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ORIGINAL ARTICLE

# Skewed X inactivation in Lesch–Nyhan disease carrier females

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**X chromosome inactivation (XCI) ratios of normal females can range from a highly skewed ratio of 0:100 to a 50:50 ratio. In several X-linked disorders, female carriers present skewed X inactivation. Hypoxanthine–guanine phosphoribosyltransferase (HPRT) deficiency is an X-linked disorder. Males are affected and present with the complete Lesch–Nyhan disease (LND) or with a partial phenotype (Lesch–Nyhan variant, LNV). Female carriers are usually asymptomatic. The aim of the present study was to analyze the XCI pattern of HPRT-deficiency carrier females. As a group, 75% of HPRT-deficiency carrier females presented skewed XCI. Moreover, skewed XCI is significantly more frequent in LND carriers (83%) than in LNV (0–50%, depending on the phenotype severity). The ratios of the preferentially inactivated allele of carrier females were significantly higher than the ratios of the preferentially inactivated allele of noncarrier females ($89.4 \pm 15$, $n = 52$ vs $65.2 \pm 12$, $n = 52$; $P < 0.0001$). For carrier diagnosis, the presence of skewed XCI presents a sensitivity of 75% with a specificity of 85%. In LND families, the presence of skewed XCI is more sensitive for carrier diagnosis than in LNV families; however, we believe that this test is not accurate for carrier diagnostic purposes.**
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## INTRODUCTION

In mammalian females, inactivation of one of the two X chromosomes occurs in early embryonic life, about the time of implantation. X chromosome inactivation (XCI) derives in the transcriptional silencing of the majority of genes (about 15% of genes on the X chromosome escape from inactivation) on one of the two X chromosomes and is random and permanent for all descendants of a cell.[1–3]

Every female is mosaic for two cell lines, one with the maternal X and one with the paternal X as the active chromosome. X inactivation is usually a stochastic event, but some females will have skewed XCI due to chance. The XCI ratios of normal females can range from a highly skewed ratio of 0:100, in which the same X chromosome is active for all cells, to a 50:50 ratio, in which each X chromosome is active in an equal number of cells.[4] Skewed XCI is a marked deviation from the 50:50 ratio and is arbitrarily defined as preferential inactivation of either the maternally or paternally inherited X chromosome in 80% or more of the cells. Extreme skewing of XCI is the preferential inactivation of one X chromosome in $\geqslant 90$ of cells or $\geqslant 95\%$ of cells.[4] In several X-linked disorders, female carriers are asymptomatic and have extremely skewed X inactivation, probably because of selectively mediated favorable skewing.[5,6] In these disorders, skewed XCI in the mother of a possibly affected male could be diagnostically helpful.

Lesch–Nyhan syndrome is an inborn error of purine metabolism characterized by hyperuricemia, severe action dystonia, choreoathetosis, ballismus, cognitive and attention deficit, and self-injurious behavior.[7,8] The syndrome is associated with a complete deficiency of hypoxanthine–guanine phosphoribosyltransferase (HPRT, EC 2.4.2.8) activity. Partial HPRT deficiency is present in Lesch–Nyhan variant (LNV) patients and they present with HPRT-related gout and a variable degree of neurological involvement, but without the complete Lesch–Nyhan syndrome.[9,10] They are classified as having HPRT-related neurological disease (HND), if they present clinically apparent neurological manifestations, or as HPRT-related hyperuricemia, the less severe form in which patients do not present clinically apparent neurological manifestations.[11]

Human HPRT is encoded by the *HPRT1* gene, located on the long arm of the X chromosome,[12] and HPRT deficiency is inherited as a recessive X-linked trait. Thus, men are generally affected and women are generally asymptomatic carriers, although some women with Lesch–Nyhan syndrome, due to a variety of molecular mechanisms, have been described in the literature.[13] Female carriers are usually asymptomatic and HPRT activity is most often normal in their hemolysate,[14] probably due to selection against HPRT-deficient erythrocyte precursors.[15] HPRT-deficient cells can be selected based on their resistance to 6-thioguanine. In females who are heterozygous for the *HPRT1* mutation, there is mosaicism of peripheral blood lymphocytes and cultured fibroblasts with regard to sensitivity to 6-thioguanine.[16] Mosaicism for HPRT activity has been also described in hair follicles or cultured fibroblasts in carrier females. The proliferation assay of peripheral blood T-lymphocytes, in the presence

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of 6-thioguanine, measures the frequency of HPRT-deficient cells to determine carrier status. The frequency of 6-thioguanine-resistant cells from HPRT-deficiency carrier females is higher than that of noncarrier females, but is $<0.5$ expected for non-random XCI.[17] Currently, faster and more accurate carrier diagnoses are usually performed using molecular methods.

