Gene Expression Patterns in Relation to the Clinical Phenotype in Klinefelter Syndrome

Michael Zitzmann, Rebecca Bongers, Steffi Werler, Nadja Bogdanova, Joachim Wistuba, Sabine Kliesch, Jörg Gromoll,* and Frank Tüttelmann*

Centre of Reproductive Medicine and Andrology (M.Z., R.B., S.W., J.W., S.K., J.G.), and Institute for Human Genetics (N.B., F.T.), University of Münster, University Clinics, D-48149 Münster, Germany

Context: Klinefelter syndrome (KS) is the most common chromosome disorder in men (47,XXY), exhibiting a phenotype with marked variation and increased morbidity. The pathophysiological link between the supernumerary X chromosome and the clinical phenotype remains unknown.

Objective: To elucidate whether differential gene expression patterns can be detected in KS patients and whether these are related to inherent clinical features.

Design, Setting, Participants: EXAKT (Epigenetics, X-chromosomal Features and Clinical Applications in Klinefelter Syndrome Trial) is a Münster-based prospective project involving 132 Klinefelter men and their parents. A range of cardiovascular, inflammatory, and metabolic factors, in comparison to age-matched male (n = 50)/female controls (n = 50) and in relation to genetic features, is assessed.

Main Outcomes and Measures: Our predefined hypothesis was that differential gene expression patterns in blood cells exist in KS patients vs male controls and are related to the clinical phenotype.

Results: Differential expression of 36 X-chromosomal and autosomal genes put KS patients into a unique genetic setting vs male and female controls. The KS cohort exhibited increased insulin resistance, enhanced inflammatory and procoagulatory status, higher waist circumference, dyslipidemia, and a markedly shorter 12-lead electrocardiogram QTc interval (partly located within the pathological range) vs male controls (all $P < .001$). Clinical dyshomeostasis was associated with expression patterns of dysregulated genes (all $P < .01$). Parental origin of the supernumerary X chromosome was a confounder regarding insulin resistance and cardiac phenotype ($P < .05$). Results are considered preliminary because gene expression was measured in blood cells.

Conclusions: The supernumerary X chromosome contributes to a number of pathologies in KS. The pattern of gene expression is altered in KS, and the degree of differential gene expression is associated with the clinical phenotype. **(*J Clin Endocrinol Metab* 100: E518–E523, 2015)**

The most prevalent chromosomal disorder in men, Klinefelter syndrome (KS; 1/500 of males, karyotype 47,XXY), presents a complex pathology that is assumed to have underlying mechanisms in hormonal as well as genetic disbalances, exhibiting marked phenotypical variation (1).

It is speculated that the pathophysiology including increased mortality of KS patients is determined by X-chromosomal factors and not T deficiency alone. First, the parental origin of the additional X chromosome may affect clinical features (2–5). Second, genes of the apoptotic cascade, glucose metabolism, and inflammation are located on the X chromosome; transcriptional dysregulation of such genes might be responsible for the increased metabolic morbidity in KS (6, 7).

The aim of this study was to investigate prospectively how KS patients relate to male and female controls in

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* J.G. and F.T. contributed equally to this study and should be considered shared last authors.

Abbreviations: DEG, differentially expressed gene; ECG, electrocardiogram; HOMA1-IR, homeostasis model of assessment for insulin resistance 1; KS, Klinefelter syndrome; PAI-1, procoagulatory plasminogen activator inhibitor type 1; PAR, pseudoautosomal region.

regard to cardiovascular, inflammatory, and metabolic parameters and to focus on the extent to which these pathological differences are associated with gene expression profiles and/or the parental origin of the supernumerary X chromosome.

Patients and Methods

EXAKT (Epigenetics X-chromosomal Features and Clinical Applications in Klinefelter Syndrome Trial) is a Münster-based cross-sectional prospective project involving a large cohort of KS patients and their parents and assessing cardiovascular, inflammatory, and metabolic factors in comparison to age-matched male and female controls. For selection of patients, sample size determinations/statistics, blood sampling, and conventional assays, see Supplemental Data.

