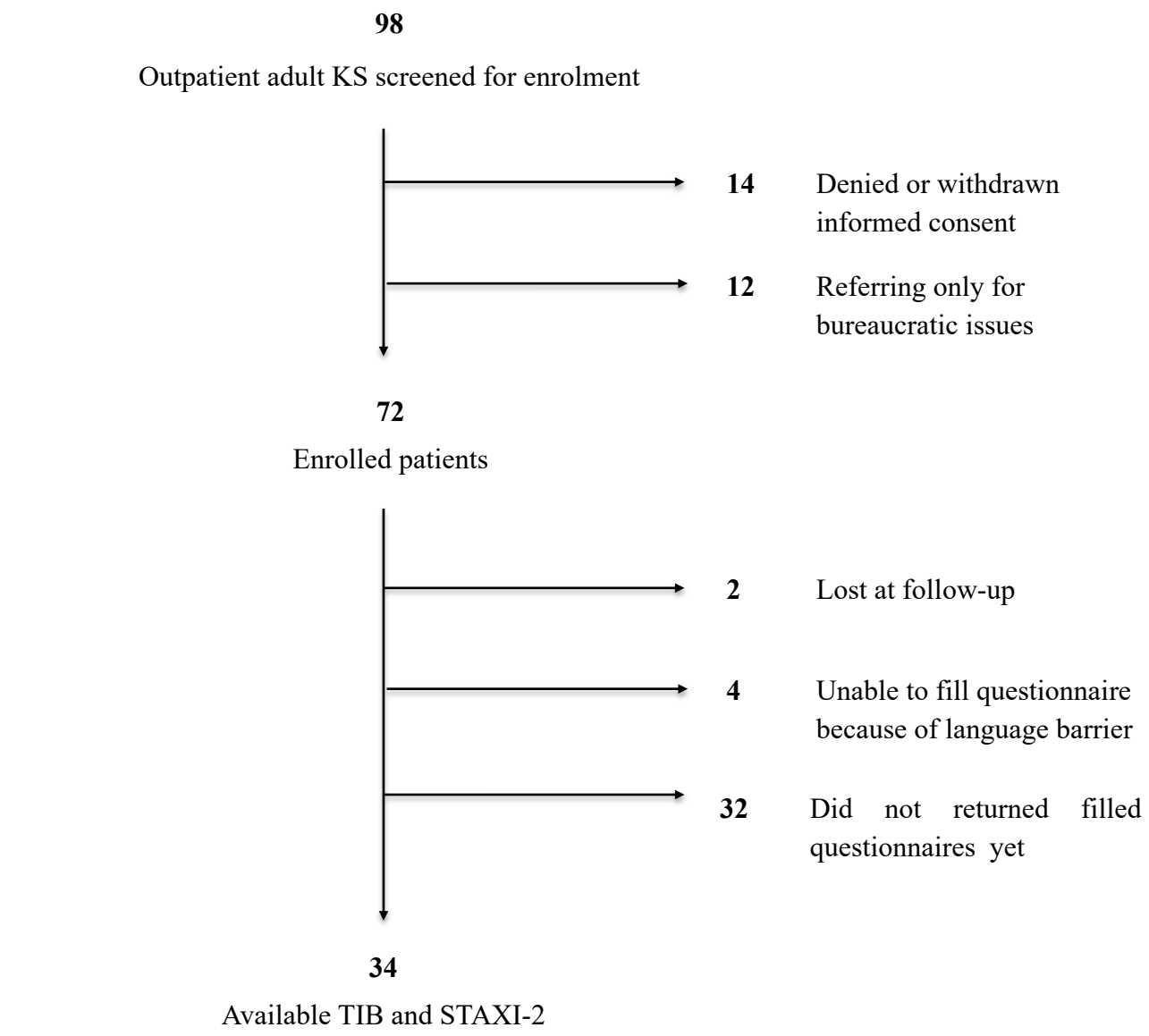

Table 1. General characteristics and andrological features of the examined cohort.

Age at diagnosis (*years*)	27.5 (17.0 – 39.0)
Age at evaluation (*years*)	45.0 (32.5 – 53.0)
Karyotype	
47,XXY	69 (95.8%)
46,XY/47,XXY	3 (4.2%)
Bi-testicular volume (*ml*)	5.3 (3.0 – 7.0)
Gynecomastia	28 (38.9%)
Surgically removed	6 (21.4%)
Varicocele	11 (15.3%)
TRT	65 (90.3%)
no	7 (9.7%)
Transdermal	22 (30.6%)
Intramuscular	41 (56.9%)
Withdrawn	2 (2.8%)
T on target	49 (67.1%)
Erythrocytosis	26 (36.1%)
Phlebotomy	8 (11.1%)
BPH	12 (16.4%)
ED	17 (23.3%)

Table 1 bis. Fertility in the examined cohort.