Since 1984, we have diagnosed 41 patients with HPRT deficiency at La Paz University Hospital in Madrid, Spain.[9,18] Molecular diagnosis has permitted a carrier diagnosis in 109 women from these HPRT-deficient families. The aim of the present study was to analyze the XCI pattern of HPRT-deficient carrier females versus noncarrier females, to confirm whether skewed XCI is associated with carrier status and whether the XCI assay is helpful as a carrier diagnosis tool.

## MATERIALS AND METHODS

### Patients

We analyzed 109 women belonging to 31 HPRT-deficient families, whose propositus was diagnosed at La Paz University Hospital.[9,18] These patients were diagnosed as HPRT-deficient on the basis of the following criteria: (1) clinical symptoms and signs typical of an HPRT-deficient state, with or without neurological manifestations including self-injurious behavior; (2) decreased HPRT activity in erythrocyte lysates and simultaneously increased adenine phosphoribosyltransferase (EC 2.4.2.7) activity; and (3) mutations in the *HPRT1* gene or decreased HPRT1 mRNA.[19]

All the studies were conducted according to the Declaration of Helsinki and were approved by the Institutional Research and Ethics Review Committees of La Paz University Hospital. In all the women, the carrier diagnosis was performed by molecular methods including PCR amplification followed by DNA automated sequencing ($n=53$), restriction fragment length polymorphism analysis ($n=43$), multiplex ligation-dependent probe amplification ($n=12$) and quantitative real-time PCR ($n=1$).

### Methods

The female RNA-free genomic DNA samples were isolated from whole blood using a DNA Purification Kit (Puragene, Gentra Systems, Minneapolis, MN, USA). To determine the XCI pattern, we examined the differential methylation status in the human androgen receptor gene (*HUMARA*). This XCI assay is currently the most widely used and it evaluates differential methylation of a CpG dinucleotide near the highly polymorphic CAG repeat in exon 1 of *HUMARA* in the active and the inactive X chromosome. The advantage of this PCR-based method is that it can be used on small amounts of genomic DNA and it distinguishes the two X chromosomes from each other in ~90% of females.[20] Briefly, 50–150 ng of DNA was digested for 16 h with the methyl-sensitive enzyme *Hpa*II. The digestion preferentially degrades activated (unmethylated) over inactivated (methylated) DNA. After digestion, the enzyme was inactivated at 80 °C for 10 min. One quarter of the resulting digest and an undigested DNA sample were simultaneously used for PCR amplification with primers designed in the (CAG)*n* flanking sequences of the *HUMARA* gene intron 1.[20] The forward primer was fluorescently labeled and the reverse primer was unlabeled. The resulting products from digested and undigested PCR were run on an ABI 3130 genetic analyzer and were analyzed by GeneMapper 4.0 software (Applied Biosystems Inc, Foster City, CA, USA). Samples were corrected for amplification efficiency by using the data from the undigested samples. Raw peak height values of the digested DNA (d1 from the smaller allele and d2 for the longer allele) were normalized using peak height values of the undigested DNA (u1 from the smaller allele and u2 for the longer allele) for each female.[21] The percentage of the XCI was calculated using the formula: (d1/u1)/[(d1/u1)+(d2/u2)] [21]. The X-inactivation pattern was expressed arbitrarily as a ratio of the smaller to larger allele. In addition, one digested and undigested control male sample was included in every batch of samples, to control for complete digestion and amplification efficiency.

Statistical tests were performed using the Statview software package (SAS Institute, Inc., Cary, NC, USA). A value of $P<0.05$ was considered significant.

The sensitivity, specificity, positive predictive value and negative predictive value for HPRT-deficiency carrier diagnosis, of the presence of a preferentially inactivated allele $\geqslant 80\%$, were calculated in all carriers as a group and in Lesch–Nyhan disease (LND) and LNV carriers separately. Besides, we calculated the sensitivity, specificity, positive predictive value and negative predictive value for HPRT-deficiency carrier diagnosis, of the presence of a preferentially inactivated allele $\geqslant 90\%$ and of the presence of a preferentially inactivated allele $\geqslant 95\%$, in all carriers as a group and in LND and LNV carriers separately. For calculation, we employed the following formulas:

(a) Number of carrier females (all carrier females as a group, LND carriers or LNV carriers) that test positive for the presence of a preferentially inactivated allele $\geqslant 80\%$ or the presence of a preferentially inactivated allele $\geqslant 90\%$, or the presence of a preferentially inactivated allele $\geqslant 95\%$, in each case.

