# Letters to the Editor

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## Limb-girdle muscular dystrophy with apparently different clinical courses within sexes in a large inbred kindred

EDITOR—The autosomal recessive limb-girdle muscular dystrophies (AR-LGMD) are clinical entities characterised by primary and progressive muscle degeneration, mainly at the pelvic and shoulder girdles, with great variability in the clinical course. Some patients present a severe course similar to Duchenne muscular dystrophy, while others maintain the capacity to walk even in adult life.[1–3] At least eight autosomal recessive genes have been mapped. The chromosome localisation of these genes and their products, and a brief comment on the clinical course of each type of AR-LGMD are summarised in table 1. Of these mapped genes, six have been cloned: the gene responsible for LGMD2A which encodes calpain 3, a muscle specific protease,[30] the genes that cause the known sarcoglycanopathies (LGMD2C-LGMD2F),[11 15 20 22 26] and, recently, the gene for LGMD2B which encodes a protein called "dysferlin" by the investigators.[8]

LGMD2C is a Duchenne-like muscular dystrophy particularly prevalent in North Africa,[10–12 31 32] but rare in other geographical regions; its prevalence in north eastern Italy was estimated to be $1.72 \times 10^{-6}$ inhabitants.[33] This phenotype, which affects both sexes equally, was first described by Ben Hamida *et al*[12] in 93 patients belonging to 28 Tunisian families. A few large kindreds, with many affected persons, have been described. Here we report the results of a clinical and molecular study in a large inbred kindred from the north east of Brazil with LGMD2C, which is unusual because the male patients appear to have a more severe clinical course than the affected females.

The genealogical data from five generations (fig 1) were obtained and confirmed by different family members. The dates of birth, marriage, and death, causes of death, and abortions were documented. A total of 56 subjects, including all living affected persons, were clinically examined. Muscle strength was evaluated in 13 patients according to the manual muscle test (based on the Medical Research Council scale). The diagnosis of AR-LGMD was based on clinical examination, course of the disease, family history, serum creatine kinase (CK) levels, muscle histopathology, and muscle protein and DNA analyses.

A muscle sample was obtained from a biceps biopsy of one male patient (V.5). Immediately after removal, the muscle sample was frozen in liquid nitrogen and stored at −70°C until analysis. Dystrophin was analysed by immunofluorescence (IF) and western blotting (WB) with rabbit polyclonal N-terminal 303-8 and C-terminal monoclonal Dy8/6C5 antibodies.[34 35] The amount of dystrophin on WB was estimated by densitometric analysis.[36] The patient's band was compared with a normal control and corrected for the myosin content in the muscle extract. On immunohistochemical staining of frozen muscle sections using double labelling reactions for dystrophin + γ-SG, α-SG + β-SG, and γ-SG + δ-SG, the following antibodies were used: α-SG, monoclonal Ad1/20A6[18]; β-SG, rabbit polyclonal antibody[21]; γ-SG, rabbit polyclonal antibody[13] and monoclonal 35DAG/21B5[37]; and δ-SG, rabbit polyclonal raised against a glutathione S-transferase (GST)-δ-sarcoglycan fusion protein.[26]

DNA samples from 28 members of the family, including 11 affected persons, were extracted from whole blood (after informed consent) according to the method of Miller *et al*.[38] Microsatellite markers corresponding to genes involved in AR-LGMD were amplified by PCR and the products were visualised on 6.5% denaturing gels, which were dried and exposed to *x* rays. Two point linkage analysis involving the mutant gene and the microsatellite markers D13S232, D13S115, and D13S143 (at chromosome 13q12) was performed using the MLINK software program.[39] An estimated gene frequency of 0.001 for the disease allele and an equal recombination for both sexes were assumed. For characterising the mutation, exon 6 of the γ-SG gene was amplified by PCR from DNA samples of affected patients and sequenced. Primers were purchased from Research Genetics.

