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# *SOX13* gene downregulation in peripheral blood mononuclear cells of patients with Klinefelter syndrome

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SOX13 and Klinefelter syndrome

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## Abstract

Klinefelter syndrome (KS) is the most common sex-chromosome disorder in men. It is characterized by germ cell loss and other variable clinical features, including autoimmunity. The *sex-determining region of Y(SRY)-BOX 13 (Sox13)* gene is expressed in mouse spermatogonia. In addition, it has been identified as islet cell autoantigen 12 (ICA12), which is involved in the pathogenesis of autoimmune diseases, including type 1 diabetes mellitus (DM) and primary biliary cirrhosis. *SOX13* expression has never been investigated in patients with KS. In this age-matched case-control study performed in 10 patients with KS and 10 controls, we found that *SOX13* is significantly downregulated in peripheral blood mononuclear cells of patients with KS compared to controls. This finding might be consistent with the germ cell loss typical of patients with KS. However, the role of *SOX13* in the pathogenesis of germ cell loss and humoral autoimmunity in patients with KS deserves to be further explored.


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**Keywords:** Klinefelter syndrome; Rare disease; intellectual disability; SOX13; germ cells

## 1.1 Introduction

Klinefelter syndrome (KS) is the most common sex-chromosome disorder in men, with an estimated prevalence of 1:500 to 1:1000 newborns [1]. The most widespread karyotype in men with KS is 47,XXY (the so-called "classic" nonmosaic karyotype). Classic nonmosaic karyotype occurs in approximately 80-90% of men with KS [2] and is due to paternal meiotic nondisjunction in 50% of cases [3]. Otherwise, mosaic KS (e.g., 47,XXY/46,XY) and other nonmosaic forms, such as complex karyotype or other numeric sex chromosome abnormalities (e.g., 48,XXXY, 48,XXYY and 49,XXXXY), can be found in the remaining patients [2,4].

The abnormal karyotype leads to progressive germ cell degeneration starting from mid-puberty, impaired Sertoli cell (SC) function [5], total tubular atrophy or hyalinizing fibrosis and relative hyperplasia of Leydig cells [6]. Occasionally, foci of spermatogenesis have been observed in the testes of men with KS [6]. Clinically, azoospermia occurs in the majority of patients with nonmosaic KS. In addition, sperm has been found in 7.7-8.4% of patients with (apparently) nonmosaic KS [2].

Several other clinical manifestations can be associated with the syndrome, such as learning and developmental disability, personality disorder and behavioral problems, intelligence quotient (IQ) lower by 10-15 points but not in the intellectual disability range, increased risk for mitral valve prolapse, lower-extremity varicose veins, venous stasis ulcers, deep vein thrombosis, pulmonary embolism, autoimmune diseases, 20-fold-higher risk of developing breast cancer, type 2 diabetes mellitus (T2DM), metabolic syndrome, extragonadal germ cell tumors and non-Hodgkin lymphoma [1,7-9].

Despite an increasing number of studies investigating the gene expression profile in both peripheral blood mononuclear cells (PBMCs) and, when available, in the testicular tissue of patients with KS [10-20], the molecular mechanisms responsible for germ cell degeneration remain elusive. It has been hypothesized that the escape of inactivation of genes on the supernumerary X chromosome might affect germ cell development and/or meiosis [21]. However, transcriptome analysis of testicular tissue of men with KS resulted in normal expression of X-linked genes [20]. By contrast, deregulation of gene mapping on autosomes has been shown in men with KS, and therefore, the supernumerary X chromosome has been suggested to influence the regulation of these genes [14].

The *sex-determining region of Y*(*SRY*)*-BOX 13* (*SOX13*) maps to the 1q32.1 chromosome. It belongs to the family of SRY-related high mobility group (HMG)-BOX genes, which, in turn, encode a group of

transcription factors with an HMG-type DNA-binding domain. The latter consists of three α-helixes whose binding to specific DNA sequences influences DNA structure and transcription [22,23]. In mice, members of the Sox transcription factor family play a role in fetal development in multiple tissues, including the testis [24]. Accordingly, SRY, required for male sex determination in both humans and mice, targets *sex-determining region of Y(SRY)-BOX 9 (Sox9)* expression, which initiates Sertoli cell differentiation [25,26].

