Metabolic Effects of Increasing Doses of Nitisinone in the Treatment of Alkaptonuria

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Abstract Alkaptonuria is an autosomal recessive disease involving a deficiency of the enzyme homogentisate dioxygenase, which is involved in the tyrosine degradation pathway. The enzymatic deficiency results in high concentrations of homogentisic acid (HGA), which results in orthopedic and cardiac complications, among other symptoms. Nitisinone (NTBC) has been shown to effectively treat alkaptonuria by blocking the conversion of 4-hydroxyphenylpyruvate to HGA, but there have been concerns that using doses higher than about 2 mg/day could cause excessively high levels of tyrosine, resulting in crystal deposition and corneal pathology. We have enrolled seven patients in a study to determine whether higher doses of NTBC were effective at further reducing HGA levels while maintaining tyrosine at acceptable levels. Patients were given varying doses of NTBC (ranging from 2 to 8 mg/day) over the course of between 0.5 and 3.5 years. Urine HGA, plasma tyrosine levels, and plasma NTBC were then measured longitudinally at various doses. We found that tyrosine concentrations plateaued and did not reach significantly higher levels as NTBC doses were increased above 2 mg/day, while a significant drop in HGA

continued from 2 to 4 mg/day, with no significant changes at higher doses. We also demonstrated using untargeted metabolomics that elevations in tyrosine from treatment resulted in proportional elevations in alternative tyrosine metabolic products, that of *N*-acetyltyrosine and γ -glutamyltyrosine.

Abbreviations

3,4-DPA	3,4-Dihydroxyphenylacetate
4-HPPD	4-Hydroxyphenylpyruvate dioxygenase
ACN	Acetonitrile
FA	Formic acid
HGA	Homogentisic acid
LC-MS-Q-TOF	Liquid chromatography-quadrupole-time of flight mass spectrometry
mg/d	Milligrams per day
NTBC	Nitisinone

Introduction

Alkaptonuria (OMIM 203500) is an autosomal recessive disease that leads to ochronosis, joint deterioration, and cardiac valve disease, generally presenting in adulthood. The late-onset clinical manifestations are a result of a defective enzyme in the tyrosine degradation pathway, homogentisate dioxygenase, that prevents the metabolism of the intermediate homogentisic acid (HGA) to maleylacetoacetate (Pollak et al. 1993; Janocha et al. 1994; Fernandez-Canon et al. 1996). The result is a high concentration of HGA that polymerizes after oxidation to the benzoquinone form (Milch 1961; Zannoni et al. 1962, 1969). The polymers deposit in the connective tissue and are responsible for the bulk of the symptoms. The most effective treatment for lowering HGA in this disease is the drug nitisinone (Orfadin[®]), which is also the most common and effective form of treatment for type I

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tyrosinemia (Anikster et al. 1998; Suwannarat et al. 2005; Introne et al. 2011; Schlune et al. 2012). NTBC blocks the enzyme 4-hydroxyphenylpyruvate dioxygenase that produces HGA from 4-hydroxyphenylpyruvate (Ellis et al. 1995; Kavana and Moran 2003).

The reduction of HGA and its corresponding polymers with NTBC treatment may have potential in reducing complications associated with alkaptonuria, including the progression of aortic valve disease (Introne et al. 2011), a correlation that still requires further study. NTBC doses of 0.5–2 mg/day were shown to reduce HGA levels nearly 95%, but the three-year study was not able to show a change in the progression of symptoms, including joint deterioration (Introne et al. 2011). There has been caution about using higher doses out of concern that high tyrosine levels associated with NTBC treatment will lead to corneal crystal deposits and ocular complications, though several studies have shown that high tyrosine itself does not appear correlated to ocular problems and that some patients are able to tolerate plasma tyrosine levels of over 1,000 μM (Ney et al. 1983; Holme and Lindstedt 1998; Gissen et al. 2003).

