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Clinical Research Article

From Klinefelter Syndrome to High Grade Aneuploidies: Expanding the Gene-dosage Effect of Supernumerary X Chromosomes

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Abstract

Objective: High-grade aneuploidies of X and Y sex chromosomes (HGAs) are exceedingly rare and complex conditions. We aimed to investigate the effect of supernumerary X chromosomes (extra-Xs) on the clinical, hormonal, metabolic, and echocardiographic features of patients with HGAs.

Design and Methods: In a cross-sectional study, we compared 23 subjects with HGAs and 46 age-matched subjects with 47,XXY Klinefelter syndrome (KS), according to the number of extra-Xs: two (47,XXY and 48,XXYY), three (48,XXXY and 49,XXXYY), or four supernumerary Xs (49,XXXXY). A second cohort consisting of 46 pubertal stage-matched KS subjects was employed for validation. Clinical, hormonal, metabolic and ultrasonographic parameters were collected and analyzed.

Results: The increase in the number of extra-Xs was associated with a progressive adverse effect on height, pubertal development, testicular volume and function, adrenal steroidogenesis, and thyroid function. A progressive linear increase in ACTH and a decrease in cortisol/ACTH ratios were found. Weight and body mass index, Sertoli cell function, lipid profile, and glucose tolerance post-oral glucose tolerance test were all worse in the HGA cohort compared to KS. Cardiac evaluation revealed a linear association with reduced left and right end-diastolic diameters and reduced ejection fraction.

Conclusion: The increase in the number of extra-Xs is associated with a "dose-dependent" progressive impairment in steroid producing glands, thyroid function, cardiac structure, and performance.

Key Words: sex chromosomes, aneuploidy, HGA, Klinefelter syndrome, X chromosome, 47,XXY, 48,XXXY, 48,XXYY, 49,XXXXY, 49,XXXYY

Sex chromosome aneuploidies represent the most frequent form of chromosome number abnormality in humans (1). Among them, high-grade aneuploidies (HGAs) with more than 47 chromosomes and a male phenotype are extremely rare. Tetrasomies and pentasomies with supernumerary X and/or Y chromosomes arise from a paternal or maternal non-disjunction event during the first and/or second meiosis (2), generating, among others, the following karyotypes: 48,XXYY, with a prevalence of 1:18.000-1:50.000 (3); 48,XXXY, with a prevalence of 1:50.000; 49,XXXXY, with a prevalence of 1:85.000-1:100.000; and 49,XXXYY, with <10 cases reported (3-5), and other even rarer sex chromosomes combinations (6). HGAs may also present with karyotype mosaicism (7).

The first male HGA case was reported in 1960 in a 7-year-old patient with 49 chromosomes (3). For a long time, HGAs were considered more severe variants of the classic 47,XXY Klinefelter syndrome (KS), the most common sex chromosome disorder in men, with a considerably higher incidence of 1:520 among prenatal diagnoses (8). Although HGAs share with KS some common features, such as the eunuchoid habitus and the hypergonadotropic hypogonadism, accompanied by testicular failure and infertility, patients with HGAs display specific facial features and multiple skeletal anomalies, including radio-ulnar synostosis, cardiac and renal defects, hypotonic musculature, and tremors (9, 10). Main characteristic traits also include hypertelorism with epicanthal folds, cubitus varus, clubfoot, pes planus, clinodactyly, scoliosis, and dental problems (10, 11). Finally, severe cognitive and behavioral dysfunction and neurologic complications are extremely frequent (12).

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As our cohort of patients with HGAs increased over time, the phenotypic differences with KS subjects became increasingly apparent, which led our group to illustrate the endocrine and metabolic differences between the KS and HGAs cohorts with regards to the earlier timing of gonadal failure in HGAs (13). These findings are consistent with previous case reports, which pointed toward poorer outcomes in HGAs compared to 47,XXY males (12, 14, 15). However, because of the rarity of the conditions, comprehensive and long follow-up studies are lacking, little is known about the mechanisms involved, and recommendation on clinical management are missing.

For these reasons, we are now performing a much more comprehensive clinical, hormonal, and imaging evaluation of patients with HGAs. Specifically, we were interested in exploring extra-gonadal complications and testing whether the phenotypic variance among HGAs could be ascribed to the number of supernumerary X chromosomes (extra-Xs), rather than Y chromosomes, as the latter contain just 63 protein-coding genes compared with the 856 expressed by the X chromosome, based on Ensembl data (v107) (16). As such the present study aims to assess the presence of a direct X-related gene dosage effect on the phenotype of patients with HGAs in one of the largest cohort of HGAs with male phenotype reported so far.

Materials and Methods

Study Participants

We enrolled 23 patients with HGAs followed at the Endo-ERN and Regional Rare Endocrine and Andrological Disease Centre at the Department of Experimental Medicine of Sapienza University of Rome, Italy ("Policlinico Umberto I" Hospital). All HGA patients with available data were included and randomly matched by age (±1 year), at a 1:2 ratio, with 46 patients with KS followed in our center. An additional cohort of 46 subjects with KS, matched by pubertal status at a 1:2 ratio, was included for validation of significant findings, as well as gonadal development and function parameters. In all patients the diagnosis was established by postnatal peripheral blood karyotype analysis, conducted on at least 40 metaphases, thus excluding mosaicism (13, 17). All patients underwent a thorough clinical examination, a hormonal and metabolic evaluation, and a detailed ultrasonographic study of the testes, according to the European Academy of Andrology ultrasound Standard Operating Procedures (18) and of the heart. Pubertal stage was evaluated according to Tanner original description for external genitalia appearance (ie, scrotum, penis, pubic hair) (19). At the time of the evaluation, none of the patients was taking testosterone replacement therapy or any other hormonally or metabolically active drugs.

Written informed consent was obtained from all the participants of legal age able to do so, whereas in minors it was obtained from the participant's parents (Local Ethics Committee Approval Ref. CE 7155, Protocol no. 0414/23 of March 5, 2023).

Hormonal and Metabolic Evaluations

Pituitary, thyroid, gonadal, and adrenal function was assessed by fasting, early morning venipuncture and measurement of TSH, free T3 (fT3), free T4 (fT4), FSH, LH, sex hormone-binding globulin (SHBG), dehydroepiandrosterone sulfate (DHEAS), 17β-estradiol (E2), total testosterone (T), inhibin B (INHB), Δ4-androstenedione (Δ4), cortisol, and ACTH.