The pedigree of this large, white, consanguineous kindred, of Portuguese ancestry, is shown in fig 1. The total number of affected persons, including five subjects who had died, is 20 (11 males and nine females).

According to the patients' parents, the first clinical manifestations were difficulty in running and climbing stairs and frequent falls. The ages of onset, wheelchair confinement, and death (five patients) are listed in table 2. Physical examination showed that all living affected persons had muscle atrophy (more severe in the older patients) and had lost the ability to walk. Prominence of calves was seen only in the brothers V.4 and V.5. The tendon reflexes were abolished and the facial muscles were spared. Clinical cardiological evaluation performed in all patients and electrocardiogram/echocardiogram examinations, performed in five of them (IV.20, IV.22, IV.34, V.4, and V.5), showed no evidence of cardiac

*Table 1 The autosomal recessive limb-girdle muscular dystrophies*

| | *Chromosome localisation* | *Gene product* | *Clinical course* | *References* |
|---|---|---|---|---|
| LGMD2A | 15q15 | Calpain 3 | Onset ranging from early childhood to adulthood, but often before 15 years. Progression variable, with WC often at 20-30 years | 4 5 6 |
| LGMD2B | 2p13 | Dysferlin | Onset around 20 years. Progression often slow, with WC usually after 40 years | 7 8 9 |
| LGMD2C | 13q12 | γ-sarcoglycan | Onset 3-12 years. Progression usually fast, with WC often at 10-30 years. A slowly progressive form has been reported in some families | 10 11 12 13 14 |
| LGMD2D | 17q21 | α-sarcoglycan | Onset around 10 years. Progression ranging from WC aged 10 years to ambulation at least into 30s. Null mutation associated with the most severe form | 15 16 17 18 19 |
| LGMD2E | 4q12 | β-sarcoglycan | Severe and mild forms have been reported, with progression from fast to slow, and probable correlation with type of mutation | 20 21 22 23 |
| LGMD2F | 5q33-34 | δ-sarcoglycan | Onset 4-10 years and WC aged 9-16 years (four families, seven patients) | 24 25 26 |
| LGMD2G | 17q11-12 | Not known | Only one family so far reported (six affected sibs), with a slowly progressive dystrophy | 27 |
| LGMD2H | 9q31-33 | Not known | Recently described in Manitoba Hutterites. Onset often after 15 years, with patients still ambulant in their 20s, 30s, and 40s, and others asymptomatic after 20 years | 28 29 |

WC= wheelchair confinement.

