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ORIGINAL ARTICLE

Urine metabolomics reveals novel physiologic functions of human aldehyde oxidase and provides biomarkers for typing xanthinuria

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Abstract Classical xanthinuria is a rare inherited metabolic disorder caused by either isolated xanthine dehydrogenase (XDH) deficiency (type I) or combined XDH and aldehyde oxidase (AO) deficiency (type II). XDH and AO are evolutionary related enzymes that share a sulfurated molybdopterin cofactor. While the role of XDH in purine metabolism is well established, the physiologic functions of AO are mostly unknown. XDH and AO are important drug metabolizing enzymes. Urine metabolomic analysis by high pressure liquid chromatography and mass spectrometry of xanthinuric patients was performed to unveil physiologic functions of XDH and AO and provide biomarkers for typing xanthinuria. Novel endogenous products of AO, hydantoin propionic acid, N1-methyl-8-oxoguanine and *N*-(3-acetamidopropyl) pyrrolidin-2-one formed in the histidine, nucleic acid and spermidine metabolic pathways, respectively, were identified as being lowered in type II xanthinuria. Also lowered were the known AO products, N1-methyl-2-pyridone-5-carboxamide and N1-methyl-4-pyridone-5-carboxamide in the nicotinamide degradation pathway. In contrast to the KEGG annotations, the results suggest minor role of human AO in the conversion of pyridoxal to pyridoxate and gentisaldehyde to gentisate in the vitamin B6 and tyrosine metabolic pathways, respectively. The perturbations in purine degradation due to XDH deficiency radiated further from the previously known metabolites, uric acid, xanthine and hypoxanthine to guanine, methyl guanine, xanthosine and inosine. Possible pathophysiological implications of the observed metabolic perturbations are discussed. The identified biomarkers have the potential to replace the allopurinol-loading test used in the past to type xanthinuria, thus facilitating appropriate pharmacogenetic counseling and gene directed search for causative mutations.

Keywords Inherited disorders · Urine metabolomics · Molybdo-flavo-enzymes · Pharmacogenetics · Fourier transform mass spectrometry

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Abbreviations

XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
HMCS	Human molybdenum cofactor sulfurase
MS^2	Tandem mass spectra
KEGG	Kyoto encyclopedia of genes and genomes
2PY	N1-methyl-2-pyridone-5-carboxamide
4PY	N1-methyl-4-pyridone-5-carboxamide
NMN	N1-methylnicotinamide
HPA	Hydantoin propionic acid
NMOG	*N*-methyl-8-oxoguanine
APP	*N*-(3-acetamidopropyl) pyrrolidin-2-one
ADH	Aldehyde dehydrogenase
TPMT	Thiopurine methyl transferase
6-MP	6-Mercaptopurine

1 Introduction

Classical xanthinuria is a rare inherited metabolic disorder characterized by either isolated xanthine dehydrogenase/oxidase (XDH/XO, EC 1.17.1.4/EC 1.17.3.2) deficiency due to mutations in the XDH gene (type I), or combined XDH and aldehyde oxidase (AO, EC 1.2.3.1) deficiencies due to mutations in the human molybdenum cofactor sulfurase (HMCS) gene (type II) (Simmonds et al. 1995, http://www.ncbi.nlm.nih.gov/omim/278300). The main clinical manifestation of classical xanthinuria is xanthine urolithiasis and symptoms include hematuria, crystalluria, renal colic, acute renal failure, recurrent urinary tract infections that may lead to hydronephrosis, chronic renal failure, and even death from uremia. The second most frequent symptom, muscle pain and cramps is due to xanthine deposition in the muscles and may be precipitated by strenuous exercise.

XDH and AO are evolutionary related molybdo-flavo-enzymes that share a sulfurated molybdopterin cofactor and catalyze similar oxidative hydroxylation reactions, yet differ in their substrate specificities and inhibitors (Hille et al. 2011). The allopurinol-loading test, based on specific allopurinol inhibition of XDH, was used in the past to type xanthinuria (Ichida et al. 1998). While XDH acts mainly on purines and its role in the formation of uric acid in humans is well established, the physiological substrate(s) and function(s) of human AO are still mostly unknown. Both enzymes are known as important drug metabolizing enzymes (Kitamura et al. 2006) yet other pathophysiological functions, mostly in animals have been suggested. Experiments in mouse knockouts and in murine and human cell-lines suggested the involvment of XDH and AO in physiological processes like milk fat goblet secretion (Vorbach et al. 2002), innate immunity (Vorbach et al. 2003), adipogenesis and lipid transport linked to peroxisome proliferator-activated receptors, PPARα and PPARγ activities (Cheung et al. 2007; Weigert et al. 2008), modulation of ATP-binding cassette transporter-1 (ABC-1) mediated lipid efflux and reverse cholesterol transport (Sigruener et al. 2007), kidney development and cyclooxygenase-2 (COX-2) activity (Ohtsubo et al. 2004; Ohtsubo et al. 2009).

