Physiological levels of folic acid reveal purine alterations in Lesch-Nyhan disease

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Lesch-Nyhan disease (LND), caused by a deficient salvage purine pathway, is characterized by severe neurological manifestations and uric acid overproduction. However, uric acid is not responsible for brain dysfunction, and it has been suggested that purine nucleotide depletion, or accumulation of other toxic purine intermediates, could be more relevant. Here we show that purine alterations in LND fibroblasts depend on the level of folic acid in the culture media. Thus, physiological levels of folic acid induce accumulation of 5-aminoimidazole-4-carboxamide riboside 5′-monophosphate (ZMP), an intermediary of de novo purine biosynthetic pathway, and depletion of ATP. Additionally, Z-nucleotide derivatives (AICAr, AICA) are detected at high levels in the urine of patients with LND and its variants (hypoxanthine-guanine phosphoribosyltransferase [HGprt]-related neurological dysfunction and HGprt-related hyperuricemia), and the ratio of AICAr/AICA is significantly increased in patients with neurological problems (LND and HGprt-related neurological dysfunction). Moreover, AICAr is present in the cerebrospinal fluid of patients with LND, but not in control individuals. We hypothesize that purine alterations detected in LND fibroblasts may also occur in the brain of patients with LND.

purines | folic acid | Lesch-Nyhan disease | ZMP | AICAr

Lesch-Nyhan disease (LND) is an X-linked recessive disorder caused by deficiency of hypoxanthine-guanine phosphoribosyltransferase (HGprt), an enzyme involved in the salvage synthesis of purines. Patients with LND have hyperuricemia, macrocytic anemia, and severe neurological problems including dystonia, spasticity, cognitive deficit, and self-injurious behavior (1–3). Milder forms of this disease, named variants, exhibit fewer neurological problems and correlate with residual levels of HGprt activity. These variants are grouped into two categories: HGprt-related neurological dysfunction (HND), and HGprt-related hyperuricemia (HRH). HND is characterized by hyperuricemia, motor impairment, and neurocognitive abnormalities. The mildest phenotype, HRH, has hyperuricemia, nephrolithiasis, and gout, but no neurological symptoms (4).

HGprt catalyzes the transfer of the 5-phosphoribosyl group from 5-phosphoribosyl-1-pyrophosphate (PRPP) to a purine base (hypoxanthine or guanine) to form a nucleoside monophosphate (IMP or GMP, respectively) (Fig. 1*A*). Patients with LND have hyperuricemia because hypoxanthine and guanine cannot be recycled, and they are instead degraded to uric acid (1, 2). Moreover, there is a pronounced activation of de novo purine synthesis, further increasing the production of uric acid (5, 6) (Fig. 1*A*).

Uric acid toxicity has been discarded as a significant contributor to these neurological problems for several reasons. The suppression of uric acid levels in LND by the administration of allopurinol from birth does not attenuate the neurobehavioral problems; there are other clinical disorders with excessive production of uric acid, but without the neurobehavioral problems of LND, and there are no significant differences in serum uric acid levels among LND, HND, and HRH (4, 7). Many investigators instead have proposed that the failure of purine recycling results in purine deficiency. However, multiple studies have failed to reliably show any significant purine aberration in numerous types of HGprt-deficient cells in culture (8) or in any tissue from *HPRT1* gene knockout mice (9). Only erythrocytes from patients with LND have a decrement in ATP and GTP levels (10, 11). However, erythrocytes lack de novo purine synthesis (12, 13) and, therefore, are not representative of most other cell types, which may compensate for HGprt deficiency with an accelerated de novo pathway.

Another mechanism that has been proposed involves toxic accumulation of 5-aminoimidazole-4-carboxamide riboside 5′-monophosphate (ZMP), an intermediate of the purine biosynthetic pathway (14) (Fig. 1*A*). High levels of ZMP and its triphosphate derivative ZTP have been detected in erythrocytes from patients with LND, but not in normal control individuals (11, 15). ZTP can be synthetized in an unusual one-step reaction from ZMP and PRPP catalyzed by PRPP synthetase (16). However, erythrocytes cannot synthesize ZMP de novo, and paradoxically, other cell types from patients with LND maintained in regular culture medium do not accumulate any Z-nucleotides (ZNT).

In a small study, AICA (a metabolite of AICAr) was increased in urine samples from 5 patients with LND (17), and AICAr has been

Significance

Fibroblasts from patients with Lesch-Nyhan disease (LND) do not show any significant nucleotide alteration when they are maintained in regular culture medium, which typically contains artificially high levels of folic acid. However, they show ATP depletion and accumulation of 5-aminoimidazole-4-carboxamide riboside 5′-monophosphate (ZMP; an intermediary of the novo purine biosynthetic pathway) when the culture medium has physiological levels of folic acid. A derivative of ZMP (AICAr) is present in the urine and the cerebrospinal fluid of patients with LND, but not in control individuals, suggesting that a similar situation may occur in vivo. The manipulation of folic acid levels may therefore provide a valuable strategy to further study the pathogenesis of LND.

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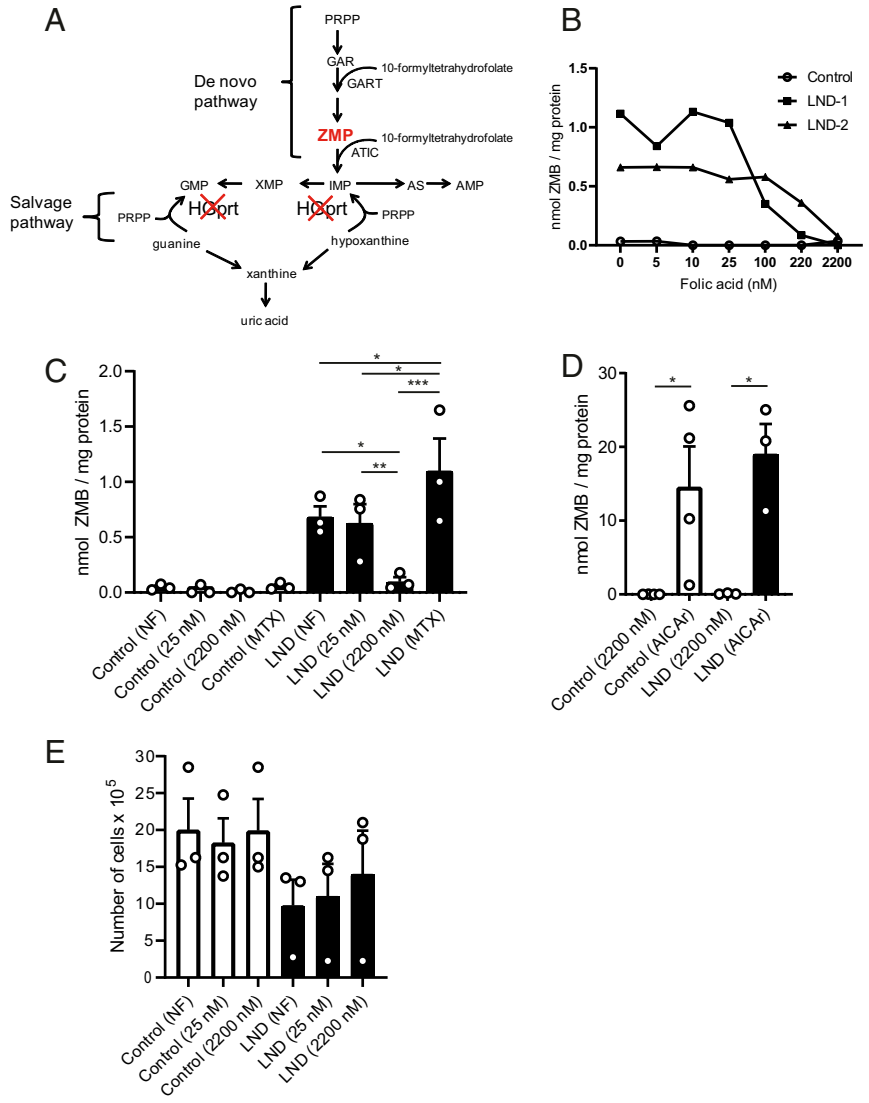

