Discovery of anti-inflammatory dihydroxylated phenolic acids in patients with severe cardiac symptoms and conditions associated with inflammation and hypoxia

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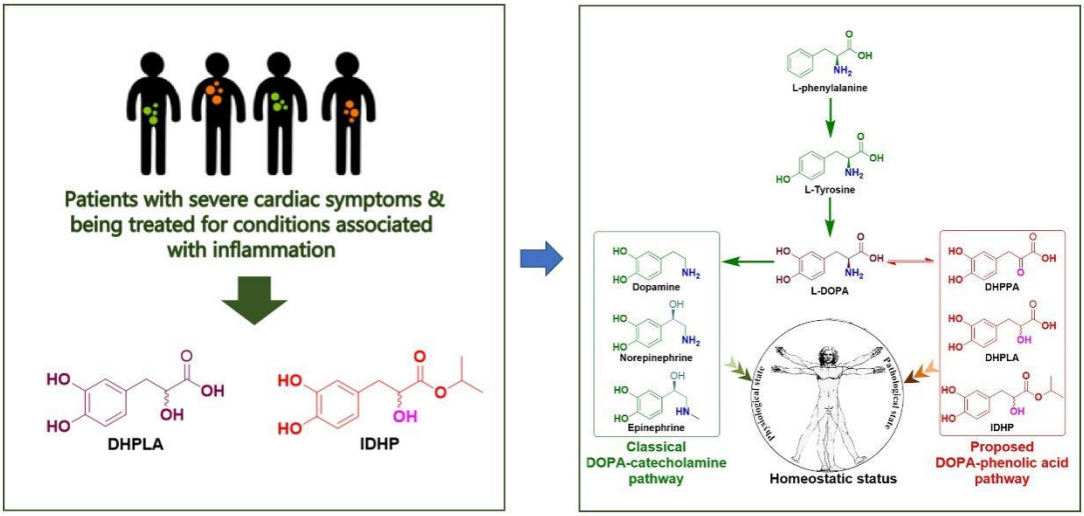
ABSTRACT

Our initial studies detected elevated levels of 3,4-dihydroxyphenyllactic acid (DHPLA) in urine samples of patients with severe cardiac symptoms when compared with healthy subjects. In view of the reported anti-inflammatory properties of DHPLA and related dihydroxylated phenolic acids (DPAs), we embarked on a multi-centre investigation to establish the possible pathophysiological significance and therapeutic implications of these findings. Chinese and Caucasian patients with severe cardiac symptoms and those being treated for conditions associated with inflammation (WBC $\geq 10\times10^9$/L or hsCRP $\geq$ 3.0 mg/L) and/or hypoxia ($PaO_2 \leq 75$ mmHg) were enrolled, their urine samples were analyzed by HPLC, HPLC-MS, GC-MS and biotransformation assays. DHPLA was detected in urine samples of patients, but undetectable in healthy volunteers. Dynamic monitoring of inpatients undergoing treatment showed their DHPLA levels declined in proportion to their clinical improvement. *Proteus vulgaris* and *P. mirabilis* were significantly more abundant in DHPLA-positive patients' fecal samples than healthy volunteers. In culture, these bacteria were capable of reversible interconversion between DOPA and DHPLA. Furthermore, porcine and rodent organs were able to metabolize DOPA to DHPLA and related phenolic acids. The elevated levels of DHPLA in these patients suggest anti-inflammatory DPAs are generated *de novo* as part of a human's defense mechanism against disease. Given that DHPLA isolated from Radix *Salvia miltiorrhizae* has multi-dimensional pharmacological activities, these data demonstrate not only scientific basis of the ethnopharmacological uses of this medicinal plant but also highlight the therapeutic potential of endogenous, natural or synthetic DPAs and their derivatives in humans.

Graphical Abstract



Key words

3,4-dihydroxyphenylalanine (DOPA); dihydroxylated phenolic acids; 3,4-dihydroxyphenyllactic acid (DHPLA); isopropyl 3-(3,4-dihydroxyphenyl)-2-hydroxypropanoate (IDHP); inflammation and/or hypoxia; human microbiota, mammalian tissues.

Introduction

Research on bioactive substances and their metabolites from microorganisms (e.g. penicillin, cyclosporine), plants (e.g. salicylic acid, morphine) and animals (e.g. insulin and heparin) has improved our knowledge of drug molecules, chemical mediators and their receptors, mechanisms of action and metabolic pathways in the context of health and disease. For example, phyto-cannabinoids have a similar pharmacological effect as the endocannabinoid anandamide on the CB1 receptor in the brain or the CB2 receptor in the immune system (1, 2). Such discoveries are fundamental in promoting the development of safe and effective therapeutics.

3,4-dihydroxyphenyllactic acid (DHPLA, Figure 1A) is a dihydroxylated phenolic acid derived from Radix *Salvia miltiorrhizae.* Because this medicinal plant is known as Danshen in China, DHPLA is also named Danshensu (3), exhibiting a multitude of pharmacological activities. In addition to protecting vascular endothelial cells against homocysteine-induced dysfunction (4) and inhibiting proliferation of cardiac fibroblasts (5), DHPLA has been shown to have anti-inflammatory (6), antioxidant (7), cardiovascular protective effects (6, 8, 9) as well as attenuating neuroinflammation mediated by advanced glycation end products (10). Recently, preventive DHPLA treatment has been shown to significantly reduce the elevation of right ventricle systolic pressure and right ventricle hypertrophy index in rats exposed to hypoxia (11).

In our previous metabolic studies of oral botanical formulations containing Radix *Salvia miltiorrhizae*, we found DHPLA and its active metabolite isopropyl 3-(3,4-dihydroxyphenyl)-2-hydroxypropanoate (IDHP) not only in human plasma (12, 13), but also in rabbit hearts (12, 13). Like DHPLA, IDHP has also been shown to possess many pharmacological activities: vasorelaxation (14), protection against myocardial ischemia/reperfusion injury (15), attenuation of β-adrenoceptor mediated cardiac fibrosis (16).

Following our initial discovery of DHPLA in urine samples of patients with severe cardiac symptoms, we sought to measure DHPLA in urine samples of Chinese patients being treated for conditions associated with inflammation and/or hypoxia but

not receiving drugs known to contain DHPLA. Furthermore, we carried out a multi-centre clinical investigation in China and the Netherlands to confirm the presence of DHPLA and IDHP in both Chinese and Caucasian patients, as well as increasing our understanding of these molecules' physiological significance and possible origin in humans. Given the fact that DHPLA is similar to DOPA (3,4-dihydroxyphenylalanine) in chemical structure, we hypothesized that DHPLA can be synthesized from endogenous DOPA in patients as part of a human's defense mechanism against inflammation and/or hypoxia. Thus, homogenates of porcine hearts and various rat organs were used as a proxy for testing the ability of mammalian tissues/cells to metabolize DOPA to dihydroxylated phenolic acids *in vitro*. Furthermore, because some bacterial species are known to express enzymes involved in catechol metabolism (17), and gut microbiota play a crucial role in the host's health (18), we also examined the ability of the five dominant microbes in DHPLA-positive patients' feces to convert DOPA to DHPLA and IDHP.