All patients, parents, and controls signed specific informed consent forms. The study was conducted according to the Declaration of Helsinki, was approved by the local authorities and the Ethics Committee of the State Medical Board (code 2009-164-S), and was registered (ClinicalTrials.gov Identifier: NCT01703676).

X-Inactivation was determined by methylation analysis of the promoter region of *XIST* by pyrosequencing. Microsatellites were analyzed in buccal swabs of mothers, fathers, or both from 99 KS patients to evaluate the parental origin of the supernumerary X chromosome (Supplemental Data).

Transcriptome profiling was performed using Human Genechip 1.0 ST arrays on whole blood samples (Supplemental Data). Previous research has demonstrated that blood RNA profiling can serve as readout for different diseases (8, 9); however, relevance at the tissue level might be challenged.

Results

A total of 132 KS patients (mean age, 37.7 ± 12.2 y), 50 male controls (mean age, 42.4 ± 14.2 y), and 50 female controls (mean age, 32.6 ± 11.9 y) participated. Ninety-seven KS patients (73.5%) received T substitution. Because the focus of the study was to determine putative relations of gene expression patterns with clinical pictures in KS patients vs controls, KS patients both receiving T and being treatment-naive were needed, controlling for treatment status as a dichotomous variable in regression analyses (for treatment modalities, see Supplemental Data).

For anthropometric results and metabolic parameters exhibiting marked differences of KS patients vs controls, see Table 1.

Gene expression

X-Inactivation in KS resembled the pattern found in female controls, see Supplemental Figure 1. We identified 21 X-chromosomal genes that were differentially expressed in the KS group vs male controls. In addition, 15 further differentially expressed genes (DEGs) between KS patients and male controls were found on autosomes 1, 3, 5, 9, and 15 and on the Y chromosome. Among these 36 DEGs, all but two genes were up-regulated in KS patients compared to male controls. The two down-regulated genes were the autosomal genes *LAMB2* and *PRLR* (prolactin receptor). Findings regarding DEGs were confirmed by quantitative RT-PCR. For a complete display of all DEGs between KS patients and male controls, see Supplemental Figure 2.

KS patients also displayed a differential gene expression compared to female controls: 86 DEGs were found, 46 were located on the Y chromosome and 10 on the X chromosome, and 30 were of autosomal origin (Supplemental Figure 3).

Clinical phenotype in relation to genetic parameters

Clinical parameters were largely altered in KS patients compared to male controls (Table 1) and significantly associated with DEGs and the parental origin of the supernumerary X chromosome.

Waist circumference of KS patients was markedly associated with expression levels of *KDM6A* ($P = .006$), *CSF2RA* ($P = .002$), *CD99* ($P = .001$), *EIF1AX* ($P = .001$), and *DDX3X* ($P = .008$) (for location of genes, see Supplemental Figure 2). Serum concentrations of IL-6, TNF, high-resolution C-reactive protein, and procoagulatory plasminogen activator inhibitor type 1 (PAI-1) were significantly associated with these DEGs. KS patients with the metabolic syndrome had markedly higher *CD99* expression values than male controls with the metabolic syndrome ($P < .001$).

Fifty-one patients (51.5%) carried an additional X chromosome from their fathers and 48 (48.5%) from their mothers. An error during maternal meiosis I was causative in 25 (25.3%) cases and in meiosis II in 21 (21.2%) cases. Age of mothers at birth was significantly higher, with 30.6 ± 6.0 years for KS of maternal origin vs 27.9 ± 5.7 years for KS of paternal origin ($P = .027$). A relationship of the parental origin of the additional X chromosome to insulin resistance and 12-lead electrocardiogram (ECG) QTc time was demonstrated, in addition to DEGs associated with these parameters (Figure 1).

Insulin resistance (homeostasis model of assessment for insulin resistance 1 [HOMA1-IR]) was significantly ($P = .007$) related to the overexpression of *CSF2RA*, a gene located in the pseudoautosomal regions (PARs) of p-arms [PAR1] of both sex chromosomes (Xp22.32 and Yp11.3), and thus in KS expressed from three copies. Not only DEG expression, but also paternal origin of the X chromosome augmented the finding (Figure 1).