Azoospermia (n = *59*)	57 (96.6%)
TESE/microTESE	19 (32.2%)
Refused TESE/microTESE	9 (15.3%)
Sperm retrieval	2 (10.5%)
Fatherhood (n = *72*)	7 (9.7%)
Spontaneous	2 (2.8%)
Heterologous ART	4 (5.6%)
Adoption	1 (1.4%)

Data are expressed ad median (interquartile range) or absolute number (percentage).

Abbreviations: **TRT**: *Testosterone Replacement Therapy*; **T**: *Testosterone*; **BPH**: *Benign Prostatic Hyperplasia*; **ED**: *Erectile Dysfunction*; **TESE**: *Testicular Sperm Extraction*; **ART**: *Assisted Reproductive Techniques*.

Table 2. Correlation analysis: Andrological features.

		Age	Age $_{diagnosis}$	BMI	T $_{on\ target}$	TRT	Bitest. volume
Gynecomastia	ρ	0.118	0.014	0.128	- 0.064	- 0.180	0.002
	p	*0.353*	*0.909*	*0.286*	*0.590*	*0.130*	*0.990*
Varicocele	ρ	0.150	- 0.011	- 0.048	0.208	- 0.048	- 0.070
	p	*0.236*	*0.926*	*0.687*	*0.079*	*0.688*	*0.660*
Azoospermia	ρ	0.014	0.066	- 0.041	- 0.114	**0.330**	**- 0.380**
	p	*0.918*	*0.635*	*0.756*	*0.389*	***0.011***	***0.016***
TESE	ρ	- 0.050	**- 0.336**	- 0.036	0.207	- 0.035	0.167
	p	*0.695*	***0.004***	*0.761*	*0.080*	*0.770*	*0.290*

		Age	Smoke	BMI	T $_{on\ target}$	AH
Serum T on target	ρ	- 0.034	- 0.192	**- 0.337**	-	- 0.147
	p	*0.779*	*0.106*	***0.004***	-	*0.216*
Erythrocytosis	ρ	0.071	0.157	0.202	- 0.167	0.153
	p	*0.555*	*0.188*	*0.090*	*0.161*	*0.198*
ED	ρ	**0.466**	- 0.085	0.054	- 0.040	**0.509**
	p	***< 0.0001***	*0.479*	*0.655*	*0.739*	***< 0.0001***
BPH	ρ	**0.559**	**- 0.297**	0.056	0.067	**0.474**
	p	***< 0.0001***	***0.011***	*0.637*	*0.578*	***< 0.0001***

Data are expressed as Spearman coefficient (ρ) and statistical significance (***p***).

Abbreviations: **TESE**: *Testicular Sperm Extraction*; **T**: *Testosterone*; **ED**: *Erectile Dysfunction*; **BPH**: *Benign Prostatic Hyperplasia***; BMI**: *Body Mass Index*; **TRT**: *Testosterone Replacement Therapy*; **Bitest. Volume**: *Bitesticular Volume*; **AH**: *Arterial Hypertension*.

Table 3. Cardio-metabolic, Hormonal, Neuropsychological and Oncological comorbidities in the examined cohort.

Comorbidities in KS patients

Cardio-Metabolic		*Hormonal*		*Others*	
BMI (*Kg/m²*)	26.5 (23.1 – 29.5)	Vitamin D deficiency	60 (82.2%)	Vascular	11 (15.1%)
- Normal	30 (41.7%)	Vitamin D Replacement therapy	60 (82.2%)	Autoimmune	5 (6.9%)
- Overweight	27 (37.5%)			Inflammatory (*pericarditis*)	1 (1.4%)
- Obese	15 (20.8%)	**BMD**		Endocrine-metabolic	4 (5.6%)
		- Under investigation	15 (20.8%)	OSAS	4 (5.6%)
Glucose metabolism		- Normal	37 (51.4%)	Malformations	3 (4.2%)
- Normoglycemia	44 (61.1%)	- Osteopenia	14 (19.4%)	Others	1 (1.4%)
- IFG/IGT	17 (23.6%)	- Osteoporosis	6 (8.2%)		
- Diabetes Mellitus	11 (15.3%)			**Neoplasm**	9 (12.3%)
HbA_1c on target	9 (81.8%)	**Thyroid disease**	5 (6.9%)		
		TSH (*$\mu U/ml$*)	1.9 (1.2 – 2.8)	**Neurologic**	17 (23.6%)
Hypertriglyceridemia	17 (23.6%)	L-T4 replacement therapy	1 (1.4%)	- essential tremor	8 (11.0%)
Hypercholesterolemia	31 (42.5%)				
LDL-c on target	7 (21.6%)			**Psychiatric/Psychological**	17 (23.6%)
Arterial Hypertension	24 (32.9%)			- anxiety and/or depression	13 (18.1%)
CVD	1 (1.4%)				
Arrythmia	8 (11.0%)			Active Smoking	22 (30.1%)
Valvulopathy					
- no	15 (20.5%)				
- yes	6 (8.2%)				
- under investigation	51 (69.9%)				

Data are expressed ad median (interquartile range) or absolute number (percentage).