Fig. 1. Accumulation of ZMB in LND fibroblasts depend on folic acid levels. (*A*) Scheme of the de novo and salvage pathways of purine biosynthesis. The deficiency of HGprt increases the levels of guanine, hypoxanthine, and PRPP and accelerates the de novo pathway producing high levels of uric acid. ZMP is an intermediate of the de novo pathway. (*B*) Fibroblasts from two patients with LND and one control individual were incubated for 5 d in RPMI medium with different concentrations of folic acid (0, 5, 10, 25, 100, 220, 2,200 nM). Physiological levels of folic acid in human plasma are around 25 to 50 nM, whereas 2,200 nM is the concentration in regular culture media. Cell extracts were obtained with 0.4 N PCA and ZMB determined with the Bratton-Marshall test. (*C*) Fibroblasts were incubated for 5 d in RPMI medium without folic acid (NF), 25 nM folic acid, 2,200 nM folic acid, or 2,200 nM folic acid plus 5 μM MTX, and ZMB determined with the Bratton-Marshall test. The graph represents the mean ± SEM of 3 control individuals and 3 patients with LND, expressing the results as nanomoles ZMB per milligram protein. The differences obtained were significant, comparing LND (NF), LND (25 nM), or LND (MTX) with LND (2,200 nM), and comparing LND (MTX) with LND (NF) or LND (25 nM). ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. Two-way ANOVA, Newman-Keuls test. (*D*) Fibroblasts were incubated in RPMI medium with 2,200 nM folic acid with or without 0.5 mM AICAr for 24 h, and ZMB determined with the Bratton-Marshall test. Results are the mean ± SEM of 4 control individuals and 3 LND, expressed as nanomoles ZMB per milligram protein. The differences obtained were significant comparing control or LND with AICAr treatment. Note the different scales for the graphs *C* and *D*. *$P < 0.05$. Two-way ANOVA, Bonferroni's test. (*E*) Effect of folic acid levels and HPRT deficiency in the proliferation of human fibroblasts. Fibroblasts (1.8×10^5 cells) were seeded in 100-mm plates on day 0 and were incubated for 5 d, as reported in *C*, and cells were counted at day 5 with a Neubauer chamber. Results are the mean ± SEM of 3 control individuals and 3 patients with LND, expressing the results as number of cells $\times 10^5$.

detected in the urine from 1 patient with LND (18). Although the number of patients analyzed is very small, the available data suggest that ZNT or their metabolites accumulate in LND. Presumably, erythrocytes accumulate ZNT because they use metabolic precursors synthesized by other tissues. Interestingly, ZMP accumulation has been detected recently in the brain of *HPRT1* knockout mice (19).

In the current studies, we show that ZMP accumulates and ATP drops in LND fibroblasts in culture only when folic acid is reduced to physiological levels. To assess the potential in vivo relevance of this finding, we analyzed ZNT and their metabolites in a large cohort of patients to demonstrate that significant levels of AICAr are detected in cerebrospinal fluid (CSF) from patients with LND, but not in control individuals. Moreover, AICAr and AICA are excreted at high levels in urine samples from LND or its variants (HND and HRH), and the ratio of AICAr/AICA seems to increase with the severity of the disease (LND > HND > HRH).

Results

The Bratton-Marshall Test Reveals That Accumulation of Z-Metabolites in LND Fibroblasts Depends on Folic Acid Levels. The abnormal accumulation of ZNT reported for LND erythrocytes (11, 15) has not been

described for any other cell type. Indeed, in preliminary studies, we were unable to detect any ZNT or any of their metabolites in LND fibroblasts or other HGprt-deficient cells in culture (6). This discrepancy may reflect differences in purine metabolism among different cell types, because the de novo pathway is intact in most cells but absent in erythrocytes (12, 13). Alternatively, it may reflect differences in the environment in vitro compared with in vivo. A prior study demonstrated that LND fibroblasts grew poorly in tissue culture medium with low folic acid, a key intermediate in the de novo pathway (20). Since most currently available tissue culture media have nonphysiologically high levels of folic acid (2,200 nM in most tissue culture media compared with 25 to 50 nM in vivo), we addressed whether ZNT or their metabolites might be detected in human fibroblasts cultured in media containing more physiologically relevant folate concentrations.

As a screening method, we used the Bratton-Marshall test, a colorimetric method used to detect AICA in the urine of normal people (21) and succinyl-AICAr in the urine of patients with adenylosuccinate lyase deficiency (22). This method is based on the reaction of an amine group of AICA with nitrous acid and a second reaction with *N*-naphthylethylenediamine to form a compound with maximum absorbance at 540 nm (22). This method allows the combined detection of AICA, AICAr, ZNT, succinyl-AICAr, and succinyl-ZMP. We verified that the method was reproducibly capable of detecting low concentrations of AICA, AICAr, and ZMP standards, with linearity in a range of 1 to 100 μM, and we adapted the method for the detection of metabolites in perchloric acid (PCA) extracts from cultured human fibroblasts (*Methods*). Since the Bratton-Marshall test detects both ZNT and their derivatives, data obtained with this test are referred to as Z-metabolites (ZMB).