Table 1. Summary of Clinical Parameters

Parameter	KS Patients			Effect Size[e]	% of Non-Overlap[e]	Male Controls	Female Controls
	No TRT	On TRT	Total				
n	35	97	132			50	50
Height, cm	186.9 ± 8.1 *188.0, IQR 12*	186.4 ± 8.4[ns] *186, IQR 12*	186.5 ± 8.4 *187.0, IQR 12*	0.9	52	179.0 ± 6.9*** *178.5, IQR 12*	168.8 ± 5.2*** *170.0, IQR 7*
Weight, kg	91.7 ± 8.4 *90, IQR 23*	93.6 ± 19.9[ns] *94, IQR 25*	93.0 ± 19.7 *92.0, IQR 26*	0.9	52	77.7 ± 11.0 *** *76.5, IQR 16*	65.7 ± 14.3 *** *61.0, IQR 12*
Body mass index, kg/m^2	26.3 ± 5.4 *25.5, IQR 8.0*	26.8 ± 4.8[ns] *26.2, IQR 5.6*	26.6 ± 5.0 *26.0, IQR 6.1*	0.5	33	24.2 ± 3.0*** *23.7, IQR 3.4*	23.0 ± 4.4*** *22.2, IQR 4.8*
Waist circumference, cm	101.9 ± 16.4 *99.5, IQR 24*	101.4 ± 14.9[ns] *100.0, IQR 18*	101.4 ± 15.3 *100.0, IQR 20*	0.7	43	91.0 ± 11.4*** *89.0, IQR 13*	81.7 ± 12.3*** *79.5, IQR 12*
Triglycerides, mg/dL	127 ± 77 *99, IQR 78*	136 ± 81[ns] *117, IQR 94*	134 ± 81 *114, IQR 92*	0.4	27	100 ± 69* *78, IQR 66*	89 ± 44*** *78, IQR 67*
HDL-cholesterol, mg/dL	49.3 ± 7.6 *48.0, IQR 10*	45.8 ± 9.3* *45.0, IQR 13*	46.6 ± 9.1 *46.0, IQR 12*	0.8	47	54.3 ± 11.7*** *54.0, IQR 14*	72.8 ± 18.9*** *71.0, IQR 24*
LDL-cholesterol, mg/dL	134 ± 41 *138, IQR 58*	141 ± 38[ns] *138, IQR 58*	139 ± 39 *138, IQR 57*	0.2	15	130 ± 36[ns] *130, IQR 52*	119 ± 30** *119, IQR 38*
HOMA1-IR, mU/mL∗mg/dL	4.9 ± 1.6 *4.3, IQR 2.3*	3.4 ± 2.7*** *3.3, IQR 2.6*	3.7 ± 2.2 *3.6, IQR 2.8*	1.0	55	1.8 ± 1.0*** *1.6, IQR 1.1*	1.7 ± 1.8*** *1.2, IQR 1.2*
Presence of metabolic syndrome, n (%)[a]	12 (34)	42 (43)[ns]	54 (41)			5 (10)***	5 (10)***
IMT, mm[b]	0.74 ± 0.16 *0.7, IQR 0.2*	0.79 ± 0.18 [ns] *0.8, IQR 0.1*	0.78 ± 0.17 *0.7, IQR 0.2*	0.7	43	0.67 ± 0.12*** *0.6, IQR 0.1*	0.61 ± 0.12*** *0.5, IQR 0.1*
FMD, %[c]	16 ± 9 *17, IQR 14*	15 ± 10[ns] *14, IQR 11*	15 ± 9 *15, IQR 11*	0.0	0	15 ± 8[ns] *16, IQR 14*	20 ± 10** *18, IQR 15*
TNFα, pg/mL	1.53 ± 1.45 *1.4, IQR 1.0*	1.36 ± 0.72[ns] *1.2, IQR 1.0*	1.41 ± 0.97 *1.4, IQR 1.0*	0.5	33	0.95 ± 0.47*** *0.9, IQR 1.0*	1.02 ± 0.32** *0.9, IQR 1.0*
IL-6, pg/mL	1.33 ± 1.09 *1.4, IQR 0.7*	1.62 ± 1.73[ns] *1.7, IQR 0.8*	1.54 ± 1.58 *1.5, IQR 0.9*	0.6	38	0.66 ± 0.68*** *0.6, IQR 0.4*	1.09 ± 1.84[ns] *0.8, IQR 0.5*
IL-10, pg/mL	0.19 ± 0.13 *0.2, IQR 0.1*	0.19 ± 0.37[ns] *0.2, IQR 0.1*	0.19 ± 0.32 *0.2, IQR 0.1*	0.1	8	0.21 ± 0.16[ns] *0.2, IQR 0.2*	0.14 ± 0.15[ns] *0.1, IQR 0.1*
PAI-1, ng/mL	1.92 ± 1.24 *1.8, IQR 1.7*	2.04 ± 1.40[ns] *1.9, IQR 1.7*	2.01 ± 1.36 *1.9, IQR 1.7*	0.8	47	1.09 ± 0.54*** *1.1, IQR 0.8*	0.66 ± 0.38*** *0.6, IQR 0.4*
hsCRP, mg/dL	0.19 ± 0.01 *0.17, IQR 0.12*	0.24 ± 0.04*** *0.25, IQR 0.23*	0.23 ± 0.04 *0.21, IQR 0.11*	5.2	>80	0.05 ± 0.01*** *0.06, IQR 0.09*	0.18 ± 0.11** *0.17, IQR 0.12*
12-lead ECG QTc time, ms[d]	398 ± 21 *399, IQR 33*	396 ± 18[ns] *397, IQR 28*	397 ± 19 *397, IQR 29*	1.1	59	417 ± 18*** *417, IQR 24*	421 ± 21*** *427, IQR 39*