(b) Number of carrier females (all carrier females as a group, LND carriers or LNV carriers) that test negative for the presence of a preferentially inactivated allele $\geqslant 80\%$ or the presence of a preferentially inactivated allele $\geqslant 90\%$, or the presence of a preferentially inactivated allele $\geqslant 95\%$, in each case.

(c) Number of noncarrier females that test positive for the presence of a preferentially inactivated allele $\geqslant 80\%$ or the presence of a preferentially inactivated allele $\geqslant 90\%$, or the presence of a preferentially inactivated allele $\geqslant 95\%$, in each case.

(d) Number of noncarrier females that test positive for the presence of a preferentially inactivated allele $\geqslant 80\%$ or the presence of a preferentially inactivated allele $\geqslant 90\%$, or the presence of a preferentially inactivated allele $\geqslant 95\%$, in each case.

Sensitivity $= a/a+b$
Specificity $= d/c+d$
Positive predictive value $= a/a+c$
Negative predictive value $= d/b+d$
Values are expressed as percentages.

## RESULTS

Of all the studied women, 55 out of 109 were diagnosed as carriers and 54 as noncarriers. Only five women (3.6%) were uninformative, due to *HUMARA* homozygosity, and were excluded from the study.

Of the informative women, 52 out of 104 were diagnosed as carriers and 52 as noncarriers. A total of 78 women belonged to a family in which the propositus presented the complete LND phenotype (41 were diagnosed as carriers and 37 as noncarriers), 22 women belonged to a family in which the propositus presented the HND phenotype (10 were diagnosed as carriers and 12 as noncarriers) and 4 women belonged to a family in which the propositus presented the HPRT-related hyperuricemia phenotype (1 was diagnosed as a carrier and 3 were diagnosed as noncarriers).

Figure 1a shows the X-inactivation patterns of noncarrier females. XCI patterns were assigned to 20 'bins' with increments of 5%. The patterns were normally distributed, with the mean of the distribution residing at 35%:65% and the median at 37%:63% (s.d. of the mean = 12%). Figure 1b shows the X-inactivation patterns of carrier females. XCI patterns were assigned to 20 'bins' with increments of 5%. The patterns appeared skewed, with the mean of the distribution residing at 11%:89% and the median at 3%:97% (s.d. of the mean = 15%).

For analysis purposes, the X-inactivation patterns were expressed arbitrarily as the ratio of the preferentially inactivated allele. The ratios of the preferentially inactivated allele in the 104 females were $77.3 \pm 18$, (mean $\pm$ s.d.). The ratios of the preferentially inactivated allele of carrier females were significantly higher than the ratios of the preferentially inactivated allele of noncarrier females ($89.4 \pm 15$, $n=52$ vs $65.2 \pm 12$, $n=52$; $P<0.0001$). The ratios of the preferentially