In the present work advantage was taken of the relatively large number of xanthinuric patients identified in Israel (Levartovsky et al. 2000; Peretz et al. 2007, Peretz et al. unpublished) and a metabolomic approach was applied to obtain new insights into the physiologic roles of XDH and AO in humans. In addition, identification of enzyme specific biomarkers, may facilitate the differential diagnosis of type I and type II classical xanthinuria, thus allowing for tailored pharmacogenetic counseling and orientation of the search for the causative mutations towards the respective genes.

2 Materials and methods

2.1 Subjects

Patients and their non-carrier family members were recruited from 11 families affected by classical xanthinuria. The study protocol was approved by the Institution Ethics (Helsinki) Committee. Written informed consent was obtained from all participants. Demographic and clinical details of the studied subjects are summarized in Table 1. In order to avoid ascertainment bias as much as possible, the subjects were chosen in such a way that the age and sex distribution in each category, type I, type II and wild type, were similar and included children and adults of both sexes. The dietary habits and the climate in which the investigated families live were also similar. Xanthinuria typing was achieved by allopurinol-loading tests and/or homozygosity mapping and confirmed by identification of the causative mutations in the respective genes as described previously (Levartovsky et al. 2000; Peretz et al. 2007).

2.2 Sample collection and preparation

Subjects were instructed to collect first-morning, middle stream urine samples following an overnight fast. The samples were kept refrigerated for no more than 8 h, than kept frozen at −80°C until sent from Tel Aviv to Glasgow on dry ice for metabolomic analysis. For some individuals, two urine samples were collected about 1 year apart (Table 1). Immediately prior to MS analysis samples were thawed and aliquots of 0.2 ml were added to acetonitrile (0.8 ml) in a protein crash plate attached to a vacuum manifold (Biotage, Sweden), the samples were left for 10 min and then filtered into a collection tube by applying vacuum.

2.3 General biochemistry tests

Urine biochemistry tests were performed to confirm diagnosis of xanthinuria and to assess basic renal function. General urine analysis was performed on an (AJ-4270) instrument (Arkray, Japan) using 10EA Aution sticks and sediments were examined under a bifocal Olympus BX41 microscope. Uric acid, creatinine and blood urea nitrogen (BUN) levels in serum and uric acid, creatinine and total protein levels in urine samples were measured by routine clinical laboratory testing on an Advia 1650 (Siemens, USA) analyzer.

2.4 Metabolomic analysis

Experiments were carried out using an Orbitrap Exactive (ThermoElectron, Hemel Hempstead, UK) fitted with a

Table 1 Demographic, clinical and biochemistry data of studied subjects

Subject[a]	Age (years)	Sex	Clinical findings	Serum			Urine		
				UA	Cre	BUN	UA	Cre	Prot
I-1	10	M	Asymptomatic	–	–	–	0	–	8
	11			0.2	0.67	15	1	116	6
I-2	12	F	Down's syndrome	0.1	0.39	10	0	50	–
I-3	9	F	Asymptomatic	0.2	0.57	9	0	109	8
I-4	13	M	Renal stone at age 4	0.1	0.94	11	0	90	–
I-5	24	F	Congenital glaucoma	0.2	0.80	16	0	395	19
I-6	8	F	Abdominal pain, irritation of the urinary tract, microscopic hematuria	0.1	0.67	15	0	267	
II-1	9	F	Nephrolithiasis, muscle pain	–	–	–	1	110	10
	10			0	0.75	14	2	108	11
II-2	15	M	Asymptomatic				1	87	7
	16			0	0.98	15	1	135	6
II-3	19	F	Muscle pain				1	165	9
	20			0.1	1.00	16	2	215	10
II-4	58	M	Arthralgia, nephrolithiasis, diabetes mellitus, scleroderma	0.2	1.21	15	1	98	10
II-5	77	M	Carcinoma of urinary ladder	0.1	1.19	20	1	95	92
WT-1	16	M	Asymptomatic	6.1	0.84	11	50	73	–
WT-2	7	F	Asymptomatic	5.0	0.65	9	78	65	–
WT-3	2	F	Asymptomatic	5.2	0.53	9	107	138	–
WT-4	54	F	Asymptomatic	5.1	1.03	14	24	48	3
WT-5	25	M	Asymptomatic	6.2	0.95	14	45	119	12
WT-6	30	F	Low back pain	4.9	1.01	14	51	161	9
WT-7	12	M	Asymptomatic	2.7	–	–	43	100	8