Fibroblasts from patients with HGprt deficiency and control individuals were maintained for 5 d in medium with different concentrations of folic acid. As illustrated in Fig. 1*B*, the dose–response experiment showed that more physiological concentrations of folic acid (25 to 50 nM) induced accumulation of ZMB in fibroblasts from patients with LND, but not in control individuals.

To further explore the role of folic acid, fibroblasts from patients with HGprt deficiency and control individuals were maintained for 5 d in standard tissue culture medium (2,200 nM folic acid), medium without folic acid (NF), medium with physiological folic acid (25 nM), or standard medium (2,200 nM folic acid) with the folic acid antagonist methotrexate (MTX). As a positive control for detection of intracellular ZMB, fibroblasts also were treated with AICAr, which can be incorporated into the cells and phosphorylated to ZNT. No ZMB were observed in control fibroblasts under any condition, except for the addition of AICAr. No ZMB were observed in LND fibroblasts in standard tissue culture medium. However, ZMB were significantly elevated in LND fibroblasts cultured without folic acid, with physiological folic acid, or with MTX or AICAr (Fig. 1 *C* and *D*). Control fibroblasts cultured for 5 d with different amounts of folic acid did not show any change in cell proliferation, whereas LND fibroblasts showed a reduced cell proliferation with decreasing concentrations of folate, although the differences were not statistically significant (Fig. 1*E*).

In summary, these results indicate that LND fibroblasts are more sensitive than controls to folate levels, resulting in the accumulation of ZMB at physiological folate levels, or when the folate antagonist MTX is added.

ZMP Accumulation and ATP Depletion in LND Fibroblasts Grown with Physiological Concentrations of Folic Acid. Although LND fibroblasts had more ZMB than controls, the Bratton-Marshall test cannot distinguish among AICA, AICAr, or ZNT. Fibroblasts were therefore reanalyzed using a high-performance liquid chromatography (HPLC) method capable of discriminating all these compounds separately. As shown in Fig. 2*A* and *SI Appendix*, Table S1, folate depletion (NF) increased ZMP levels in LND fibroblasts, but not in controls. Increased ZMP was also observed in LND fibroblasts cultured with physiological folate (25 nM) or with standard folate (2,200 nM) plus MTX, but not with high levels of folic acid (2,200 nM).

The ZMP levels measured by HPLC were highly correlated ($R^2 = 0.98$) with the ZMB values obtained with the Bratton-Marshall test (Fig. 2*B*). LND and control fibroblasts did not have significant levels of AICA, AICAr, ZDP, or ZTP, thus explaining the high correlation between ZMB and ZMP.

In addition to accumulation of ZMP, there was a significant reduction of ATP in LND fibroblasts cultured without folate, with physiological folate, or with standard folate (2,200 nM) plus MTX (Fig. 2*C*). There was no reduction of ATP in LND fibroblasts maintained with standard folate (2,200 nM) or in control fibroblasts under any condition (Fig. 2*C* and *SI Appendix*, Table S1). Overall, the ATP reduction in LND cells with low folate (either by reduction of folic acid or by incubation with MTX) was ~30% (Fig. 2*D*). No statistically significant differences were seen among culture conditions for levels of ADP, AMP, GTP, or GDP (*SI Appendix*, Table S1). The adenylate energy charge, defined as the ratio ([ATP] + 1/2 [ADP])/([ATP] + [ADP] + [AMP]) by Atkinson and Walton (23) was significantly lower in LND fibroblasts incubated with MTX (Fig. 2*E*). There were no significant differences between groups in the AMP/ATP ratio (*SI Appendix*, Fig. S1).

Measurement of Urinary ZMB with the Bratton-Marshall Test in Patients with HGprt Deficiency and Normal Control Individuals. The previous results indicate that ZMP accumulates in the cells of patients with LND at physiological levels of folic acid. To address the in vivo relevance of this finding, we measured ZMB in the urine of 28 patients with HGprt deficiency and 28 normal control individuals. Patients with HGprt deficiency were classified into three groups according to the severity of the disease (LND, HND, and HRH). Patients with HGprt deficiency, either pooled or separated in the three groups, had significantly higher ZMB levels compared with control individuals, expressing the results as micromoles ZMB/L (Fig. 3 *A* and *B*). No significant differences were observed among LND, HND, and HRH.

ZMB were also analyzed by normalizing the results to urine creatinine, to correct for any potential effects of urine concentration. Creatinine levels were not significantly different between controls and HGprt deficiency, as a group (Fig. 3*E*), but LND presented lower creatinine levels (5,598 ± 826 μmol/L) than controls (9,343 ± 1,107 μmol/L) or HRH (13,393 ± 133 μmol/L), and these differences were statistically significant (Fig. 3*F*). Despite any potential differences in creatinine, patients with HGprt deficiency again showed significantly higher ZMB levels than control individuals (Fig. 3*C*). Patients with LND and HND presented a statistically significant higher content of ZMB than control individuals (Fig. 3*D*).

High Levels of AICAr and AICA in the Urine of Patients with HGprt Deficiency, and Increased Ratio of AICAr/AICA in Patients with Neurological Dysfunctions. Although patients with LND and the variants had more urinary ZMB than control individuals, the Bratton-Marshall test cannot distinguish AICA, AICAr, or ZNT. The same urine samples were therefore reanalyzed using HPLC to discriminate these compounds separately. As shown in *SI Appendix*, Table S2, uric acid levels, expressed as micromoles per liter, did not show a significant difference between groups, since all the patients were treated with allopurinol to correct this metabolic alteration (*Methods*). Instead, significantly higher levels of the uric acid precursors, hypoxanthine and xanthine, were detected in the urine of patients with LND and HND compared with the control individuals. No significant differences were observed