As previous studies have not reported patient outcomes when increasing NTBC doses higher than 2 mg/day, we set out to investigate whether using NTBC at doses between 4 and 8 mg/day would further reduce HGA levels, while keeping tyrosine levels below critically high levels, as established for tyrosinemia type 1 patients who are also using NTBC. The seven patients who were enrolled in the study were not placed on diet restriction, but did receive an amino acid supplement called Tyrex-2[®] (Abbott Laboratories, Columbus, Ohio), which lacks tyrosine and phenylalanine. The hypothesis for using this supplement was that, given a formula with all amino acids except tyrosine and phenylalanine, the natural process of anabolism would consume the endogenous pools, as observed in treatment of maple syrup urine disease with an amino acid mixture lacking branched-chain amino acids (Saudubray et al. 1984; Nyhan et al. 1991). In addition to measuring the levels of HGA, tyrosine, and NTBC over the course of 0.5–3.5 years during this longitudinal study, we further explored the biochemical consequence of high plasma tyrosine using untargeted metabolomics methods (LC-MS-Q-TOF) to compare the plasma metabolome from patients prior to NTBC treatment with their posttreated samples and potentially identify unknown biochemical implications of excessive tyrosine and NTBC treatment.

Methods

Patient Enrollment

The protocol is an IRB-approved open-label study (listed in [clinicaltrials.gov# NCT01390077](https://clinicaltrials.gov/ct2/show/study/NCT01390077)), which was approved by

the UCSD Human Research Protections Program. Patients of any age with the diagnosis of alkaptonuria and documented increased excretion of homogentisic acid were eligible; exclusion criteria included significant laboratory abnormalities unrelated to alkaptonuria or significant comorbidities. Eight patients were enrolled (two women and six men), though only seven have participated in treatment. Age at enrollment ranged from 31 years to 61 years. All patients had arthritic joint damage with resulting pain. Four patients had experienced arthroscopic debridement or replacement surgeries of one or more joints. Echocardiograms, radiographs, eye examination, and routine laboratory panels were obtained. Treatment with 2 mg/day NTBC (once daily) was initiated, and plasma tyrosine levels were measured one week and one month after. Routine chemistry and hematology analyses were obtained at month 3 and then annually. Plasma tyrosine, trough NTBC, and 12-hour urine HGA measurements were obtained at months 3 and 6 and then every 6 months, more often if the NTBC dose was changed. Patients 1–3 have been monitored over the course of 3–3.5 years, while patients 4–7 have been monitored between 0.5 and 1 year (Table 1). Patient 3 spent the majority of the study at a 2 mg/day NTBC dosage, while patients 1 and 2 have been on 4 mg/day NTBC or higher for the majority of the study. No changes were made to ongoing medications or dietary intake, though five of the patients (patients 3–7) took a supplement of Tyrex-2[®] (35 g, twice daily) approximately 1 month after starting treatment. Patient 1 began taking Tyrex-2[®] 6 months after starting NTBC treatment, while patient 2 began ten months after treatment initiation. Patient 3 discontinued Tyrex-2[®] several months after starting supplement, while several of the other patients had discontinued taking supplement for only portions of the study (patients 1, 2, and 4).

Sample Storage

Urine and plasma samples were stored in -80°C freezer. Urine samples were extracted and analyzed within 1–3 months after sample collection. HGA is known to be unstable in alkaline conditions (Hughes et al. 2014). We tested stability under our own storage times and conditions by comparing the HGA concentration from five different urine samples extracted shortly after collection, compared to aliquots of the same samples stored for 3 months at -80°C prior to extraction and analysis (Supplementary Table 1). We separately acidified three urine samples within the same day of sample acquisition (using a final concentration of either 250 mM final perchloric acid or 3% sulfosalicylic acid) and compared their endogenous HGA levels (normalized to a 3,4-DPA internal standard) with sample aliquots that were stored at -80°C for a month prior to acidification. The relatively small change in HGA between these sample sets was within the acceptable

Table 1 Tyrosine and HGA (in parentheses) concentrations are shown in μM for the seven different patients following treatment at different dose levels of NTBC. The time in months (m) that each patient remained on the respective dose level is shown below these values. At dose levels where multiple measurements were taken (time between repeat measurements varied for the seven patients and ranged from 1 to 6 months between measurements), the standard deviation is displayed. The total time that each patient has been monitored in the study is shown below the patient number in parenthesis