Abbreviations: TRT, T replacement therapy; IQR, interquartile range; hsCRP, high-resolution C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; [ns], not significant. Data are expressed as means ± SD or median IQR (in italics). Differences: KS patients (total) vs controls, and untreated vs treated KS patients, Mann-Whitney *U* test (*, $P < .05$; **, $P < .01$; ***, $P < .001$). Effect size (Cohen's D) for KS patients (total) vs male controls and respective percentage of non-overlap between these two groups. χ^2 test for metabolic syndrome. All data are unadjusted and not corrected for confounders before analysis.

Testicular size was a strong discriminator between KS patients and male controls; receiver operating characteristics revealed a cutoff of 3 ml mean testicular size to distinguish between KS patients and controls with 100% sensitivity and 97% specificity. In comparison to male controls, average values of total T in KS patients were comparable/similar because of substitution therapy in most of them (19.4 ± 11.6 vs 19.3 ± 7.2 nmol/L; $P = .298$). Nevertheless, concentrations of estradiol were higher in KS patients: 104.2 ± 39.3 vs 83.6 ± 20.6 pmol/L; $P < .001$). In addition to the unadjusted data presented and analyzed in Table 1, multivariate models revealed the presence of KS (which was used as a dichotomous variable) as an independent risk factor for waist circumference, HOMA1-IR, triglyceride levels, and HDL-cholesterol concentrations (models were controlling for advancing age). For the prevalence of the metabolic syndrome, presence of KS and TRT (both as dichotomous variables) were significant factors in a binomial regression model controlling for advancing age. This finding is further reported in Supplemental Figure 5, revealing the expression level of the gene *CD99* as pivotal variable.

[a] Metabolic syndrome according to harmonized criteria (11).

[b] Mean carotid artery intima media thickness. Within the age group of men older than 40 years, 24.5% of KS patients (total n = 53) and 6.5% of male controls (total n = 31) had an intima media thickness ≥0.9 mm ($P = .037$), which is considered a threshold value for cardiovascular damage (10).

[c] Flow-mediated vasodilatation of the brachial artery of the dominant arm according to standardized protocols (12).