Abbreviations. **KS**: Klinefelter Syndrome; **BMI**: *Body Mass Index*; **IFG**: *Impaired Fasting Glucose;* **IGT**: *Impaired Glucose Tolerance;* **HbA_1c**: *Glycosylated Hemoglobin;* **LDL-c**: *calculated Low Density Lipoprotein;* **CVD**: *Cardiovascular Disease;* **BMD**: *Bone Mineral Density*; **TSH**: *Thyroid Stimulating Hormone;* **L-T4**: *Levotiroxine;* **OSAS**: *Obstructive Sleep Apnea Syndrome.*

Table 4. Correlation analysis: Hormonal, Neuropsychological and Oncological comorbidities.

		Age	Age $_{diagnosis}$	BMI	Bitest. volume	T $_{on\ target}$	Smoke
Vitamin D deficiency	ρ	**0.289**	0.078	- 0.040	- 0.283	- 0.047	0.119
	p	***0.015***	*0.545*	*0.741*	*0.073*	*0.698*	*0.325*
Reduced BMD	ρ	**0.386**	**0.415**	0.011	- 0.073	0.104	0.145
	p	***0.003***	***0.003***	*0.934*	*0.678*	*0.441*	*0.282*
Thyroid diseases	ρ	0.143	**0.285**	- 0.037	0.133	- 0.072	- 0.052
	p	*0.284*	***0.043***	*0.784*	*0.453*	*0.591*	*0.698*
Neoplasm	ρ	- 0.027	- 0.160	0.027	- 0.055	0.079	0.023
	p	*0.820*	*0.207*	*0.820*	*0.730*	*0.511*	*0.849*
Neurologic disease	ρ	0.120	- 0.006	0.037	0.160	0.085	- 0.069
	p	*0.317*	*0.961*	*0.757*	*0.311*	*0.478*	*0.563*
Essential Tremor	ρ	0,176	0,005	0,116	0,128	0,147	- 0,139
	p	*0,140*	*0,970*	*0,332*	*0,419*	*0,216*	*0,246*
Psychiatric/Psychological disease	ρ	- 0.017	- 0.220	- 0.062	- 0.112	**- 0.250**	0.128
	p	*0.890*	*0.081*	*0.604*	*0.482*	***0.034***	*0.283*

Data are expressed as Spearman coefficient (ρ) and statistical significance (***p***).

Abbreviations: **BMD**: *Bone Mineral Density*; **BMI**: *Body Mass Index*; **Bitest. Volume**: *Bitesticular Volume*; **T**: *Testosterone*.

Table 5. Correlation analysis: Cardiovascular and Metabolic comorbiodities.

		Age	Age $_{diagnosis}$	BMI	Smoke	T $_{on\ target}$	Hypercol.	AH
BMI	ρ	**0.257**	0.217	-	0.142	**- 0.337**	**0.376**	**0.320**
	p	***0.029***	*0.085*	-	*0.233*	***0.004***	***0.001***	***0.006***
Glucose Metabolism	ρ	**0.499**	**0.336**	**0.343**	- 0.220	- 0.064	**0.305**	**0.645**
	p	***< 0.0001***	***0.007***	***0.003***	*0.063*	*0.590*	***0.010***	***< 0.0001***
Hypertriglyceridemia	ρ	**0.234**	**0.263**	0.195	- 0.019	- 0.105	**0.371**	**0.246**
	p	***0.049***	***0.038***	*0.103*	*0.874*	*0.382*	***0.001***	***0.038***
Hypercholesterolemia	ρ	**0.489**	**0.394**	**0.376**	- 0.037	- 0.179	-	**0.483**
	p	***< 0.0001***	***0.001***	***0.001***	*0.758*	*0.134*	-	***< 0.0001***
Hypertension	ρ	**0.637**	**0.395**	**0.320**	- 0.085	- 0.147	**0.483**	-
	p	***< 0.0001***	***0.001***	***0.006***	*0.476*	*0.216*	***< 0.0001***	-
CVD	ρ	0.163	-	0.163	0.179	- 0.173	0.136	0.168
	p	*0.172*	-	*0.172*	*0.133*	*0.146*	*0.259*	*0.159*
Arrythmia	ρ	0.210	0.106	- 0.014	- 0.139	0.147	0.046	0.219
	p	*0.077*	*0.404*	*0.908*	*0.246*	*0.216*	*0.706*	*0.065*
Valvulopathy	ρ	0.174	0.108	- 0.052	0.230	0.038	0.030	0.000
	p	*0.450*	*0.671*	*0.822*	*0.316*	*0.869*	*0.897*	*1.000*

Data are expressed as Spearman coefficient (ρ) and statistical significance (***p***).