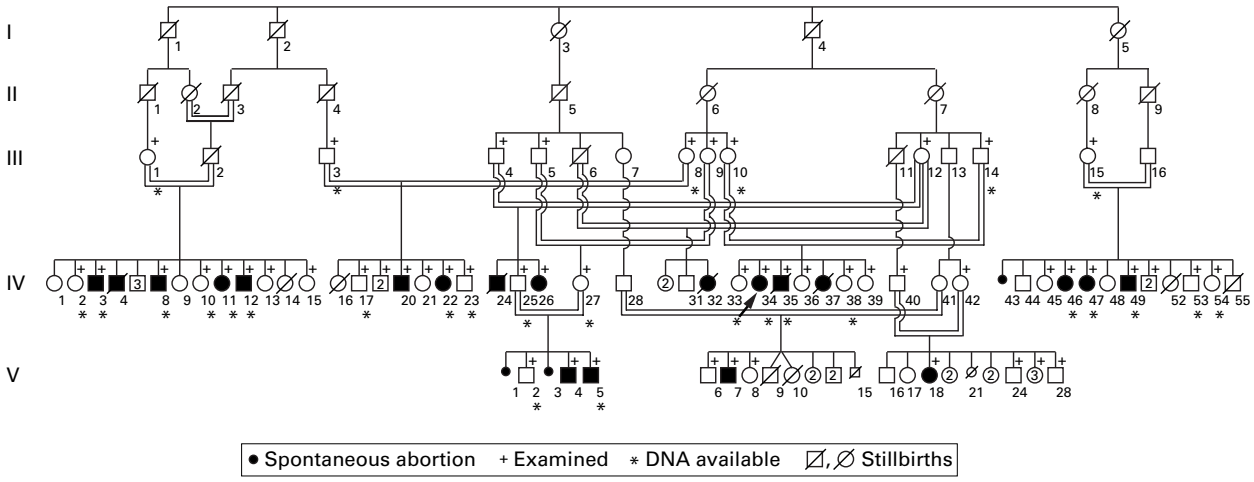


*Figure 1 Pedigree of kindred.*

disease. Respiratory function (spirometry) was investigated in these five patients. A mild restrictive lung disease pattern was diagnosed in the children V.4 and V.5 and the three adult patients had a moderate to severe pattern. Intellectual development was normal in all affected persons. Serum CK level in V.5 (the youngest patient) was 340 IU/ml (normal ⩽20). CK levels in the oldest patients (IV.3, IV.26, and IV.34) were normal.

The disease seemed to be more severe, with a more rapid rate of clinical progression, in the affected males than in the female patients. The mean ages of onset were 3.18 (SD 1.08) and 4.56 (SD 1.13) years respectively for male and female patients, and the difference between these mean values is significant at the 5% level (p=0.013). Regarding wheelchair confinement, the mean ages were 13.91 (SD 2.47) years (affected males) and 21.67 (SD 3.32) years (affected females), and the significance is p=0.0002. The manual muscle test performed in 13 patients (table 3) showed that the proximal muscles of the four limbs were much more affected than the distal ones, but no significant sex difference was observed. The degree of contractures in these patients ranged from mild to severe and involved the elbow, hip, and knee joints. The most severe contractures were observed in affected males IV.8, IV.12, IV.20, and IV.49. In addition, IV.8 and IV.20 had contractures of the wrist joints. Scoliosis was seen in only one patient (IV.8). The cause of death of the five dead affected subjects (three men and two women) was pneumonia. IV.35 died some months after our clinical evaluation.

*Table 2 Main clinical features in the 20 patients with LGMD2C*

| *Subject* | *Sex* | *Current age (y)* | *Age at onset (y)* | *Age at wheelchair confinement (y)* | *Age at death (y)* |
|---|---|---|---|---|---|
| IV.3 | M | 40 | 3 | 18 | — |
| IV.4 | M | — | 3 | 13 | 30 |
| IV.8 | M | 34 | 3 | 18 | — |
| IV.11 | F | 28 | 5 | 20 | — |
| IV.12 | M | 26 | 3 | 13 | — |
| IV.20 | M | 36 | 3 | 15 | — |
| IV.22 | F | 31 | 5 | 30 | — |
| IV.24 | M | — | 3 | 13 | 21 |
| IV.26 | F | 40 | 5 | 19 | — |
| IV.32 | F | — | 4 | 21 | 36 |
| IV.34 | F | 38 | 6 | 23 | — |
| IV.35 | M | — | 6 | 14 | 35 |
| IV.37 | F | — | 6 | 21 | 29 |
| IV.46 | F | 29 | 3 | 20 | — |
| IV.47 | F | 27 | 3 | 20 | — |
| IV.49 | M | 24 | 3 | 14 | — |
| V.4 | M | 12 | 2 | 11 | — |
| V.5 | M | 10 | 2 | 10 | — |
| V.7 | M | 29 | 4 | 14 | — |
| V.18 | F | 27 | 4 | 21 | — |

The muscle biopsy from patient V.5 showed histopathological changes of a primary myopathic process, characterised by a marked variation in fibre diameter, round shaped fibres, split fibres, proliferation of endomyseal and perimyseal conective tissue, and fat infiltration. The histoenzymological studies showed a predominance of type I fibres.

IF staining with dystrophin antibodies showed a mosaic pattern of positive and negative fibres, and the IF pattern with antibodies directed at each of the four known SG proteins was negative (fig 2). WB analysis showed a reduction in the amount of dystrophin (about 20% of normal).

