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REVIEW

Italian experience with Lesch-Nyhan patients and animal models of the disease

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Abstract

Lesch-Nyhan Disease (LND) is a rare X-linked genetic disease with hypoxanthine-guanine phosphoribosyltransferase (HGPRT) deficiency, due to mutation in the encoding gene, located on the X-chromosome. LND patients exhibit hyperuricemia with stones due to unrecycled purine accumulation and increased synthesis, and a devastating neurological syndrome with dystonia and self-injurious behaviour, choreoathetosis and spasticity. In spite of biochemical and molecular research, the fine connection between the neurological syndrome and HGPRT deficiency is still unclear, though there is consensus regarding brain neurotransmitter dysfunction with few dopaminergic neuron terminals in the striatum. The rarity of the disease makes it difficult to obtain homogeneous population of patients to study.

The aim of this paper is to contribute to the understanding of the connection between genotype and phenotype in a cohort of Italian patients, to propose a reliable method of identifying carrier women in affected families, and to provide evidence of a possible link between HGPRT deficiency and altered adenosinergic and serotonergic neurotransmission.

Biochemical and mutation analysis is reported in 28 LNS Italian patients from 25 families, with virtually no HGPRT activity and typical LNS phenotype. Genetic analysis identified 24 HPRT mutations, nine of which had never previously been reported, and no mutation hotspots. Carrier females were identified by a new semiquantitative real-time PCR.

Studies performed by real-time PCR on knockout mice demonstrated altered adenosinergic and serotonergic pathways, with greatly increased ADORA1A receptor expression, slightly decreased ADORA2A expression and unchanged ADORA2B expression. Increased HTRC2 expression with no significant difference in mRNA editing suggested serotonergic involvement.

The different approaches used allowed us to study certain aspects of LND, focusing on mutation analysis in patients and carriers and on simultaneous analysis of biochemical and genetic features. Mouse models elucidated the possible involvement of adenosine and serotonine receptors in the neurotransmission aberration occurring in HGPRT deficiency.

Keywords: Lesch-Nyhan disease, LND, X-linked

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Introduction

Lesch-Nyhan Disease (LND) is a rare X-linked genetic disease with hypoxanthine-guanine phosphoribosyltransferase (HGPRT, EC 2.4.2.8) deficiency, due to mutation in the encoding gene *HPRT*, a constitutively expressed housekeeping gene on X-chromosome (1,2). HGPRT is a purine salvage enzyme and its deficiency causes accumulation of unrecycled hypoxanthine and guanine, readily oxidized to uric acid; increased purine synthesis has also been reported. LND patients exhibit uric acid urinary stones and a devastating neurological syndrome with dystonia, self-injurious behavior, choreoathetosis and spasticity (3). The mechanism of uric acid overproduction has been clarified, but the connection between HGPRT deficiency and the neurobehavioral manifestations remains unclear, though there is some evidence of dysfunction of basal ganglia dopamine systems (4,5,6).

The biochemical aspects of LND have been explored extensively in cells of patients (red blood cells, fibroblasts, lymphoblasts), autopsy brain specimens and different cultured HGPRT$^-$ cell lines. The results are often inconsistent, suggesting significant differences related to species and tissue source (7).

Depending on residual HGPRT activity, neurological disability may be severe (LND full syndrome) or milder (LND variants) (8). Despite much research (9), the fine link between the neurological syndrome and HGPRT deficiency is still unclear, though there is general consensus regarding brain neurotransmitter dysfunction with fewer dopaminergic neuron terminals in the striatum and increased amounts of serotonin and 5-hydroxyindolacetate (4).

Metabolic abnormalities known to occur in LND include grossly increased *de novo* purine synthesis with elevated uric acid production and excretion. Peculiar metabolic alterations also accompany HGPRT deficiency in erythrocytes, such as GTP depletion, increased phosphoribosylpyrophosphate and UDP-glucose, and grossly increased NAD. A number of related enzymes, such as adenine phosphoribosyltransferase (APRT, E.C.2.4.2.7) (10) and two enzymes involved in NAD synthesis, nicotinate phosphoribosyltransferase (NAPRT; E.C. 2.4.2.11) and NAD synthetase (NADs, E.C. 6.3.5.1) are reported to display abnormally high activities in HGPRT-deficient erythrocytes (11). These are accompanied by lower NADglycohydrolase (NADase; E.C. 3.2.2.5) activity (12).

In contrast, LND fibroblasts exhibit NAD, ATP and GTP depletion (13). Together these findings suggest that disturbed pyridine metabolism accompanies altered purine metabolism in different cell types.