Abbreviations. **BMI**: *Body Mass Index*; **CVD**: *Cardiovascular Disease*; **T**: *Testosterone*; **Hypercol**: *Hypercholesterolemia*; **AH**: *Arterial Hypertension*.

Table 6. TIB – *"Test Intelligenza Breve"*

	KS-males (*n = 34*)	**Non KS-males** (*n = 34*)	*p*
Age (*years*)	46.5 (35.75 – 54.75)	48 (35.75 – 54.75)	*0.934*
Age $_{\text{diagnosis}}$ (*years*)	27.0 (18.0 – 35.0)		
Years of study (*years*)	15.0 (8.0 – 15.0)	15.0 (8.0 – 15.0)	*0.275*
N accentuation errors	7.5 (2.75 – 14.25)	2.0 (1.0 – 5.0)	*< 0.001*
N pronunciations errors	1.0 (0.0 – 2.0)	1.0 (0.0 – 1.0)	*0.119*
TIB score	9.0 (3.0 – 16.25)	3.0 (1.0 – 5.25)	*< 0.001*
IQ $_{\text{verbal}}$	102.5 (94.0 – 109.4)	110.6 (105.4 – 113.4)	*< 0.001*
IQ $_{\text{performance}}$	101.9 (93.0 – 111.5)	110.5 (107.7 – 114.8)	*< 0.001*
IQ $_{\text{total}}$	102.8 (93.2 – 111.7)	110.9 (107.8 – 114.9)	*< 0.001*

Data are expressed ad median (interquartile range) or absolute number (percentage).

Abbreviations: **KS**: Klinefelter Syndrome; **TIB***: "Test Intelligenza Breve" (Brief Intelligence Test);* **IQ**: *Intelligence Quotient*.

Table 7. Correlations: TIB – *"Test Intelligenza Breve"*

		Age	**Age** $_{\text{diagnosis}}$	**Years of study**	**T** $_{\text{on target}}$
	ρ	- 0.267	0.132	- 0.256	0.189
	p	*0.126*	*0.480*	*0.144*	*0.285*
N pronunciations errors	ρ	0.042	- 0.211	- 0.065	- 0.145
	p	*0.814*	*0.255*	*0.715*	*0.413*
TIB score	ρ	- 0.228	0.070	- 0.250	0.130
	p	*0.194*	*0.709*	*0.154*	*0.464*
IQ $_{\text{verbal}}$	ρ	0.310	0.009	**0.468**	- 0.089
	p	*0.075*	*0.961*	***0.005***	*0.615*
IQ $_{\text{performance}}$	ρ	**0.464**	0.061	0.166	- 0.176
	p	***0.006***	*0.746*	*0.349*	*0.320*
IQ $_{\text{total}}$	ρ	**0.377**	0.006	0.325	- 0.145
	p	***0.028***	*0.972*	*0.061*	*0.413*

Data are expressed as Spearman coefficient (**ρ**) and statistical significance (***p***).

Abbreviations: **TIB***: "Test Intelligenza Breve" (Brief Intelligence Test);* **IQ**: *Intelligence Quotient;* **T**: *Testosterone*.

Table 8. STAXI-2

KS subjects (*n = 26*)				
		Scale / Subscale	**Score**	**cases > 75th centile**
Age	45.0 (34.5 – 53.3)	**R/S**	15.0 (15.0 – 19.0)	2 (7.7%)
Age at diagnosis	28.0 (17.0 – 36.5)	RS/S	5.0 (5.0 – 6.5)	4 (15.4%)
		RS/V	5.0 (5.0 – 5.0)	1 (3.8%)
BMI	26.7 (23.5 – 30.0)	RS/F	5.0 (5.0 – 5.0)	1 (3.8%)
- normal	9 (34.6%)	**R/T**	17.5 (12.0 – 21.0)	2 (7.7%)
- overweight/obese	17 (63.4%)	RT/T	6.5 (4.0 – 8.0)	2 (7.7%)
		RT/R	7.5 (5.9 – 9.0)	4 (15.4%)
Hypertension	10 (38.5%)	**ER/out**	14.0 (10.0 – 17.25)	5 (19.2%)
P.D.	5 (19.2%)	**ER/in**	14.0 (12.75 – 17.0)	4 (15.4%)
TRT	23 (88.5%)	**CR/out**	19.5 (15.75 – 23.25)	2 (7.7%)
T on target	17 (65.4%)	**CR/in**	23.0 (16.0 – 24.5)	7 (26.9%)
ED	6 (23.1%)	**ER/Index**	33.5 (27.0 – 45.25)	6 (23.1%)

Data are expressed ad median (interquartile range) or absolute number (percentage).