[d] QTc times shorter than 370 milliseconds (which is considered a lower threshold to the short-QT syndrome in men [female threshold, 360 ms] presenting with increased risk for cardiac instability) (13) were observed in 11 KS patients, ranging from 344 to 369 milliseconds, and in none of the controls.

[e] Effect size (Cohen's D) describes the magnitude of a statistical result between two groups. It helps to estimate the clinical meaning of data differences because results can become significant by conventional tests if the number is large enough, but the clinical importance may still remain unclear. Cohen's D is independent from the number of subjects, and it is generally accepted that it should be at least 0.4 to have clinical impact (19). The non-overlap between the two investigated distributions can be derived from Cohen's D to further elucidate the effect size (14). The pooled variance was used to calculate Cohen's D (14). Note that, due to the exploratory nature of the comparisons, a correction for multiple testing was not applied. Effect sizes are independent from α-error accumulation and *P* values of significant results are mostly below .001.

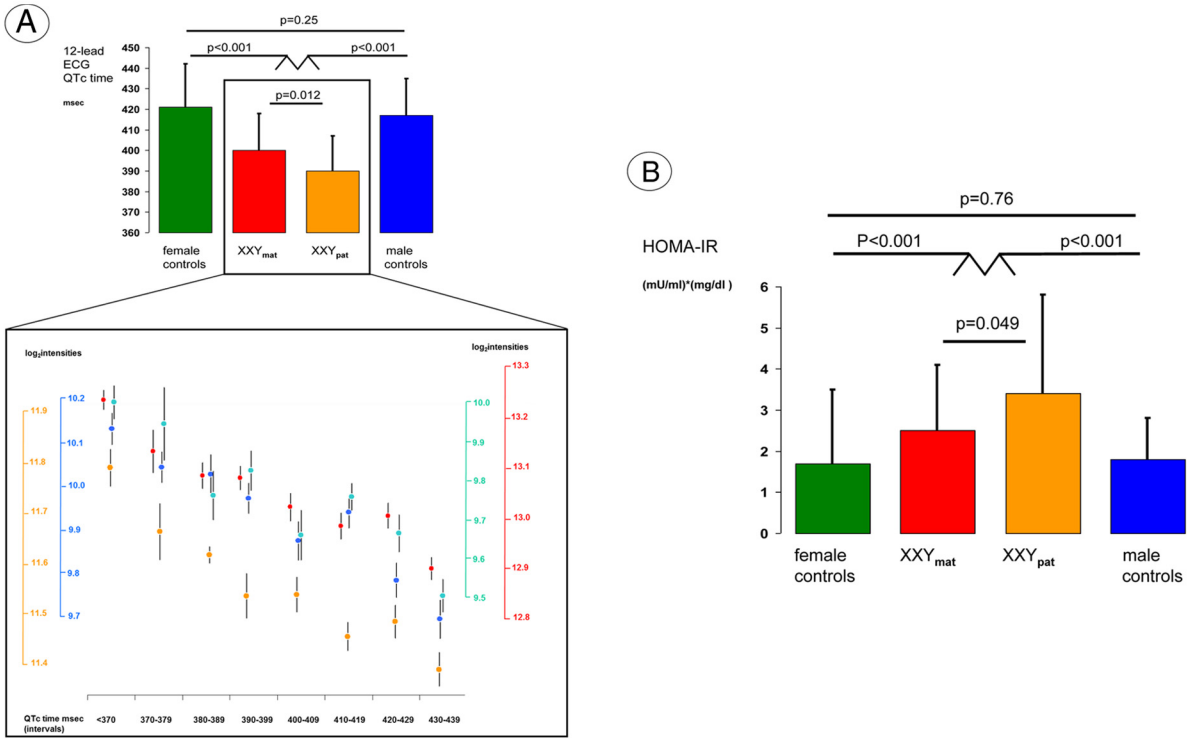

Figure 1. A, Top, QTc time (ms) was overall significantly lower in KS patients vs male and female controls (see Table 1). An even more pronounced phenotype was seen in KS patients with paternal (n = 50) vs maternal origin (n = 46) of the supernumerary X chromosome (391 ± 17 vs 400 ± 18; $P = .012$). Pathologically short QTc times (<370 ms) were observed in 11 KS patients but in none of the controls. Serum T levels were not associated with QTc times. Bottom, QTc time was inversely associated with the level of aberrant gene expression in KS patients (see *Results*). This figure shows 12-lead QTc time within the KS patient cohort in relation to mRNA expression of four selected DEGs (log_2 intensities, corresponding y-axes, and scales for each expression level/gene) detected with Human Gene 1.0 ST Array (Affymetrix). (Also see *Results* for relationship of all DEGs related significantly to QTc time.) Expression of each gene is shown as mean and SEM within the respective interval of QTc time (grouped in 10-ms intervals—<370 ms, n = 11 patients; 370–379 ms, n = 15; 380–389 ms, n = 19; 390–399 ms, n = 28; 400–409 ms, n = 16; 410–419 ms, n = 23; 420–429 ms, n = 12; 430–439 ms, n = 5; QTc times ≥ 440 ms were not observed in KS patients). Blue: *CD99* (PAR1-gene, X and Y chromosomes), Spearman's Rho (−0.426; $P < .001$). Orange: *LOC138412* (chromosome 9), Spearman's Rho (−0.381; $P = .002$). Turquoise: *P2RY8* (PAR1-gene, X and Y chromosomes), Spearman's Rho (−0.445; $P < .001$). Red: *SLC25A6* (PAR1-gene, X and Y chromosomes), Spearman's Rho (−0.473; $P < .001$). B, The pronounced insulin resistance of KS patients determined by HOMA1-IR was not only significantly higher in all KS patients vs male and female controls (see above, Table 1) but also was higher in KS of paternal vs maternal origin. All tests: Mann-Whitney *U* test, means and SD values. In summary, the presence of the supernumerary X chromosome in KS patients is a predictor of these clinical differences between KS and controls (also see Table 1). In addition, the parental origin of the supernumerary X chromosome influences the phenotype. Within the KS cohort itself, the variance within DEG transcription is related to the phenotype and clinical variables.