UA uric acid, *Cre* creatinine, *Prot* protein. Normal reference values are: in serum, UA 2.3–8.0 mg/dl, BUN 5–25 mg/dl, Cre 0.7–1.3 mg/dl; in urine: UA 20–80 mg/dl, Cre 90–300 mg/dl, Prot 1–14 mg/dl, Prot/Cre ratio <0.2 mg/mg

[a] I-denotes patient affected by type I xanthinuria, II-denotes patient affected by type II xanthinuria, WT-wild type; Subjects I-1, I-2 and I-3 and subjects I-4, WT-1, WT-2 and WT-3 and subjects I-5, I-6, WT-4, WT-5 and WT-6 belong to 3 families of Bedouin-Arab extraction, respectively. Subjects II-1, II-2, II-3 and WT-7 are members of a Bedouin-Arab family (Peretz et al. 2007). Subjects II-4 and II-5, belong to two unrelated families of Yemenite-Jewish extraction (Peretz et al. unpublished)

Dionex HPLC pump and autosampler (Dionex, UK). Analysis was carried out in positive or negative switching mode over a mass range of 60–1000 *m/z* using 30,000 resolution. The capillary temperature was 275°C, the ion spray voltage was 4.5 kV in positive ion mode and 3.0 kV in negative ion mode and the sheath and auxiliary gas flow rates were 55 and 15, respectively (units not specified by manufacturer). The instrument was externally calibrated before analysis and internally calibrated using lock masses at *m/z* 83.06037 and *m/z* 195.08625 in positive ion and 91.00368 in the negative ion mode. The software programme Xcalibur (version 2.0) was used to acquire the LC-MS data. Samples were analysed sequentially and the vial tray was set at a constant temperature of 4°C. Tandem Mass Spectral (MS^2) experiments were carried out at 30 V on an LTQ Orbitrap using the same conditions. Two columns were used for the analyses. A ZIC-HILIC column (5 µm, 150 × 4.6 mm; HiChrom, Reading, UK) was used with a binary gradient method. Solvent A was 0.1% v/v formic acid in HPLC grade water and solvent B was 0.1% v/v formic acid in acetonitrile. A flow rate of 0.3 ml/min was used and the injection volume was 10 µl. The gradient programme used was 80% B at 0 min to 50% B at 12 min to 20% B at 28 min to 80% B at 37 min, with total run time of 46 min. Also a ZICpHILIC column (5 µm, 150 × 4.6 mm; HiChrom, Reading, UK) was used with a binary gradient. Solvent A was 20 mM ammonium carbonate adjusted to pH 9.2 with 0.1% ammonia hydroxide and B was acetonitrile. The gradient was the same as was used for the ZICHILIC column. The raw Xcalibur data files from control and treated samples were processed using Sieve, version 1.2 (Thermo Fisher). The masses of the

metabolites were pasted into Excel and searched against a mass list comprised of the exact masses of 10,000 biomolecules using a macro written in house.

3 Results and discussion

3.1 Clinical and general biochemistry data

Demographic, clinical and biochemistry data of the studied subjects are shown in Table 1. Five out of the eleven patients presented with disease specific symptoms, four at young age. The other affected subjects came to the attention of the treating physicians due to various congenital or acquired conditions or during routine biochemical screening. As expected, serum and urinary uric acid concentrations of patients were very low, 0–2 mg/dl. Normal serum BUN and creatinine levels together with normal urine protein/creatinine ratios indicated normal renal function in all individuals except one (subject II-5). This individual suffered from carcinoma of the bladder and was undergoing chemotherapy. General urine tests by dry chemistry and microscopic inspection of the urine sediments showed no remarkable differences in patients compared to wild-type subjects (not shown).