All tests were performed in duplicate in the laboratory of the Department of Experimental Medicine, Sapienza University of Rome. TSH, fT3, fT4, FSH, LH, SHBG, DHEAS, E2, and T were measured with chemiluminescent microparticle immunoassay (CMIA, Architect System; Abbott Laboratories, IL, USA) with limits of detection (LOD) of .0025 μIU/mL, 1.0 pg/mL, .4 ng/dL, .05 mIU/mL, .07 mIU/mL, .1 nmol/L, 3.0 μg/dL, 10 pg/mL, and .28 nmol/L respectively. Intra- and interassay coefficients of variation (CVs) for our laboratory were 3.6% and 5.4% at 2.71 μIU/mL (TSH), 2.8% and 5.2% at 2.76 pg/mL (fT3), 3.1% and 5.1% at 1.07 ng/dL (fT4), 3.6% and 5.4% at 3.2 mIU/mL (FSH), 3.8% and 5.5% at 4.1 mIU/mL (LH), 5.65% and 9.54% at 8.8 nmol/L (SHBG), 2.68% and 7.41% at 10.7 μg/dL (DHEAS), 5% and 7% at 190 pg/mL (E2), and 2.1% and 3.6% at 10.08 nmol/L (T) (20). INHB was measured by enzyme-linked immunosorbent assay (GEN II, Beckman Coulter Laboratories, Brea, CA, USA) with LOD of 4.0 pg/mL, whereas intra- and interassay CVs were 3.3% and 7.2%, respectively, at 122 pg/mL (21). Δ4 was measured with radioimmunoassay (Cisbio Bioassays, Codolet, France) with LOD 5 ng/dL, whereas the intra- and interassay CVs were 7.4% and 8.1%, respectively, at 168 ng/dL. Cortisol was measured by radioimmunoassay (Beckman Coulter): the LOD was 5 nmol/L, and the intra- and interassay CVs were 4.9% and 7.8%, respectively, at 447.8 nmol/L. Immunoradiometric assay (Cisbio Assays, France) was used to measure ACTH levels, with LOD of 2 pg/mL, and intra- and interassay CVs of 5.9% and 6.5% at 48 pg/mL. Calculated free T (cfT) was obtained using Vermeulen's formula, assuming a fixed albumin concentration of 4.3 g/dL. We further derived the T/LH and cortisol/ACTH ratios as markers of Leydig cell function and adrenal sensitivity, respectively (22, 23).

Total and high-density lipoprotein (HDL) cholesterol, and triglyceride levels were measured by an enzymatic colorimetric assay, whereas low-density lipoprotein (LDL) cholesterol was derived by using the Friedewald formula. Glucose was measured by the hexochinase-G6PD method, whereas insulin was determined by an electrochemiluminescent assay. Both glucose and insulin were measured under basal conditions and after 120 minutes from an oral glucose tolerance test (oGTT) with 75 g of glucose. Glycated hemoglobin was assessed by high-performance liquid chromatography. Transaminases and γ-GT were measured by the International Federation of Clinical Chemistry and Laboratory Medicine method at 37 °C (24). The Homeostatic Model Assessment index (HOMAi) was calculated as a hepatic insulin-resistance marker (25).

Clinical and Ultrasonographic Evaluations

Anthropometric evaluations were performed using a Harpenden stadiometer to measure height and calibrated scales to determine weight. For each patient, we derived the body mass index (BMI) and calculated the body surface area according to Haycock's formula. Height, weight, and BMI values were transformed into standard deviation scores (SDS) according to 2006 Italian reference growth parameters (26) using the free software Growth4 (http://www.weboriented.it/gh4/) by the Italian Society for Pediatric Endocrinology and Diabetology to allow for comparisons among children and adults. Ultrasonographic bitesticular volume (US-BTV) was assessed through color Doppler evaluation (Philips iU22),

performed using linear transducers (5-12 MHz) and employing Lambert's formula, given its higher accuracy with regard to reduced bitesticular volumes (27). Finally, the stretched penile length was measured from the pubic symphysis to the tip of the glans using a standard ruler.

Echocardiographic examinations were performed with a 2.5-MHz transducer (Esaote MyLab™X7), using standardized techniques in accordance with the European Association of Cardiovascular Imaging recommendations (28), evaluating left ventricular end-diastolic diameter (LV-EDd), LV end-systolic diameter (LV-ESd), right ventricular EDd (RV-EDd), interventricular septum EDd (IVS-EDd), posterior wall EDd (PW-EDd), left atrial diameter (LAd), aortic root diameter (ARd), ascending aorta diameter (AAd), ejection fraction (EF), and epicardial fat thickness (EFT) measured on the free wall of the RV from both parasternal long-axis and short-axis views at ED in 3 cardiac cycles. The maximum value at any site was measured, and the average value was considered (29). LV mass was calculated according to the linear method, cube formula, and indexed to the body surface area. Relative wall thickness (RWT) was calculated as follows: (2 * PW-EDd)/LV-EDd.

Statistical Analysis

Data are expressed as medians or means adjusted for the number of Y chromosomes and as 95% bias-corrected and accelerated (BCa) confidence intervals (CI) or interquartile ranges at 25°–75° percentiles, as appropriate. To assess the effect of extra-Xs on phenotype, patients with HGAs and KS were classified as follows: group 1, XX/- karyotypes (ie, 47,XXY; 48,XXYY); group 2, XXX/- karyotypes (ie, 48,XXXY; 49,XXXYY); and group 3, XXXX/- karyotypes (49,XXXXY). In view of the numerosity of some groups, we employed bootstrapping of 2000 samples as a resampling technique for the calculation of the adjusted means and the respective BCa 95% CI, as recommended in rare diseases (30, 31). The groups were compared using independent samples *t*-tests for unequal variances with bootstrapping. We further conducted linear trend analyses to assess the additive effect of the number of extra-Xs (predictive variable), evaluating the different variables of interest (outcome variables), adjusting for the number of Y chromosomes (covariate). Given the exploratory nature of the study, as well as the relatively limited numerosity due to the extreme rarity of HGAs, we adjusted for multiple comparisons employing the false discovery rate (FDR) method according to Benjamini-Hochberg, Dunn-Šidàk version, and report both nonadjusted and FDR-significant *P*-values in tables (32, 33), taking into account the following familywise comparisons: anthropometric parameters, gonadal and penile development, thyroid, testicular and adrenal function, glucose and lipid metabolism, cardiac parameters. We also conducted a sensitivity analysis, excluding prepubertal subjects, with regards to parameters concerning gonadal and penile development and testicular function, which confirmed the results of the primary analysis. Statistical comparisons for categorical variables were conducted with the χ^2 tests, and for dichotomic variables Fisher's two-tailed exact test was employed. Data are presented in figures as violin plots (KS data) alongside the means adjusted for the number of Y chromosomes and their respective BCa 95% CI (HGA data). The level of statistical significance was set at .05 (two-sided). Statistical analyses were performed using IBM SPSS Statistics for Windows, version 27 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 8.3.0 (GraphPad Software, LLC).