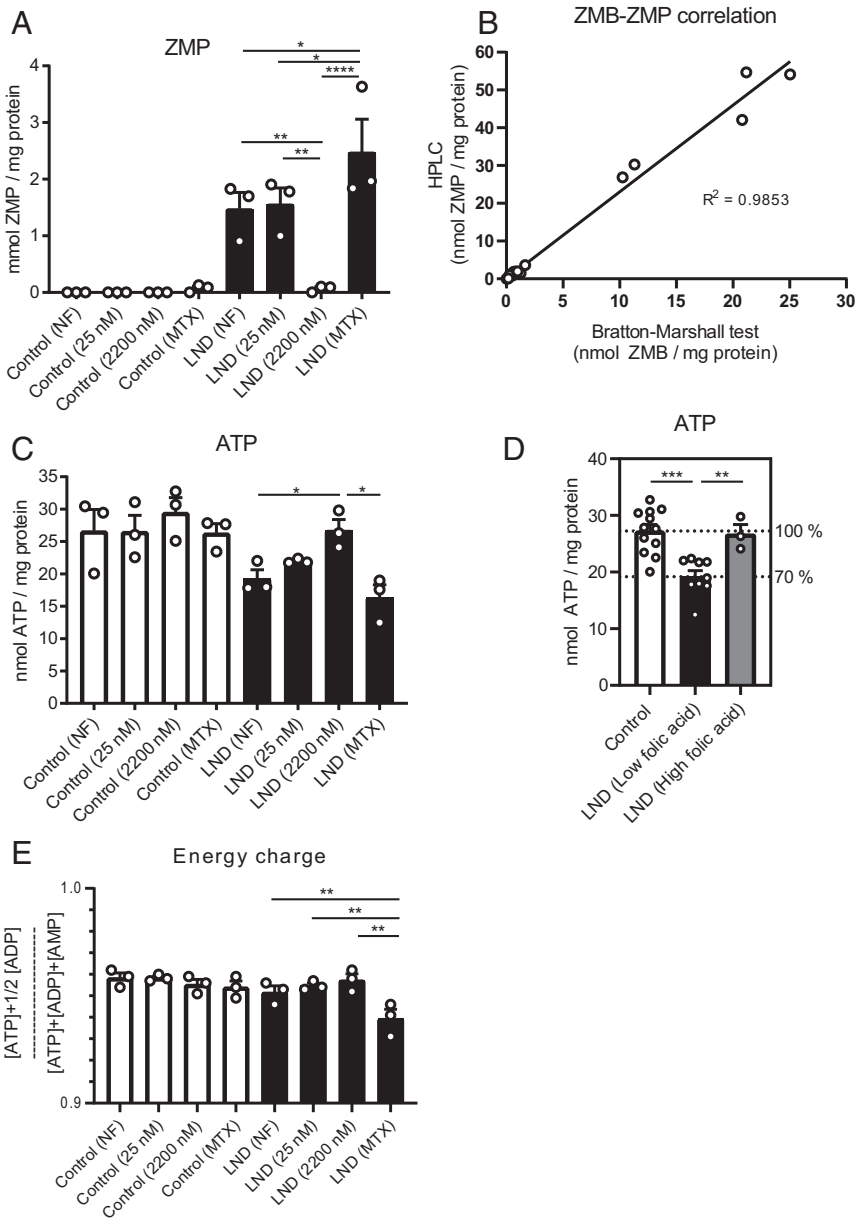

Fig. 2. ZMP accumulation and ATP depletion in LND fibroblasts depend on folic acid levels. (*A*) Fibroblasts were incubated with different media, as reported in Fig. 1*C*: cell extracts obtained with 0.4 N PCA and ZMP levels determined by HPLC. The graph represents the mean ± SEM of 3 control individuals and 3 patients with LND, expressing the results as nanomoles ZMP per milligram protein. The differences obtained were significant comparing LND (NF), LND (25 nM) or LND (MTX) with LND (2,200 nM), and comparing LND (MTX) with LND (NF) or LND (25 nM). $^{****}P < 0.0001$; $^{**}P < 0.01$; $^{*}P < 0.05$. Two-way ANOVA, Newman-Keuls test. (*B*) Correlation between ZMB levels, quantified by the Bratton-Marshall test, and ZMP levels determined by HPLC. LND ($n = 15$) and control fibroblasts ($n = 15$) were cultured under different conditions and analyzed as reported earlier. The results are represented as nanomoles per milligram protein, and the correlation coefficient obtained is depicted in the graph. (*C*) Fibroblasts were incubated with different media, as reported before, and ATP concentrations determined by HPLC and expressed in nanomoles per milligram protein as the mean ± SEM of 3 control individuals and 3 patients with LND. $^{*}P < 0.05$. Two-way ANOVA, Newman-Keuls test. (*D*) Data from Fig. 2*C* were grouped as follow: control individuals (regardless of the treatment received), LND with low folate content (either by reduction of folic acid in the medium or by incubation with methotrexate), and LND incubated in medium with high folic acid content (2,200 nM). $^{***}P < 0.001$; $^{**}P < 0.01$. One-way ANOVA, Newman-Keuls test. (*E*) Adenylate energy charge, expressed as ([ATP] + 1/2 [ADP])/([ATP] + [ADP] + [AMP]), is represented for LND and control fibroblasts incubated with different media. Purine nucleotides were determined by HPLC and the results expressed as the mean ± SEM of 3 control individuals and 3 patients with LND. $^{**}P < 0.01$. Two-way ANOVA, Newman-Keuls test.

between patients with HRH and control individuals (Fig. 4 *A* and *B* and *SI Appendix*, Table S2).

Purine nucleotides, including ZNT, were not detected in any of the control or patient urines. AICA and AICAr were not detectable in the control individuals, but high levels of both were present in all subjects with HGprt deficiency (Fig. 4 *C* and *D* and *SI Appendix*, Table S2). There were no significant differences in the total content of AICA+AICAr among LND, HND, and HRH (Fig. 4*E*). Interestingly, AICA levels were very high in the HRH group, intermediate in the HND group, and lower in the LND group (Fig. 4*C*). Conversely, AICAr appeared to be increased in patients with LND and HND compared with HRH (Fig. 4*D*). Therefore, the ratio AICAr/AICA is ranked as follows: LND > HND > HRH, with statistically significant differences