3.2 Metabolomic analysis

3.2.1 Identification of marker compounds of XDH deficiency

Metabolomic analysis of urine sample of xanthinuric patients was performed for detection and identification of compounds that are specific products of each of the enzymes, XDH and AO in humans. Table 2 shows the metabolites with altered levels in type I and type II xanthinuric patients combined, as compared to wild-type subjects. As expected, lack of XDH activity in these patients resulted in very low levels of uric acid and its ribonucleoside conjugate. In contrast, highly elevated levels were observed for xanthine, the immediate precursor of uric acid, and also for more distant precursors: hypoxanthine, guanine xanthosine and inosine. Methyl guanine levels were about 2 fold higher in xanthinuric patients compared to wild type. No other changes in the urine metabolome, common to type I and type II xanthinuria were observed by the methods employed. These results confirm that the main metabolic role of XDH in humans is confined to the purine degradation pathway and show that the perturbation in this pathway radiates further from uric acid, xanthine and hypoxanthine as previously known, to the more distant metabolites, guanine, methyl guanine, xanthosine and inosine. Other tissue-specific, temporal metabolic functions of human XDH, that could not be detected by the methods employed in the present study, cannot be excluded.

3.2.2 Identification of marker compounds of AO deficiency

According to the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways database (http://www.genome.jp/kegg/pathway.html) human AO is involved in the metabolism of amino acids (tryptophan, tyrosine, valine, leucine and isoleucine), vitamin B6 and nicotinamide. But, whereas only in the nicotinamide, vitamin B6 and tyrosine metabolic pathways are the relevant reactions catalyzed by AO alone, in all the other pathways the reactions involving AO can be catalyzed by competing enzymes. Thus, the missing metabolites in the type II xanthinuria samples compared to that of type I patients and

Table 2 Marker compounds common to type I and type II xanthinuria

Metabolite	*m/z*[b]	Elemental composition	ppm dev[c]	Rt Min[d]	Ratio[e]	*P* value[f]
Hypoxanthine	137.0458	$C_5H_5N_4O$	0.22	9.9	3.90	2.00E−04
Guanine	152.0567	$C_5H_6N_5O$	0.02	12.9	4.08	6.10E−05
Xanthine	153.0406	$C_5H_5N_4O_2$	−0.47	9.6	15.93	6.60E−11
Methylguanine	166.0724	$C_6H_8N_5O$	0.32	12.2	1.77	8.80E−04
Uric acid	169.0356	$C_5H_5N_4O_3$	−0.16	12.1	0.02	2.10E−04
Methyloxoguanine	180.0529	$C_6H_6N_5O_3$	1.1	11.3	0.68	2.30E−01
Inosine	269.0878	$C_{10}H_{13}N_4O_5$	−0.25	9.8	4.14	2.30E−02
Xanthosine	285.0828	$C_{10}H_{13}N_4O_6$	−0.60	11.0	8.21	3.30E−09
Urate ribonucleoside[a]	299.0636	$C_{10}H_{11}N_4O_7$	0.63	11.3	0.02	7.50E−04

[a] Negative ion mode; [b] Mass to charge ratio; [c] Deviation of measured mass from theoretical exact mass for the listed elemental composition; [d] Retention time on the HPLC column in minutes; [e] Ratio of mean abundance of metabolite in patients' samples over wild type samples; [f] *P* value of <0.01 (1.00E−02) is considered statistically significant