Cardiac rhythmogenic stability expressed as 12-lead ECG QTc time was markedly altered in KS patients; QTc time was significantly shorter in those patients expressing higher levels of DEGs: *CD99* ($P = .002$), *SLC25A6* ($P < .001$), *LOC138412* ($P = .001$), *P2RY8* ($P < .001$), *ZBED1* ($P = .005$), *GTPBP6* ($P = .001$), *SMCA1* ($P = .009$), *DOCK7* ($P = .003$), and *GSTM2* ($P = .008$) (Figure 1). A QTc time of 370 milliseconds is considered the lower threshold of the short-QT syndrome in men (13). QTc times < 370 milliseconds, ranging from 344 to 369 milliseconds, were observed in 11 KS patients (none of controls).

In summary, an association of hormonal disbalance, proinflammatory status, and aberrant gene expression with clinical pathologies was described using a signaling pathway analysis in KS, interlinking elevated serum levels of TNF with a set of DEGs (Supplemental Figure 4).

T replacement seemed to have little effect on risk factors (Table 1) in KS patients receiving T substitution (n = 97; age, 38 ± 12 y) vs those not on treatment (n = 35; age, 34 ± 11 y), although this is a cross-sectional approach with respective limitations. Within the subcohort of KS patients receiving T substitution, the prevalence of the metabolic syndrome was higher in those men exhibiting higher expression levels of the DEG *CD99*, irrespective of the treatment modality (Supplemental Figure 5).

Discussion

The cross-sectional EXAKT study revealed that in men with KS, the pattern of gene expression, and not hypogonadism alone, is associated with altered clinical features and phenotype. These patients are in a state of genetic

expression with X-chromosomal genes transcribed similarly to female controls and Y-chromosomal genes expressed in patterns as seen in male controls. Moreover, autosomal genes are differentially expressed in comparison to male and female controls.

It remains to be elucidated whether DEG expression patterns translate into relevant changes at protein levels and functions in specific tissues. Although the described alterations in gene expression refer to changes in blood cells, previous research has demonstrated that gene expression patterns derived from blood RNA can serve as a measure for several pathological conditions (8, 9). The validity of using blood as a potential readout/measure for transcriptional profiling is further underlined by a publication in which peripheral blood cells from KS patients were used to determine the microRNA expression profile. That study revealed >70 differentially regulated microRNAs in KS (15).