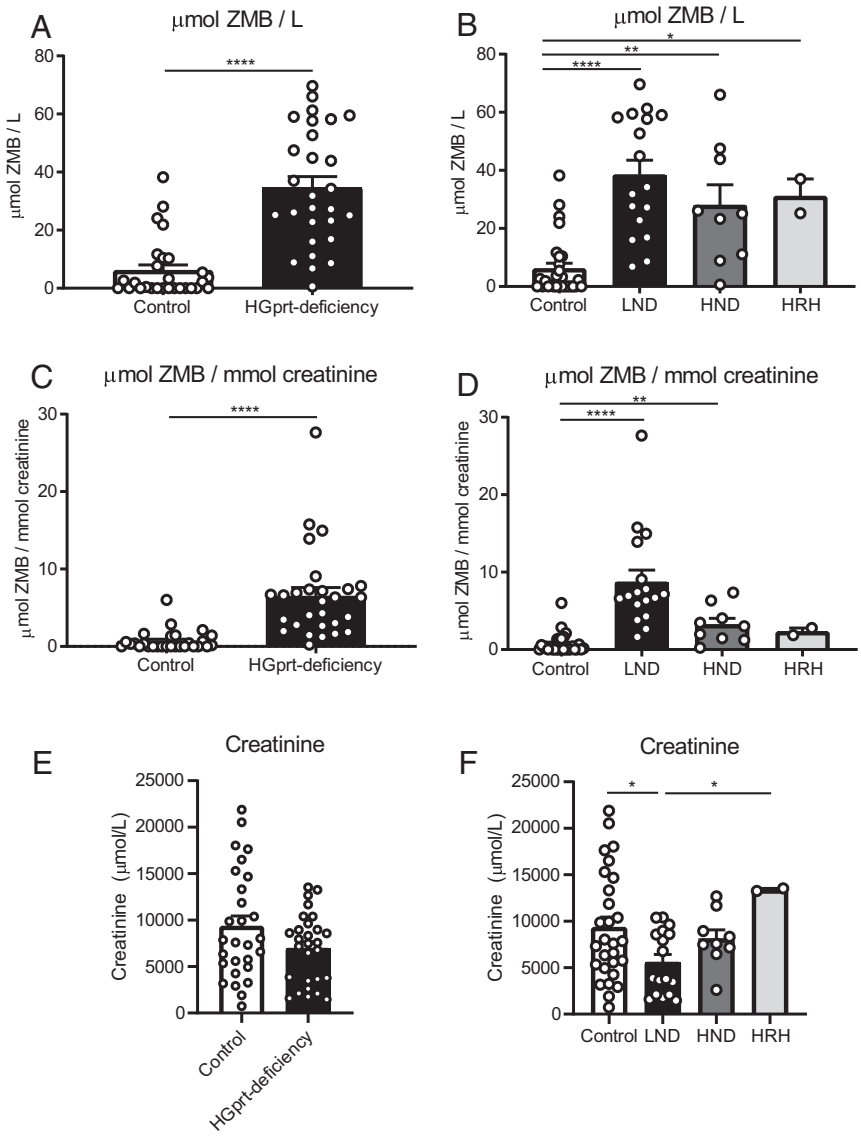

Fig. 3. Increased levels of ZMB in the urine of patients with LND and their variants (HND, HRH) with respect to control individuals. (*A* and *C*) The concentration of ZMB was determined in urine samples with the Bratton-Marshall test and the results expressed as micromoles ZMB per liter (*A*) or as micromoles ZMB per millimole creatinine (*C*). Graphs represent the mean ± SEM for HGprt deficiency ($n = 28$) and control individuals ($n = 28$). ****$P < 0.0001$. Mann–Whitney test. (*B* and *D*) Concentration of ZMB in urine samples from control individuals ($n = 28$), and patients LND ($n = 17$), HND ($n = 9$), HRH ($n = 2$). There was a significant difference between control and LND, HND, or HRH when the results are expressed as micromoles ZMB per liter, and between control and LND or HND when the results are expressed as micromoles ZMB per millimole creatinine. ****$P < 0.0001$; **$P < 0.01$; *$P < 0.05$. Kruskal-Wallis, Uncorrected Dunn's test. (*E* and *F*) Creatinine concentration in control individuals and HGprt deficiency. The results, expressed as micromoles creatinine per liter, are the mean ± SEM of subjects described earlier. There was a significant difference between control and LND, or between HRH and LND. *$P < 0.05$. One-way ANOVA, uncorrected Fisher's LSD test.

between LND or HND and control individuals (Fig. 4*F* and *SI Appendix*, Table S2). Similar results were obtained when normalizing the data to urine creatinine (*SI Appendix*, Fig. S2).

In summary, AICA and AICAr were absent in urine from normal subjects, but present at high levels in all patients with HGprt deficiency, with an increased AICAr/AICA ratio in the patients presenting neurological alterations (LND and HND).

AICAr Is Detected in the CSF of Patients with LND, but Not in Control Individuals. Since patients with LND have severe neurological alterations, purine measurements from the brain are of great relevance to understanding the pathophysiology of the disease. However, it is not feasible to obtain reliable measures of purines in brain samples from human subjects. To address whether ZMB might be produced in the LND brain, we measured purines in the CSF from 17 patients with LND and 17 normal control individuals. Purine nucleotides, including ZNT, were not detected in any CSF sample from patients or control individuals. Similar to urine samples, uric acid levels did not show a significant difference between control and LND CSF (Fig. 5*D*). Patients with LND showed a 5-fold increase in hypoxanthine and xanthine levels compared with control individuals (Fig. 5 *A* and *B*). All patients with LND had detectable AICAr in the CSF, with values ranging from 0.039 µmol/L to 3.187 µmol/L and an average of 0.278 ± 0.182 µmol/L, but it was undetectable in 15 of 17 control individuals (Fig. 5*C*), where the overall average was 0.006 ± 0.004 µmol/L.

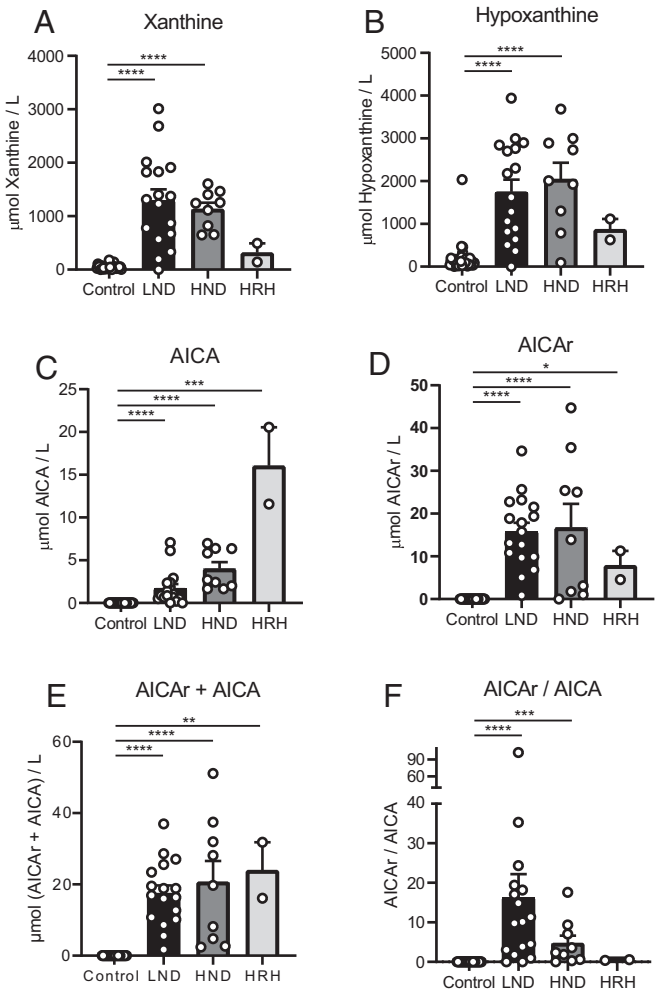

Fig. 4. AICAr and AICA are highly elevated in the urine of patients with HGprt deficiency. (*A–D*) Xanthine, hypoxanthine, AICA, and AICAr concentration in urine samples of control individuals ($n = 28$) and HGprt-deficient patients (LND, $n = 17$; HND, $n = 9$; HRH, $n = 2$) were determined by HPLC. The results are expressed as micromoles per liter as the mean ± SEM. (*E*) High levels of AICAr+AICA in the urine of LND, HND, and HRH patients. (*F*) The ratio AICAr/AICA is increased in the patients with neurological problems (LND and HND). ****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. Kruskal-Wallis, uncorrected Dunn's test.