Like cardiovascular diseases, chronic obstructive pulmonary disease (COPD), rheumatoid arthritis and coronavirus disease (COVID-19) (19) are also associated with inflammation and/or hypoxia. Here, our findings suggest a new biological perspective of these novel dihydroxylated phenolic acids and classical catecholamines in mediating the crosstalk between the nervous, cardiovascular and immune systems in health and these clinical conditions. This hypothesis warrants further confirmatory studies which can possibly lead to development of future treatment strategies.

Results

Discovery of DHPLA in urine samples of patients with severe cardiac symptoms

Ten Chinese patients with severe cardiac symptoms and ten healthy Chinese volunteers (5 males and 5 females) were enrolled in the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China).These patients had not consumed products or been treated with agents known to contain DOPA or 3,4-dihydroxyphenyllactic acid （DHPLA） for 14 days prior to the analytic studies. First morning urine

samples of patients were analyzed by LC-MS and ^{1}H NMR to identify the produced metabolites. A chromatographic peak with the same retention time ($t_R \approx 25.5$ min) as that of DHPLA standard was detected in urine samples of patients, but not in samples from healthy individuals (the typical LC-MS chromatogram is shown in Figure 1B No.1 and No. 4). The eluted component of this peak was isolated and collected by preparative HPLC (Supplementary Figure 1) and then subjected to mass spectrometric and ^{1}H NMR identification. The LC-MS and ^{1}H NMR data of the compound isolated from urine were consistent with those of DHPLA reference standard (Figure 1B, 1C and Supplementary Table 1 for MS results; Supplementary Figure 2 for ^{1}H NMR results). These results confirmed that DHPLA was present in urine samples of patients with severe cardiac symptoms (Table 1).

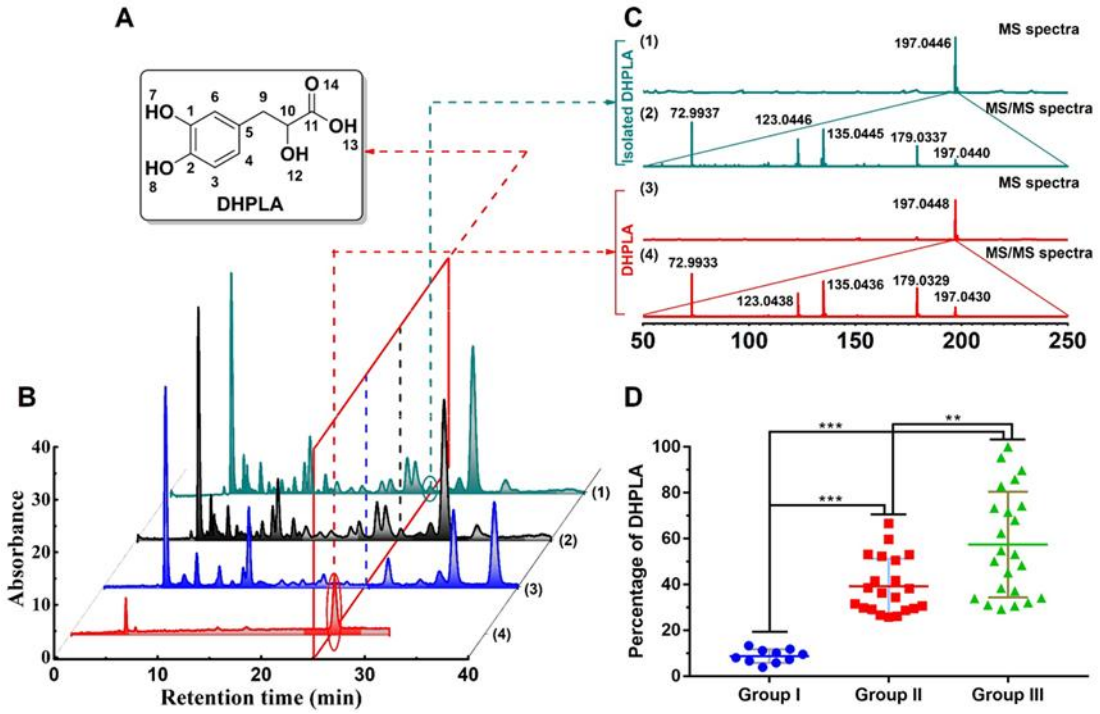


Figure 1. Identification and quantitation of DHPLA in urine samples of Chinese patients with inflammation and/or hypoxia.

A. Chemical structure of DHPLA. **B.** Analysis and identification of DHPLA in typical urine samples by HPLC-FLD: (1) representative clinical trace of a patient's urine sample, (2) representative clinical trace of a patient's urine sample spiked with DHPLA reference standard (DHPLA-RS), (3) representative trace of a healthy volunteer's urine sample, (4) trace of DHPLA-RS (t_R = 25.5 min.). **C.** LC-Q-TOF-MS and MS/MS results for a patient's urine sample obtained in negative mode: (1) MS spectrum of DHPLA separated from a patient urine sample with accurate masses of $[M-H]^-$ ions, (2) MS/MS spectrum of the ion with an accurate mass of 197.044 Da, (3) MS spectrum of DHPLA-RS with accurate masses of $[M-H]^-$ ions, (4) MS/MS spectrum of

DHPLA-RS ion with an accurate mass of 197.044 Da. **D.** Urinary DHPLA levels were measured in 43 Chinese patients (40 males and 3 females) and ten healthy Chinese volunteers (5 males and 5 females). The sample with the highest DHPLA level detected in this study was designated as 100%, with all other samples being calculated as its percentage. Data are expressed as mean ± s.d. ** $p < 0.01$, *** $p < 0.001$ (One-way ANOVA). Group I: healthy volunteers (n = 10); Group II: patients meeting any one of the three clinical criteria (n = 21); Group III: Chinese patients meeting any two or three clinical criteria (n = 22).

Table 1 The detection of DHPLA in the urine samples of ten Chinese patients with severe cardiac symptoms

Age	Gender	DHPLA (ng/mL)	hsCRP	PaO_2	WBC
74	M	> 104, < 313	+	+	-
62	M	> 104, < 313	-	-	+
65	M	> 104, < 313	+	+	-
63	M	> 104, < 313	+	-	-
86	F	> 104, < 313	+	+	-
85	M	> 104, < 313	-	+	-
86	M	> 104, < 313	-	+	-
78	M	> 104, < 313	-	+	-
52	M	> 104, < 313	+	-	-
64	F	> 104, < 313	+	-	+

"+": WBC ≥ 10×10^9/L or hsCRP ≥ 3.0 mg/L or $PaO_2 \leq 75$ mmHg;
"–": WBC < 10×10^9/L or hsCRP < 3.0 mg/L or PaO_2 > 75 mmHg.