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Recently, Sox13 has been found to be expressed in mouse type A and B spermatogonia [24]. Interestingly, SOX13 is also a diabetes autoantigen expressed in pancreatic cells [27]. No data are currently available on its expression in men with KS. Therefore, this study was undertaken to evaluate whether differential *SOX13* gene expression occurs in peripheral blood mononuclear cells (PBMCs) of men with KS compared with healthy controls.

## 2. Materials and Methods

### 2.1 Patients, controls and RNA extraction

Ten men with KS with the nonmosaic KS karyotype 47,XXY (as confirmed by cytogenetic investigation performed on at least 50 metaphases) and ten healthy age-matched controls with 46,XY karyotype, no clinical history of genetic diseases, normal testicular volume and normal reproductive hormone (gonadotropins and total testosterone) serum levels were recruited. Patients and controls were Italians. They were evaluated for gonadotropins, total testosterone (TT) levels, body mass index (BMI), glycemia and serum insulin levels. Insulin resistance was calculated using the homeostasis model assessment index (HOMA-IR).

Fitting with the diagnosis, all patients with KS had azoospermia, increased follicle stimulating hormone (FSH) serum levels and low testicular volumes. The clinical and biochemical parameters of each man with KS and control have already been reported [14]. Patients and controls were age-matched (32.4±8.1 vs. 33.1±7.9 years, p>0.1) and did not differ in BMI, glycemia, insulin or HOMA-IR. As expected, serum gonadotropins and TT levels were significantly different in patients with KS compared to controls ($p<0.05$) (**Table 1**). Among patients with KS, 5 were on testosterone replacement therapy (TRT). No KS or control was diabetic. An increased HOMA index, consistent with insulin resistance, was found in 42.9% (3/7) of men with KS and 20.0% (1/5) of controls (p>0.1).

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Two blood samples were withdrawn from each patient and control, the first for next-generation sequencing (NGS) analysis and the second to validate the results obtained. PBMCs were separated from each blood sample using Ficoll-Paque (Ficoll Plaque PLUS – GE Healthcare Life Sciences, Piscataway, New Jersey, USA), and RNA was extracted using TRIzol reagent (TRIzol Reagent, Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA concentration in each sample was assayed with an ND-1000 spectrophotometer (NanoDrop, Thermo Fisher, Waltham, MA, USA), and its quality was assessed with a TapeStation 4200 (Agilent Technologies, Santa Clara, CA, USA). All RNA had an RNA Integrity Number (RIN) >8 on Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA).

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### 2.2 Ethical statement

The present study belongs to a broad project designed to evaluate any difference in the transcriptome of men with KS compared with healthy controls [13,14,28]. This project has been approved by the Ethical Committee of the University Teaching Hospital "Policlinico-Vittorio Emanuele", Catania, Italy: trial registration number 49/2015/PO (Register of the Ethics Committee opinions). All the participants in the study signed an informed consent form to participate and to publish.

### 2.3 RNA sequencing and data analysis

Indexed libraries were prepared from 1 µg of purified RNA with the TruSeq Stranded Total RNA (Illumina, Eindhoven, The Netherlands) Library Prep Kit according to the manufacturer's instructions. Libraries were quantified using the Agilent 2100 Bioanalyzer (Agilent Technologies) and pooled such that each index-tagged sample was present in equimolar amounts, with a final concentration of the pooled samples of 2 nM. The pooled samples were subjected to cluster generation and sequencing using an Illumina HiSeq 2500 System (Illumina) in a 2x100 paired-end format. The raw sequence files generated (.fastq files) underwent quality control analysis using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

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Bioinformatics analysis was performed by Genomix4Life Srl ("Schola Medica Salernitana", Baronissi, SA, Italy). The quality checked reads were trimmed with cutadapt v.1.10 (https://cutadapt.readthedocs.io/en/v1.10/changes.html#v1-10) and then aligned to the human genome (hg19

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assembly) using STAR v.2.5.2 [29] with standard parameters. Differentially expressed mRNAs were identified using DESeq2 v.1.12 [30].