Discussion

The fundamental metabolic defect in LND involves failure of HGprt-mediated purine recycling, with a secondary up-regulation of purine synthesis de novo (5, 6). The neurobehavioral features of the disorder have been hypothesized to arise because this increase in purine synthesis cannot fully compensate for the failure of purine recycling in the brain. These metabolic changes should result in reduced purine nucleotide levels. Such abnormalities have never been demonstrated, because it is not feasible to measure purine nucleotides in the brains of living subjects. Purine nucleotides also cannot be measured in tissue samples taken from these subjects because they begin to degrade within seconds after disruption of the blood supply. As a result, many investigators have sought to delineate the relevant purine abnormalities in cultured cells (8). Quite surprisingly, these in vitro studies have revealed no consistent abnormalities of purine nucleotides. Recently, Cantor et al. have stressed the importance of using culture media with physiological concentrations of some metabolites to detect metabolic alterations (24, 25).

Here we demonstrate that the failure to detect purine nucleotide abnormalities in HGprt-deficient cells in culture reflects an artifact of the standard tissue culture environment. Specifically, the nonphysiologically high levels of folic acid present in almost all standard tissue culture media mask the abnormalities in purine pools. With physiological levels of folic acid, two alterations become apparent: accumulation of ZMP and a decrement in ATP. These abnormalities can be replicated in standard medium with high levels of folate when the folate antagonist methotrexate is added. The accumulation of ZMP in culture may also be physiologically relevant in vivo, because we could measure substantially elevated levels of ZMP derivatives in urine and CSF samples from patients with HGprt deficiency.

The accumulation of ZMP when folate is lowered to physiological levels most likely results from the fact that its conversion to IMP by ATIC is folate dependent (Fig. 1*A*). When the de novo pathway is accelerated in HGprt deficiency, physiological levels of folate may become limiting, especially when there is an increased demand for purines. What is not so obvious is why 5′-phosphoribosyl-glycinamide (GAR), instead of ZMP, is not accumulated, since the enzyme GAR transformylase also requires 10-formyltetrahydrofolate (Fig. 1*A*). Interestingly, this effect has been observed before in cancer cells incubated with pemetrexed (an antifolate drug) inducing an inhibition of ATIC, but not GART (26). The authors hypothesized that it could be due to the differential binding mechanisms of the substrates to the enzymes: GART can bind its two substrates in a random manner (27), whereas ATIC obeys an ordered sequential binding mechanism, with 10-formyltetrahydrofolate binding first before ZMP can bind (28). Alternatively, it has been reported that human ATIC and GART do not utilize the same 10-formyltetrahydrofolate pool (29, 30). Thus, ATIC can use 10-formyltetrahydrofolate formed from tetrahydrofolate and histidine or formate, whereas GAR transformylase, which is in a complex with the trifunctional folate-metabolizing enzyme, can use 10-formyltetrahydrofolate formed from tetrahydrofolate and glycine or serine. Therefore, the lower concentrations of histidine or formate in humans compared with serine or glycine (nonessential amino acids) could explain the higher sensitivity of ATIC to folic acid depletion. Whatever the mechanism, ZMP accumulation is a consequence of folic acid depletion, as shown by numerous examples in bacteria (31), plants (32), and human cancer cells (26). ZMP was proposed as an alarmone for 10-formyltetrahydrofolate deficiency in bacteria (31), and recently it has been reported that ZMP binds and activates a riboswitch class that is associated with genes related to de novo purine biosynthesis and one-carbon metabolism (33).

The decrement in ATP in the LND fibroblasts likely results from the folate-dependent block of de novo synthesis at the ATIC step combined with a low ability of purine recycling. Essentially, both sources for purine nucleotides are compromised. Alternatively, ZMP has been reported to inhibit mitochondrial oxidative phosphorylation through complex I (34). Thus, ATP depletion observed in LND fibroblasts could also be an indirect consequence of ZMP accumulation. Additional experiments are necessary to test this hypothesis.

We have shown in this study that ZNT derivatives (AICAr and AICA) are detected by HPLC in the urine of patients with LND, HND, and HRH, but they are undetectable in the control individuals. However, it has been reported that AICA can be measured in normal human urine (17, 21). We probably did not detect AICA in the control urine because we used urine samples directly, whereas the previous works used highly concentrated urine (17, 21). Interestingly, we found that the ratio AICAr/AICA in the patients was progressively increased with the severity of the disease (LND > HND > HRH).

Our study also includes the largest survey of CSF purines for LND. Purine nucleotides are rarely detected in CSF because most are retained intracellularly and those that are released are degraded by nonspecific phosphatases. Hypoxanthine and xanthine were markedly increased in LND samples, in accordance with prior