Confirmation of linkage (lod score >3) was obtained between the disease gene and D13S232, D13S115, and D13S143 microsatellite markers. The rare D13S232-3 allele (122 bp) was found in a homozygous state in the 11 patients studied. Amplification by PCR followed by sequencing of exon 6 of the γ-SG gene in these persons showed the deletion of a thymine from the span of 521-525 bp (Δ521-T mutation).

The great majority of the patients with severe childhood onset progressive muscular dystrophy had mutations on the X chromosome, with autosomal recessive inheritance involved in only about 5% of the cases.[40] In the kindred with LGMD2C described here, this pattern is well illustrated, since the affected persons had unaffected consanguineous parents and comprised approximately 25% of their offspring of both sexes. Because of the lack of reliable information about more remote antecedents, it is difficult to establish the origin of the abnormal recessive gene in this inbred kindred, but it was probably introduced by one of the parents of the sibs I.1/I.5 who were heterozygotes. The genetic homogeneity of LGMD2C in North African populations (where this disease was initially studied), derived from the same ancestral population, suggests a common origin for the mutant gene in that geographical region.[10 31 32]

The immunohistochemical analyses of SGs in muscle biopsy specimens from patients with any of the types of sarcoglycanopathy have shown deficiencies of all components of the SG complex, suggesting that pathogenic mutations in a single SG gene disturb the organisation of the whole glycoproteic complex and lead to secondary deficiency of the other SGs.[11 13 25 36 41] In agreement with these features, our patient V.5 showed absence of the four known components of the SG complex. In addition, dystrophin WB showed a reduced quantity in this patient. In a study on muscle proteins in six types of AR-LGMD

*Table 3 Manual muscle strength evaluation in 13 patients with LGMD2C: strength rating for each patient*

| *Movement tested* | *IV.3 (M)* | *IV.8 (M)* | *IV.11 (F)* | *IV.12 (M)* | *IV.20 (M)* | *IV.22 (F)* | *IV.26 (F)* | *IV.34 (F)* | *IV.46 (F)* | *IV.47 (F)* | *IV.49 (M)* | *V.4 (M)* | *V.5 (M)* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Neck flexion | 4 | 3 | 4 | 3 | 3 | 4 | 3 | 3 | 3 | 4 | 4 | 4 | 4 |
| Neck extension | 4 | 4 | 4 | 4 | 4 | 4 | 3 | 4 | 4 | 4 | 4 | 4 | 4 |
| Shoulder abduction | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 2 | 1 | 2 | 3 |
| Shoulder external rotation | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 2 | 1 | 2 | 3 |
| Elbow flexion | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 2 | 2 | 2 | 2 | 3 |
| Elbow extension | 3 | 3 | 3 | 3 | 2 | 3 | 0 | 0 | 3 | 3 | 3 | 3 | 3 |
| Wrist flexion | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Wrist extension | 4 | 4 | 4 | 4 | 2 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Thumb abduction | 4 | 4 | 4 | 4 | 3 | 4 | 3 | 4 | 4 | 4 | 4 | 5 | 5 |
| Hip flexion | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 2 | 2 | 2 | 2 |
| Hip extension | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 2 | 2 |
| Knee flexion | 4 | 4 | 4 | 4 | 2 | 4 | 4 | 3 | 4 | 4 | 4 | 3 | 4 |
| Knee extension | 4 | 4 | 4 | 4 | 2 | 4 | 3 | 3 | 4 | 4 | 4 | 3 | 4 |
| Ankle dorsiflexion | 4 | 4 | 4 | 4 | 2 | 4 | 3 | 4 | 4 | 4 | 4 | 4 | 4 |
| Ankle plantar flexion | 4 | 4 | 4 | 4 | 2 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Toe flexion | 5 | 5 | 5 | 5 | 4 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| Toe extension | 5 | 5 | 5 | 5 | 4 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |

0=no movement; 1=flicker of movement; 2=movement of the joint when the effect of gravity is eliminated; 3=movement through full range of the joint, against gravity but not against resistance; 4=movement of the joint, against gravity and against added resistance; 5= full strength; M=male; F=female.