Detection of DHPLA is associated with ischemia, hypoxia stress and inflammation.

What can be the biological meaning for the detection of DHPLA in urine of patients with severe cardiac symptoms? Through the examination of the patient's clinical parameters, it is found that the white blood cell count and hsCRP levels of critically ill patients with heart disease were significantly high, we are questioning if these clinical parameters may represent a class of pathological conditions, namely ischemia, hypoxia, stress, inflammation, etc., and the detection of DHPLA can be associated with these type of clinical conditions. We further extended our analytic work of DHPLA to more board spectrum of diseases but using following clinical parameters for selection of patients criteria: inflammation (WBC ≥ 10×10^9/L; hsCRP ≥ 3.0 mg/L) and/or hypoxia ($PaO_2 \leq 75$ mm Hg).

A total of 43 Chinese patients (40 males and 3 females) and 10 healthy Chinese volunteers (5 males and 5 females) who had not consumed products or been treated with agents known to contain DOPA or DHPLA for 14 days prior to the analytic studies， and met at least one of the following clinical criteria: (a) WBC ≥ 10×10^9/L (b) hsCRP ≥ 3.0 mg/L and (c) PaO_2 ≤ 75 mm Hg. 100 % of Chinese patients'urine samples contained detectable levels of DHPLA and/or IDHP. The urinary DHPLA levels of patients with any two or three abnormal clinical indices WBC, hsCRP and PaO_2 (Figure 1D, Group III) were significantly higher than those of patients with only one abnormal clinical index (Figure 1D, Group II), while the urinary DHPLA levels in healthy volunteers were below the limit of detection (LOD) (Figure 1D, Group I). Thus, there was a good correlation between the urinary levels of DHPLA and the severity of inflammation and/or hypoxia, as indicated by clinical indices.

Correlation between DHPLA level and disease progression

To follow the dynamic changes in urinary DHPLA levels in response to medical treatment, we monitored 9 Chinese inpatients for 8 consecutive days. Patients were given diuretics and vasodilators for symptomatic treatment, or antiplatelet drugs and cholesterol lowering drugs for etiological treatment. Over a 6-day period, the clinical indices of inflammation (WBC, hsCRP) and hypoxia (PaO_2) in these inpatients improved and returned to normal range (defined as WBC = 4×10^9-10×10^9/L; hsCRP = 0-3 mg/L; PaO_2 = 75-100 mm Hg) while their urinary DHPLA levels gradually declined to below the limit of detection (LOD) between Day 3 to Day 8. Collectively, the results showed a positive correlation between urinary DHPLA levels (Figure 2D) with WBC and hsCRP (Figure 2A and 2B), showed an inverse correlation with PaO_2 (Figure 2C). The level of DHPLA was positively correlated with the degree of inflammatory and hypoxia injury

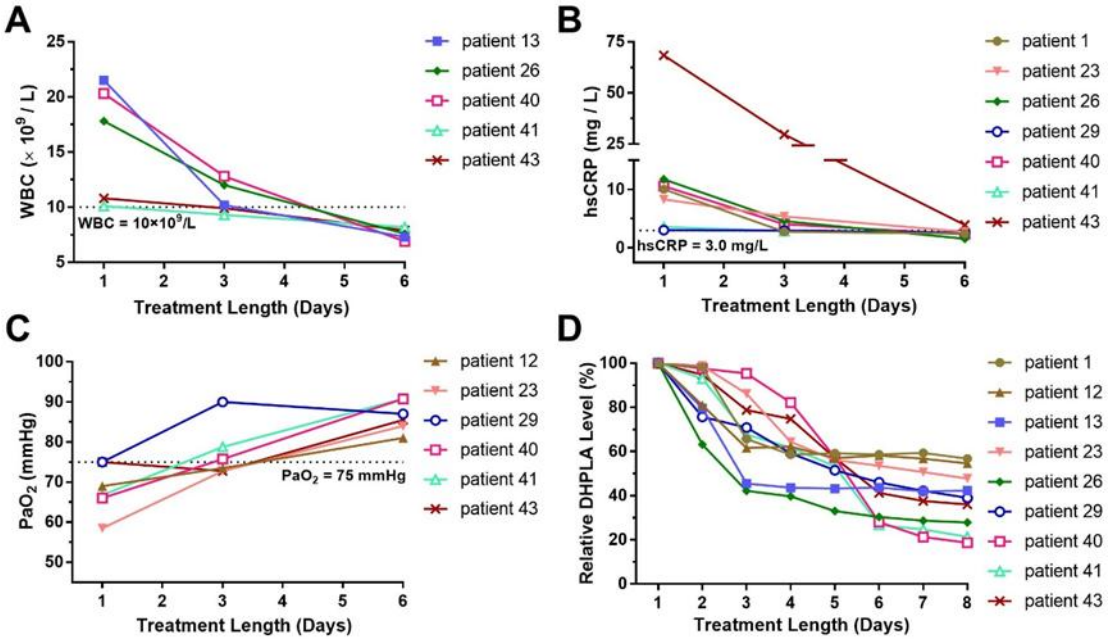


Figure 2. Correlation between dynamic changes in urinary DHPLA levels and clinical indices of 9 Chinese inpatients receiving treatment.

For 8 consecutive days, the first morning urine samples of 9 Chinese in patients undergoing medical treatment were collected daily for measurement of DHPLA levels by HPLC-FLD. The WBC, hsCRP and PaO_2 levels of these patients were monitored on Days 1, 3 and 6. As the patients responded to treatment, their clinical indices returned to normal range on Day 6 (**A**, **B** and **C**. The urinary DHPLA levels (**D**) in these 9 inpatients (expressed as relative DHPLA abundance % of their individual peak levels on Day 1) gradually declined to below the limit of detection (LOD) between Day 3 to Day 8. Thus, there was a positive correlation between patients' urinary DHPLA levels (**D**) with WBC (**A**, 5 inpatients) and hsCRP (**B**, 7 inpatients), but an inverse correlation with PaO_2 (**C**, 6 inpatients).

Quantitative analysis of urinary DHPLA and IDHP in Caucasian patients with inflammation and/or hypoxia

A total of 34 Caucasian patients (26 males and 8 females) admitted to ICU were enrolled in Department of Intensive Care Medicine, VUmc (Amsterdam, the Netherlands). These patients met at least one of the following clinical criteria: (a) WBC $\geq 10\times10^9$/L (b) hsCRP $\geq$ 3.0 mg/L and (c) $PaO_2 \leq$ 75 mm Hg. All patients had received oxygen therapy. Five Chinese patients' urine samples also subjected to the same analytical procedure. In this study, a more sensitive new analysis method with GC-QQQ-MS/MS was developed (Supplementary Tables 2-4). DHPLA was detected in 13 Caucasian patients' urine (detection rate = 38.2 %), with 6 samples in the range of 1.3-2.0 ng/mL, other 7 samples in the range of 0.2-0.7 ng/mL. Furthermore, IDHP

was also detected in 28 Caucasian patients' urine (detection rate = 82.4%), with 11 samples in the range of 0.9-2.76 ng/mL, other 11 samples in the range of 0.4-0.7 ng/mL. IDHP was not detected in the healthy controls. Thus, 88.2 % of urine samples contained detectable levels of DHPLA and/or IDHP. The DHPLA and IDHP detection rates in the 5 Chinese patients with this new analytic method were 80 % and 100 %, and the concentrations ranged from 1.3-1.9 ng/mL and from 1.5-1.9 ng/mL, respectively (Table 2, Supplementary Figure 3).