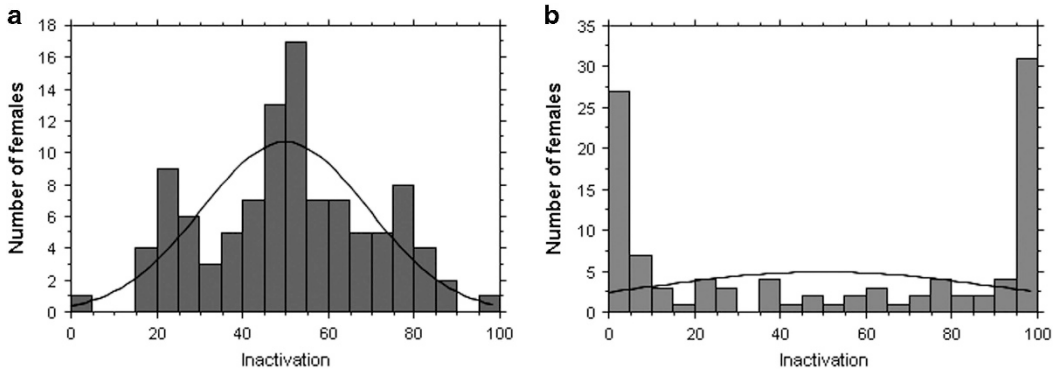


**Figure 1** (**a**) X chromosome inactivation (XCI) patterns of noncarrier females. XCI patterns were assigned to 20 'bins' with increments of 5%. The patterns were normally distributed, with the mean of the distribution residing at 35:65 and the median at 37:63 (SD of the mean = 12). (**b**) X-inactivation patterns of carrier females. XCI patterns were assigned to 20 'bins' with increments of 5%. The patterns appeared skewed, with the mean of the distribution residing at 11:89 and the median at 3:97 (s.d. of the mean = 15).

**Table 1 Ratio of the preferentially inactivated allele and percentage of study females presenting skewed XCI (ratio of preferentially inactivated allele ⩾80), and extreme skewed XCI (ratio of preferentially inactivated allele ⩾90 or 95)**

| | *n* | *Ratio of the preferentially inactivated allele Mean ± s.d.* | *Ratio of preferentially inactivated allele ⩾ 80 (%)* | *Ratio of preferentially inactivated allele ⩾ 90 (%)* | *Ratio of preferentially inactivated allele ⩾ 95 (%)* |
|---|---|---|---|---|---|
| All females | 104 | 77 ± 23 | 45.2 | 34.6 | 30.5 |
| Carrier | 52 | 89.4 ± 10.6 | 75.0 | 67.0 | 59.6 |
| LND | 41 | 92.6 ± 11.1 | 83.0 | 73.0 | 65.8 |
| HND | 10 | 79.7 ± 19.5 | 50.0 | 50.0 | 40.0 |
| HRH | 1 | 56 | 0 | 0 | 0 |
| Noncarrier | 52 | 65.2 ± 12.07 | 15.0 | 1.9 | 1.9 |
| LND | 37 | 64.1 ± 10.9 | 13.5 | 0 | 0 |
| HND | 12 | 68.1 ± 15.3 | 16.0 | 8.3 | 8.3 |
| HRH | 3 | 66.7 ± 13.8 | 33 | 0 | 0 |
| Control* | 415 | 52 ± 19.3 | 14.2 | 3.6 | 1.7 |

Abbreviations: HND, hypoxanthine–guanine phosphoribosyltransferase-related neurological disease; HRH, hypoxanthine–guanine phosphoribosyltransferase-related hyperuricemia; LND, Lesch–Nyhan disease; LNV, Lesch–Nyhan variant; XCI, X chromosome inactivation.
Ratios of the preferentially inactivated allele are expressed as mean ± s.d. Data are compared with data from 415 adult women controls obtained from the study of Amos-Landgraf *et al.*[4] Carrier and noncarrier females were divided according to their membership in a family where the propositus presented the complete LND phenotype or an LNV phenotype (HND or HRH).

inactivated allele of the LND carrier females were significantly higher than the ratios of the preferentially inactivated allele of the HND carrier females (92.6 ± 11.1, $n = 41$ vs 79.7 ± 19.5, $n = 10$; $P < 0.01$) (Table 1).

Table 1 shows the percentage of our study females presenting skewed XCI (ratio of preferentially inactivated allele ⩾80%) and extremely skewed XCI (ratio of preferentially inactivated allele ⩾90 or 95%), compared with data from 415 control women obtained from the study of Amos-Landgraf *et al.*[4] The percentage of noncarrier females presenting skewed XCI (⩾80%) were 15%, similar to that of the control women (14.2%). The percentage of noncarrier females presenting extreme skewed XCI (⩾90 or ⩾95%) was 1.9%, similar to that of the control women (3.6% and 1.7%, respectively). However, the percentage of carrier females presenting skewed XCI (⩾80%) or extremely skewed XCI (⩾90 or 95%) were much higher than that of the control women (75%, 67% and 59.6% vs 14.2%, 3.6% and 1.7%, respectively) (Table 1). Nevertheless, it is noteworthy that 25% of total carrier females, 17% of LND carrier females and 50% of HND carrier females presented no skewed X inactivation. For carrier diagnosis, the presence of skewed XCI (defined as a preferentially inactivated allele ⩾80%) presents a sensitivity of 75% (95% CI 61–86%), with a specificity of 85% (95% CI 72–93%), a positive predictive value of 83% (95% CI 72–90%) and a negative predictive value of 77% (95% CI 67–85%) (Table 2).