Results

Anthropometric and US-BTV Data

Participants' karyotypes, age, anthropometric data, and US-BTV for HGAs and age-matched KS subjects are reported in Table 1 and Fig. 1. Age was similar between groups. Pubertal stage was significantly delayed in HGAs subjects compared to age-matched KS individuals ($P = .023$). A negative trend was observed for height SDS with an increasing number of X chromosomes, whereas the HGA group demonstrated higher weight and BMI SDS than the KS group (Fig. 1A–1C).

A difference was observed in US-BTV between the KS and HGA age-matched groups, with a decreasing linear trend as the number of supernumerary Xs increased, ie, 5.93 mL [5.24, 6.27] for group 1 vs 3.89 mL [2.02, 6.33] for group 2 vs 1.26 mL [.56, 1,98] for group 3 (Fig. 1D). No group differences or linear trends were apparent in the other anthropometric parameters analyzed in this study.

Hormonal Profile

Several differences were observed in basal hormonal values between age-matched participants grouped according to the number of extra-Xs (Table 1; Fig. 2). A reduction in fT4 levels and a respective increase in the fT3/fT4 ratio were observed with the increasing number of Xs, but no compensatory rise in TSH levels was found (Fig. 2A–2D). Serum T levels showed a linear decrease with the increasing number of extra-Xs, although LH, FSH, and SHBG levels were not different (Fig. 2E–2H). Furthermore, the HGA group showed lower cfT concentrations and INHB levels, often at or below the LOD (Fig. 2I and 2K). We also found a positive linear trend for ACTH values, accompanied by decreasing DHEAS and Δ4 levels (Fig. 2M, 2P, and 2Q). Consistently, the cortisol/ACTH ratio, a marker of adrenal responsiveness, was affected by the number of extra-Xs and, as such, was lower in the HGA group than in the KS group, although serum cortisol levels were not different between the groups (Fig. 2O and 2N). No differences were noted in the other hormonal parameters analyzed.

Metabolic Parameters

Participants' metabolic parameters are reported in Table 2 and Fig. 3. No linear trends were observed with an increasing number of extra-Xs, although the HGA group showed higher LDL and lower HDL cholesterol levels than the age-matched KS group (Table 2; Fig. 3B and 3C). Concerning glucose homeostasis, the HGA group showed higher glucose and insulin levels, at both basal and 2-h post oGTT, and a higher HOMAi (Fig. 3E–3I). By contrast, hemoglobin $A1_C$ levels showed no difference or trend (Fig. 3J). No differences were also observed for the other metabolic data.

Echocardiographic Data

Age-matched participants' cardiac US data are reported in Table 2 and Fig. 3. As regards the cardiac structure, a linear trend was observed toward reduced LV and RV-EDd, with the increasing number of extra-Xs. A less evident trend

Table 1. Anthropometric data, US-BTV, and hormonal parameters according to the number of supernumerary X chromosomes