The actual physiological concentration of DHPLA in urine may be varied from the different detection methods, this may due to systemic variations of analytic methodology and possible complex formation between endogenous proteins and DHPLA/IDHP. To summarize, the urinary levels of DHPLA and IDHP in Chinese and Caucasian patients are in the same range by using GC-QQQ-MS/MS analytic method, suggesting an universal *de novo* production of these dihydroxylated phenolic acids in patients responding to inflammation and/or hypoxia, irrespective of their race. Since most of the patients under their circumstance will be given oxygen therapy during the rescue process, so the corelation of low oxygen partial pressure with the level of DHPLA is less significant.

Table 2. Levels of DHPLA and IDHP in the urine samples of Caucasian and Chinese patients detected by GC-QQQ-MS/MS

Classification	Age	Gender	DHPLA (ng/mL)	IDHP (ng/mL)
Caucasian patients (n = 34)	53	F	2.0	> 0.4, < 0.7
	86	F	1.9	1.51
	66	M	1.4	2.76
	71	M	1.4	not detected
	77	F	1.3	> 0.4, < 0.7
	76	M	1.3	> 0.4, < 0.7
	54	M	> 0.2, < 0.7	> 0.4, < 0.7
	68	F	> 0.2, < 0.7	< 0.4
	45	M	> 0.2, < 0.7	> 0.4, < 0.7
	77	F	> 0.2, < 0.7	> 0.4, < 0.7
	68	F	> 0.2, < 0.7	1.08
	87	M	> 0.2, < 0.7	0.88
	59	M	< 0.2	not detected
	78	M	< 0.2	> 0.4, < 0.7
	71	M	< 0.2	> 0.4, < 0.7

	61	M	< 0.2	0.90
	81	M	> 0.2, < 0.7	1.09
	76	M	< 0.2	1.16
	79	M	< 0.2	> 0.4, < 0.7
	50	M	< 0.2	> 0.4, < 0.7
	71	M	< 0.2	1.02
	71	M	< 0.2	< 0.4
	69	M	< 0.2	> 0.4, < 0.7
	29	F	< 0.2	1.04
	86	F	< 0.2	> 0.4, < 0.7
	83	M	< 0.2	1.11
	67	F	< 0.2	not detected
	70	M	< 0.2	< 0.4
	57	M	< 0.2	> 0.4, < 0.7
	61	M	< 0.2	> 0.4, < 0.7
	77	M	< 0.2	> 0.4, < 0.7
	60	M	< 0.2	> 0.4, < 0.7
	75	M	not detected	1.92
	81	M	not detected	> 0.4, < 0.7
Chinese patients (n = 5)	50	M	1.9	1.5
	71	M	1.8	1.7
	42	M	1.5	1.8
	55	M	1.3	> 0.4, < 0.7
	69	F	not detected	> 0.4, < 0.7

Metabolism of DOPA, DHPPA and DHPLA in homogenates of mammalian organs

Because the chemical structure of DHPLA is similar to DOPA, we hypothesize that DHPLA may be one of the new metabolites derived from DOPA. As a proxy for testing the ability of mammalian tissues/cells to metabolize DOPA to dihydroxylated phenolic acids, we incubated homogenates of porcine hearts with DOPA in a water bath shaker at 37°C for 6 hours. The samples were then prepared for analysis by HPLC-MS (20). Three independent experiments showed that porcine heart homogenates were able to metabolize DOPA to DHPPA (3,4-dihydroxyphenylpyruvic acid) and DHPLA (Supplementary Table 5A). In a separate series of experiments, the homogenates of rat heart, liver, spleen, lungs and kidneys were shown to convert DHPPA to DHPLA and DHPLA to IDHP, respectively (Supplementary Table 5B, n = 3). These data demonstrated that the DOPA can also be metabolized to dihydroxylated phenolic acids such as DHPLA in mammalian tissues.

Dominant species of bacteria in fecal samples of Chinese patients and their ability to convert DOPA to DHPLA

Recent development points out that microbiota play an important role is healthy and disease conditions, we are questioning, if microbiota is also involved in the formation of DHPLA? The microorganisms in fecal samples of Chinese patients and healthy volunteers were isolated, amplified and tested. Five dominant species of bacteria (*Proteus vulgaris*, *Proteus mirabilis*, *Enterococcus faecium*, *Enterococcus faecalis* and *Escherichia coli*) were determined to be significantly more abundant in DHPLA-positive patients' samples than healthy volunteers' samples ($P < 0.01$). Figure 3A shows the morphology of *P. vulgaris* and *P. mirabilis*, with their identity being confirmed by 16S rRNA gene identification (Supplementary Table 6 and 7). Figure 3B shows the $\log_{10}$ CFU/g of these two species isolated from patients (n = 3) were significantly higher ($P < 0.01$) than healthy volunteers (n = 3). To test whether these five intestinal microbes were able to metabolize DOPA and DHPLA, DOPA or DHPLA were added to each species of bacteria isolated from patients' fecal samples respectively, The culture media were analyzed by LC-MS after 24 h. Pilot transformation tests demonstrated that only *P. vulgaris* and *P. mirabilis* exhibited the ability to metabolize DOPA to DHPLA *in vitro*, while the other three species of bacteria did not (Supplementary Table 8).

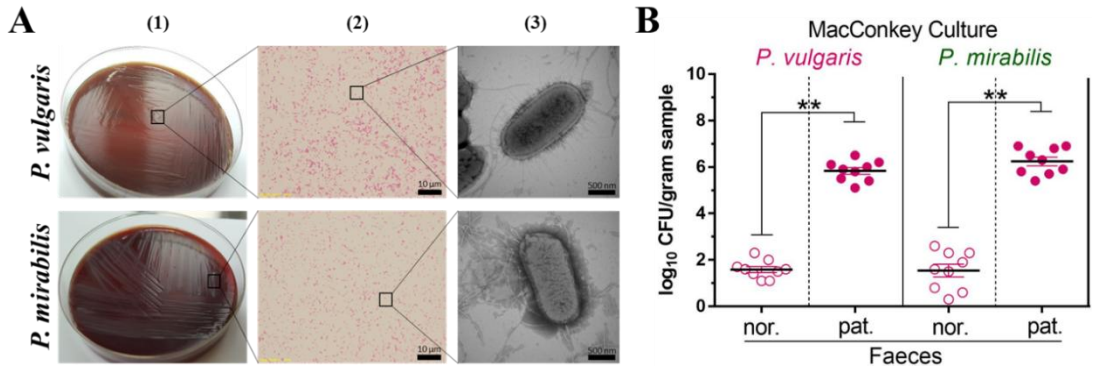


Figure 3. Identification of *P. vulgaris and P. mirabilis* and their relative abundance in the fecal samples of Chinese healthy volunteers and patients.