Gene annotation, as provided by Ensembl (GRCh37) (https://grch37.ensembl.org/index.html), was obtained for all known genes in the human genome. We calculated the number of reads mapping to each transcript with HTSeq-count v.0.6.1. These raw read counts were then used as input to DESeq2 for calculation of normalized signal for each transcript in the sample, and differential expression was reported as the fold change along with associated adjusted p-values (computed according to Benjamini-Hochberg). Differential expression data were further confirmed using Cuffdiff36).

**2.4 Validation with Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)**

To validate the results obtained by NGS analysis, we compared the RT-PCR results from 10 patients with KS and 10 normal subjects. qRT-PCR was performed as described elsewhere [14].

cDNA reverse transcription was carried out for each sample using a cDNA synthesis kit (Thermo Scientific Maxima First Strand cDNA Synthesis Kit for RT-qPCR, Waltham, MA, USA) according to the manufacturer's instructions. Real-time PCR analysis for SOX13 was performed using TaqMan Gene Expression Assay primers. Briefly, total RNA was extracted from samples using TRIzol reagent (Sigma-Aldrich, Milan, Italy) and quantified by reading the optical density at 260 nm. In particular, 2.5 µg of total RNA was subjected to reverse transcription (RT, Thermo Scientific, Waltham, MA, USA) in a final volume of 20 µl. qPCR was performed using 25 ng of cDNA prepared by RT and SYBR Green Master Mix (Stratagene, Amsterdam, The Netherlands – Agilent Technology). This was performed in an Mx3000P cycler (Stratagene), using FAM for detection and ROX as the reference dye. The mRNA level of each sample was normalized against GAPDH mRNA and expressed as the fold change versus the level in the control samples. The *SOX13* and the reference gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) primers were obtained from Applied Biosystems (Carlsbad, CA, USA) (catalog number ID Hs00232193_m1 and ID Hs99999905_m1, respectively). The mean was obtained with the Software Version 1.5 supplied with the LightCycler 480, as previously reported [31].

Distribution analysis of measured gene transcript levels was performed using the Shapiro–Wilk test, and statistical analysis of the results was carried out using paired two-tailed *t*-test and bivariate linear

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regression analysis. Graph Pad Prism 5 software (https://www.graphpad.com/scientific-software/prism/) was used for statistical analysis. A $p$ value <0.05 was accepted as significant.

Differential expression data were further confirmed using Cuffdiff36. Raw data are available in the ArrayExpress database repository (https://www.ebi.ac.uk/arrayexpress/) with accession number E-MTAB-6107.

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## 3. Results

Integrative Genomics Viewer for SOX13 in three KS patients and three controls (**Fig. 1, panel a**), revealed a quantitatively reduced expression of SOX3 in patients than controls, as confirmed by the analysis of expression of the 20 consecutive samples (**Fig. 1, panel b**). Overall, NGS transcriptome analysis revealed that the *SOX13* gene (locus 1:204042242-204096863) was downregulated in patients with KS by -3.701-fold (q-value<0.05) compared with controls. (**Fig. 1, panel c**).

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The raw data of this research project are available in the ArrayExpress database repository (https://www.ebi.ac.uk/arrayexpress/) with accession number E-MTAB-6107.

In our case–control study with qRT-PCR, we used all KS cases and controls, and specifically, we obtained a mean ± standard deviation FC of cases of 0.48±0.25 (**Fig. 2**). Statistical analysis revealed a significant difference between the control and KS groups ($p$<0.05). The mean of KS cases was obtained with the Software Version 1.5 supplied with the LightCycler 480. We can conclude that the results confirmed the data obtained by NGS analysis, and differences in values reflect the diversity of the methods.

## 4. Discussion

Impaired spermatogenesis with total tubular atrophy or hyalinizing fibrosis is the most common histological testicular feature of men with KS [1]. Although a number of transcriptome studies have been performed both in PBMCs and in testicular tissue from patients with KS, the molecular mechanism responsible for germ cell degeneration in KS is not yet understood. Its acknowledgment would be of great interest to address future target gene therapies.