(2A-2F), involving 35 patients, Vainzof *et al*[36] also found reduced quantities of dystrophin in the severe cases, but described a variable IF pattern for the sarcoglycans.

The localisation of the gene for LGMD2C on chromosome 13q12 was made initially by Ben Othmane *et al*[10] in three Tunisian kindreds based on the observation of segregation of the disease with markers in this chromosome region. Further studies with Algerian[31] and Moroccan[32] families confirmed the mapping. Linkage disequilibrium between the LGMD2C locus and the rare allele 3 (122 bp) of marker D13S232 was described in nine Tunisian and one Egyptian families, suggesting that the two loci are very close to one another.[42] A homozygous deletion of one thymine base from the span of 521-525 bp of the γ-SG gene (Δ521-T mutation) was found in affected persons from three of these kindreds.[11] In our

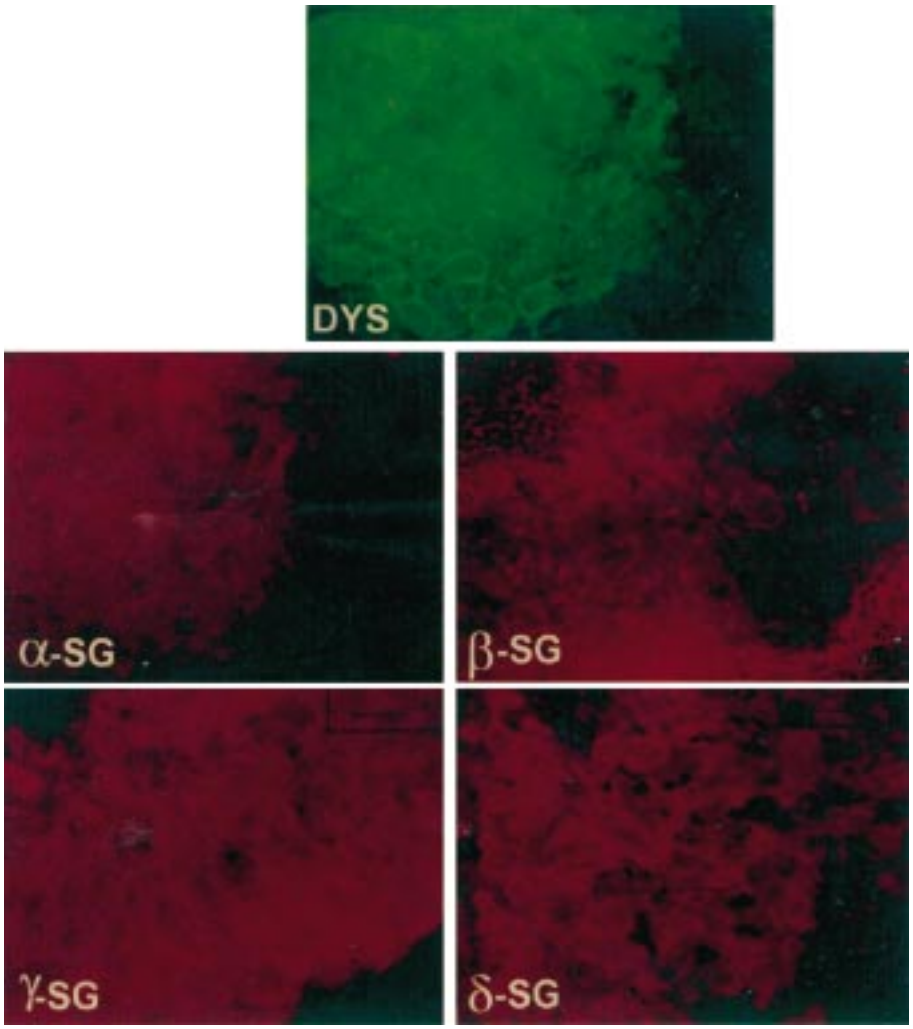


*Figure 2 Double immunofluorescence labelling for dystrophin (DYS) and sarcoglycans (SG) in patient V.5, showing a pattern of positive and negative fibres for dystrophin and a complete absence of staining for all sarcoglycans.*