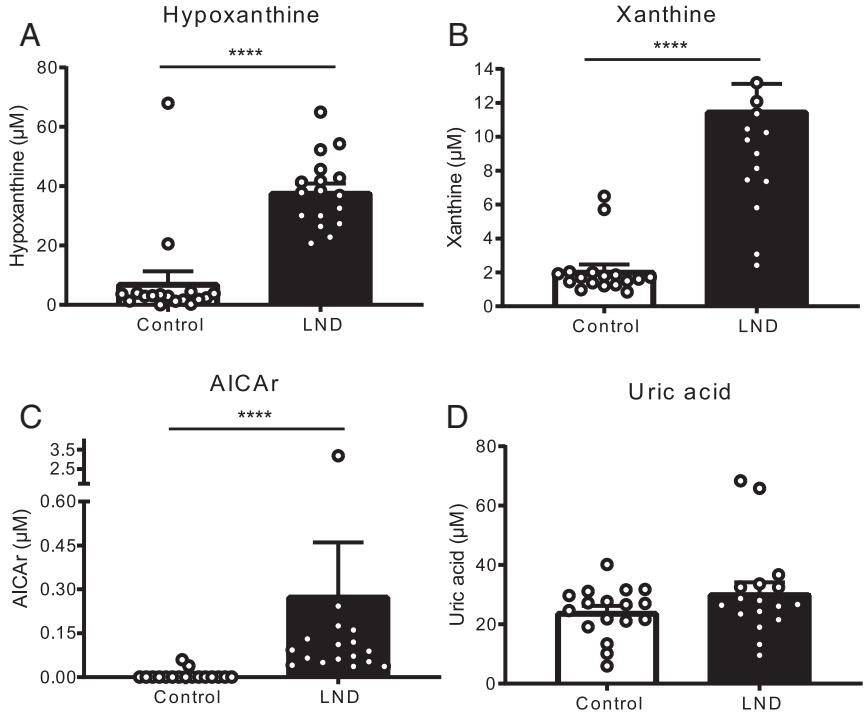

Fig. 5. AICAr, hypoxanthine, and xanthine are increased in CSF of patients with LND. (*A–D*) Hypoxanthine, xanthine, AICAr, and uric acid concentration in CSF samples of control individuals ($n = 17$) and patients with LND ($n = 17$) were determined by HPLC. The results are expressed as micromoles per liter as the mean ± SEM ****$P < 0.0001$. Mann–Whitney test.

studies (35). Uric acid was not significantly increased in LND CSF, either because patients were taking allopurinol or because uric acid is not produced in CSF due to relatively low brain xanthine oxidase. Most important, AICAr was found in the CSF from all patients with LND, but it was rare in control individuals. This finding suggests that ZMP might accumulate in the brain, because AICAr normally arises from dephosphorylation of ZMP. Although we have not measured purine nucleotides in the brain of patients with LND, it is interesting to note that *HPRT1* knockout mice accumulate ZMP in the brain (19).

Our results lead to two distinct hypotheses regarding the pathogenesis of neurobehavioral abnormalities in LND. The first is that ATP depletion may impair either the function or the development of the brain. The adult brain is heavily dependent on ATP, even at rest, for maintaining axonal ion gradients and synaptic function required for neuronal signaling (36, 37). The proliferation and differentiation of neurons during early development also is dependent on ATP, and several investigators have provided evidence that development may be abnormal in HGprt-deficient neurons (38–41). Although ATP cannot be measured in the brains or CSF of human subjects, CSF from patients with LND has increased levels of lactate, pyruvate, phosphate, citrate, and glycolate. Elevations of these metabolites are similar to changes occurring after acute ATP depletion due to cardiac arrest (42). However, no study has yet demonstrated an ATP defect in the brain of patients with LND. Further studies will be needed to address the hypothesis of ATP depletion as a potential cause for neuropathogenesis in LND.

Another hypothesis for neurobehavioral dysfunction in LND relates to the accumulation of ZMP, which could also affect the function or development of the brain. As noted earlier, ZMP inhibits mitochondrial activity (34). ZMP is an allosteric activator of the AMP-activated protein kinase (AMPK), a master regulator of cell metabolism (43, 44). ZMP also has other targets. It inhibits the bifunctional enzyme adenylosuccinate lyase (45, 46), and deficiency of adenylosuccinate lyase in humans causes autistic behavior, mental retardation, and severe psychomotor retardation (47). Therefore, ZMP could act on different targets to promote important alterations in the cell (14). Recently, it has been reported that ZMP accumulation is toxic in yeast, and several proteins, different from AMPK, can bind ZMP (48). Interestingly, AICAr alters gene expression (sonic hedgehog and Wnt/β-catenin pathways) in the human embryonic carcinoma cell line NT2/D1 during neural differentiation (49). Further studies are needed to address any hypotheses regarding a role for ZMP or AICAr in the pathogenesis of neurobehavioral symptoms.

Our results with fibroblasts suggest that supplements of folic acid in patients with LND could have a therapeutic effect. However, if the neurological features of LND result from a defect in early brain development, then folic acid supplements may be ineffective, unless they are given before neurodevelopment defects become permanent. In fact, a study conducted in 1970 with two patients who were given folic acid from birth caused only slight improvements reducing the severity of neurological symptoms (50). Additional clinical studies will be needed to reach a conclusion about the effectiveness of folic acid supplements. But even in the case that folic acid supplements could not solve the neurological problems, they might be useful to improve some cellular alterations. For instance, patients with LND frequently present macrocytic anemia in adulthood (51, 52), and folic acid deficiency induces macrocytic anemia in humans (53). It is possible that the high rate of de novo purine biosynthesis in patients with LND limits the availability of folic acid in the erythroblasts, which in turn would induce ZMP accumulation and ATP depletion to cause macrocytic anemia.

In summary, the results presented here demonstrate accumulation of ZMP and reduction of ATP in LND fibroblasts when folate in the tissue culture medium is lowered to normal physiological levels. They also demonstrate accumulation of AICA and/or AICAr in the urine and CSF of subjects with HGprt deficiency, with an increased ratio of AICAr/AICA in the patients with neurological alterations. Whether the neurobehavioral

abnormalities in HGprt deficiency result from accumulation of ZMP or depletion of ATP, or both, will require further studies. If either of these abnormalities disrupts brain function, then folate supplementation could have therapeutic value. If either of these abnormalities disrupts brain development, then such supplementation may need to occur during early brain development.

Materials and Methods

Research Subjects. Subjects with HGprt deficiency were diagnosed according to accepted criteria. Subjects with LND had <2% HGprt enzyme activity, or a genetic variant in the *HPRT1* gene predicting null activity, along with evidence for overproduction of uric acid and a characteristic neurobehavioral phenotype (2). Milder forms of the disease, named Lesch-Nyhan variants, exhibited some residual HGprt activity, along with a less severe clinical phenotype (4). The variants were divided into two groups: those with HND had overproduction of uric acid along with some neurobehavioral problems, while those with HRH had overproduction of uric acid without obvious neurobehavioral problems. All of the subjects from which urine samples were obtained were male, with the following average ages, in years, for each group: control (16.86 ± 1.97), LND (12.53 ± 1.98), HND (28.22 ± 4.82), HRH (14.50 ± 5.50). All of the patients, except one with HRH, were receiving allopurinol, while none of the control individuals were receiving this drug. Allopurinol doses ranged from 30 to 300 mg/day, and the mean allopurinol dose was 110 ± 77 mg/day. All subjects from which CSF samples were obtained were male with the following average ages in years: control individuals (30.00 ± 3.04) and LND (37.44 ± 2.85). All these patients were taking allopurinol, while none of the control individuals were taking this drug.