Abbreviations. **KS**: Klinefelter Syndrome; **BMI**: *Body Mass Index*; **P.D:** *Psychiatric or Psychological disease*; **TRT**: *Testosterone Replacement Therapy*; **T**: *Testosterone*; **ED**: *Erectile Dysfunction*. **R/S**: *State Anger*; **RS/S**: *Feeling Angry*; **RS/V**: *Feel Like Expressing Anger Verbally*; **RS/F**: *Feel Like Expressing Anger Physically*; **R/T**: *Trait Anger*; **RT/T**: *Angry Temperament*; **RT/R**: *Angry Reaction*; **ER/out**: *Anger Expression-Out*; **ER/in**: *Anger Expression-In*; **CR/out**: *Anger Control-Out*; **CR/in**: *Anger Control-In*; **ER/Index**: *Anger Expression Index*.

Table 9. STAXI-2

	Age 20-29 years			**Age > 30 years**		
	KS males (*n = 5*)	**non-KS males** (*n = 251*)	*p*	**KS males** (*n = 21*)	**non-KS males** (*n = 323*)	*p*
R/S	**16.00 ± 2.24**	**21.62 ± 9.28**	***0.002***	17.19 ± 3.52	18.81 ± 7.06	*0.070*
RS/S	5.80 ± 1.79	7.06 ± 3.08	*0.193*	6.14 ± 1.82	6.49 ± 2.64	*0.416*
RS/V	**5.20 ± 0.45**	**7.46 ± 3.63**	***<0.0001***	**5.48 ± 1.25**	**6.41 ± 2.95**	***0.006***
RS/F	**5.00 ± 0.00**	**7.09 ± 3.76**	***<0.0001***	5.57 ± 1.36	5.92 ± 2.39	*0.291*
R/T	20.40 ± 5.50	20.60 ± 5.31	*0.940*	16.62 ± 5.46	18.96 ± 6.28	*0.072*
RT/T	7.20 ± 3.14	7.32 ± 2.3	*0.936*	6.33 ± 2.16	6.94 ± 3.59	*0.244*
RT/R	9.20 ± 3.27	9.48 ± 2.85	*0.858*	**7.29 ± 2.83**	**8.73 ± 2.88**	***0.034***
ER/out	15.4 ± 5.32	16.07 ± 4.2	*0.793*	13.81 ± 4.09	14.50 ± 3.71	*0.459*
ER/in	16.00 ± 1.23	16.99 ± 4.51	*0.158*	15.05 ± 4.13	16.51 ± 4.38	*0.132*
CR/out	17.80 ± 3.70	20.51 ± 5.11	*0.178*	19.7 ± 5.52	21.49 ± 5.36	*0.163*
CR/in	21.20 ± 5.81	21.21 ± 4.76	*0.997*	21.33 ± 6.67	22.21 ± 4.85	*0.558*
ER/Index	40.40 ± 10.60	39.35 ± 12.71	*0.837*	35.81 ± 15.58	35.27 ± 12.30	*0.878*

Data are expressed ad mean ± standard error. Data of "non-KS males" were derived from the Italian validation cohort of STAXI-2 questionnaire.

R/S: *State Anger*; **RS/S**: *Feeling Angry*; **RS/V**: *Feel Like Expressing Anger Verbally*; **RS/F**: *Feel Like Expressing Anger Physically*; **R/T**: *Trait Anger*; **RT/T**: *Angry Temperament*; **RT/R**: *Angry Reaction*; **ER/out**: *Anger Expression-Out*; **ER/in**: *Anger Expression-In*; **CR/out**: *Anger Control-Out*; **CR/in**: *Anger Control-In*; **ER/Index**: *Anger Expression Index*.