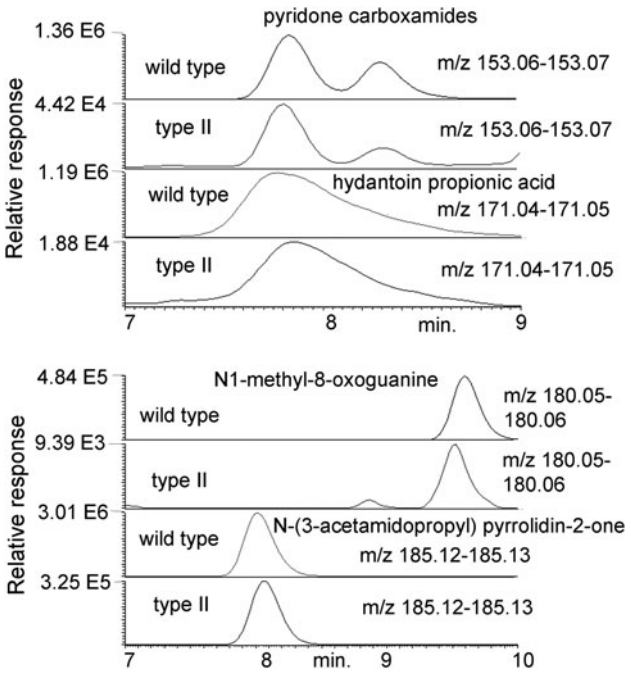

Fig. 1 Extracted ion traces of type II xanthinuria marker compounds in urine

wild type subjects combined, reflect products that are exclusively specific to AO activity. Extracted ion traces and a summary of the data obtained for the marker compounds of AO deficiency are presented in Fig. 1 and Table 3, respectively. Two known, N1-methyl-2-pyridone-5-carboxamide (2PY) and N1-methyl-4-pyridone-5-carboxamide (4PY) and three novel products of AO activity, hydantoin propionic acid (HPA), *N*-methyl-8-oxoguanine (NMOG) and *N*-(3-acetamidopropyl) pyrrolidin-2-one (APP), were identified.

3.2.3 N-methylpyridone carboxamides

Almost complete absence (around 3% of wild type) of two isomers of *N*-methylpyridone carboxamide, separated by HPLC (Fig. 1) but producing identical fragments in their MS^2 analysis (Table 4), was observed in the urine samples of type II patients. These findings together with the elevated levels of *N*-methylnicotinamide (NMN) (Table 3) are consistent with the role of AO in the conversion of N1-methylnicotinamide to 2PY and 4PY in the final steps of the nicotinamide degradation pathway (Fig. 2). Pyridone carboxamides were first demonstrated in human serum and urine in the early 1960s (Abelson et al. 1963). Their concentration in serum increases about 10 fold in patients with renal insufficiency and 2-PY was suggested to be an inhibitor of poly(ADP-ribose) polymerase (PARP-1) and thus a uremic toxin (Rutkowski et al. 2003). A similar compound, 4-pyridone-3-carboxamide ribonucleoside triphosphate, accumulating in erythrocytes in end stage renal failure was proposed to be formed via the tryptophan degradation pathway by the action of AO on riboside and ribotide intermediates of NAD turnover (Laurence et al. 2007). It is expected that in type II xanthinuric patients suffering from renal insufficiency the formation of these toxic metabolites will be avoided. But, accumulation of NMN in xanthinuric individuals may possibly increase their risk for developing type 2 diabetes according to recent studies in humans and rodent models (Delaney et al. 2005; Salek et al. 2007; Zhou et al. 2009). This risk may be aggravated by renal insufficiency and/or over-consumption of nicotinamide enriched foods (Zhou et al. 2009). On the other hand, NMN was found to limit platelet-dependent thrombosis in experimental thrombosis in rats, (Chlopicki et al. 2007). This effect, if it holds true in humans, might be an advantage for type II xanthinuric patients.

3.2.4 Hydantoin propionic acid

Hydantoin propionic acid is an intermediary metabolite of histidine formed as shown in Fig. 2. The identification of the peak at 8 min (Fig. 1) was further supported by the fragments generated in MS^2 mode where losses of CO_2 and HCN indicate the proposed structure (Table 4). The level of this metabolite in urine samples of type II patients was

Table 3 Marker compounds specific for type II xanthinuria

Metabolite	*m/z*[b]	Elemental Composit	ppm dev	Rt Min	Ratio	*P* value
N-methylnicotinamide	137.0709	$C_7H_9N_2O$	0.52	15.7	2.264	5.60E−05
gentisic acid	153.0193	$C_7H_5O_4$	−0.41	7.0	0.77	5.60E−01
pyridone carboxamide I	153.0658	$C_7H_9N_2O_2$	−0.03	8.1	0.006	4.30E−04
pyridone carboxamide II	153.0658	$C_7H_9N_2O_2$	−0.03	8.7	0.006	9.50E−05
hydantoin propionic acid	171.0414	$C_6H_7N_2O_4$	0.88	8.0	0.011	6.60E−05
N-methyl-8-oxoguanine[a]	180.0529	$C_6H_8NO_4$	1.0	9.8	0.006	1.40E−06
pyridoxate	182.0453	$C_8H_6N_5O_3$	−0.99	4.6	0.222	7.30E−03
N-(3-acetamidopropyl) pyrrolidin-2-one	185.1284	$C_9H_{17}N_2O_2$	0.19	8.5	0.071	8.40E−06