In the present study, we report, for the first time, that the *SOX13* gene is downregulated in PBMCs from patients with KS compared to controls. The *SOX13* gene belongs to the *SOX* family, whose members are involved in testicular differentiation in most vertebrates. In mice, the *Sox* gene family encodes a group of

transcription factors with an HMG-box DNA-binding domain that is similar to that of the *sex-determining region of the Y* (Sry) protein. Sox genes are classified into 8 groups, named from A to H. In particular, the SoxD group includes *Sox5*, *Sox6* and *Sox13* in most vertebrates [32]. Sox proteins are known to be involved in testicular differentiation. In particular, Sox9 is tightly associated with SC differentiation [33-35] and might also influence testosterone production by Leydig cells. Furthermore, Sox4, Sox11 and Sox12 protein expression has been found in the mouse testis during development [36], while Sox9, Sox5 and Sox13 have been found in the seminiferous tubules of the postnatal mouse testis [24]. Sox proteins are likely involved in spermatogenesis. Accordingly, Sox4, Sox8, Sox9 and Sox12 proteins are highly expressed in SCs and Sox5, Sox6 and Sox30 in spermatocytes and spermatids, whereas Sox3, Sox4, Sox12 and Sox13 have been detected in spermatogonia of both mice and rats [37].

The role that Sox proteins display in spermatogenesis has been proven by knockout studies. Accordingly, Sox30 knockout mice show infertility due to arrested spermatogenesis at the spermatid phase. This protein seems to address haploid gene transcription in the late meiosis and spermiogenesis phases. In contrast, this role has not been observed in mouse female gametogenesis [38-40]. Additionally, Sox4, which is known to be involved in gonadal morphogenesis, is involved in germ cell differentiation in male mice. Indeed, Sox4 deficiency results in the reduction in mouse germ cell differentiation markers, such as *Nanos c2hc-type zinc finger 2* (*Nanos2*) and DNA methyltrasferase 2-like protein (*Dnmt3l*), and increased pluripotency gene expression. Instead, female germ cells normally enter meiosis [41].

SoxD proteins have two conserved functional domains: the family-specific HMG box DNA-binding domain in the C-terminal part and the group-specific coiled coil in the N-terminal region of the protein. In mouse proteins, these domains share 87% and 76% homology with the N-terminal and C-terminal domains of the human SOXD proteins, respectively [32]. This likely supports that Sox proteins may have a conserved function among vertebrates, including humans, where their role in spermatogenesis cannot be excluded.

SoxD proteins are involved in transcriptional activation and repression. In particular, *Sox13* has been shown to modulate canonical Wingless-type MMTV integration site family (Wnt) signaling [42,43]. Interestingly, FSH enhances type A undifferentiated spermatogonia ($A_{und}$) proliferation via Leydig cell-derived Wingless-type MMTV integration site family, member 5a (Wnt5a) production [44]. Accordingly, an *in vitro* study reported that Wnt5a maintains the number of murine spermatogonial stem cells (SSCs) by activating the c-Jun N-terminal kinases (JNK) pathway [45]. In addition, FSH-induced secretion of insulin-

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like growth factor 3 (Igf3) in Sertoli cells from zebrafish induces $A_{und}$ differentiation into type A spermatogonia ($A_{diff}$) via β-catenin, which is a component of Wnt signaling [46]. Therefore, by modulating the Wnt signaling pathway, Sox13 might be involved in the maintenance of the SSC number and in $A_{und}$ differentiation.

Overall, these data point to a role for Sox13 in mouse and rat spermatogenesis [37]. The homology of Sox domains with the human SOX proteins [32] indicates that, being highly conserved in vertebrates, they might likely display similar functions among species. Furthermore, the strong similarity between mice and human spermatogenesis has led to a rapid increase in the list of genes recently discovered to be involved in human spermatogenetic failure, mainly based on mouse and rat studies [47]. Although no data have been provided on the role of the *SOX13* gene in human spermatogenesis, it may likely be involved in human spermatogenesis due to its expression in mouse and rat spermatogonia [37].

We found *SOX13* downregulation in PBMCs from patients with KS. Some studies have recently addressed a diagnostic role of NGS analysis in PBMCs of patients with apparently idiopathic nonobstructive azoospermia, since the mutation of genes involved in spermatogenesis can be detected in the blood [47,48]. It cannot be excluded that SOX13 downregulation found in PBMCs may also occur in KS germ cells, leading to their apoptosis. Recent research has highlighted the role of *SOX13* in cell proliferation. In greater detail, it has been found to enhance Paired box gene 8 (PAX8) protein expression, in turn promoting the proliferation of gastric carcinoma cells [49]. In addition, *SOX13* upregulates angiogenesis in gliomas [50]. Taken together, these findings may suggest a role for *SOX13* in cell proliferation. In view of its expression at the spermatogonial level [37], *SOX13* may also be involved in germ cell proliferation.