Animal models, such as HPRT knock-out and 6-hydroxydopamine-treated rats, have been developed to reproduce the disease (14, 15). Such animals do not develop the neurological symptoms associated with complete deficiency of HGPRT in humans, but their brains are not entirely normal[16]. HPRT knock-out mice have metabolic aberrations, such as increased *de novo* purine synthesis and dopamine depletion in basal ganglia (17, 18). HPRT knockout mice have been used as a model of the disorder, and though not fully replicating the human disease, they are valuable for studying LND pathogenesis. Knockout mice do not show obvious behavioral abnormalities, but drugs acting on the brain dopamine system, such as amphetamine, can stimulate locomotor or stereotyped behavior similar to that found in human LND patients (14). They are therefore considered a useful model for studying metabolic abnormalities caused by HPRT deficiency, but not for expression of neurobehavioral defects.

The present review summarizes our efforts to identify biochemical and genetic features in a group of Italian patients. We also describe a new molecular method for identifying mutation carriers, and provide an overview of biochemical and genetic research conducted in certain Italian laboratories on specimens from LND patients and on animal models of the disease. Metabolic and molecular pathways were explored in an attempt to understand the pathogenesis of the disease and the role of NAD in patients and animal models. The involvement of adenosine and serotonin receptors was also investigated.

Study of families: biochemical and cell studies

Our case studies included about one sixth of all Lesch-Nyhan patients in Italy. Spread over various regions, their identification and management is rather difficult. A network was needed to manage these patients and voluntary associations were invaluable, making it possible to unite a rather large sample of Italian Lesch Nyhan families (25 families and 28 patients).

Genotype–phenotype correlations were recorded accurately for all families by biochemical and genetic study. Those with phenotypic variants also underwent biochemical and cell tests.

Correlation between residual HPRT activity and neurological involvement was investigated (19, 20), although values overlapped for patients with very different phenotypes. In this review we report biochemical studies (metabolite analysis and enzyme activity of intact red blood cells and lysates) performed by HPLC-linked methods (21) in 16 patients with typical LNS (**Table 1**). In all cases, HPRT activity was found to be virtually absent in haemolysates and intact erythrocytes. Concomitantly, APRT activity was grossly increased. Such findings, together with abnormally high NAD levels in erythrocytes, also found in LNS patients (11), enabled full biochemical diagnosis of the syndrome.

Mutation analysis

Mutation analysis of the 28 patients (**Table 2**) revealed seven missense mutations due to single base substitutions leading to single amino acid substitutions (25%), two nonsense point mutations leading to premature stop codons and truncated proteins (7%), four mutations in splicing sites (14%), six large deletions leading to loss of one or more exons (21%), one deletion of two bases, three insertions of one base (10.7%), and one combined mutation consisting of a four-base deletion and a three-base insertion.

Nine of these 24 mutations had not previously been described: 74C>G (Pro25Arg) in exon 2, 194-195delTC in exon 3, 329-332delCAAC insTCT in exon 4, 418G>C (Gly418Arg) in exon 4, 506insC in exon 7, IVS2þ1G>C in the donor site of intron 2, IVS8-1G>C in the acceptor site of intron 8, and IVS9-1G>A in the acceptor site of intron 9. Only two mutations, E2-9 and 212insG, were found in unrelated families. Overall, the study did not find any mutation hotspots. All missense mutations identified in the present study, which only included typical LNS cases with no detectable HPRT activity, led to non conservative changes.

Diagnostic test for identification of carriers

Diagnosis of a healthy carrier cannot rely on HPRT activity measured in blood cells, because this activity does not differ from that of non-carriers. Genetic tests can help reveal mutations only when both alleles can be amplified by PCR (from genomic DNA or cDNA), while gross deletions involving the ends of cDNA are not detectable by PCR (22). Several approaches have been used to diagnose healthy carriers bearing large deletions: 1) DNA analysis by southern blot and RFLP linkage analysis (23, 24), 2) mRNA analysis by amplification of HPRT cDNA

Table 1. Biochemical data in 16 patients

Subjects	HPRT	[C^{14}] Hypoxanthine incorporation	APRT	[C^{14}] Adenine incorporation	PRPS	NAD
8/00	0.4	ND	49	354	29.8	159
6/00	ND	ND	59	468	29.9	179
46/00	ND	ND	65	264	35.3	147
158/01	ND	ND	70	264	33.2	206
56/00	ND	0.2	54	339	37.1	171
20/00	ND	0.2	62	582	37.1	101
24/01	ND	ND	66	379	44.9	204
5/00	ND	Not determined	89	Not determined	72.6	ND
2027124	0.6	15	63	88	34.4	217
2037024	ND	ND	49	Not determined	ND	230
2037023	ND	ND	48	Not determined	ND	228
2037039	ND	ND	59	Not determined	ND	223
12/01	ND	ND	60	389	ND	181
10/00	ND	ND	44	236	ND	158
12/00	ND	ND	50	388	ND	210
110/01	ND	ND	52	321	ND	132
Controls (mean ± SD)						
Adults	119 ± 12	178 ± 55	22 ± 4	101 ± 18	40 ± 5	49 ± 8
Children	129 ± 30	156 ± 4	23 ± 3	168 ± 33	44 ± 9	54 ± 11