study, the two point linkage analysis between the disease gene and each of the markers at 13q12 showed the presence of the D13S232-3 allele in all patients. The occurrence of the Δ521-T mutation associated with this allele in the affected subjects is consistent with the linkage disequilibrium previously reported.[42]

It was surprising to observe that the disease appeared to be more severe in our male patients, since the mean ages of onset and wheelchair confinement were significantly lower in these patients than in the affected females. The pattern of muscle involvement (table 3) showed no evidence of differences within sexes, but this pattern was investigated only when all affected persons had already lost independent walking (data on different stages of the disease are not available because this family had never been previously studied). Recently, in a large study of facioscapulohumeral muscular dystrophy involving 173 affected persons from 53 families, Zatz *et al*[43] also described a more severe clinical phenotype in the male patients. In their original description of Duchenne-like muscular dystrophy, Ben Hamida *et al*[12] found significant intrafamilial and interfamilial variability in the severity of manifestation of the disease, with loss of independent walking varying between the ages of 10 and 31 years, but without differences within sexes. The Δ521-T mutation was previously described in four Brazilian families (with a total of 14 patients) of Negroid ethnicity[13 44] with no biological relationship with the (white) kindred reported here. Three of them manifested the classical (severe) form of LGMD2C. However, in the fourth family, the three affected sibs (a 23 year old woman and two males aged 20 and 14 years respectively) had a mild phenotype with preservation of ambulation, particularly the older sister who was almost asymptomatic at that age, possibly reflecting a slight difference of clinical expression favouring the female sex. These observations suggest that the Δ521-T mutation can lead to a milder phenotype or a more severe form of the disease in one sex in some families.

Further investigations in a larger series of LGMD2C patients are necessary for a complete delineation of the spectrum of variation in the clinical expression of this and other mutations in the γ-SG gene, and for understanding the underlying molecular mechanisms, since they have implications for genetic counselling.

We would like to express our gratitude to the members of the family described in this work for their collaboration. Our special thanks to Drs Maria R Passos-Bueno, Mayana Zatz, Mariz Vainzof, and Eloísa S Moreira for their valuable help in the laboratory work at the Department of Biology, University of São Paulo, São Paulo, Brazil. Drs Zatz and Passos-Bueno also critically read the manuscript. This investigation was supported by grant APQ 0168-2.02/94 from FACEPE (Fundação de Amparo à Ciência e Tecnologia de Pernambuco).

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# Rescue from the effects of trisomy 13q32→qter owing to skewed X inactivation in a der(X)t(X;13)(p21;q32) carrier

EDITOR—X;autosome translocations are very rare and occur at an estimated frequency of 1:300 000.[1] According to the hypothesis of Lyon,[2] there is a random and irreversible inactivation of one of the two X chromosomes in the female, occurring at an early stage of development. In patients with an X;autosome translocation, X inactivation occurs at random but is followed by cellular selection, favouring the better genetic balance.[3] Accordingly, nearly 95% of patients with balanced X;autosome translocations show a skewed inactivation of the normal X chromosome in almost all cells, thereby avoiding somatic monosomy or X chromosome disomy, while patients with unbalanced X;autosome translocations have the der(X) constantly inactivated in 91% of the cases in order to obtain the most optimal balance of the genome.[1] We report here a woman who was referred for chromosome analysis because of four consecutive first trimester spontaneous miscarriages following the birth of a healthy daughter.