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The evidence suggests a role for SOX13 dysregulation in the development of autoimmune diseases. By modulating the Wnt signaling pathway, Sox13 protein is involved in the emergence of gamma-delta T-cells in the thymus, opposing alpha-beta T cell differentiation, as the analysis of fetuses with *Sox13* gene gain-of-function and loss-of-function mutations suggests [32]. Accordingly, SOX13 has been identified as islet cell autoantigen 12 (ICA12), which is involved in the pathogenesis of autoimmune diseases, including type 1 DM and primary biliary cirrhosis [32]. It is noteworthy that endocrine organ-specific humoral autoimmunity is not rare in patients with KS. Data from 61 patients with KS and 122 controls indicate that it is more frequently directed against type 1 diabetes-related autoantigens [insulin, glutamate decarboxylase (GAD), Islet Antigen 2 (IA-2) and Zinc transporter 8 (Znt8) antibodies] [51], although the prevalence of type

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250 1 DM is low in these patients (few cases have been reported so far) [52,53]. Therefore, the possible role of
251 *SOX13* downregulation in the pathogenesis of autoimmune disorders in patients with KS deserves to be
252 examined.

253 Our results must be taken with care since no data from testicular tissue was available in the present
254 study. Accordingly, none of the patients gave his consent to proceed with testicular biopsy. We are aware
255 that this represents a limit for understanding the role of *SOX13* in spermatogenesis. However, the vast
256 majority of transcriptome studies on KS patients have analyzed the transcriptome from the blood [10,12-15,
257 54] due to the limitation in having testicular tissue. This is particularly true nowadays when the testicular
258 biopsy is used to retrieve spermatozoa for assisted reproductive techniques (ART). We think that the results
259 of the present study may prompt to develop further focused analysis in centers (or Countries) where
260 testicular biopsy of KS patients is readily available.

261 On the other hand, it could be speculated that the study of SOX13 expression in testicular tissue from
262 adults with KS would not be effective in finding *SOX13* downregulation since this tissue already lacks of
263 germ cells. Therefore, testicular *SOX13* expression would reflect germ cell loss in KS patients. By contrast,
264 blood downregulation might hypothetically reveal a molecular dysfunction possibly occurring in germ cells,
265 prior to and, maybe, favoring, their loss. However, further studies should be performed in aborted fetuses
266 with KS with the aim of assessing *SOX13* expression in KS germ cells.

267 Another reason to take with care our results is that, unfortunately, no data on testicular histology
268 could be provided as patients did not give their consensus. Indeed, total testicular volume is low in patients
269 with KS. Testicular fine needle biopsy would further reduce this volume, thus reducing the success rate of
270 ART in patients willing to undergo to this procedure later in life. However, the most typical histologic
271 feature of KS patients is Sertoli Cell Only Syndrome (SCOS) and, since all the enrolled patients were
272 azoospermic, it could be supposed that they had SCOS.
273

274 5. **Conclusions**

275 In conclusion, the present study reports, for the first time, a downregulation of the *SOX13* gene in the
276 PBMCs of patients with KS compared to controls. Data from animal studies indicate a role for *Sox13* in SSC
277 maintenance and in immune system regulation. Further studies are needed to establish whether *SOX13* is

involved in the pathogenesis of germ cell loss and in endocrine organ-specific humoral autoimmunity in patients with KS.

**Competing interests**

The authors declare that there are no conflicts of interest regarding the publication of this study. None of the authors have financial competing interests.

**Ethics approval and consent to participate**

This study was approved by the Ethical Committee of the University Teaching Hospital "Policlinico-Vittorio Emanuele," Catania, Italy: trial registration number 49/2015/PO (Register of the Ethics Committee opinions). All the participants in the study signed an informed consent form to participate.

**Consent for publication**

All the participants in the study signed an informed consent form to publish.