Activities are expressed in nmol/h per mg Hb; radiolabel incorporation is expressed in nmol/h per ml RBC; NAD concentration is expressed in nmol/ml RBC; ND: not detectable

(25) (insufficient to detect deletion of the whole *HPRT* gene or deletions including its extremities), 3) biochemical test of HPRT activity in hair bulbs (26) (completely abandoned due to false-negative results (21)), 4) cell-growth test in selective medium containing 6-thioguanine (O'Neill test (27)) in which only HPRT-deficient lymphocytes can grow, allowing very accurate, though technically and analytically demanding, identification of carriers. In the present study the above test was performed in families where the genetic test was problematical, as in those showing large deletions (28). Moreover there is a new method to identify carriers with large deletions based on semiquantitative real-time PCR, never previously used in the diagnosis of LNS. Results were validated with a linkage study. Semiquantitative real-time PCR is a very powerful technique for diagnosis of deletions, enabling quantification of target gene amplification by comparison with a reference gene by the ΔCt method (29). Our experiments were validated in a blind manner, comparing results obtained in Italian families previously analyzed with approved diagnostic methods (i.e. O'Neill's test).

Real-time PCR

The newly developed real-time PCR method was used to identify carrier females in two families. The first analysis was conducted in family 1 (**Fig. 1A**) carrying a deletion between exons 1 and 3. Male and female controls were separated (CN-M1 and CN-F1) to show that when the *FANCB* gene was used as genomic reference, the results were not influenced by gender. Each control bar in **Fig. 1** is the result of three different control-patient results repeated in four experiments. The other bars represent values obtained from analysis of other family 1 members in the four experiments. All the proband's relatives showed a normal pattern.

Results of the analysis conducted on family 2, which carries a deletion involving exon 9 of the *HPRT* gene, are shown in **Fig. 1B**. All the probands' relatives showed a normal pattern. All $2^{-\Delta\Delta Ct}$ results were collected to calculate the mean value of each group of samples (male and female negative controls, positive controls and carriers) and the range (**Table 3**). Taken together, data shown in the tables confirms the reliability of real-time PCR results.

Thirty-three females from the 25 families, variously related to the probands, were analyzed genetically to evaluate their carrier status. Carrier status was ascertained in 19 out of 33, while 14 were found to be non carriers. *De novo* mutations were found in two patients whose mothers were not carriers. The O'Neill test was performed in female members of one family, characterized by the mutation E1-3. The test demonstrated that the proband's mother and sister were carriers of the mutation, while the aunt and the female cousin were not (data not shown).

Microsatellite analysis

To validate the diagnostic pathway and results obtained by real-time PCR, blind haplotype analysis was performed using SNPs and microsatellite analysis. The heterozygosity rate of all SNPs allowed us to monitor transmission of affected HPRT pedigree.

Experimental studies using *HPRT* gene knockout mice

Dopaminergic and serotonergic abnormality in HPRT knockout mice is suggested by severe dopamine depletion and increased serotonin levels in the striatum, while other

Table 2. List of mutations in the 28 patients described in the text

Sample	Mutation	Base	Exon	Codon	Effect on protein
Missense point mutations					
8/00	G>T	208	3	70	G>W
15/00	**C>G**	**74**	**2**	**25**	**P>R**
1/01	C>A	191	3	64	A>D
27/01	**G>T**	**606**	**8**	**202**	**L>F**
58/01	A>T	590	8	197	E>V
106/01	G>A	212	3	71	G>D
2027124	**G>C**	**418**	**4**	**140**	**G>R**
Nonsense point mutations					
5/00	C>T	508	7	170	R>X
53/00	C>T	151	3	51	R>X
54/00	C>T	151	3	51	R>X
Deletions					
6/00	E2-9				8 exons deleted
12/00	E1-3				3 exons deleted
46/00	E2-9				8 exons deleted
8/01	E1				1 exon deleted
11/01	E1				1 exon deleted
24/1	E4				1 exon deleted
69/01	**194-195delTC**				**Frame shift E3 (stop after 8 aa)**
110/01	E1-9				All exons deleted
2037023	E9				1 exon deleted
2037024	E9				1 exon deleted
Splice site mutations					
3/00	IVS7-1G>A				E8 skipped
17/00	**IVS8-1G>C**				**?**
12/01	**IVS2+1G>C**				**?**
158/01	**IVS9-1G>A**				**?**
Insertions					
20/00	212insG				Frame shift in E3
56/00	**506insC**				**Frame shift in E7**
2037039	212insG				Frame shift in E3
Combined mutation					
10/00	**329-332delCAAC insTCT**		**4**		**?**

Novel mutations are indicated in bold type; the others are additional cases of mutations already reported in the Human Gene Mutation Database (URL: http://archive.uwcm.ac.uk/uwcm/mg/ hgmd0.html). Cases 8/00 and 6/00 were reported by Gathof et al. (1998).