	KS subjects	HGA subjects	*P* KS vs HGAs	Group 1 (XX/-)	Group 2 (XXX/-)	Group 3 (XXXX/-)	FDR[a] Linear trend
Karyotypes and number of subjects	n = 46	n = 23		47,XXY: n = 46 48,XXYY: n = 10	48,XXXY: n = 4 49,XXXYY: n = 1	49,XXXXY: n = 8	
Age, years	19.0 [14.0, 23.9]	18.5 [11.3, 28.1]	.934	19.3 [14.0, 25.7]	25.9 [11.6, 32.0]	13.7 [7.1, 22.3]	/
Height, SDS	.60 [−.38, 1.29]	.74 [−.54, 1.37]	.899	.61 [.31, .89]	.56 [−.42, 1.87]	−.34 [−1.11, .42]	.049
Weight, SDS	−.15 [−.88, .74]	1.20 [.08, 1.77]	**.002**	.13 [−.15, .40]	1.49 [.05, 2.73]	−.41 [−1.53, 1.20]	.722
BMI, SDS	−.55 [−1.47, .42]	1.03 [.51, 1.60]	**<.001**	−.25 [−.59, .08]	1.41 [.29, 2.56]	.15 [−1.95, 1.60]	.464
Penile length, cm*	4.75 [4.00, 6.63]	5.25 [4.08, 7.25]	.470	5.67 [4.85, 6.50]	5.07 [3.31, 6.72]	5.44 [4.29, 7.04]	.784
US-BTV, mL*	5.90 [4.60, 8.10]	3.00 [1.70, 5.55]	**<.001**	5.93 [5.20, 6.67]	3.89 [2.02, 6.33]	1.26 [.56, 1.98]	**<.001**
TSH, µIU/mL	1.55 [1.14, 2.23]	1.89 [.88, 2.55]	.561	1.73 [1.55, 1.92]	2.34 [.84, 3.77]	1.62 [1.07, 2.14]	.715
fT3, pg/mL	3.28 [3.07, 3.43]	3.32 [3.12, 3.63]	.376	3.25 [3.16, 3.34]	3.54 [3.14, 3.84]	3.33 [3.11, 3.61]	.564
fT4, ng/dL	1.01 [.94, 1.10]	.91 [.84, .99]	**.001**	1.01 [.97, 1.05]	.98 [.81, 1.14]	.86 [.82, .91]	**.010**
fT3/fT4 ratio	3.24 [2.81, 3.60]	3.64 [3.38, 3.96]	**.002**	3.30 [3.15, 3.45]	3.67 [3.20, 3.98]	3.87 [3.54, 4.30]	**.009**
LH, mIU/mL*	10.28 [5.30, 16.79]	8.34 [.16, 13.43]	.105	10.42 [8.28, 12.53]	10.14 [3.08, 15.94]	7.40 [2.77, 12.23]	.284
FSH, mIU/mL*	23.10 [12.37, 34.67]	21.79 [3.60, 29.22]	.315	21.92 [17.98, 26.29]	22.28 [6.83, 34.55]	21.41 [13.68, 28.08]	.902
T, nmol/L*	12.92 [8.53, 16.08]	5.55 [.46, 13.18]	**.020**	12.10 [10.19, 14.00]	7.02 [2.03, 13.09]	5.48 [.07, 12.14]	.021
SHBG, nmol/L*	38.7 [28.6, 58.0]	39.3 [28.6, 60.2]	.984	49.1 [40.4, 59.1]	48.8 [35.1, 58.0]	45.0 [24.9, 65.9]	.753
cfT, nmol/L*	.24 [.16, .32]	.09 [.01, .24]	**.035**	.23 [.19, .26]	.11 [.03, .21]	.16 [.01, .35]	.259
T/LH ratio*	1.26 [.62, 2.16]	.84 [.39, 1.51]	.053	1.53 [1.26, 1.83]	.71 [.45, .95]	1.10 [.03, 2.57]	.371
E2, pg/mL*	23.5 [12.5, 33.3]	18.0 [10.0, 35.0]	.305	25.4 [21.9, 29.1]	20.9 [11.3, 32.6]	26.2 [11.2, 45.7]	.887
INHB, pg/mL*	8.65 [4, 59.18]	4.0 [4.0, 8.0]	**.009**	41.0 [26.8, 58.2]	4.90 [0, 18.0]	7.61 [0, 17.6]	.113
DHEAS, µg/dL	199.2 [88.0, 327.6]	92.3 [29.4, 183.6]	**.006**	214.5 [176.9, 252.6]	147.1 [50.4, 267.3]	30.4 [9.9, 82.4]	**.001**
Δ4, ng/dL	174.0 [77.0, 241.5]	101.0 [38.0, 179.0]	.084	157.0 [128.9, 184.3]	138.1 [48.1, 218.1]	73.9 [24.4, 136.6]	**.025**
ACTH, pg/mL	42.2 [24.7, 59.8]	62.3 [45.2, 77.5]	**.009**	50.5 [41.1, 60.9]	58.5 [41.1, 75.3]	74.3 [54.9, 103.3]	.044
Cortisol, nmol/L	402.0 [302.0, 581.0]	372.0 [267.0, 544.0]	.259	432.3 [380.2, 486.4]	506.6 [403.1, 612.9]	334.0 [233.0, 458.5]	.149
Cortisol/ACTH ratio	13.03 [7.64, 17.45]	6.83 [2.94, 8.38]	**.002**	11.67 [9.58, 13.83]	9.84 [5.02, 16.44]	5.28 [2.35, 8.67]	**.006**

Abbreviations: Δ4-androstenedione; BMI, body mass index; cfT, calculated free testosterone; DHEAS, dehydroepiandrosterone sulfate; E2, 17β-estradiol; EF, ejection fraction; FDR, false discovery rate; fT3, free T3; fT4, free T4; HGAs, high-grade sex chromosome aneuploidies; INHB, inhibin B; KS, Klinefelter syndrome; SDS, standard deviation score; T, total testosterone; US-BTV, ultrasound-derived bitesticular volume. Data are reported as means adjusted for the number of Y chromosomes and their bias-corrected and accelerated 95% confidence intervals for group comparisons of 47,XXY vs HGA subjects, and the linear trend according to the number of supernumerary X chromosomes, except for data concerning the KS group and for age, which are reported as median and 25° to 75° interquartile range. Significant *P*-values are presented in bold. A sensitivity analysis was conducted after the exclusion of prepubertal subjects for the parameters marked by *, confirming statistical significance.
[a]For linear trend analyses nonadjusted *P*-values are reported, with significant values after FDR adjustment presented in bold.

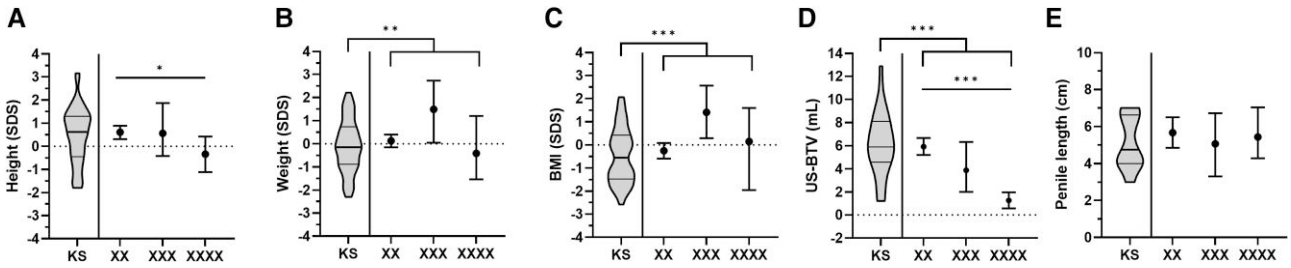

Figure 1. Anthropometric and US-BTV in patients with KS and HGAs. Note: Data of patients with KS are shown by violin plots, whereas the means adjusted for the number of Y chromosomes and their respective BCa 95% CI are reported for patients with HGAs. The bar indicates statistical significance for linear trend analyses, whereas the brackets indicate comparisons across groups. The single, double, and triple stars represent statistical significance levels below .05, .01, and .001, respectively, before adjustment by FDR.
Abbreviations: BCa, bias-corrected and accelerated; CI, confidence interval; FDR, false discovery rate; HGA, high-grade aneuploidies of X and Y sex chromosomes; KS, Klinefelter syndrome; US-BTV, ultrasonographic bitesticular volume.

toward reduced LV-ESd and left ventricular mass index with the increase in extra-Xs was also observed. Reduced RV-EDd and increased RWT were observed in the HGA group (Fig. 3K, 3L, 3M, 3P, and 3Q).