	Pre-NTBC	1 mg NTBC	2 mg NTBC	4 mg NTBC	6 mg NTBC	8 mg NTBC
Patient 1 (3.5 years)	56 (1,046)		460 $\pm$ 48 (42) 8 m	465 $\pm$ 24 (20) 8 m	510 $\pm$ 113 (30 $\pm$ 13) 16 m	413 (19 $\pm$ 4) 8 m
Patient 2 (3.5 years)	57 (1,465)	869 6 m	850 $\pm$ 269 (126) 6 m	793 (48) 12 m	736 $\pm$ 73 (34 $\pm$ 13) 18 m	
Patient 3 (3 years)	51 (1,025)		646 $\pm$ 127 (101 $\pm$ 28) 36 m	734 (42) 1 m		
Patient 4 (1 year)	44 (1,251)		694 (121 $\pm$ 6) 4 m	775 $\pm$ 60 (40 $\pm$ 13) 4 m	654 (32) 1 m	
Patient 5 (1 year)	35 (2,192)		704 (22) 2 m	749 (16) 10 m		
Patient 6 (6 months)	47 (1,645)		664 (143) 3 m	706 (38) 3 m		
Patient 7 (6 months)	82 (2,357)		663 (238) 6 m			

analytical variation of the method ($<15\%$ CV) (Supplementary Table 2).

Sample Preparation

Prior to extraction of urine samples, the concentration of creatinine was determined in each sample, and an aliquot was transferred to a fresh reaction tube and diluted to 100 μL with $\text{H}_2\text{O}/0.1\%$ formic acid at a final concentration of 1 mM creatinine. The Jaffe method for creatinine determination was used, as preferred for alkaptonuria study (Loken et al. 2010). Diluted urine aliquots were extracted with a final concentration of 80% cold methanol. For the quantitation of HGA, an internal standard of 3,4-dihydroxyphenylacetate ((3,4-DPA) ring- $^2\text{H}_3$, 2,2- $^2\text{H}_2$, Cambridge Isotope) was added to each extraction at a final concentration of 1 μM . 3,4-DPA was used for its chemical, chromatographic (Supplementary Fig. 1), and ionization similarities to HGA. Samples were vortexed for 30 s, left at -20°C for an hour, and centrifuged at 16 kg at 4°C for 15 min. The supernatant was removed and dried in a SpeedVac (Thermo Fisher) coupled with a lyophilizer (Labconco). Dried supernatants were resuspended in 300 μL of 5% acetonitrile (ACN)/0.1% formic acid (FA).

For plasma samples analyzed by mass spectrometry, 100 μL of each sample was treated as reported previously (Gertsman et al. 2014). In this method, a stable isotopic mixture of 50 compounds is spiked into each sample extraction, permitting absolute quantification of many intermediates in major metabolic energy pathways and enabling potential normalization of chemically similar analytes discovered by untargeted analysis. In addition, the internal standard mesotrione (Sigma-Aldrich) was added to each extraction (final concentration of 2.4 μM /

sample extraction) for the quantitation of NTBC, which was previously used for NTBC quantitation by LC-MS/MS (Sander et al. 2011).

Sample Acquisition Methods

An LC-MS/MS method was developed to enable quantification of HGA, tyrosine, and nitisinone from urine in a single run, though only the urine HGA values are reported in the current study. An 8-pt calibration curve and three levels of quality control samples containing freshly prepared HGA were processed and run with each batch of urine samples. A control urine sample that had no detectable HGA (<1 μM based on a comparison with an LLOQ sample containing an additional 3 μM of spiked HGA) was used as the matrix for all standard curve and QC samples. Analytical validation for four days measuring intra- and inter-day accuracy and precision was performed and reported in Supplementary Fig. 1. We used a Kinetex pentafluorophenyl (PFP) stationary phase column (150 $\times$ 2.1 mm, 2.6 micron particle diameter, Phenomenex, Torrance, CA) in-line with an API 4000 mass spectrometer for this analysis (AB SCIEX, with Agilent 1200 HPLC and Leap Technologies autosampler). We used the following LC method: 0% mobile phase B (mobile phase A 0.1% FA, mobile phase B 100% ACN/0.1% FA) for 3 min, a gradient from 0% to 45% B over 1 min, 2 min of isocratic flow at 45% B, a gradient from 45% to 70% B over 1 min, 3 min of isocratic flow at 70% B, a gradient from 70% to 100% B over 1 min, followed by a 5-min isocratic flow of 100% B, and finally re-equilibration at 0% B for 10 min. A flow rate of 300 $\mu\text{L}/\text{min}$ was used, and 5 μL of each sample resuspension was injected per run. The parent to daughter ion (MRM) transition of 167 $\rightarrow$ 123 was

used for HGA, while a transition of 172 → 128 was monitored for 3,4-DPA. The run was acquired in negative ionization mode using −4,500 V for the cone voltage and a collision energy of −20 V. The nebulizing gas pressure was set to 30 psi, and the heating gas pressure was 70 psi at a temperature of 300°C. Curtain gas was set to 15 psi. Declustering potential was set to −80 V and a dwell time of 0.15 s for each transition.