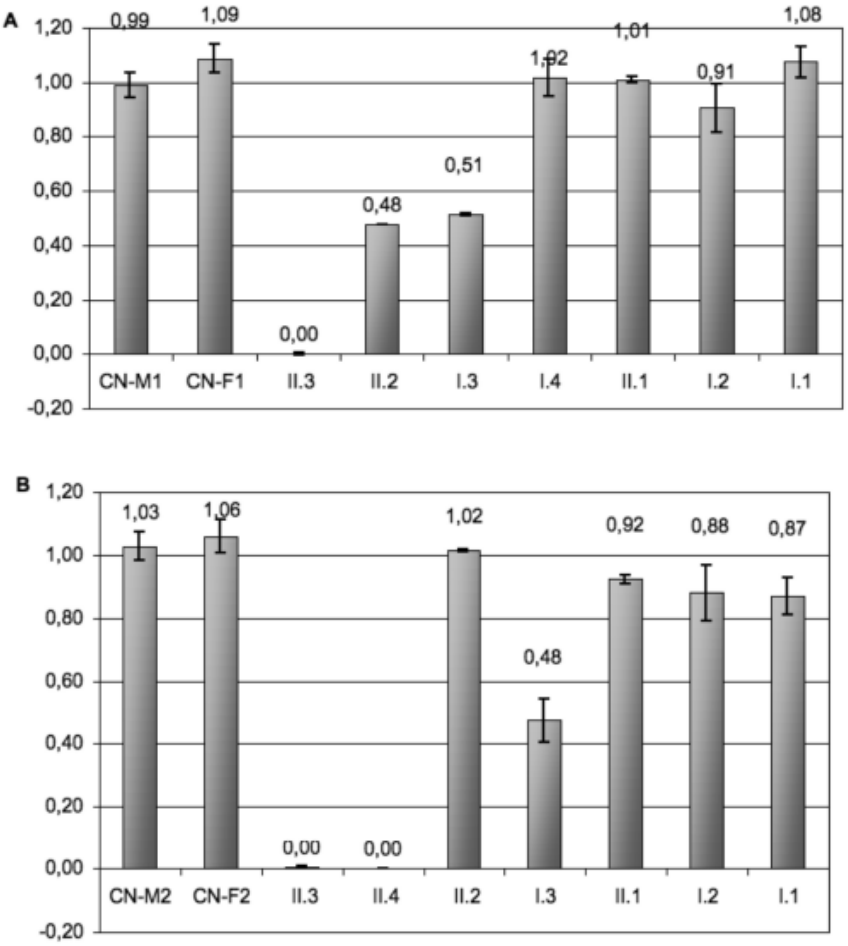

Figure 1. Semiquantitative real-time PCR results. Bars represent $2^{-\Delta\Delta Ct}$ values calculated by the ΔCt method. Data is normalized to control DNA. Control samples were divided into male and female controls (CN-M and CN-F); bars of male and female controls are the result of three different control patients. Male and female controls showed the correct number of copies of *HPRT* in the region analyzed, whereas LNS-positive controls (CP) did not show any copies of *HPRT*. (A) Real-time semiquantitative PCR results of *HPRT* exon 2 analysis. II.3 is the male proband carrier of the deletion between exons 1 and 3; II.2 and I.3 are the proband's sister and mother, both carriers of the deletion; I.4 is the proband's father, showing a pattern similar to that of normal controls. On the mother's side, the female cousin, the aunt, and the aunt's husband (II.1, I.2, and I.1, respectively) show a pattern typical of unaffected individuals. (B) Real-time semiquantitative PCR results of *HPRT* exon 9 analysis. II.3 and II.4 are the two affected brothers; II.2 is their sister, who shows a pattern similar to that of normal controls; I.3 is the probands' mother, who is a carrier of the deletion. The probands' relatives (the female cousin, the maternal aunt, and uncle by marriage, II.1, I.2, and I.1, respectively) show a normal pattern.

neurotransmitter systems appear relatively unaffected (14, 17).

Several studies on mouse and rat models of LND suggest that reduced dopamine accompanied by increased serotonin turnover may be prerequisites for self-injurious behavior (15, 30, 31, 32). We studied the possible link between HPRT deficiency and dopaminergic or serotonergic dysfunction to verify the hypothesis of an association with abnormalities in purinergic neurotransmission, and the role of purine compounds.

- Adenosinergic hypothesis - The possible role of adenosinergic transmission in LND pathogenesis was investigated. Adenosine receptors colocalize with dopaminergic receptors in the nigrostriatal pathway. Adenosine regulates dopamine release at pre-synaptic level through ADORA1A receptors, modulating membrane refractoriness to

Table 3. Real-time PCR results

Negative male control	6	1.01	0.94–1.08	0.0449
Negative female control	6	1.07	1.00–1.14	0.0602
Positive control	3	0	-	-
Carrier	3	0.49	0.48–0.51	0.0173

Average value of $2^{-\Delta\Delta Ct}$ is the result of each experiment. Results fluctuate in a small range of values. We show standard deviation as calculated for each sample in each experiment.