Table 10. Correlations: STAXI-2

		Age	Age $_{diagnosis}$	BMI	AH	P.D.	TRT	T $_{on\ target}$	ED
R/S	ρ	0.073	0.132	- 0.034	0.018	- 0.372	0.120	0.277	- 0.021
	p	*0.723*	*0.528*	*0.869*	*0.930*	*0.062*	*0.560*	*0.171*	*0.919*
R/S ^{T}	ρ	**0.390**	0.315	0.161	0.206	- 0.354	0.220	0.149	0.119
	p	***0.049***	*0.125*	*0.431*	*0.313*	*0.076*	*0.281*	*0.466*	*0.563*
RS/S	ρ	0.122	0.171	- 0.131	- 0.056	- 0.346	0.122	0.235	- 0.007
	p	*0.553*	*0.413*	*0.524*	*0.786*	*0.084*	*0.5530*	*0.247*	*0.972*
RS/S ^{T}	ρ	**0.438**	0.337	0.170	0.193	- 0.334	0.235	0.073	0.045
	p	***0.025***	*0.100*	*0.407*	*0.344*	*0.095*	*0.247*	*0.722*	*0.829*
RS/V	ρ	0.064	0.085	- 0.243	0.015	- 0.237	0.161	0.353	- 0.018
	p	*0.755*	*0.687*	*0.231*	*0.941*	*0.244*	*0.431*	*0.077*	*0.932*
RS/V ^{T}	ρ	**0.542**	0.333	0.194	0.341	- 0.214	0.252	0.130	0.087
	p	***0.004***	*0.104*	*0.342*	*0.088*	*0.294*	*0.215*	*0.527*	*0.674*
RS/F	ρ	- 0.094	0.103	0.332	0.101	- 0.207	0.025	0.086	0.048
	p	*0.649*	*0.626*	*0.098*	*0.624*	*0.310*	*0.903*	*0.677*	*0.814*
RS/F ^{T}	ρ	0.057	0.153	**0.428**	0.153	- 0.142	0.171	0.023	0.088
	p	*0.781*	*0.465*	***0.029***	*0.455*	*0.490*	*0.403*	*0.909*	*0.667*
R/T	ρ	**- 0.456**	- 0.255	- 0.106	- 0.158	0.078	- 0.336	**0.421**	0.165
	p	***0.019***	*0.219*	*0.605*	*0.439*	*0.704*	*0.093*	***0.032***	*0.421*
R/T ^{T}	ρ	- 0.320	- 0.199	0.003	- 0.037	0.085	- 0.291	**0.407**	0.202
	p	*0.111*	*0.341*	*0.990*	*0.857*	*0.679*	*0.148*	***0.039***	*0.322*
RT/T	ρ	- 0.170	- 0.174	0.039	0.038	0.073	- 0.109	0.263	0.198
	p	*0.406*	*0.406*	*0.849*	*0.856*	*0.724*	*0.595*	*0.194*	*0.332*
RT/T ^{T}	ρ	- 0.031	- 0.082	0.149	0.107	0.053	- 0.025	0.218	0.234
	p	*0.879*	*0.696*	*0.467*	*0.604*	*0.799*	*0.903*	*0.285*	*0.250*
RT/R	ρ	**- 0.478**	- 0.143	- 0.090	- 0.170	0.026	- 0.376	**0.430**	0.203
	p	***0.013***	*0.495*	*0.663*	*0.406*	*0.899*	*0.059*	***0.028***	*0.321*
RT/R ^{T}	ρ	**- 0.392**	- 0.089	- 0.017	- 0.122	0.046	- 0.372	**0.424**	0.215
	p	***0.048***	*0.674*	*0.933*	*0.552*	*0.824*	*0.061*	***0.031***	*0.292*
ER/out	ρ	- 0.108	- 0.255	0.191	0.281	0.177	- 0.227	0.260	0.055
	p	*0.601*	*0.218*	*0.349*	*0.164*	*0.388*	*0.264*	*0.199*	*0.789*
ER/out ^{T}	ρ	0.032	- 0.168	0.274	0.361	0.151	- 0.179	0.239	0.092
	p	*0.875*	*0.422*	*0.176*	*0.070*	*0.463*	*0.380*	*0.240*	*0.655*
ER/in	ρ	- 0.296	- 0.297	0.081	- 0.005	- 0.236	**- 0.442**	0.217	- 0.203
	p	*0.142*	*0.150*	*0.696*	*0.979*	*0.245*	***0.024***	*0.286*	*0.321*
ER/in ^{T}	ρ	- 0.221	- 0.235	0.128	0.048	- 0.210	**- 0.455**	0.207	- 0.190
	p	*0.277*	*0.259*	*0.535*	*0.816*	*0.303*	***0.019***	*0.311*	*0.352*
CR/out	ρ	0.327	**0.418**	0.252	0.095	- 0.059	0.125	**- 0.541**	- 0.116
	p	*0.103*	***0.038***	*0.214*	*0.644*	*0.776*	*0.543*	***0.004***	*0.573*
CR/out ^{T}	ρ	0.251	0.364	0.194	0.053	- 0.065	0.091	**- 0.520**	- 0.128
	p	*0.217*	*0.074*	*0.342*	*0.797*	*0.751*	*0.657*	***0.007***	*0.532*
CR/in	ρ	0.286	**0.529**	0.149	- 0.005	- 0.092	0.121	- 0.250	- 0.043
	p	*0.157*	***0.007***	*0.467*	*0.979*	*0.656*	*0.557*	*0.219*	*0.835*
CR/in ^{T}	ρ	0.189	**0.471**	0.100	- 0.053	- 0.098	0.062	- 0.243	- 0.079
	p	*0.355*	***0.017***	*0.627*	*0.797*	*0.634*	*0.764*	*0.231*	*0.700*
ER/Index	ρ	- 0.308	**- 0.445**	- 0.089	0.063	0.059	- 0.257	0.378	0.055
	p	*0.126*	***0.026***	*0.664*	*0.759*	*0.776*	*0.205*	*0.057*	*0.790*
ER/Index ^{T}	ρ	- 0.156	- 0.381	- 0.007	0.169	0.078	- 0.198	**0.395**	0.110
	p	*0.448*	*0.061*	*0.972*	*0.409*	*0.704*	*0.332*	***0.046***	*0.593*