LV hypertrophy, as defined by a left ventricular mass index >115 g/m^2, was present in 2 KS patients and in none in the HGA group, whereas an increased RWT was observed in 3 KS patients and 5 HGAs patients. Regarding cardiac geometry 2 KS patients showed concentric remodeling as compared with 5 in the HGA group and 1 each presenting with concentric and eccentric hypertrophy among KS patients.

Cardiac function was found affected, with a tendency toward a reduced EF with the increase in the number of extra-Xs (Fig. 3S), linking cardiac structure alterations with functional changes. No differences were found in the other echocardiographic parameters, including atrial and aortic diameters, as well as EFT.

Pubertal Stage-matched Validation Cohort

We compared HGAs subjects with an additional validation cohort composed of 46 pubertal stage-matched KS individuals. Significant findings by linear trend analyses with the age-matched cohort were investigated, alongside gonadal development and function parameters, and are shown in Supplementary Table 1 (34). Statistically significant trends were confirmed with the increase in the number of X chromosomes with regards to gonadal development (US-BTV and INHB), thyroid function (fT4 and fT3/fT4 ratio), adrenal function (DHEAS and cortisol/ACTH ratio), and echocardiographic parameters (LV-EDd, EV-EDd, and EF). No significant trends were observed in testicular endocrine function parameters when matching by Tanner stage.

Discussion

The X chromosome-gene dosage effect is key to understand the pathogenesis of HGAs, but the mechanisms linking it to the clinical phenotype are far from understood. The hypothesis beneath the present research builds upon our previous study, where we demonstrated premature testicular damage in HGAs compared to classic 47,XXY KS (13). In the present paper we establish a link between the number of extra-Xs and both gonadal and extra-gonadal clinical, endocrine, metabolic, and cardiac characteristics, untangling the complexity behind multiple karyotypes.

With regards to height, HGAs patients with pentasomies show a lower stature than patients with tetrasomies and 47,XXY KS. Specifically, 48,XXYY patients present an above-average stature and are generally taller than 180 cm. In fact, the 75th° percentile is usually reached between the ages of 11 and 19, whereas the 95th° percentile is frequently reached after the age of 20 years. An important role in determining the higher stature could be played by the *SHOX* gene, involved in bone maturation and skeletal growth, especially of the long bones: *SHOX* is located in the PAR 1 region of the X chromosome and escapes X chromosome inactivation (35). In this regard, it has to be taken into account the possible explanation of the lower stature of pentasomies to also partly reside in the higher degree of musculoskeletal complications, especially scoliosis (36). The existence of an additional gene influencing the final height has been hypothesized on the Y chromosome because patients with two Y chromosomes tend to have a higher average height (37).

In our study, after adjusting for the number of Y chromosomes, a borderline effect was apparent for a negative linear trend on height SDS with increasing extra-Xs, although not statistically significant after FDR adjustment. The most striking clinical data was the progressive decrease of the US-BTV with the increase in the number of extra-Xs. Although testicular volumes were at pathologically reduced levels in all groups, this evidence unequivocally suggests more severe gonadal damage as the X chromosome-gene dosage increases and is consistent with our recent publications. The trend for lower T values also supported this hypothesis. Neither the (elevated) gonadotropins levels nor the T/LH ratio showed a clear trend; this finding possibly indicates a hypothalamic–pituitary–gonadal axis that is already driven to its maximum activation or possibly a central dysfunction. Altogether, the evidence points toward a progressive worsening of Leydig cells' activity, which results in the reduction of the androgen steroidogenesis. Indeed, in daily clinical practice, patients with HGAs frequently need testosterone replacement therapy to induce or complete pubertal development, unlike what is most commonly observed in KS, in which the pubertal process starts spontaneously and is often autonomously completed (38, 39). Accordingly, in our HGAs cohort pubertal development was significantly delayed compared to age-matched KS individuals. Serum INHB, a marker of the Sertoli cell function, also demonstrated reduced values in patients with HGAs, although a linear trend was not apparent because the values of many patients are equal to the LOD.