A. Morphological identification of *P. vulgaris* and *P. mirabilis* in patients' urine samples: (1) colony morphology on blood agar plates, (2) morphology under optical microscopy (Gram staining, ×1,000), (3) morphology under electron microscopy (×50,000). **B.** Quantitative analysis of *P. vulgaris* and *P. mirabilis* in the fecal samples of 3 healthy Chinese volunteers (nor.) and 3

patients (pat.). Each sample was tested in 3 technical replicates to ensure the reliability of single values. Data (log_{10} CFU/g sample) are expressed as mean ± s.d. $^{**}p < 0.01$ compared with healthy volunteers (One-way ANOVA).

Detailed analysis revealed that *P. vulgaris* and *P. mirabilis* were not only able to convert DOPA to DHPLA, but could also convert DHPLA to DOPA. Figure 4A and 4B show representative results of the LC-MS analysis of *P. vulgaris*-mediated transformation, and similar results were obtained for *P. mirabilis* (Figure 4C and 4D). The results illustrated that DOPA and DHPLA could be reversibly interconverted in *P. vulgaris* or *P. mirabilis*. In *P. mirabilis* cultures, an intermediate compound 3,4-dihydroxyphenylpyruvic acid (DHPPA) was also detected. These data provide evidence in support of the hypothesis that in response to inflammation and/or hypoxia, the *Proteus* species in patients' gut microbiota increased in abundance and metabolized DOPA to generate DHPLA. The reversible interconversion from DHPLA to DOPA shown are consistent with the observation that in response to medical treatment, the patients' urinary DHPLA levels gradually declined to below the LOD.

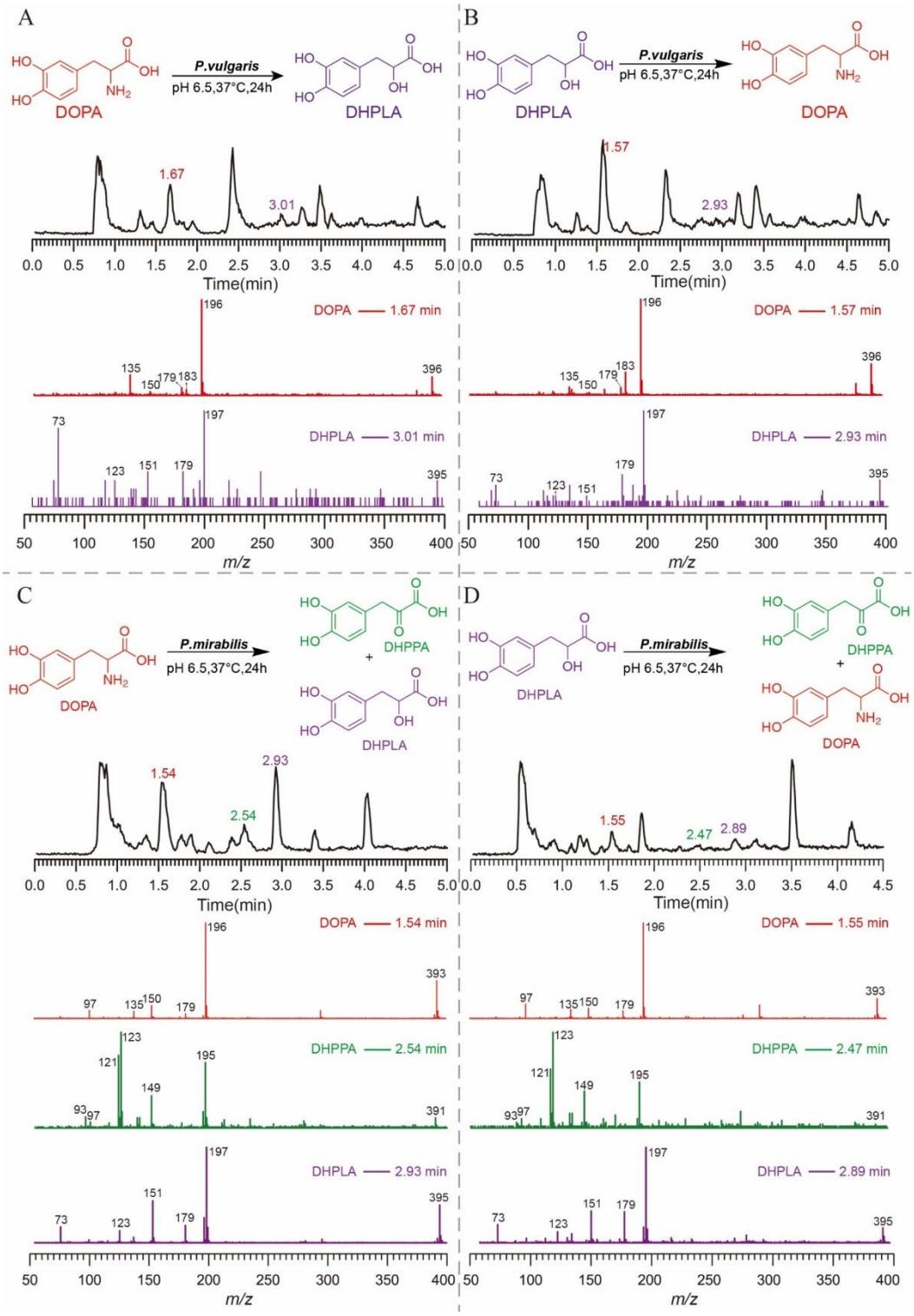


Figure 4. Reversible interconversion between DOPA and DHPLA in *P. vulgaris* or *P. mirabilis* isolated from patients.

A. Typical LC-MS spectrogram of culture medium of *P. vulgaris* incubated with DOPA (negative ion mode), showing the biotransformation of DOPA to DHPLA; **B.** Typical LC-MS spectrogram of culture medium of *P. vulgaris* incubated with DHPLA (negative ion mode) showing the biotransformation of DHPLA to DOPA; **C.** Typical LC-MS spectrogram of culture medium of *P. mirabilis* incubated with DOPA (negative ion mode) showing the biotransformation of DOPA to

DHPPA and DHPLA; **D.** Typical LC-MS spectrogram of culture medium of *P. mirabilis* incubated with DHPLA (negative ion mode) showing the biotransformation of DHPLA to DHPPA and DOPA. DHPPA: 3,4-dihydroxyphenylpyruvic acid.