CSF Samples from LND and Control Individuals. A CSF sample was obtained in patients and control individuals via lumbar puncture, and aliquots were kept at −80 °C. CSF aliquots were thawed on ice and 100 μL were centrifuged at 12,000 × *g* for 5 min at 4 °C. The supernatant obtained was transferred to a new tube, and 10 μL PCA was added to obtain a final concentration of 0.1 N. The samples were kept on ice for 15 min, and then centrifuged at 12,000 × *g* for 5 min at 4 °C. The supernatant was neutralized with 5 M potassium carbonate and the samples were kept on ice for 15 min. The samples were clean by centrifugation at 10,000 × *g* for 10 min at 4 °C, and the supernatants were kept at −80 °C for HPLC determination. For HPLC assay the samples were thawed and filtered through a 0.22-μm spin filter (catalog #F2517-5, Fisher) by centrifugation at 5,000 × *g* for 5 min at 4 °C.

Urine Samples from LND and Control Individuals. A morning urine sample (spot) was obtained in patients and control individuals, and aliquots were kept at −80 °C. The day of the assay the urine samples were thawed on ice and 300 μL were centrifuged at 12,000 × *g* for 5 min at 4 °C. For the Bratton Marshall test, 100 μL of the supernatant obtained were diluted 1/10 with H_2O and used for determinations as described here. For purine determination by HPLC, 150 μL of the supernatant were used and PCA added to obtain a final concentration of 0.2 N. The samples were kept on ice for 15 min, and then centrifuged at 12,000 × *g* for 5 min at 4 °C. The supernatant was neutralized with 5 M potassium carbonate and filtered through a 0.22 μm spin filter (catalog #F2517-5, Fisher) by centrifugation at 10,000 × *g* for 10 min at 4 °C. The supernatants filtered were kept at −80 °C for HPLC determination. Creatinine in urine was measured in a multichannel autoanalyzer (Modular P800, Roche, Mannheim, Germany; and Hitachi 704, Hitachi, Tokyo, Japan) or with a creatinine colorimetric assay kit (catalog #500701, Cayman Chemical).

Culture of Human Fibroblasts. Primary skin fibroblasts from patients with LND and control individuals were collected as previously described (6) and maintained in regular RPMI with folic acid (Sigma, R8758) supplemented with 15% FBS, 1% L-Glutamine, and 1% Pen/Strep. The day before treatment, plates were prepared with 180,000 cells per 100 mm dish, and maintained in regular medium. The next morning, plates were washed several times with PBS and maintained in RPMI without folic acid (Sigma, R1145) supplemented with 15% FBS, 1% L-Glutamine, 1% Pen/Strep, 0.2% $NaHCO_3$, and different concentrations of folic acid. A stock of folic acid (5 mg/mL) was prepared by dissolving folate in 1 M NaOH. This solution was filtered, stored at 4 °C, and protected from light. Fibroblasts were incubated in medium with different concentrations of folic acid for 3 d, changed, and incubated 2 d more with these media. Some fibroblasts were maintained in regular RPMI with folic acid (Sigma, R8758) and incubated with 0.5 mM AICAr, or 5 μM methotrexate for 24 h. Cells were collected from 100-mm plates by trypsinization, counted with a Neubauer chamber, and centrifuged at 1,200 × *g* for 5 min at room temperature, and the pellets were resuspended in 100 μL of 0.4 N PCA by pipetting up and down. The homogenates were kept on ice for 15 min and then centrifuged at 12,000 × *g* for 5 min at 4 °C. The pellet was kept at −80 °C for protein determination, and the supernatant was neutralized with 5 M potassium carbonate and filtered through a 0.22-μm spin filter (catalog #F2517-5, Fisher) by centrifugation at 10,000 × *g* for 10 min at 4 °C. The supernatants filtered were kept at −80 °C for the Bratton-Marshall test or HPLC determinations.

Bratton-Marshall Test. The Bratton-Marshall test (54), modified with prior addition of acetic anhydride for acetylation of amines (55), was performed as follows. For urine samples, 10 μL acetic anhydride was added in 500 μL diluted urine (1/10 with H_2O) and incubated for 30 min at room temperature. Next, 50 μL 4N HCl plus 50 μL 0.1% sodium nitrite were added, and this mixture was incubated on ice for 3 min. Next, 50 μL of 0.5% ammonium sulfamate was added and incubated on ice for an additional 2 min, at which time 50 μL of 0.1% *N*-1-naphthylethylenediamine dihydrochloride solution was added. Absorbance at 540 nm was measured after 5 min. As a reference blank for each urine sample, all the reagents were added in 500 μL diluted urine, as described earlier, but 50 μL H_2O was added in the last step instead of *N*-naphthylethylenediamine. A 96-multiwell plate was used to read the absorbance, loading 200 μL of each sample per well and by duplicate. A standard curve prepared with different concentrations of AICA, AICAr, or ZMP (0, 2.5, 5, 10, 25, 50 μM) was used for quantification. For human fibroblasts, 50 μL of the PCA extract obtained were diluted 1/2 with H_2O and assayed with the Bratton-Marshall test, as described earlier, adjusting the amounts of reagents added for 100 μL.

Quantification of Purines, AICAr, and AICA by HPLC. Purines were measured using a HPLC with photodiode array UV detection (HPLC-PDA), as previously described (8). Briefly, this method resolves and quantifies the most abundant biologically relevant adenine-based purines (ATP, ADP, AMP, adenosine, adenine) and guanine-based purines (GTP, GDP, GMP, guanosine, guanine), and several additional purine metabolites (IMP, hypoxanthine, inosine, xanthine, and uric acid). For the current studies, we verified that this method was also capable of resolving and quantifying Z-derivatives (AICA, AICAR, ZMP, ZDP, ZTP) by testing purified standards. All compounds were identified by comparing their retention times and spectral profiles to known standards, quantified at a detection wavelength of 254 nm.

Statistics. All data are presented as mean ± SEM. Statistical analysis was performed with GraphPad Prism 8.30 program, using one-way ANOVA when more than 2 groups were compared, or two-way ANOVA when 2 conditions were involved. If the data have Normal distribution, an unpaired two-tailed Student's *t* test was used when only 2 groups of data were concerned. When the data did not have Normal distribution, a nonparametric test was used: Mann–Whitney test when 2 groups of data were concerned and Kruskal-Wallis when more than 2 groups were compared. $P < 0.05$ was considered statistically significant.

Study Approval. Written informed consent was received from participants before inclusion in the study. 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 and by the Emory ethics board.

Data Availability. All the graphics presented in the manuscript contain individual data presented as dot plots showed next to the average for the group with appropriate error bars. The dot plot clearly reveals the distribution of the data. The authors would be happy to supply the numeric values on Excel format on request.

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