Extremely skewed XCI (defined as a preferentially inactivated allele ⩾90% or ⩾95%) presents higher specificity (98%, with 95% CI 90–99.95% for both ⩾90 and ⩾95), but with less sensitivity (67%, 95% CI 53–78% for ⩾90 and 60%, 95% CI 45–73% for ⩾95). In LND families, the presence of skewed XCI is more sensitive for carrier diagnosis than in HND families (83%, 95% CI 68–93% in LND vs 50%, 95% CI 19–81% in HND) (Table 2).

## DISCUSSION

In this study, we found that most HPRT-deficiency carrier females presented skewed XCI. As a group, 75% of HPRT-deficiency carrier females presented skewed X-inactivation, probably due to preferring selection of cells expressing the wild-type allele.

Moreover, skewed XCI is significantly more frequent in LND carriers (83% skewed XCI) than in HND carriers (50% of skewed XCI) or in HPRT-related hyperuricemia carriers (0% of skewed XCI). In general, the overall severity of the disease in HPRT deficiency depends on how *HPRT1* mutations influence HPRT enzyme activity, allowing or not some residual activity. In our experience, missense point mutations are the main cause of partial deficiency of the enzyme activity and LNV phenotypes (including HND and HPRT-related

**Table 2 Utility of the XCI test for the carrier diagnosis of HPRT deficiency**

| | *Sensitivity (95% CI)* | *Specificity (95% CI)* | *PPV (95% CI)* | *NPV (95% CI)* |
|---|---|---|---|---|
| *Preferentially inactivated allele ⩾ 80%* | | | | |
| All carriers | 75% (61–86%) | 85% (72–93%) | 83% (72–90%) | 77% (67–85%) |
| LND carriers | 83% (68–93%) | 85% (72–93%) | 81% (69–89%) | 87% (76–93%) |
| HND carriers | 50% (19–81%) | 85% (72–93%) | 38% (20–60%) | 90% (82–94%) |
| *Preferentially inactivated allele ⩾ 90%* | | | | |
| All carriers | 67% (53–78%) | 98% (90–99.95%) | 97% (83–99.6%) | 75% (67–82%) |
| LND carriers | 73% (57–86%) | 98% (90–99.95%) | 98% (81–99.6%) | 82% (74–88%) |
| HND carriers | 50% (19–81%) | 98% (90–99.95%) | 83% (39–97%) | 91% (85–95%) |
| *Preferentially inactivated allele ⩾ 95%* | | | | |
| All carriers | 60% (45–73%) | 98% (90–99.95%) | 97% (81–99.6%) | 71% (64–77%) |
| LND carriers | 66% (49–80%) | 98% (90–99.95%) | 96% (79–99.5%) | 78% (70–85%) |
| HND carriers | 40% (12–74%) | 98% (90–99.95%) | 80% (33–97%) | 89% (84–93%) |

Abbreviations: CI, confidence interval; HND, HPRT-related neurological disease; HPRT, hypoxanthine–guanine phosphoribosyltransferase; LND, Lesch–Nyhan disease; LNV, Lesch–Nyhan variant; NPV, negative predictive value; PPV, predictive positive value; XCI, X chromosome inactivation.
Sensitivity, specificity, PPV and NPV for the preferentially inactivated allele ⩾80%, preferentially inactivated allele ⩾90% and preferentially inactivated allele ⩾95% in the diagnosis of HPRT deficiency carrier, LND carrier and LNV carrier.

hyperuricemia), whereas mutations that modify the size of the predicted protein are usually related to LND.[18,22] These results suggest that in HPRT-deficient carrier females, the selection of cells expressing the wild-type allele is favored if the mutated allele is more deleterious.

XCI is usually a stochastic event; however, due to the small number of cells at the time of X inactivation, some females will have a skewed XCI due to chance. According to Amos-Landgraf *et al.*,[4] an extremely unbalanced inactivation pattern is particularly rare in the normal population, with a ratio >90:10 present in ~3.6% of adult women and a ratio >80:20 present in ~14.2% of adult women. In our study, we found a ratio >90:10 in 1.9% of noncarrier females and a ratio >80:20 in 14% of noncarrier females. Thus, noncarrier females presented similar XCI patterns to control women employed in other studies. Skewed XCI is more common in elderly women,[23] with age-related skewing starting around age 55 years and increasing until age 100 years. In the present study, the age of all the women was not available. However, we compared the ratios of the preferentially inactivated allele in three noncarrier groups with several different age ranges: grandmothers and great-grandmothers (mean ± s.d., 60 ± 13, $n = 15$), mothers and aunts (mean ± s.d., 69 ± 13, $n = 21$), and sisters and cousins of the propositus (mean ± s.d., 62 ± 10, $n = 24$), and we have found no significant differences.