Discussion

Here we report for the first time that the anti-inflammatory dihydroxylated phenolic acids DHPLA and IDHP are detected in the urine samples of both Chinese and Caucasian patients being treated for both severe cardiac symptoms as well as conditions associated with inflammation (WBC $\geq 10\times10^9$/L; hsCRP $\geq$ 3.0 mg/L) and/or hypoxia ($PaO_2 \leq$ 75 mm Hg), but undetectable in healthy volunteers. Our data suggest that these clinical findings are racially and geographically independent. More importantly, Figure 2 clearly shows that the urinary levels of DHPLA in those 9 inpatients were linked to their clinical indices. However, due to the patients suffering from different clinical conditions (e.g. sepsis, respiratory insufficiency, pneumonia), the DHPLA and IDHP levels in some samples were below LOD or LOQ. To improve such clinical studies, future work should apply stratification to control for confounding variables due to patient heterogeneity. For example, it will be useful to focus specifically on cardiovascular diseases, COPD, rheumatoid arthritis and COVID-19, for which clinical and laboratory parameters are well documented in the literature.

DHPLA is structurally composed of a catechol and a lactic acid, therefore chemically similar to catecholamines. However, these two groups of catechol-containing compounds have opposing activities in inflammation. Flierl and colleagues not only reported the ability of phagocytes to produce and release catecholamines *de novo* (21, 22), but also showed that both epinephrine and norepinephrine are potent inflammatory activators of macrophages, upregulating NF-κB and further downstream cytokine production (TNF-α, IL-1β, IL-6) of these cells (22). In contrast, DHPLA has been shown to inhibit beta-adrenergic receptor-mediated cardiac fibrosis (5) and attenuate advanced glycation end product-mediated neuroinflammation (10). IDHP exerted anti-inflammatory activity by abolishing TNF-α and IL-1β secretion from BV-2 mouse microglial cells stimulated by LPS (23), as well as attenuating

cardiac fibrosis induced by isoprenaline, via a NOX2/ROS/p38 pathway (16). Furthermore, Monagas *et al.*(24) showed a series of dihydroxylated phenolic acids derived from microbial metabolism present marked anti-inflammatory properties, inhibiting the secretion of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) from lipopolysaccharide (LPS)-stimulated human peripheral blood mononuclear cells.

We reasoned that DHPLA (and IDHP) might also be derived from DOPA like catecholamines, but through a yet unidentified pathway activated by pathological signals as part of a human self-protective and regenerative repertoire. To ensure that the DHPLA being measured in our study was actually generated from endogenous sources, we recruited only patients who had not received DOPA or drugs known to contain DHPLA two weeks prior to this study. Figure 2 demonstrates a positive correlation between DHPLA levels with WBC and hsCRP in these patients, and an inverse correlation with their PaO_2. Furthermore, dynamic monitoring of inpatients undergoing treatment clearly demonstrated their DHPLA levels declining in proportion to clinical improvement. In support of the postulate that DOPA can be metabolized to DHPLA in mammals, our experiments using mammalian tissue homogenates and different precursor substrates confirmed the ability of porcine heart homogenates to metabolize DOPA to DHPPA and DHPLA (Supplementary Table 5A), while the homogenates of various rat organs were able to metabolize DHPPA to DHPLA and DHPLA to IDHP (Supplementary Table 5B). In this context, Maini Rekdal *et al.* (25) have provided compelling evidence for enzymes that dihydroxylate host- and plant-derived catechols. Work is in progress in our laboratories to identify, isolate and characterize the corresponding enzymes that convert DOPA to dihydroxylated phenolic acids.

It has been shown that gut microbiota play a crucial role in the host's health and this complex community has the ability to interact with each other and with the host's immune system (18). A sizeable number of bacterial species in the gut are capable of metabolizing catechol-containing compounds. Indeed, Maini Redkal and colleagues reported *Enterococcous faecalis* completely decarboxylated DOPA to dopamine,

while *P. mirabilis* to be unable to do so (26). Thus, we investigated the possibility that the 5 dominant bacterial species (*Proteus vulgaris*, *Proteus mirabilis*, *Enterococcus faecalis*, *Enterococcus faecium* and *Escherichia coli*) identified in the patients' feces might be able to metabolize DOPA to DHPLA. In our hands, only *Proteus vulgaris* and *P. mirabilis* were able to metabolize DOPA to DHPLA and its active metabolite IDHP. Thus, while some species of gut microbes are able to metabolize DOPA to catecholamines via the classical pathway, other species may produce DHPLA and IDHP via an alternative DOPA-phenolic acid pathway under certain physiological and pathological conditions (Figure 5). Taken together, the work of Maini Rekdal *et al.* (26) and our findings here provide a good example for the important role of gut microbes in metabolizing DOPA into bioactive molecules for the maintenance of health (27).