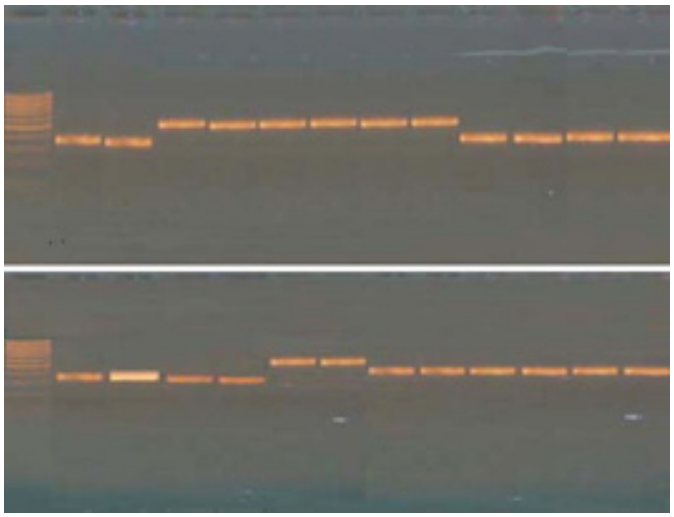

Figure 2. Target genes amplified by RT-PCR from control (cn) and knockout mice (ko). First lane: molecular weight markers; following lanes: single amplified genes from cn and ko, from left to right *ADA, ADK, ADAR, ADARB1, ADARB2, HTR2C, ADORA1A, ADORA2A, ADORA2B, ADORA3, ENTPD1, SLC29A*. Simultaneous amplification of the housekeeping gene GAPDH was also performed. Amplification kinetics was obtained by analyzing PCR samples at different steps (25, 27, 29, 30, 33 cycles). The expression level of each gene was calculated from the fluorescence intensity of the corresponding band on agarose gel as described in Materials and methods; data was normalized on the basis of amplification of the housekeeping gene and then compared.

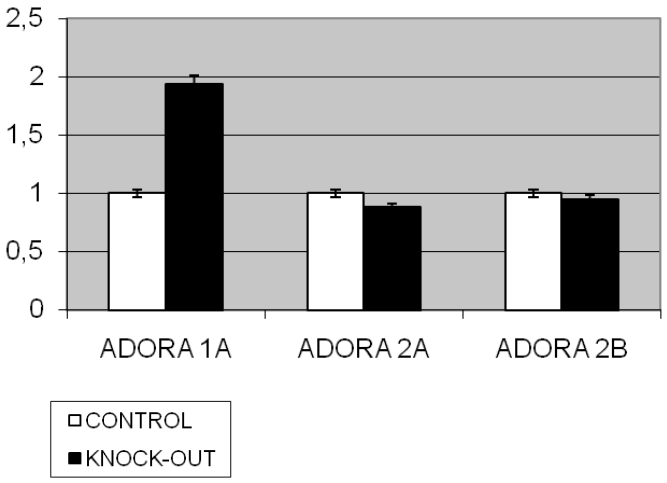

Figure 3. Expression level of adenosinergic receptors ADORA1, ADORA2A and ADORA2B analyzed by real-time PCR.

depolarizing stimuli from activated dopamine receptors. At post-synaptic level, adenosine modulates excitability through ADORA2A and ADORA2B receptors, causing membrane hyperpolarization and refractoriness. Adenosinergic receptors ADORA1A and ADORA2A are also reported to modulate the release of serotonin in hippocampal rat synapses (33-35). Though not directly involved in adenosine metabolism, HPRT deficiency may conceivably affect adenosine levels and distribution indirectly. Adenosine transport across cell membranes is reported to be strongly reduced in HPRT-deficient lymphocytes after incubation with hypoxanthine (36-40). Studies using agonists of adenosine receptors A1 failed to demonstrate altered regulation of adenylyl cyclase by such receptors in HPRT-deficient cells of B103 rats (41).

In this study the expression of adenosine receptors, adenosine transporters and adenosine-related enzymes was compared in the brain of HPRT knockout mice. Expression levels of a series of target genes were tested by semiquantitative RT-PCR. Only adenosine receptor types *ADORA1A* and *ADORA2A*, and serotonin receptor *2C* genes were expressed differently in knockout and control mice (**Fig. 2**). Real-time PCR showed a 95% increase in *ADORA1A* expression, a 15% decrease in *ADORA2A* expression, and no change in *ADORA2B* expression in knockout mice compared to controls (**Fig. 3**).