Plasma tyrosine levels reported in the manuscript were measured using a Biochrom 30 ion exchange automated amino acid analyzer with ninhydrin detection (Shapira et al. 1989). A hybrid targeted/untargeted metabolomics method using LC-MS-Q-TOF (AB SCIEX 5600 mass spectrometer with Shimadzu Prominence UFLC) and a C18-PFP (MAC-MOD Analytical, Inc.) was used for untargeted analyte profiling while also quantifying NTBC and tyrosine. This method was previously validated for the quantification of tyrosine (Gertsman et al. 2014), but these values are not reported here for purposes of consistency, as not all of the plasma samples had been quantified by LC-Q-TOF, while all were evaluated by the Biochrom 30 system. The C18-PFP column exhibited suitable linearity for the quantification of NTBC in the range of 0.5–20 µM as well (6- to 8-pt calibration curves were prepared and run along with each batch, with correlation coefficients of linear regression exceeding 0.99).

Statistical Analysis

Quantification from mass spectrometry runs was performed using MultiQuant Software (AB SCIEX). Untargeted analysis from Q-TOF runs was performed using Marker-View Software (AB SCIEX). Runs from pre-NTBC treated samples along with post-NTBC treated samples were aligned, integrated, and statistically compared between these two distinct cohorts. Unpaired *t*-test analysis of all aligned peaks was evaluated to find statistically significant changes corresponding to NTBC treatment. Statistical correction for multicomponent null hypothesis testing using the Q-value software was applied to limit false discovery (Storey and Tibshirani 2003). Analytes of interest were initially identified by MS/MS matching with existing database entries. Standards for γ-glutamyltyrosine and glu-tyr dipeptide were purchased (Sigma-Aldrich) and analyzed to confirm analyte identity.

Results

HGA in Urine

Aliquots from pooled 12-hour (overnight) urine samples were evaluated for HGA and normalized to creatinine concentrations from the same sample. Values reported in

Table 1 for all patients are reported in µM HGA/mM creatinine from each patient's pooled 12-h sample. The average decrease in HGA following a 2 mg/day dosage of NTBC was 93% (pre-NTBC: 1538 ± 483 µM, 2 mg/day NTBC: 110 ± 70 µM). Six of the seven enrolled patients were further evaluated at a 4 mg dose and showed an additional 2.7-fold decline in mean HGA concentrations (*p*-value <0.05 from paired *t*-test analysis (Fig. 1a)). Three patients had doses increased to 6 mg/day, and one of those patients later had her dose increased further to 8 mg. As shown in Figure 1, though the HGA levels dropped significantly from a 2 mg to 4 mg/day NTBC dosage, there was no significant change when increasing to 6–8 mg/day NTBC in the three patients taking those higher doses. Plasma NTBC concentrations and NTBC dose levels were correlated to urine HGA in Fig. 1c, d.

Tyrosine and NTBC in Plasma

As reported in previous studies, the levels of tyrosine dramatically increase during treatment with NTBC (Suwannarat et al. 2005; Introne et al. 2011). There was a mean 13-fold increase in tyrosine from pretreated plasma to samples following 2 mg of NTBC treatment. However, as patient doses were increased to 4 mgs, there was not a significant change in tyrosine levels, as shown in Fig. 2a (1.1 mean fold change, *p*-value >0.05 following paired *t*-test analysis). Though there were only three patients in whom tyrosine levels were measured at NTBC doses >4 mg/day, tyrosine levels were not further elevated in those samples.

Four of the seven patients in the study have had tyrosine levels measured with and without taking the supplement Tyrex-2® while on the same NTBC dose level. Several of these patients showed a decrease in plasma tyrosine using the supplement, while for others the reverse was measured, with no overall statistical significance to the change in this limited sampling (Supplementary Fig. 2). Patient 3 spent the majority of the study without taking the supplement and did not show further elevations in tyrosine following discontinuation. Further studies are required to determine if Tyrex-2® can reduce tyrosine levels, which may also require diet restriction for a pronounced effect.