Data are expressed as Spearman coefficient (**ρ**) and statistical significance (***p***). **^{T}** refers to normalized T-scores.

Abbreviations: **R/S**: *State Anger*; **RS/S**: *Feeling Angry*; **RS/V**: *Feel Like Expressing Anger Verbally*; **RS/F**: *Feel Like Expressing Anger Physically*; **R/T**: *Trait Anger*; **RT/T**: *Angry Temperament*; **RT/R**: *Angry Reaction*; **ER/out**: *Anger Expression-Out*; **ER/in**: *Anger Expression-In*; **CR/out**: *Anger Control-Out*; **CR/in**: *Anger Control-In*; **ER/Index**: *Anger Expression Index*. Abbreviations: **BMI**: *Body Mass Index*; **AH**: *Arterial Hypertension*; **P.D**.: *Psychiatric or Psychological Disease*; **TRT**: *Testosterone Replacement Therapy*; **T**: *Testosterone*; **ED**: *Erectile Dysfunction*.

Table 11. Partial Correlations: STAXI-2

		Age [a]	Age $_{diagnosis}$ [a]	BMI [b]	AH [c]	P.D. [b]	TRT [b]	T $_{on\ target}$ [d]	ED [a]
R/S	ρ	0.204	0.292	- 0.057	- 0.046	- 0.246	0.193	0.293	0.061
	p	*0.327*	*0.166*	*0.791*	*0.834*	*0.247*	*0.367*	*0.165*	*0.772*
R/S [T]	ρ	**0.418**	0.377	- 0.017	- 0.087	- 0.258	0.248	0.381	0.130
	p	***0.037***	*0.070*	*0.938*	*0.694*	*0.223*	*0.242*	*0.066*	*0.536*
RS/S	ρ	0.189	0.281	- 0.249	- 0.015	- 0.251	0.199	0.237	- 0.029
	p	*0.364*	*0.184*	*0.241*	*0.948*	*0.237*	*0.351*	*0.264*	*0.891*
RS/S [T]	ρ	0.306	0.342	- 0.225	- 0.039	- 0.272	0.212	0.262	- 0.014
	p	*0.137*	*0.102*	*0.291*	*0.858*	*0.198*	*0.321*	*0.217*	*0.947*
RS/V	ρ	0.258	0.201	- 0.104	- 0.115	- 0.123	0.093	0.312	0.035
	p	*0.213*	*0.346*	*0.629*	*0.602*	*0.566*	*0.665*	*0.138*	*0.868*
RS/V [T]	ρ	**0.435**	0.287	- 0.057	- 0.157	- 0.147	0.171	0.354	0.060
	p	***0.030***	*0.175*	*0.792*	*0.475*	*0.492*	*0.424*	*0.090*	*0.775*
RS/F	ρ	0.034	0.182	0.285	0.000	- 0.171	0.134	0.173	0.167
	p	*0.871*	*0.394*	*0.177*	*0.998*	*0.423*	*0.532*	*0.418*	*0.425*
RS/F [T]	ρ	0.135	0.221	0.319	- 0.041	- 0.142	0.207	0.239	0.190
	p	*0.520*	*0.300*	*0.129*	*0.854*	*0.508*	*0.331*	*0.261*	*0.363*
R/T	ρ	- 0.249	- 0.115	0.373	0.142	0.187	0.090	**0.411**	0.248
	p	*0.231*	*0.592*	*0.073*	*0.519*	*0.381*	*0.677*	***0.046***	*0.233*
R/T [T]	ρ	- 0.103	- 0.059	**0.414**	0.154	0.181	0.093	**0.446**	0.285
	p	*0.626*	*0.784*	***0.044***	*0.484*	*0.396*	*0.665*	***0.029***	*0.167*
RT/T	ρ	-.088	- 0.180	0.254	0.132	0.099	0.282	0.291	0.208
	p	*0.676*	*0.400*	*0.231*	*0.548*	*0.647*	*0.182*	*0.168*	*0.320*
RT/T [T]	ρ	0.022	- 0.194	0.238	0.058	0.062	0.311	0.279	0.203
	p	*0.915*	*0.364*	*0.263*	*0.794*	*0.774*	*0.140*	*0.187*	*0.331*
RT/R	ρ	- 0.263	- 0.006	0.425	0.134	0.154	0.017	**0.443**	0.239
	p	*0.204*	*0.979*	*0.039*	*0.543*	*0.474*	*0.939*	***0.030***	*0.251*