In rare cases, there is a genetic cause of skewed XCI due to a mutation in the XCI specified transcript gene (*XIST*). This pattern can be incidental or inherited as an isolated dominant trait.[24] However, skewed XCI has more frequently been associated with a mutant gene on the X chromosome. In some cases, the mutant gene is unknown and a mutation is suspected, for example, in female carriers of some of the X-linked mental retardation syndromes presented in males, and in females with recurrent spontaneous abortions, who have increased frequency of skewed XCI.[25]

When the mutant gene on the X chromosome is known, selection will commonly favor cells expressing the wild-type allele, but there are some exceptions. In adrenoleukodystrophy, the X chromosome carrying a mutation in the *ABCD1* gene is the preferentially active chromosome in female carriers and results in disease.[26] Likewise, although HPRT deficiency is inherited as a recessive X-linked trait and men are generally affected, some women have been described with the full characteristic manifestations of Lesch–Nyhan syndrome. The first described LND female patient presented a total deletion of the *HPRT1* gene in the maternally inherited X chromosome and a non-random inactivation of the paternally inherited X chromosome with an abnormal methylation.[27] Other seven females with LND phenotype and two females who showed clinical manifestations of partial HPRT deficiency have been described. All of them presented a mutation in *HPRT1* gene in one allele and skewed inactivation of the wild-type allele of unknown cause.[22]

The mechanisms underlying selection have not been extensively investigated. Selection is not always present in X-linked disorders. There are several X-linked disorders in which selection does not take place and carrier females have a random XCI. In Fabry disease, hemophilia A, or B,[28–30] a clear relationship between XCI and clinical phenotype has not been found. In this group of disorders, carrier females are usually healthy or slightly ill, although in rare cases they can be severely affected.

However, in other X-linked disorders female carriers are also asymptomatic but present extremely skewed XCI, probably due to selectively favoring cells expressing the wild-type allele.

To date, XCI analyses have not been reported in LND carriers. In LND and LNV families, female carriers are usually asymptomatic. In our experience, most female carriers for HPRT deficiency can be differentiated from noncarriers when 24 h urine samples are analyzed after a 5-day purine-restricted diet: carriers show significantly higher mean urinary excretion rates of hypoxanthine and xanthine compared with noncarrier females from the same families.[14] However, some data suggest that female carriers would present skewed XCI. When HPRT-deficient cells are selected based on their resistance to 6-thioguanine, the frequency of 6-thioguanine-resistant cells from HPRT-deficiency carrier females is <0.5 expected for non-random XCI.[17] According to our data, we can conclude that *HPRT1* mutation carrier females present skewed XCI, and that skewed XCI is more frequent in females heterozygous for *HPRT1* mutations which cause the LND phenotype than in females heterozygous for *HPRT1* mutations that cause the variant phenotype.

Other known examples of X-linked disorders in which female carriers are also asymptomatic but present extremely skewed XCI are Wiskott–Aldrich syndrome, dyskeratosis congenita, X-linked agammaglobulinemia and severe combined immunodeficiency.[5] In these disorders, it has been postulated that a skewed XCI in the mother of a possibly affected male might be diagnostically helpful. We have found

that 25% of total carrier females, 17% of LND carrier females and 50% of HND carrier females, presented non-skewed X inactivation. For a carrier diagnosis, the presence of skewed XCI presents a sensitivity of 75% with a specificity of 85%. In LND families, the presence of skewed XCI is more sensitive for carrier diagnoses than in HND families (83%, in LND vs 50% in HND); however, we believe that this test is not accurate for carrier diagnostic purposes.

We conclude that LND is an X-linked disorder in which there is a selection favoring cells expressing the wild-type allele in carrier females. Most HPRT-deficiency carrier females presented skewed XCI, but skewed XCI is significantly more frequent in LND than in LNV. Thus, there is a correlation between skewed XCI and severity of the phenotype. Although in LND families the presence of skewed XCI is more sensitive for carrier diagnosis than in LNV families, this test is not accurate for carrier diagnostic purposes.

## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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