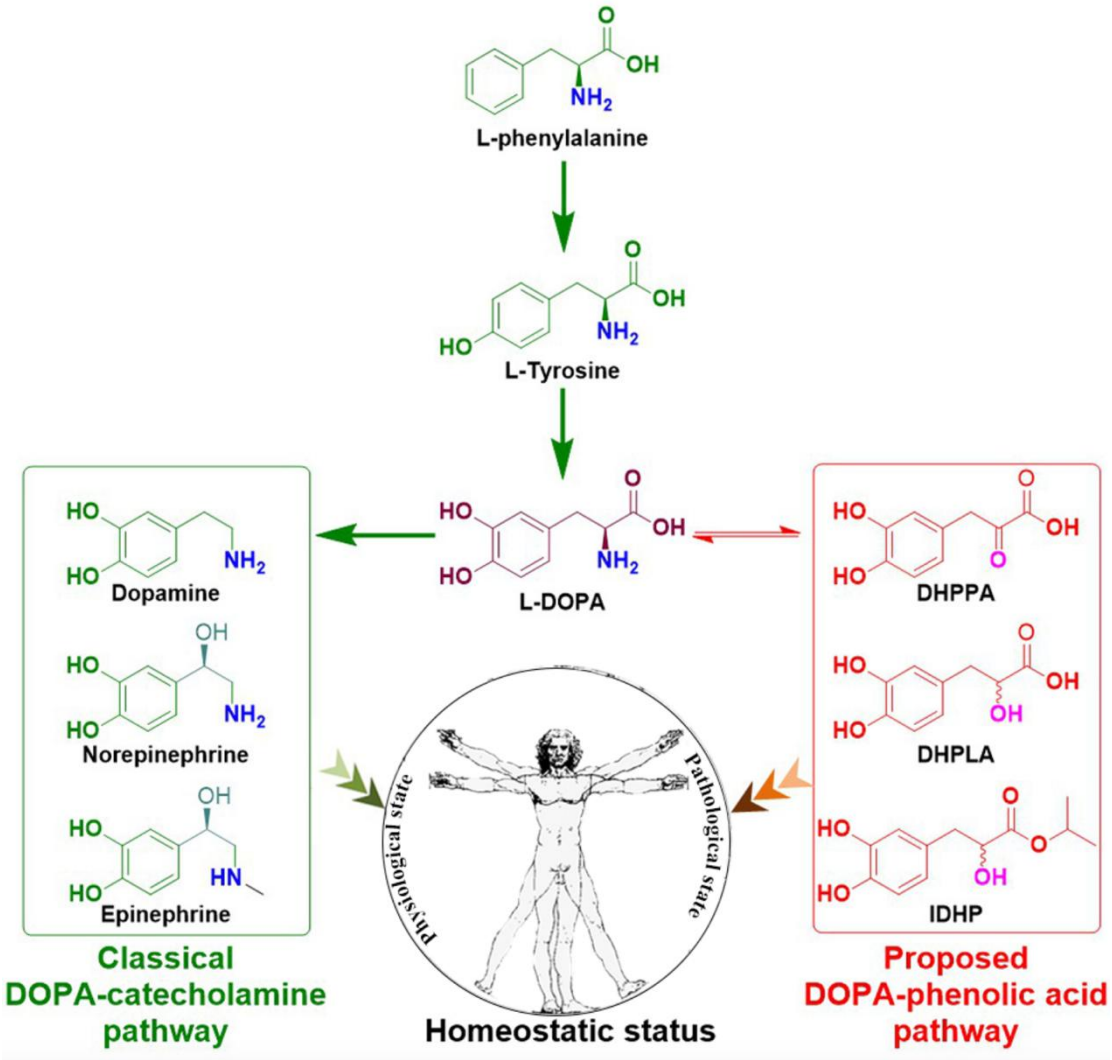


Figure 5. Hypothetical schema showing catecholamines and bioactive dihydroxylated phenolic acids derived from DOPA mediating the crosstalk between the nervous, cardiovascular and immune systems in health and disease.
The vast existing literature on dopamine shows that a Classical DOPA-catecholamine pathway is operational in adrenal medulla, postganglionic fibers of the sympathetic nervous system, mesenteric organs (gastrointestinal tract, spleen, and pancreas)(28), lymphocytes(29, 30), phagocytes(21) and microbes such as *Enterococcous faecalis*(26). Epinephrine and norepinephrine are potent inflammatory activators of macrophages, upregulating NF B and further downstream cytokine production (22). Here, the detection of DHPLA and IDHP in patients' urine, and the generation of these anti-inflammatory and vasodilator molecules by *Proteus* species as well as mammalian organs (heart, brain, liver, lungs, kidneys) suggest the existence of a DOPA-phenolic acid pathway that is activated in response to the varying physiological needs for these molecules under inflammation and/or hypoxia. The dynamic interplay between these two pathways maintains the homeostatic status of the nervous, immune and cardiovascular systems.

What is the significance of our discovery in terms of health, disease, and physiology as a whole? It is a well-known fact that cortisol is a hormone that is mainly released at times of stress. Our study provides good evidence that DHPLA, originally discovered in medicinal plants such as *Salvia miltiorrhiza* (red sage), can be generated in humans in response to stress such as inflammation and/or hypoxia. The circulating level of DHPLA might well be subject to homeostatic control. Under severe pathological conditions, the endogenous level of DHPLA may not be sufficient but can be augmented by exogenous DHPLA through the use of botanical drug formulations containing *S. miltiorrhiza.* Indeed, Compound Danshen Dripping Pills are used sublingually in China for treating acute myocardial infarction. Dantonic® (a composite Danshen formulation containing standardized extracts of *Salvia miltiorrhiza* and *Panax notoginseng* plus borneol) is in FDA Phase III trial for preventing and treating stable angina. (Clinical Trials.gov Identifier: NCT01659580).

As depicted in Figure 5, we hypothesize that through distinct but complementary pathways, catecholamines and dihydroxylated phenolic acids (DHPPA, DHPLA and IDHP) mediate the crosstalk between the nervous, cardiovascular and immune systems in maintaining the balance of physiological and pathological states. Based on this concept, we have explored the therapeutic potential of DHPLA and compounds derived from it. First, instead of extracting DHPLA from Radix *Salvia miltiorrhizae*

by laborious and costly conventional techniques, we have developed a whole-cell biotransformation method to produce DHPLA from DOPA (31). This was done by co-expressing L-amino acid deaminase, D-lactate dehydrogenase, and glucose dehydrogenase in engineered *Escherichia coli*. The large quantity of DHPLA enabled us to synthesize IDHP (32) for preclinical studies. Significantly, synthetic IDHP was shown to prevent isoprenaline-induced cardiac fibrosis through inhibiting a NOX2/ROS/p38 pathway, suggesting it to be a potential candidate drug for the treatment of heart diseases (16). Collectively, DHPLA and IDHP modulate the body's ability to mount and resolve responses to inflammation and/or hypoxia so as to ameliorate damage caused by pathological conditions. Our findings contribute to the understanding of the biology underlying the self-protective and regenerative repertoire of human defense mechanisms and shed new light on the development of treatment strategies for chronic inflammatory diseases.

As a hydrophilic molecule, DHPLA is poorly soluble in lipidic matrices (33). In our previous studies in rabbits, the bioavailability of DHPLA was found to increase, and the tissue distribution was improved by co-administration of borneol (34). According to the principle of Chinese medicine combinatorial formulations (35), borneol is considered to improve the bioavailability of principal therapeutic agents (36-38). Thus, we designed and synthesized DHPLA borneol ester (DBZ; 1,7,7-trimethylbicyclo[2.2.1]heptan-2-yl-3-(3,4-dihydroxyphenyl)-2-hydroxy-propano ate) by chemical combination of DHPLA and borneol (39). In apoE$^{-/-}$ mice, DBZ exerted anti-atherosclerotic effects that involve the inhibition of inflammation, macrophage migration, leukocyte adhesion and foam cell formation (40). Further studies revealed that DBZ promotes multiple steps of angiogenesis, at least in part through Akt and MAPK signalling pathways (41), as well as ameliorating neuroinflammation in LPS-stimulated microglia and ischemic stroke rats (42). With more rigorous pre-clinical and clinical research, we expect these DHPLA-inspired compounds to be developed into therapeutic agents for the prevention and treatment of cardiovascular and other chronic diseases.

METHODS

Study population. Healthy Chinese volunteers and patients were recruited from the First Affiliated Hospital of Xi'an Jiaotong University, Shaanxi Provincial People's Hospital and Xi'an North Hospital in China, while Caucasian patients were recruited from the Department of Intensive Care, Amsterdam UMC, location VUmc, the Netherlands. The patients met at least one of the following three clinical criteria: (a) white blood cell (WBC) count ≥ 10×10^9/L, (b) C-reactive protein (hsCRP) ≥ 3.0 mg/L and (c) oxygen partial pressure (PaO_2) ≤ 75 mmHg. For healthy volunteers, their WBC was 4×10^9-10×10^9/L, hsCRP was 0-3 mg/L, and PaO_2 was 75-100 mmHg. Those who had received agents known to contain DOPA or DHPLA two weeks prior to the study were excluded. All participants were confirmed to have no urinary tract infection.