RT/R [T]	ρ	- 0.159	0.036	**0.447**	0.141	0.136	0.024	**0.469**	0.269
	p	*0.449*	*0.866*	***0.029***	*0.521*	*0.526*	*0.913*	***0.021***	*0.193*
ER/out	ρ	- 0.001	- 0.199	0.440	0.333	0.205	- 0.047	0.376	0.026
	p	*0.995*	*0.350*	*0.031*	*0.121*	*0.337*	*0.826*	*0.071*	*0.904*
ER/out [T]	ρ	0.130	- 0.135	**0.447**	0.345	0.176	- 0.028	**0.408**	0.049
	p	*0.535*	*0.529*	***0.029***	*0.107*	*0.410*	*0.895*	***0.048***	*0.816*
ER/in	ρ	- 0.181	- 0.243	**0.468**	- 0.001	- 0.203	0.069	0.303	- 0.243
	p	*0.388*	*0.252*	***0.021***	*0.998*	*0.343*	*0.750*	*0.151*	*0.243*
ER/in [T]	ρ	- 0.136	- 0.209	**0.475**	- 0.028	- 0.197	0.097	0.298	- 0.228
	p	*0.515*	*0.327*	***0.019***	*0.900*	*0.356*	*0.652*	*0.157*	*0.273*
CR/out	ρ	- 0.045	0.347	- 0.074	- 0.042	- 0.212	- 0.110	**- 0.513**	- 0.140
	p	*0.832*	*0.097*	*0.733*	*0.847*	*0.319*	*0.610*	***0.010***	*0.504*
CR/out [T]	ρ	- 0.120	0.295	- 0.089	- 0.025	- 0.198	- 0.159	**- 0.524**	- 0.157
	p	*0.568*	*0.162*	*0.679*	*0.910*	*0.353*	*0.457*	***0.009***	*0.454*
CR/in	ρ	0.091	**0.457**	- 0.056	- 0.135	- 0.140	- 0.086	- 0.193	0.006
	p	*0.665*	***0.025***	*0.796*	*0.540*	*0.515*	*0.690*	*0.367*	*0.978*
CR/in [T]	ρ	0.024	**0.419**	- 0.061	- 0.124	- 0.146	- 0.122	- 0.192	- 0.014
	p	*0.910*	***0.042***	*0.776*	*0.573*	*0.495*	*0.570*	*0.369*	*0.949*
ER/Index	ρ	- 0.078	**- 0.435**	0.315	0.179	0.145	0.080	**0.450**	- 0.015
	p	*0.712*	***0.033***	*0.134*	*0.413*	*0.500*	*0.710*	***0.027***	*0.943*
ER/Index [T]	ρ	0.089	- 0.342	0.346	0.144	0.134	0.123	**0.489**	0.012
	p	*0.671*	*0.102*	*0.098*	*0.513*	*0.534*	*0.568*	***0.015***	*0.955*

Data are expressed as Spearman coefficient (**ρ**) and statistical significance (***p***). [T] refers to normalized T-scores.

a = adjusted for **T on Target**; **b** = adjusted for **Age** and **T on target**; **c** = adjusted for **Age**. **BMI** and **T on Target**; **d** = adjusted for **Age** and **BMI.**

Abbreviations: **R/S**: *State Anger*; **RS/S**: *Feeling Angry*; **RS/V**: *Feel Like Expressing Anger Verbally*; **RS/F**: *Feel Like Expressing Anger Physically*; **R/T**: *Trait Anger*; **RT/T**: *Angry Temperament*; **RT/R**: *Angry Reaction*; **ER/out**: *Anger Expression-Out*; **ER/in**: *Anger Expression-In*; **CR/out**: *Anger Control-Out*; **CR/in**: *Anger Control-In*; **ER/Index**: *Anger Expression Index*. Abbreviations: **BMI**: *Body Mass Index*; **AH**: *Arterial Hypertension*; **P.D**.: *Psychiatric or Psychological Disease*; **TRT**: *Testosterone Replacement Therapy*; **T**: *Testosterone*; **ED**: *Erectile Dysfunction*.

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