Untargeted Metabolomics Analysis

From untargeted metabolomics comparison of pretreated to NTBC-treated patient plasma samples, a number of different metabolite changes were detected, including metabolites of tyrosine, products of NTBC metabolism, and a class of indolic compounds (described in a separate manuscript currently under review). Here we report the identified compounds relevant to the study, which include *N*-acetyltyrosine and γ-glutamyltyrosine (MS/MS analysis

Fig. 1 HGA concentrations measured in urine. **(a)** Comparison of mean HGA levels of six patients evaluated at both 2 and 4 mg/day NTBC. The average fold change in HGA concentration was 2.7-fold (* indicates p -value of 0.01 from paired t -test analysis). **(b)** Urine HGA comparison between 4 and 6 mg/day NTBC dosage (fails null hypothesis testing, p -value > 0.05). **(c)** Urine HGA concentrations

plotted versus NTBC plasma concentration and fit to a single exponential decay curve. Plasma was acquired from the same day as the 12-h urine collection. The region of the graph corresponding to values of samples acquired after NTBC treatment is shown zoomed in. **(d)** Mean urine HGA concentrations plotted versus NTBC dosage. Standard deviations are depicted by error bars

Fig. 2 Plasma tyrosine and associated metabolites. **(a)** Plasma tyrosine concentrations compared following treatment with either 2 or 4 mg/day NTBC (1.1-fold change, not statistically significant). **(b)** Tyrosine conjugates discovered by untargeted metabolomics. The graph shows fold changes in normalized peak intensities of tyrosine, N -acetyltyrosine, and γ -glutamyltyrosine between NTBC-treated and

untreated patient plasma samples (12.5-fold, 11.5-fold, and 11.6-fold, respectively). Peak areas were normalized by the stable isotope internal standard $^2\text{H}_4$ -tyrosine, which was spiked into all extraction reactions for improved inter-batch comparisons. *Note:* * indicates $p < 0.01$, and ** indicates $p < 0.001$ from paired t -test analysis of untreated versus NTBC-treated metabolite levels

shown in Supplementary Fig. 3). These compounds were determined to be significantly altered after unpaired t -test and further analysis of Q -value (Q -value < 0.05 for both). Their

peak areas were normalized to a $^2\text{H}_4$ -(ring)-tyrosine internal standard, which was spiked into all extraction reactions and which demonstrated consistent linear proportionality to these

compounds in negative ionization mode. These compounds are conjugates of tyrosine and are elevated proportionally to the increased plasma tyrosine levels. We show that *N*-acetyltyrosine and γ -glutamyltyrosine had an 11.5- and 11.6-fold normalized peak area changes when comparing NTBC-treated to untreated patients (Fig. 2b). The presence of these compounds reveals alternative pathways for tyrosine metabolism/diversion when the oxidative pathway is blocked.

Patient Symptoms

Quantitative measurements of joint mobility, ochronosis, and other symptoms have not been evaluated in the study, though all patients completed short form health surveys (SF-36) for assessment of overall health, pain (Brief Pain Inventory), and life interference (Ware et al. 1981; Ware and Sherbourne 1992). Patient 1 reported significant improvement in ochronosis of the fingers, while patient 2 has had significant improvement in shoulder joint pain that allowed the cancellation of surgery. No patients reported worsening of the three categories in the SF-36 survey, while five of the seven patients reported improvements in at least one of these categories following treatment. The reported complications included ocular irritation in two patients. Specifically, patient 3 had a blood spot tyrosine of 1,180 μ M shortly after starting NTBC treatment, and described eye discomfort, while patient 2 had a recurrence of blurry vision, but did not have tyrosine measured during this episode. Both patients were temporarily placed on lower NTBC doses as well as Tyrex-2[®] and showed no further complications or tyrosine levels higher than 800 μ M. A third patient (patient 1) experienced abdominal discomfort at 8 mg/day NTBC, which resolved upon returning to 6 mg/day.