[a] Negative ion mode, [b] Column headings as in Table 2

Table 4 Fragments obtained in MS^2 mode for type II xanthinuria marker compounds

Metabolite	*m/z*[a]	Fragments
Pyridone carboxamide	153.0658	136.04 ($-NH_3$)
Hydantoin propionic acid	171.0414	127.05 ($-CO_2$), 100.04 ($-CO_2$, –HCN)
N-methyl-8-oxoguanine	180.0529	163.03 ($-NH_3$), 137.05 (–CONH) 135.03 ($-NH_3$–CO)
N-(3-acetamidopropyl) pyrrolidin-2-one	185.1284	167.12 ($-H_2O$), 143.11 ($-CH_2CO$), 126.09 ($-CH_2CO-NH_3$), 125.10 ($-CH_2CO-H_2O$)

[a] Mass to charge ratio

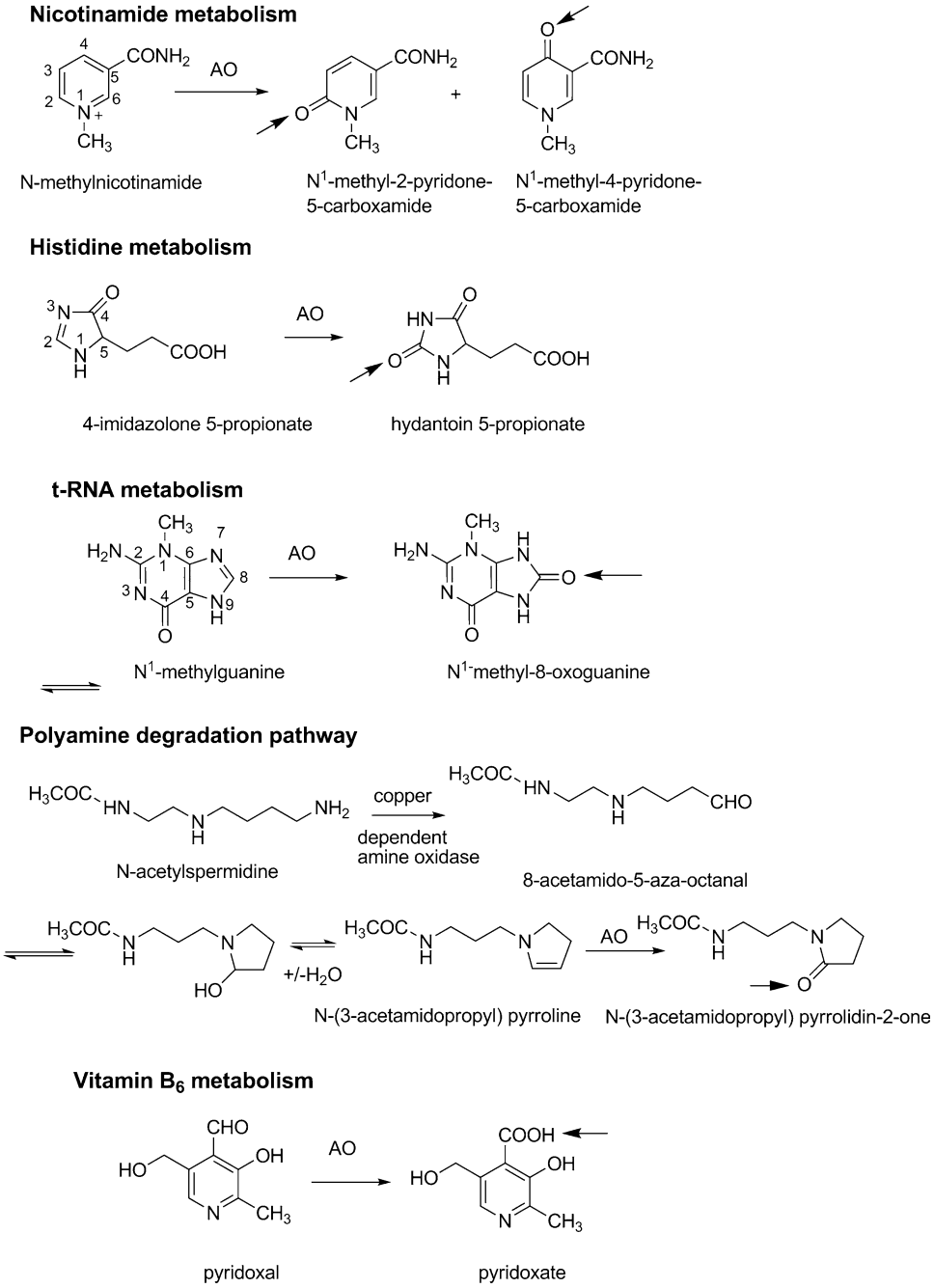

Fig. 2 Metabolic steps proposed to be catalyzed by AO in humans

around 1% compared to that found in the wild type samples (Fig. 1). HPA was detected in urine in humans about 50 years ago (Hassall and Greenberg 1963) and occurs at elevated levels in the urine of patients with folate dependent glutamate formiminotransferase deficiency (MIM#229100, EC 2.1.2.5) and disorders of folate/vitamin B12 metabolism (Human Metabolome Database, http://www.hmdb.ca, HMDB01212). In 1968, Payes and Greenberg (1968) identified AO purified from guinea pig liver as the enzyme catalyzing the oxidation of 4(5)-imidazolone-5(4)-propionic acid. Here, for the first time, we show that also in humans, hydantoin-5-propionic acid is an endogenous product of AO.

3.2.5 *N1-methyl-8-oxoguanine*

Another clear marker for type II xanthinuria, found at a level of about 2% of wild type samples, was identified as *N*-methyl-8-oxoguanine (NMOG) (Fig. 1). Nucleic acid methylation is one mechanism for regulating gene expression and methylated guanine species with modifications at three different *N* positions, 1, 2 and 7 are formed upon RNA degradation (Hou and Perona 2010) and excreted in the human urine (Ayvazian and Skupp 1965). The most common position for methylation is on N7 and this is the most abundant metabolite in urine (Ayvazian and Skupp 1965). The fragmentation pattern exhibited by the observed marker compound shows loss of an exocyclic amine group as ammonia rather than loss of methylamine, which would be expected in the case of N2-methyl-8-oxoguanine (Table 4) However, it has been shown that N7- and N2-methyl guanines are substrates of XDH as the formation of N7 and N2 isomers of NMOG in human subjects is inhibited by allopurinol (Helbock et al. 1996). Thus, the tentative identity of this marker product of AO deficiency is N1-methyl-8-oxoguanine (Fig. 2). Another unidentified isomer of NMOG, was detected at similar concentrations in urine samples of patients and controls (Table 2).