Adenosine deaminase and 5′-nucleotidase activities as well as adenosine concentration are reported to be unchanged in knockout mice with respect to controls (42). The possibility of altered adenosine availability was considered in this study, focusing on NAD and its breakdown as a source of adenosine, as demonstrated in human fibroblasts (43) and rat heart.

HPLC analysis of purine nucleotide and nucleoside concentrations in perchloric extracts of whole brains of control and knock out mice did not reveal any significant differences between control and mutant mice (data not shown), in line with previous reports (42, 44).

NAD and NADP concentrations and NADase activity were found to be significantly higher in liver of knockout mice than in controls, though not in blood and brain, demonstrating that changes in NAD metabolism respond to HPRT deficiency in relation to species and tissue type, though variations in adenosine availability through this pathway should not perturb adenosinergic transmission (**Table 4**).

Serotonergic hypothesis - Several studies on the neurobiological basis of aggressive behavior in different animal models have shown that alterations in the serotonergic system may be linked to manifestation of symptoms (45, 46).

Table 4. NAD and NADP (nmoles/mg protein) and NADase activity (nmoles/h/mg protein) in blood, brain and liver from control and HPRT-deficient mice.

	NAD	NADP	NADase
Blood			
WT	0.16 ± 0.02	0.06 ± 0.004	Nd
KO	0.15 ± 0.02	0.06 ± 0.03	Nd
Brain			
WT	1.9 ± 1.0	0.08 ± 0.03	819 ± 56
KO	1.7 ± 0.4	0.08 ± 0.03	1069 ± 350
Liver			
WT	1.1 ± 0.7	0.36 ± 0.16	2116 ± 641
KO	3.7 ± 0.9**	1.00 ± 0.30**	3158 ± 732*

*= P<0.05; ** = P<0.01; nd = not determined.

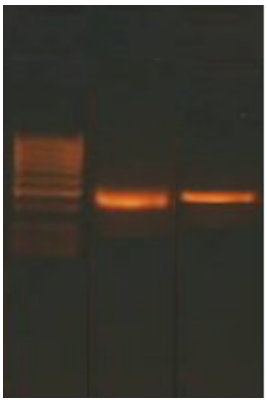

Figure 4. HTR2C genes amplified by RT-PCR from knockout mice (lane 2) and controls (lane 3). Lane 1: molecular weight markers. Simultaneous amplification of the housekeeping gene *GAPDH* was also performed (data not show). Amplification kinetics were obtained by analyzing PCR samples at different steps (25, 27, 29, 30, 33 cycles).

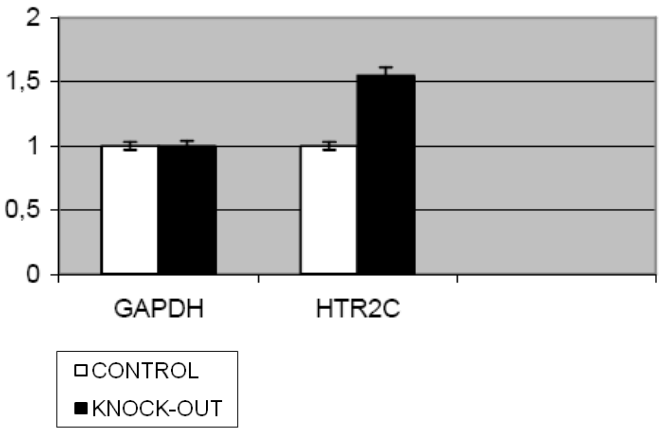

Figure 5. Expression level of HTR2C receptor analyzed by real-time PCR in control and knock-out mice.

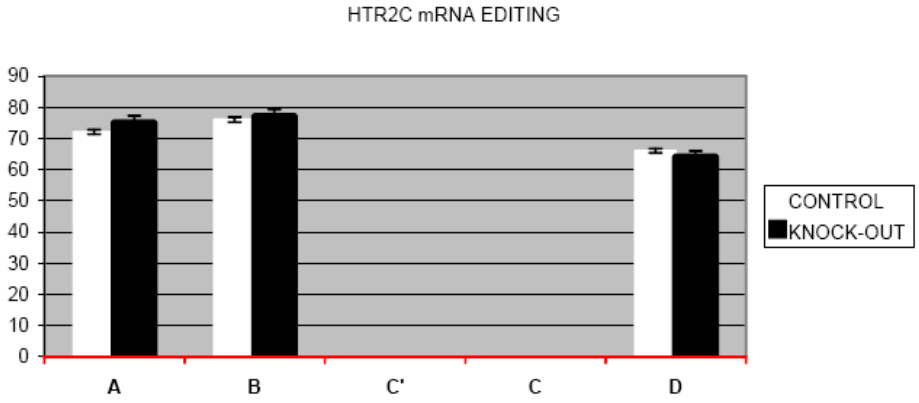

Figure 4. HTR2C genes amplified by RT-PCR from knockout mice (lane 2) and controls (lane 3). Lane 1: molecular weight markers. Simultaneous amplification of the housekeeping gene *GAPDH* was also performed (data not show). Amplification kinetics were obtained by analyzing PCR samples at different steps (25, 27, 29, 30, 33 cycles).