Discussion

NTBC, a drug that has been widely used for the treatment of tyrosinemia type 1, has been more recently used for the treatment of alkaptonuria. NTBC reduces toxic levels of HGA in patients with alkaptonuria, but, by inhibiting the 4-hydroxyphenylpyruvate dioxygenase (4-HPPD), causes a significant elevation in 4-hydroxyphenylpyruvate and tyrosine. This high elevation in tyrosine has been a concern for physicians in their choice of NTBC doses administered to patients. In this study, we demonstrate that there was a negligible change in plasma tyrosine concentrations in patients who are given a 4 mg/day as compared to a 2 mg/day dose of NTBC, while the HGA further decreased an additional 2.7-fold. In the three patients who were given doses of 6 mg/day or higher, however, there was little further change in HGA levels, and tyrosine levels continued

to remain virtually unchanged. Though we have monitored a limited patient cohort, the data suggest that though there was additional utility in prescribing 4 mg/day NTBC, there was little or no benefit to higher doses in achieving HGA reduction.

There was no major change in plasma tyrosine concentrations in the six patients who had doses of NTBC increased from the initial 2 mg/day dose. Increased levels of tyrosine (>1,000 μ M) have been reported in patients from previous NTBC dose studies, but the tyrosine fluctuation has not previously been reported beyond a 2 mg/day dose. Untargeted metabolomics uncovered two previously unreported products of excess tyrosine in alkaptonuria patients, *N*-acetyltyrosine and γ -glutamyltyrosine, which had similar peak intensity fold changes as tyrosine itself following NTBC treatment. The presence of *N*-acetyltyrosine has been identified in the urine of type II tyrosinemia patients (Macasai et al. 2001), but γ -glutamyltyrosine has never previously been reported in disorders of tyrosine metabolism. It is likely formed by γ -glutamyl transpeptidase, an enzyme critical to glutathione catabolism and regulation of cellular redox (Griffith et al. 1979; Zhang and Forman 2009). These findings help explain nontraditional pathways for the metabolism of excess tyrosine. The data show that there is no equilibrium shift between tyrosine and the concentrations of these derivative forms as plasma tyrosine levels increase, as their fold changes are nearly identical. Ocular complications have been associated with high tyrosine concentrations, but some studies have shown that high tyrosine itself does not appear correlated to ocular problems (Holme and Lindstedt 1998; Gissen et al. 2003), so it may be pertinent to investigate these and possibly other nontraditional metabolites of tyrosine in relation to the phenotype.

NTBC is known to bind tightly in the 4-HPPD-inhibitor complex, with a very slow dissociation rate (Ellis et al. 1995; Kavana and Moran 2003) and a mean half-life of 54 hours in humans (Hall et al. 2001). Therefore, the concentration of plasma NTBC should never drop below ~20% of the peak concentration in patients taking a daily dose, and at a dose of 2 mg/day, the 4-HPPD enzyme-inhibitor complex appears to approach saturation, reflected by a nearly 93% reduction of HGA. With an increase in dosage from 2 to 4 mg/day, it does appear that there is additional enzymatic inhibition, with an additional 2.4% mean decrease in HGA from pretreatment levels. Since 4-HPPD is nearly completely inhibited with 4 mg/day NTBC, it was not surprising that there was no significant additional decrease of HGA observed at higher doses. These data indicate that there may be limited rationale to utilize doses >4 mg/day. The fact that HGA levels were never measured below 15 μ M in any of the patients could be attributed to a basal amount of newly synthesized enzyme that functions

prior to the initial slow binding reaction with NTBC or potentially to an inability for NTBC to reach all affected tissues. Following this line of reasoning, only variations in dietary tyrosine should impact the levels of plasma tyrosine when treating with NTBC doses above this point of saturated 4-HPPD inhibition.

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Synopsis

Treatment of alkaptonuria with nitisinone doses greater than 2 mg/day significantly lowers homogentisic acid levels while tyrosine levels remain unchanged.

Compliance with Ethics Guidelines

Conflicts of Interest

Ilya Gertsman has no conflict of interest to declare.

Bruce Barshop has no conflict of interest to declare.

Jan Panyard-Davis has no conflict of interest to declare.

Jon Gangoiti has no conflict of interest to declare.

William Nyhan has no conflict of interest to declare.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

Author Contributions

IG: Developed methods for analysis, analyzed the data collected, principal author of the manuscript

BAB: Assisted in the design of chemometric analyses and identification of target compounds, contributed substantially to writing the manuscript

JP-D: Planned and coordinated patient treatment regimens, reviewed the manuscript

JAG: Developed chemometric methodology, reviewed the manuscript

WLN: Conceived of patient treatment regimens, contributed to writing of the manuscript

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