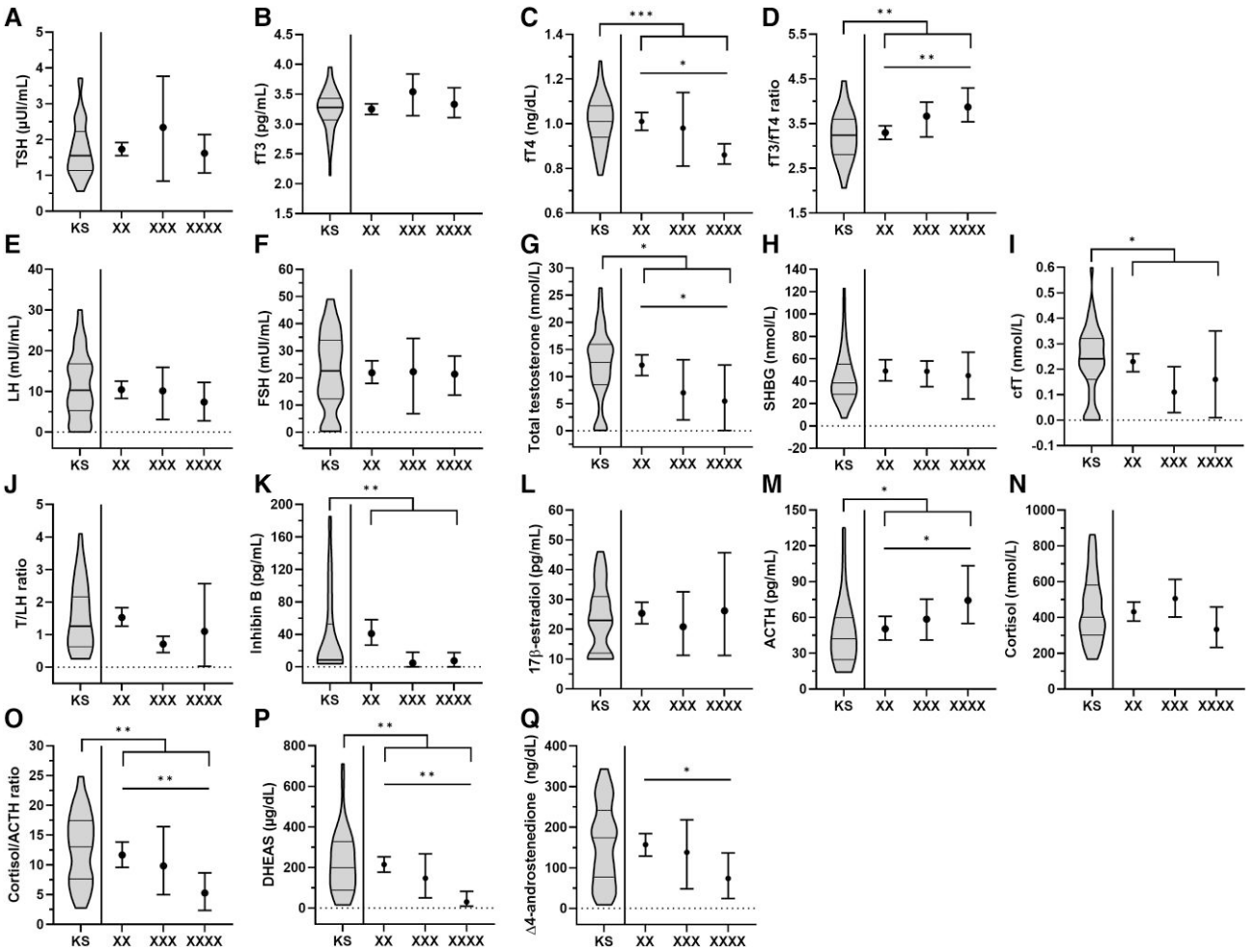

Figure 2. Hormonal parameters in patients with KS and HGAs. Note: Data of patients with KS are shown by violin plot, whereas the means adjusted for the number of Y chromosomes and their respective BCa 95% CI are reported for patients with HGAs. The bar indicates statistical significance for linear trend analyses, whereas the brackets indicate comparisons across groups. The single, double, and triple stars represent statistical significance levels below .05, .01, and .001, respectively, before adjustment by FDR.
Abbreviations: BCa, bias-corrected and accelerated; CI, confidence interval; FDR, false discovery rate; HGA, high-grade aneuploidies of X and Y sex chromosomes; KS, Klinefelter syndrome.

The study of the hypothalamic–pituitary–gonadal axis confirms a condition of consistent hypergonadotropic hypogonadism in both KS and HGAs, as demonstrated by severe testicular hypotrophy, constantly low or undetectable levels of INHB and T, and stable increase in gonadotropins. Nonetheless, the gonadal impairment is more severe in HGAs and linearly correlated with the increase in extra-Xs. This finding is in line with previous results in which we showed earlier gonadal damage in HGAs, as indicated by the anticipated FSH rise in patients aged <12 years (13).

A novel aspect of the current study is the assessment of adrenal function in supernumerary Xs. Unexpectedly, we found that as the number of Xs increased, levels of both Δ4 and DHEAS showed a progressive decreasing trend, accompanied by rising ACTH levels. These observations point toward an overall reduced steroidogenic activity at both the gonadal (T, cfT, and Δ4) and adrenal (DHEAS and Δ4) levels. In line with this hypothesis, the cortisol/ACTH ratio also showed a clear negative trend.

Finally, also according to a linear trend, thyroid function analysis showed a progressive decrease in fT4 values as the number of supernumerary Xs increased (and a symmetrical rise of the fT3/fT4 ratio). This finding is relevant, as it confirms previous data findings demonstrating lower fT4 concentrations in adults with KS compared with healthy controls, although clustering alongside the lower limit of the reference range or slightly below (40). The missing rise in TSH values is also in line with the hypothalamic–pituitary dysfunction demonstrated in KS (41), which appears to be linked, both directly through reduced T levels and higher central "sensing" of peripheral thyroid hormone levels, and indirectly to the increase in the number of X chromosomes.

As KS is associated with an increased cardiovascular risk (42), we focused on biochemical risk factors in HGAs. Concerning the metabolic profile, the increase in supernumerary Xs was not associated with progressively worse glucose or lipid metabolism, although patients with HGAs showed lower HDL and higher LDL concentrations, as well as higher fasting and 2 hours post-oGTT glucose and insulin, and HOMAi levels than KS patients. Of note, total cholesterol, triglyceride concentrations, and hemoglobin $A1_C$ were not different.

We also conducted a complete echocardiographic evaluation, which revealed a relationship between the increase in the number of extra-Xs and both lower LV- and RV-EDd, as well as LV-ESd (although not significant after FDR adjustment). Cardiac geometry was not apparently different between

Table 2. Metabolic parameters according to the number of supernumerary X chromosomes in subjects with KS and HGAs