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**Funding**

None.

**Author contributions**

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RC conceived the study, participated in data analysis and wrote the original draft. MS conceived the study, participated in genetic analysis and wrote the original draft. RAC participated in data analysis and in project supervision. LC supervised the project. GG performed the genomic studies and participated in the statistical analysis. GM participated in the genomic studies and in data analysis. AC participated in the genomic studies and in data analysis. CR participated in the genomic studies and in data analysis. SLV supervised the project. AEC conceived the study, supervised the project and edited the final version of the manuscript. All authors have read and approved the final version of the manuscript, and agree with the order of presentation of the authors.

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None.

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All authors should be listed for papers with up to five authors; for papers with more than five, the first five authors only should be quoted, followed by et al.

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**Table 1.** Clinical and biochemical parameters of men with nonmosaic Klinefelter syndrome and age-matched controls.

| Parameters | Patients (mean ± SD) | Controls (mean ± SD) |
|---|---|---|
| Age (years) | 32.4±8.1 | 33.1±7.9 |
| BMI (kg/m$^2$) | 26.0±6.7 | 25.1±2.7 |
| Glycemia (mg/dl) | 81.2±14.7 | 87.8±8.3 |
| Insulin (µIU/ml) | 29.7±44.1 | 15.2±13.7 |
| HOMA-IR | 6.2±9.3 | 3.6±3.5 |
| LH (mIU/mL) | 20.9±7.6* | 5.0±2.1 |
| FSH (mIU/mL) | 32.7±16.9* | 3.5±0.6 |
| TT (ng/mL) | 3.8±2.4* | 5.9±1.8 |
| Total sperm count (mil/ejaculate) | 0 | 270.6±132.6 |

**Abbreviations**: BMI: Body mass index; HOMA-IR: Homeostasis Model Assessment of insulin resistance; LH: Luteinizing hormone; FSH: Follicle-stimulating hormone; TT: Total testosterone. Normal ranges: Glycemia: 60-100 mg/dl; Insulin: 1.9-23 µIU/ml; LH: 1.14–8.75 IU/l; FSH: 0.95–11.95 IU/l; TT: 2.5–9.8 ng/ml. *p<0.05 vs. Controls (Student's *t* test).

**Note:** Age, BMI, LH, FSH, TT and testicular volume values for each patient and control are detailed in the study of Cimino *et al.*

**Commento [AJA42]:** Please state the objects of comparison.

## Figure Legends

**Figure 1**. **NGS analysis**. **Panel A.** Screenshot from IGV - Integrative Genomics Viewer for SOX13. Three control samples and three Klinefelter samples among the ten samples are displayed. **Panel B.** Istograms showing the expression (FPKM) in the 20 sequenced samples. In red are shown the Control samples while in blue are shown the Klinefelter samples. **Panel C.** Boxplot showing the SOX13 expression (FPKM) in Control samples (in red) and in Klinefelter samples (in blue). SOX13 expression is lower in Klinefelter samples. *indicates p-value<0.05. FPKM, Fragments Per Kilobase of transcript per Million; IGV, Integrative Genomics Viewer; *SOX13*, sex-determining region of Y(SRY)-BOX *13*.

**Figure 2:** Mean fold-change expression of SOX13 in men with Klinefelter syndrome (KS) and normal controls (NC). *indicates p-value<0.05.

**Commento [AJA43]:** As the words in the figures are not in proper format. Please provide TIFF figures with all the words and labels in editable and independent layers (do not merge them) as the guidance of figures shows. Therefore, editors can edit them when it is necessary during the next stages. Thanks.
The width of all figures can be adjusted within 8 cm, and all texts should be Arial font and 5 pt size.

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**Commento [AJA47]:** The bars are too wide, please narrow down them.

**Commento [rc48]:** This is not possible. Bars reflect the standard deviation, that is a given value.
Actually, we suggest the width of the red and blue blocks decrease, not the standard deviation. This issue is not a big deal; it is fine to keep it as is.

**Commento [AJA49]:** Please define all the abbreviations.

**Commento [AJA50]:** The bars are too wide, please narrow down them.

**Commento [rc51]:** This is not possible. Bars reflect the standard deviation, that is a given value.
it is fine to keep it as is.