3.2.6 *N-(3-acetamidopropyl) pyrrolidin-2-one*

There was one more obvious marker compound of type II xanthinuria which had a mass of 185.12. Fragmentation involving loss of acetate and ammonia sequentially suggested the presence of an acetamide group. In addition, loss of water could be observed in the MS^2 spectrum (Table 4). This compound did not correspond to any compound in the data bases of the analytical system used in the present study, however searching Pubchem (http://www.ncbi.nlm.nih.gov/pccompound) resulted in its tentative identification as *N*-(3-acetamidopropyl) pyrrolidin-2-one (APP), a metabolite of *N*-acetylspermidine (Fig. 2). The extracted ion chromatogram for APP in Fig. 2 shows that its level in type II xanthinuria is around 10% of wild type. The profound decrease in the amount of APP in urine samples of type II xanthinuria indicated that AO, and not aldehyde dehydrogenase (ADH) as previously thought (Seiler et al. 1982; Seiler 2004) is the enzyme responsible for this final step in the oxidative degradation of *N*-acetyl spermidine. The proposed reaction in the spermidine degradation pathway shown in Fig. 2 is in line with the known mechanism of action of AO to promote nucleophilic attack at an electron-deficient carbon atom adjacent to a ring nitrogen atom (Kitamura et al. 2006; Pryde et al. 2010). The involvement of AO in the terminal catabolism of polyamines, revealed in this study, may also have pathophysiological significance as polyamines affect many key areas of cell biology including gene transcription, ion channel and protein kinase activity, the cell cycle, structure/function of membranes and nucleic acids. The content and cellular distribution of polyamines is controlled by rates of synthesis, degradation, transport and excretion. Natural or artificially caused defects affecting polyamine metabolism were demostrated to induce severe disease states (Pegg and Casero 2011). One of the harmful effects of the oxidative pathway of polyamine degradation is the formation of potentially toxic aldehydes. AO, as was found in the present work, may help neutralizing such an aldehyde by creating a product that is excreted in the urine and thus helping its detoxification. To what extent and under which circumstances lack of this detoxification reaction in type II xanthinuria patients might be significant should be further investigated.