Intrastriatal injection of hypoxanthine in rats yielded a reduction in striatal serotonin content (47), and oral administration of hypoxanthine to healthy human volunteers caused a sharp reduction in urinary excretion of serotonin (48), suggesting a link between HPRT deficiency and serotonergic dysfunction. A role of the serotonergic system in HPRT deficiency is supported by the finding of altered serotonin levels in the striatum of knock-out mice (49) and in the neonatal 6-hydroxydopamine (6-OHDA)-treated rat model of LND (30); in the latter, serotonin receptor 2C becomes sensitized and administration of agonists induces self-injurious behavior (SIB). However, no further evidence suggesting that the serotonin receptor 2C is critical for SIB has so far been produced. Data in the literature indicates that serotonin receptor 2C is implicated in the regulation of mood and behavior by specific alteration of mRNA editing; indeed several studies in humans report changes in serotonin receptor 2C editing in brains of suicide victims (50). If suicide is considered an extreme form of self-aggression (51), a correlation between receptor alterations and SIB may be hypothesized. The process of mRNA editing, performed by a specific adenosine deaminase that converts adenosine to inosine (inosine nucleotides are recognized functionally as guanosine nucleotides), selectively changes the read-out of a gene in the primary RNA transcript. The editing process is a regulation system that recodes genomic information in a systematic and regulated manner. HTRC2 mRNA is known to be modified by RNA editing which changes up to three genomically encoded amino acids in the second intracellular loop of this G-protein-coupled receptor. Extensively edited receptor isoforms activate G-protein less efficiently than non-edited receptors.

In order to ascertain the role of the serotonin receptor 2C (HTRC2) in the mouse model of LND, our experimental strategy was to look for alterations in gene expression and for abnormalities in mRNA editing of *HTRC2*, generating single amino acid changes in the resulting protein, often with significant functional consequences.

Expression of *HTRC2,* tested by semi-quantitative RT-PCR was found to differ between knock-out and control mice; real-time PCR confirmed a 55% increase in expression in knock-out mice brains compared to controls (**Fig. 4 and 5**). No significant

difference in editing levels, detected in three (A, B, D) out of five possible sites in HTRC2 mRNA, was found between knock-out and control mice (**Fig. 6**).

Discussion

Lesch-Nyhan disease, characterized by hypoxanthine-guanine phosphoribosyltransferase deficiency, is very rare, which makes it difficult to find enough patients for a homogeneous study.

In this study 28 LNS patients from 25 families were collected through a survey involving two research centers, each serving several different Italian regions. Since the Italian LND population has never been studied, the present cohort was used to calculate national incidence. Based on a specific international incidence of 1/380,000 and the Italian population which is 60 million, our study presumably captured more than one quarter of all live Italian patients, already described in part (52). Based on birth incidence data (53), the birth of one or two affected subjects can be expected in Italy every year.

Overall, the unique nature of most of the mutations identified, their uniform distribution throughout the gene, and the presence of *de novo* cases confirm that LNS is not linked to any major founder mutation, at least in the Italian population, but is rather the consequence of multiple, separate, independent events randomly affecting the *HPRT* gene. This is in line with other reports. Several studies lead to the conclusion that mutation identification is a poor tool for predicting phenotype in LNS (1, 54). At biochemical level, there is evidence that some residual enzyme activity is associated with less severe clinical manifestations. Thus, large deletions and truncating mutations that cause complete enzyme deficiency lead to typical LNS, while point mutations that change a single amino acid may in principle cause either partial or complete deficiency and may therefore underlie atypical, milder cases. In particular, conservative amino acid substitutions should alter HPRT protein conformation less severely than non conservative substitutions. In line with this concept, all missense mutations identified in the present study, which only included typical LNS cases with no detectable HPRT activity, lead to non conservative changes. Several approaches are possible to diagnose LND, but the biochemical test on red blood cells is the easiest to perform; uncertain cases should be studied using cultured fibroblasts (55).