The patient is short (152 cm), but otherwise healthy, with no dysmorphic features or malformations. Chromosome analysis of peripheral blood showed an apparently pure Xp deletion using conventional banding techniques. Chromosome microdissection of the aberrant Xp was performed according to Senger *et al*,[4] using an inverted phase contrast microscope (Axiovert 135) and a micromanipulator (Narishige MMO-2YD). Six fragments containing the whole aberrant Xp were excised and transferred to a 10 nl collection drop containing 10 mmol/l TRIS-HCl, pH 7.5, 10 mmol/l NaCl, 0.1% SDS, and 0.5 mg/ml proteinase K. After digestion with proteinase K, the collection drop was transferred to a 250 µl reaction tube containing 5 µl of PCR mixture: 5 µmol/l 6-MW-primer - 5'CCG ACT CGA GNN NNN NAT GTG G 3' - , 200 µmol/l of each dNTP, 0.83 µl of Thermo sequenase buffer, and 4 U Thermo sequenase (Amesham Life Science). Degenerate oligonucleotide primed PCR (DOP-PCR) was performed in a Perkin Elmer Thermal Cycler 2400 according to Telenius *et al*[5] with minor modifications. After initial denaturation at 96°C for five minutes, eight low temperature cycles were run including annealing at 30°C for one minute 10 seconds, 37°C for one minute, and 95°C for 30 seconds. Then 45 µl PCR mixture (1.1 µmol/l 6-MW, 220 µmol/l of each dNTP, 2.5 mmol/l $MgCl_2$, 4.5 µl Stoffel buffer, and 5 U Ampli*Taq* DNA polymerase Stoffel fragment (Perkin

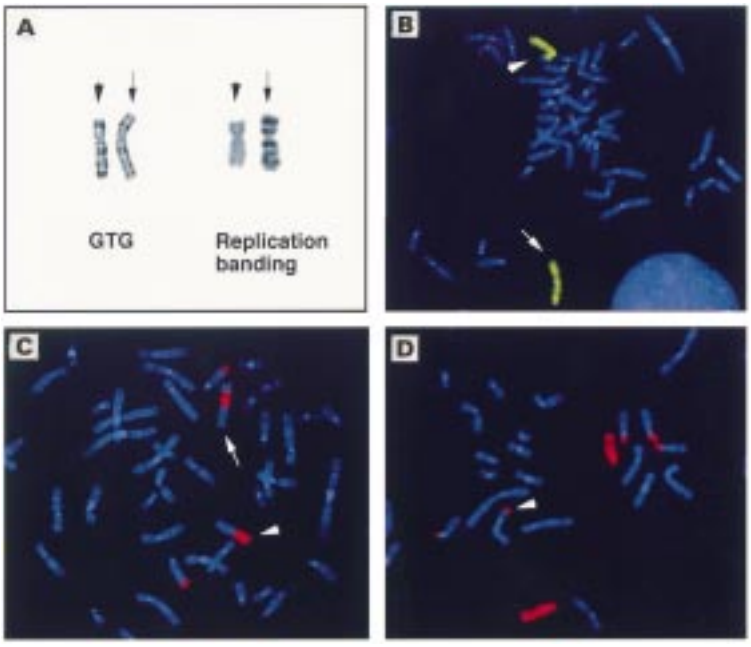


*Figure 1 The normal X chromosome is indicated by an arrow and the der(X) by an arrowhead. (A) The patient's X chromosomes, GTG banded (left) and after replication staining (right). (B) FISH on a metaphase from the patient using an X chromosome specific library. The distal part of the p arm of the der(X) is unlabelled. (C) Reverse painting on a metaphase from the patient using the microdissected library of the short arm of the der(X). As expected, the library labels the whole aberrant Xp, but also half the p arm of the normal X and the distal part of 13q. (D) FISH on a metaphase from the patient using a chromosome 13 specific library. Both chromosomes 13 are fully labelled (and there are unspecific signals on the p arms of the acrocentric chromosomes) as well as the distal part of the short arm of the der(X), indicating partial trisomy 13.*