3.2.7 *Pyridoxate and gentisate*

Two aldehyde-to-acid conversion reactions are annotated to human AO activity in the KEGG database: the conversion of pyridoxal to 4-pyridoxate in the vitamin B6 metabolic pathway and the conversion of gentisate aldehyde to gentisic acid in the tyrosine metabolic pathway. A ZIC-pHILIC column at high pH rather than the ZICHILIC column was found to better separate these acidic compounds. In contrast to what was found in the *Drosophila mal* mutant, an analog of type II xanthinuria (Kamleh et al. 2009), the levels of 4-pyridoxate, apparently a product of human AO activity in vitro (Merrill et al. 1984), were only slightly lower in type II xanthinuria than in type I and wild type samples (Table 3). Since type II xanthinuria patients lack AO activity, the main source of pyridoxal in their urine samples might be the ingested food or an enzymatic product of the intestinal flora but not human AO activity. Thus, the results obtained in the present study do not indicate a major contribution of human AO activity to the levels of pyridoxate found in urine.

The concentration of gentisic acid, an intermediary compound in the tyrosine metabolism, did not differ significantly in type II xanthinuria from that of the controls (Table 3). Contrasting the KEGG annotation these results suggest that in men, AO does not play a significant role in the conversion of gentisaldehyde to gentisate. The article cited in KEGG (Gordon et al. 1940) shows that AO isolated from pig liver has the ability to oxidize a variety of aldehydes but gentisaldehyde is not one of them. Compared to acetaldehyde as substrate, the relative AO activity toward benzaldehyde is 25% and toward salicylaldehyde (a hydroxyl derivative of benzaldehyde) is 2%. Since gentisaldehyde is a dihydroxyl derivative of benzaldehyde it is expected that it is not a good substrate of AO. In addition, it was shown that NAD-specific aromatic aldehyde dehydrogenase (ADH) is responsible for the oxidation of gentisaldehyde to gentisate in rabbit liver (Raison et al. 1966). In humans there are 19 putative functional members of of the ADH superfamily, among them some acting on aromatic substrates (Marchitti et al. 2008). All this evidence suggests that in humans one of the ADHs and not AO catalyzes the oxidation of gentisaldehyde to gentisate.

The five metabolites dramatically lowered in the urine of type II xanthinuric patients (N1-methyl-2-pyridone-5-carboxamide, N1-methyl-4-pyridone-5-carboxamide, HPA, N1-methyl-8-oxoguanine and *N*-(3-acetamidopropyl) pyrrolidin-2-one) shown in Fig. 1 and Table 3 are clear products of the enzyme and are thus valid strong markers for type II xanthinuria. Consequently, the urine metabolic profile demonstrated in the present study allows differential diagnosis of type I and type II xanthinuria by a simple urine test that may replace the classic xanthinuria typing test (Ichida et al. 1998) where allopurinol administration and repeated urine and serum sampling are needed. It is expected that this patient-friendly test will improve patient compliance and facilitate patient-tailored pharmacogenetic counseling. Many drugs are known to be metabolized either by XDH or AO alone or by both enzymes (Kitamura et al. 2006; Pryde et al. 2010; Smith et al. 2009) therefore, pharmacogenetic counseling should take into consideration the type of xanthinuria. Another benefit of an easy test for typing xanthinuria is that it facilitates the search for the causative mutation by directing it to the relevant gene.

4 Concluding remarks

In summary, urine metabolic analysis by HPLC-MS resulted in identification of novel endogenous products of AO highlighting potential pathophysiological implications of AO deficiency and providing a simple test for differential diagnosis of type I and type II xanthinuria, thus facilitating appropriate pharmacogenetic counseling and gene oriented search for causative mutations.

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