Females in LND families are at risk of being carriers, and diagnosis is very important for genetic counseling to evaluate reproductive risk. The reliable new method for carrier detection used in this study is based on semiquantitative real-time PCR and overcomes difficulties encountered by other methods, particularly in the case of gross deletions. So far, O'Neill's test, based on the 6-thioguanine-resistant phenotype of HPRT mutant cells, is the only accepted method for diagnosis of carriers of any kind of mutation, but it is difficult to perform (1, 27). Since eight of the 27 families in our cohort carry a large deletion, the national incidence of deletions in LNS patients can be estimated at around 22.8% (calculated on mutation number instead of proband number, since affected brothers are collected). Five out of eight families with deletion in *HPRT* could be of interest for this study, because their probands had deletions at the ends of *HPRT*. The O'Neill test was only available for two of these families, and was used to validate the approach used in this study. Carriers in two of the three remaining families that could not be studied by this approach were readily diagnosed with the PCR multiplex protocol on cDNA (their probands bore deletions in exons 4 and 5); the PCR multiplex protocol could not be used to quantify genomic copy number in the deleted region of *HPRT* for the proband of the third family, who carried a deletion in 5′UTR. Real-time PCR analysis (**Fig. 1**) clearly confirmed results obtained by the selective medium test. To validate the diagnostic pathway and results obtained by real-time PCR, haplotype analysis in blind was used. It confirmed the real-time PCR results, showing that the affected allele was present in female carriers and male probands. On the other hand, linkage analysis also has its limitations. A single SNP or microsatellite is clearly insufficient for analysis, even if its heterozygosity is high. It is necessary to use several markers to ensure statistical efficacy. In these cases, clinical and familial context should be studied, and pedigree and heredity should be taken into account. Moreover, to give significant results, this method requires samples from many family members, and in the case of Lesch-Nyhan syndrome, samples from the proband and mother are essential. Linkage analysis may strengthen diagnosis obtained by real-time PCR. Both techniques can be done with DNA extracted from peripheral blood (fresh or frozen), making them accessible to any laboratory. An approach based on both methods clearly gives a more reliable diagnosis, but in future it is likely that accurately set up real-time PCR will enable diagnosis of LNS by this technique alone. Based on the data presented here, this new approach can be applied to 62.5% of large deletions, that is, 22.8% of all LNS mutations.

In conclusion, genetic analysis provides a valuable tool for confirming diagnosis in probands, for identifying potential carriers, for pre-implantation testing and for prenatal diagnosis. The present results offer a survey of the mutations found in the Italian population. A new approach for detecting LNS carriers is also described: it is suitable for most LNS families with large deletions and offers an alternative to the approved selective medium method, which is not always applicable. To our knowledge, no such extensive investigations, covering most living patients in the country and almost all Italian geographical areas, have been conducted before.

The link between HGPRT deficiency and neurological and behavioral dysfunction remains unclear, though there is increasing evidence that dysfunction of dopamine systems in basal ganglia plays a role (4, 22, 29). A role of dopaminergic and serotonergic system abnormality in HPRT knockout mice is supported by the finding of severe dopamine depletion and increased serotonin levels in the striatum, while other neurotransmitter systems appear relatively unaffected (14, 16). These considerations seem to favor the hypothesis that LND is associated with an unbalanced adenosinergic system that contributes to dopaminergic dysfunction and possibly also to

serotonergic alterations in the basal ganglia.

A new finding in knockout mice reported in our study is grossly increased expression of adenosinergic ADORA1A receptor mRNA. If confirmed by protein analysis, greater expression of this receptor may reduce pre-synaptic dopamine release and contribute to the dopamine deficit previously found in knockout mice. This observation suggests that adenosinergic alteration in LND may be the initial cause of basal ganglia dysfunction, namely: reduction in number and arborization of striatal dopaminergic neurons, dopaminergic deficit, sensitization of dopaminergic receptor D1 and increased serotonin levels. The adenosinergic receptor ADORA1A is also known to be over expressed in the first weeks of neurodevelopment (56), and administration of the receptor agonist in this period provokes neural loss and fewer dendrite connections. Other observations in the literature indicate a role of adenosinergic receptors. Increased activity of ADORA2A receptors, co-localizing with D2 receptors in striatal tissue, has been found in striatal cells prone to degeneration in experimental models of this neurodegenerative disease. Moreover, reduction of ADORA2A receptor expression is one of the earliest events in Huntington's disease (57). Antagonists of ADORA2A receptors in the 6-OHDA rat reduce L-dopa-induced self-injury behavior caused by dopamine depletion (58). The 15% reduction in expression of the adenosinergic receptor ADORA2A found in knockout mice in the present study may be interpreted as compensating neurotransmitter imbalance.

Data in the literature indicates that serotonin receptor 2C is implicated in regulation of mood and behavior by specific alteration of mRNA editing (50). Grossly increased expression of *HTRC2* in the LND mouse model used in the present experimental study is a new finding. These experimental studies were limited because they used whole brain, instead of isolated basal ganglia. The higher expression of *HTRC2* found in this preliminary study seems to open a new direction for investigation of neurobehavioral abnormalities in LNS and possibly in other disorders with aggressive behavior.

In this review we described the different approaches used to study certain aspects of LND, focusing on diagnosis in patients and carriers and on simultaneous analysis of biochemical and genetic features. Clinical and molecular features need to be surveyed in as many patients as possible in order to understand the pathogenetic mechanism of this rare disease. Identification of carriers is especially important for genetic counseling.

On the experimental side, the mouse model elucidated the possible involvement of adenosine and serotonin receptors in the neurotransmission aberration occurring in HGPRT deficiency.

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