	KS subjects	HGAs subjects	*P* KS vs HGAs	Group 1 (XX/-)	Group 2 (XXX/-)	Group 3 (XXXX/-)	FDR[a] Linear trend
Karyotypes and number of subjects	n = 46	n = 23		47,XXY: n = 46 48,XXYY: n = 10	48,XXXY: n = 4 49,XXXYY: n = 1	49,XXXXY: n = 8	
Total cholesterol, mg/dL	154.5 [138.3, 173.3]	158.0 [143.5, 210,0]	.209	158.9 [151.5, 167.5]	176.0 [132.0, 231.4]	160.4 [132.9, 188.8]	.898
HDL cholesterol, mg/dL	53.0 [44.0, 64.0]	41.0 [36.5, 46.0]	**<.001**	54.4 [49.8, 59.5]	38.1 [31.7, 45.3]	47.8 [36.4, 63.3]	.389
LDL cholesterol, mg/dL	76.0 [54.0, 95.8]	101.0 [86.5, 145.0]	**.003**	88.0 [80.4, 95.1]	115.4 [70.4, 164.6]	98.4 [71.8, 125.7]	.426
Triglycerides, mg/dL	66.0 [48.3, 86.8]	74.0 [50.0, 121.5]	.222	85.9 [71.1, 104.3]	112.1 [62.9, 166.3]	72.4 [44.1, 107.5]	.616
Fasting glucose, mg/dL	85.0 [79.0, 89.0]	92.0 [86.0, 92.0]	**.001**	84.7 [82.5, 87.0]	93.6 [89.7, 98.1]	87.6 [77.7, 99.0]	.395
2-hour glucose, mg/dL	91.0 [80.0, 90.5]	105.0 [86.0, 121.0]	**.012**	93.9 [87.4, 101.3]	110.4 [56.4, 157.5]	113.2 [104.3, 125.1]	.146
Fasting insulin, µU/mL	6.50 [3.80, 10.60]	14.70 [7.20, 21.50]	**.003**	10.92 [8.18, 14.47]	26.69 [8.68, 47.33]	12.00 [7.29, 17.28]	.848
2-hour insulin, µU/mL	12.20 [7.90, 23.30]	73.60 [45.80, 187.90]	**<.001**	41.50 [24.60, 60.42]	109.64 [4.39, 236.07]	106.72 [62.75, 173.82]	.057
HOMAi	1.31 [.80, 2.20]	3.92 [1.62, 5.06]	**<.001**	2.43 [1.68, 3.39]	16.18 [2.26, 52.03]	3.15 [1.42, 4.85]	.806
$HbA1_C$, %	5.20 [5.00, 5.40]	5.20 [5.10, 5.50]	.306	5.15 [5.06, 5.24]	5.32 [5.13, 5.59]	5.31 [4.99, 5.60]	.252
LV-EDd, mm	45.0 [44.0, 48.0]	45.0 [43.3, 47.0]	.457	45.4 [44.3, 46.4]	45.2 [42.3, 47.1]	40.4 [33.6, 45.5]	**.006**
LV-ESd, mm	27.0 [25.0, 29.0]	26.0 [23.8, 28.0]	.254	26.9 [26.1, 27.6]	26.4 [23.1, 28.1]	24.0 [21.9, 25.5]	.023
RV-EDd, mm	23.0 [21.8, 25.0]	20.5 [19.3, 22.0]	**.001**	22.5 [21.5, 23.5]	22.5 [21.5, 23.5]	17.5 [14.6, 19.8]	**<.001**
IVS-EDd, mm	10.00 [8.00, 10.00]	10.00 [8.75, 10.00]	.498	9.08 [8.67, 9.43]	9.08 [8.67, 9.43]	9.02 [7.04, 10.63]	.930
PW-EDd, mm	9.00 [8.00, 9.00]	9.00 [8.75, 10.00]	.229	8.76 [8.41, 9.10]	8.76 [8.41, 9.10]	7.89 [6.13, 9.16]	.110
LVMi, g/m^2	77.5 [71.0, 86.5]	77.0 [68.3, 80.0]	.354	80.1 [75.4, 85.3]	76.0 [69.4, 83.1]	73.2 [59.0, 86.2]	.360
RWT	.38 [.36, .40]	.40 [.39, .43]	**.012**	.39 [.38, .40]	.39 [.38, .40]	.39 [.36, .41]	.964
LAd, mm	30.5 [30.0, 32.8]	31.0 [28.0, 35.0]	.768	31.0 [30.0, 31.9]	31.0 [30.0, 31.9]	27.7 [21.9, 32.5]	.067
ARd, mm	20.0 [18.0, 21.0]	20.0 [17.5, 22.3]	.790	20.1 [19.4, 20.7]	20.1 [19.4, 20.7]	19.7 [12.8, 29.2]	.834
AAd, mm	25.0 [25.0, 27.0]	26.5 [23.3, 28.0]	.575	25.7 [25.0, 26.4]	26.6 [22.7, 29.7]	25.8 [18.0, 35.2]	.939
EF, %	66.5 [65.0, 70.0]	62.0 [60.0, 62.8]	**<.001**	66.6 [65.5, 67.8]	54.1 [46.1, 59.6]	60.2 [56.8, 63.1]	**.002**
EFT, mm	7.00 [6.95, 7.23]	7.45 [6.45, 8.23]	.143	7.15 [6.92, 7.38]	7.62 [6.03, 9.11]	6.81 [5.03, 8.65]	.499

Abbreviations: Δ4, Δ4-androstenedione; AAd, ascending aorta diameter; ARd, aortic root diameter; cfT, calculated free testosterone; E2, 17β-estradiol; EFT, epicardial fat thickness; FDR, false discovery rate; $HbA1_C$, hemoglobin $A1_C$; HDL, high-density lipoprotein; HGAs, high-grade sexual chromosome aneuploidies; HOMAi, Homeostatic Model Assessment index; INHB, inhibin B; IVS-EDd, interventricular septum end-diastolic diameter; KS, Klinefelter syndrome; LAd, left atrial diameter; LDL, low-density lipoprotein; LV-EDd, left ventricular end-diastolic diameter; LV-ESd, left ventricular end-systolic diameter; LVMi, left ventricular mass index; PW-EDd, posterior wall end-diastolic diameter; RV-EDd, right ventricular end-diastolic diameter; RWT, relative wall thickness; T, total testosterone.
Data are reported as means adjusted for the number of Y chromosomes, and their respective bias-corrected and accelerated 95% confidence intervals. Significances are reported for group comparisons of 47,XXY vs HGA subjects, and for linear trend according to the number of supernumerary X chromosomes.
[a]For linear trend analyses according to the number of supernumerary X chromosomes nonadjusted *P*-values are reported, with significant values after FDR adjustment presented in bold.