The gene *KDM6A* serves as an example to approach KS pathophysiology because this X-chromosomal gene escapes X-inactivation ("escapee gene"). It is, therefore, expressed from one copy in men with normal karyotypes and two copies in women. In KS patients, we demonstrate *KDM6A* to be one of the prominently overexpressed genes. *KDM6A* might play a pivotal role in the pathophysiology of KS: it is involved in congenital anomalies/intellectual impairments and was found to be differentially expressed in the brains of KS mice (16).

Expression of the gene *CSF2RA* (located in PAR1 of both sex chromosomes) has been associated with the higher incidence of stroke in men vs women (17). In KS patients, we found the unique situation of three genetic readouts leading to an increase of expression of *CSF2RA* (vs both male and female controls); expression was associated with insulin resistance, waist circumference, and concentrations of the procoagulatory substance PAI-1 and cytokines (see *Inflammation* below).

The parental origin of the supernumerary X chromosome was identified as an additional confounder of clinically important parameters (Figure 1). Paternal imprinting may aggravate the pathological picture in regard to insulin resistance and shorter QTc time (see below).

Inflammation

In addition to the above named *CSF2RA* with three copies, overexpression of other escapee genes in KS (*EIF1AX* [$P = .001$] and *DDX3X* [$P = .008$]) was related to waist circumference, inflammation, and coagulation (concentrations of IL-6 and PAI-1). These genes, specific modifiers of inflammation, are differentially expressed in blood after ischemic stroke (8). Correspondingly, all three escapee genes were found within a general signaling pathway network in KS, interacting with the higher concentrations of estradiol and TNF (Supplemental Data). Furthermore, the overexpressed gene *CD99* located in PAR1 has been described to induce strong inflammatory conditions in a gender-dependent manner (18). This is in agreement with our description of *CD99* expression in relation to inflammatory cytokines and waist circumference in KS.

Cardiac phenotype

In KS, in addition to the putative relation of DEGs to metabolic parameters, we also demonstrate a genetic association with ECG findings, which is of pivotal clinical interest. QTc times were markedly shorter in KS patients, and this effect was even more pronounced in those men with a paternal origin of the supernumerary X chromosome (Figure 1). Short QTc time is known to be related to atrial as well as ventricular fibrillation, and association to sudden cardiac death was reported recently (19). Eleven KS patients (and none of the controls) revealed QTc times shorter than 370 milliseconds, which is considered a threshold to possible cardiac symptomatology (13).

As corroboration, a recent publication reported a phenotype of cardiovascular abnormalities in KS patients, including a chronotropic incompetence pathology (20). Cardiac pathophysiology independent from hypogonadism (treated or untreated) might therefore exist as a hitherto unknown comorbidity in KS patients; in addition, expression patterns of several DEGs in KS are related to a short QTc interval (Figure 1).

Conclusion

This is the first trial to combine clinical features of KS with inherent genetic disbalance. Sex-chromosomal gene expression patterns that are markedly different from male and female controls were described, expanding to autosomal genes. These alterations are significantly associated with clinical symptoms in KS. Moreover, the parental origin of the additional X chromosome contributed to the clinical phenotype. A confirmation of these findings, which are derived from blood cell RNA, should be the aim of tissue-specific investigations.

The presence of a supernumerary X chromosome in a male leads to a complex phenotype and multiple morbidities, which exist beyond the accompanying hypogonadism and require further investigation. Because gene expression patterns vary from patient to patient, this study might also provide a first step toward individual counseling regarding metabolic and, possibly, cardiac impairment of KS patients.

Acknowledgments

Address all correspondence and requests for reprints to: Michael Zitzmann, Centre for Reproductive Medicine and Andrology/

Clinical Andrology, Domagkstrasse 11, D-48149 Münster, Germany. E-mail: Michael.Zitzmann@ukmuenster.de.

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