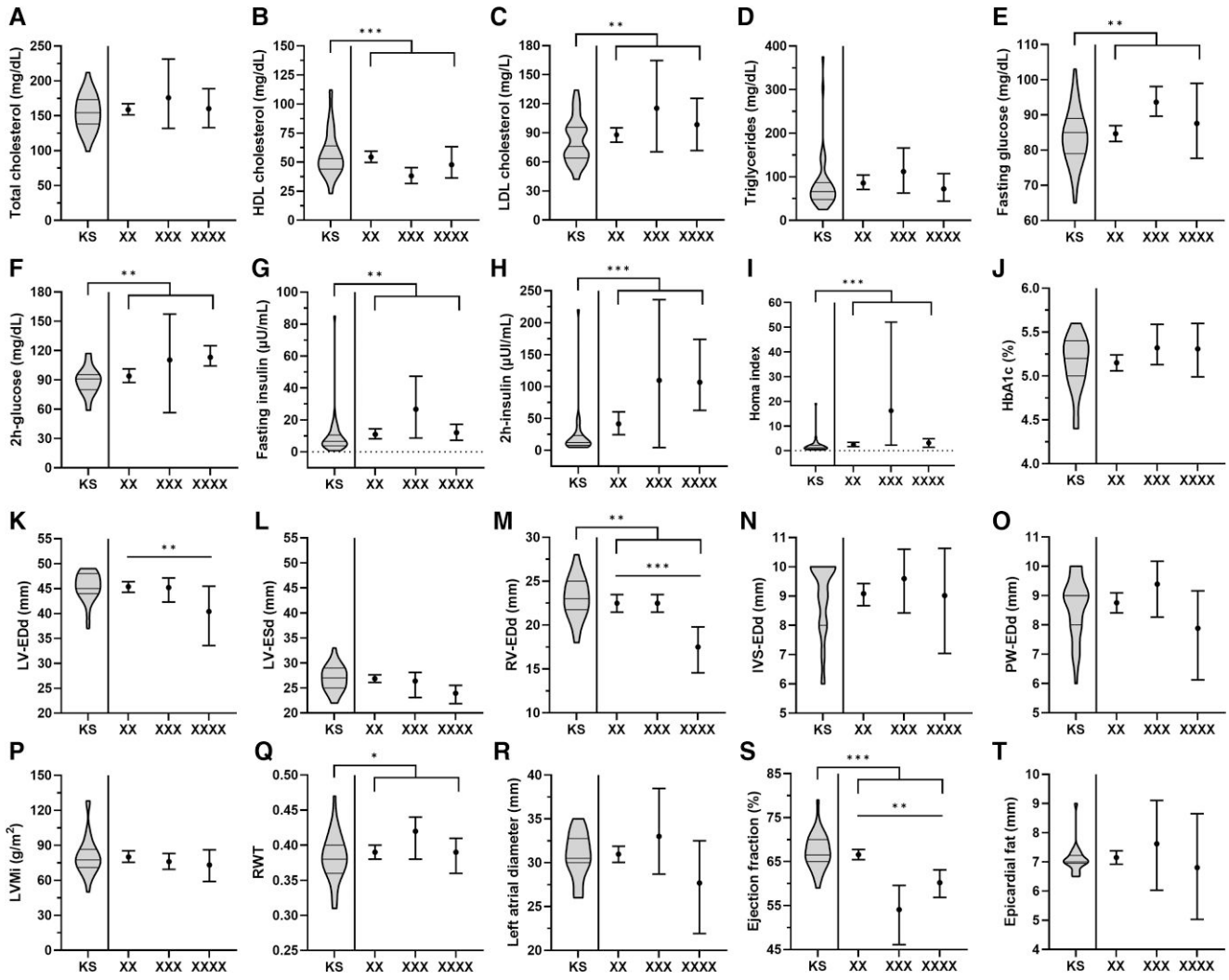

Figure 3. Metabolic parameters and echocardiographic data in patients with KS and HGAs. Note: Data of patients with KS are shown by violin plot, whereas the means adjusted for the number of Y chromosomes and their respective BCa 95% CI are reported for patients with HGAs. The bar indicates statistical significance for linear trend analyses, whereas the brackets indicate comparisons across groups. The single, double, and triple stars represent statistical significance levels below .05, .01, and .001, respectively, before adjustment by FDR.
Abbreviations: BCa, bias-corrected and accelerated; CI, confidence interval; FDR, false discovery rate; HGA, high-grade aneuploidies of X and Y sex chromosomes; KS, Klinefelter syndrome.

KS and HGAs, although the latter showed higher RWT values. Systolic function was seemingly affected, with a negative trend between supernumerary Xs and lower EF. Finally, the study of EFT, a known marker of cardiovascular risk, showed no trend or difference between the groups, although the genetic background of KS has been indicated to determine a larger contribution to EFT than serum T concentrations *per se* (24). We speculate that beyond the single supernumerary X in 47,XXY, a "gene plateau" is reached, and additional Xs apparently cannot further influence the EFT. These findings add to our understanding of altered cardiovascular features in sex chromosome aneuplodies, possibly linked to altered gonadal function (22).

Lastly, we confirmed these findings in a validation cohort composed of 46 KS individuals matched by pubertal status. A statistically significant effect for increasing number of supernumerary Xs could be confirmed for gonadal development, thyroid function, adrenal function, and echocardiographic parameters. No significant trends were present with regards to testicular function parameters when matching by Tanner stage, as expected, as the latter is directly influenced by the degree of T steroidogenic capability and "output" by the testes, with the exception of lower INHB, reflecting more severe damage to the spermatogenic compartment.

The main limitation of the present study resides in the distribution of karyotypes and intrinsic phenotype variability of the HGAs subjects, which could have limited the statistical power and the possibility of reaching significance for linear trend analyses and some comparisons. We have approached this issue by adjusting our statistical analyses by the number of Y chromosomes and employing a robust statistical resampling method. Given the exploratory nature of the present study, we further employed adjustment for multiple family-wise comparisons according to FDR. It has to be reminded that HGAs comprise a group of exceedingly rare conditions, on which almost no published clinical data are simultaneously available on the clinical, hormonal, metabolic, and cardiac components, and the present cohort is the largest presented so far in the literature. Nonetheless, we suppose that a larger population, possibly from multicentric or registry-driven

studies, could help identify additional differences and effects of the number of extra-Xs, as well as the specific role of supernumerary Y chromosomes. We also recognize the age range of our HGA cohort as a partial limit of this study, particularly with the investigation of testicular function; we approached this issue by age-matching each patient with HGAs with 2 patients with KS, and by confirming significant results in an additional pubertal stage-matched KS cohort.

In conclusion, our study shows that the increase in extra-Xs determines an adverse impact on height, pubertal development, testicular volume and function, overall steroidogenic activity, glucose and lipid homeostasis, as well as cardiac structure and function. To date, no guidelines are available for the management of these complex syndromes. As such, a better understanding of their intrinsic characteristics could guide medical practitioners toward earlier identification of complications and thus allow for more tailored management and appropriate treatment of each patient.

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Disclosures

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

Data Availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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