Ten healthy Chinese subjects (5 males and 5 females; mean age: 41.6 ± 17.6 years) were enrolled in China and designated as Group I. A total of 43 Chinese patients (40 males and 3 females; mean age: 61.7 ± 14.3 years) were enrolled, and the Chinese patients who met any one of the three clinical criteria were designated as Group II. Those who met any two or three clinical criteria were designated as Group III. A total of 34 Caucasian patients (26 males and 8 females, mean age: 68.8 ± 12.8 years) were enrolled in the Netherlands. The whole experimental process was set as an analytically-blind test. Ethical approval for this study was granted by the Institutional Human Research Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University (Approval number: 2014 ($164^{\#}$)) and the Medical Ethical Committee of Vrije Universiteit Amsterdam (METc VU Medical Center number 2017.351), respectively.

Urine collection. First morning urine samples (before any drink and food; > 50 mL) from each healthy volunteer or each patient after admission to the hospital (e.g. in the first 3 hours when they entering ICU) were collected in sealed sterile bottles. Sodium azide (0.002% final concentration) was added as an antiseptic and kept at −80°C. To monitor dynamic changes of 9 patients undergoing medical treatment, their first

morning urine samples were collected for 8 consecutive days. As directed by attending physicians, patients were given diuretics and vasodilators for symptomatic treatment, or anti-platelet aggregation drugs and cholesterol lowering drugs for etiological treatment, according to their clinical diagnosis.

Preparation of urine samples. Three hundred microliter of the urine samples was lyophilized to remove all moisture, the dried samples were re-dissolved with 350 μL of anhydrous acetone by vortex oscillation for 2 min and ultrasound treatment for 15 min at 45 kHz. 300 μL supernatant solution obtained from anhydrous acetone extract centrifugate for 10 minat 12000 × *g* was transferred to derivatization vials with 300 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA). The mixture was subjected to ultrasound treatment for 1.5 h at 45 kHz at 40°C for silylation for subsequent analysis.

Detection of DHPLA in urine samples by the GC-QQQ-MS/MS. GC-MS/MS analysis of urine samples of 34 Caucasian and 5 Chinese patients was performed with an Agilent 7890 GC system with an HP-5 UI MS column (30 m × 0.25 mm I.D., 0.25 μm film thickness) coupled to a 7010 triple-quadrupole mass spectrometer (Agilent Technologies Inc., USA). The flow rate of the carrier gas nitrogen was 1.2 mL/min. The GC oven temperature was initially increased from 60°C to 180°C at 20°C/min, then increased to 240°C at 2°C/min, held for 2 min, and finally increased to 300°C at 50°C/min. The total run time was 39.2 min. The GC was interfaced with a mass-selective detector using electron ionization with an electron energy of 70 eV. The temperatures of the auxiliary heat source, MS source and quadrupole were set at 280°C, 230°C and 150°C, respectively. The split ratio was set at 1:1. The injection volume was 1 μL. The data were analyzed using Agilent MassHunter Workstation software, version B.09.00.

The GC-QQQ-MS/MS method was established for determination of the DHPLA level in human urine (Supplementary Tables 3-5). The calibration range (0.7−11.0 ng/mL), limit of quantification (LOQ, 0.7 ng/mL), LOD (0.2 ng/mL), recovery (82.2−89.3%), precision and stability (freeze-thaw stability: RSD ≤ 10%, stability after preparation: RSD ≤ 16%) were established in accordance to the standard

biological sample analysis. DHPLA standards were purchased from the National Institutes for Food and Drug Control (NIFDC), Batch no. 110855-201614.

Preparation of urine samples and the HPLC-FLD method. Urine samples (24 mL) were acidified with 6 mol/L hydrochloric acid (12 mL) containing ascorbic acid (5% final concentration) as anti-oxidant, then were prepared for 1 minute by using vortex mixer. The mixed solution was extracted with 72 mL ethyl acetate for three times, and centrifuged for 10 min at 9000 × *g*. The combined organic layers were vacuum concentrated at 40℃, then the residue was re-dissolved in 1 mL of methanol/water (0.2% formic acid) and centrifuged for 10 min at 9000 × *g*. The supernatant solution was filtered through a 0.22 μm Nylon filter into a sample vial for HPLC-FLD analysis.

HPLC-FLD analysis of the urine samples was carried out on an Agilent 1100 HPLC system. Separation was performed on an Agilent HC-C_{18} column (4.6 × 250 mm, 5 μm) at a temperature of 25°C with a sample injection volume of 20 μL; the effluent was excited at 278 nm, and their emission was measured at 320 nm. The mixture of solvent A (water with 0.2% formic acid) and solvent B (methanol) were used as mobile phase at the flow rate of 0.6 mL/min. The gradient elution procedure and methodology investigation results were presented in Supplementary Table 9-11. Reference standard DHPLA was purchased from the National Institutes for Food and Drug Control (NIFDC), Batch no. 110855-201614.

Isolation and identification of DHPLA in urine. Urine samples were prepared as described in the section above with an Agilent HC-C_{18} column (4.6 mm × 250 mm, 5 μm). The mobile phase was composed of water with 0.2% formic acid and methanol. The fractions with a retention time from 23 min to 27 min were collected and combined, the solvent was volatilized, and the sample was re-dissolved in 1.0 mL of methanol/water (0.2% formic acid) and analyzed by an Agilent 6520 Accurate-Mass Q-TOF LC/MS system (Agilent Technologies Inc., USA) with an Agilent Eclipse XDB-C_{18} (2.1 mm × 150 mm, 3.5 μm) column. The mobile phase consisted of 0.1% acetate acid (A) and methanol (B) in water with a constant ratio of A:B = 90:10 at a flow rate of 0.20 mL/min. The injection volume was 20 μL. The other conditions were

as follows: time range: 25 min; scan range: 100-1000; ion source: dual ESI; polarity: negative; ion spray voltage: 3500 V; dry gas temperature: 350°C; dry gas: 10.0 L/min (N_2); nebulizer pressure: 45 psi (N_2); fragmentor: 130 V; collision energy: 10, 25 and 40 eV, respectively. The compound structure was analyzed by ^{1}H NMR. The nuclear magnetic resonance spectra (^{1}H NMR) were recorded on Varian Gemini 2000 (600 MHz) in DMSO-d_6 using TMS as an internal standard. The chemical shifts δ were expressed in parts per million (ppm).

Metabolism of DOPA, DHPPA and DHPLA in homogenates of mammalian organs. Fresh pig hearts were purchased from a local (super)market and subjected to the experimental procedure as for rat organs as detailed below. Male Sprague-Dawley rats (SPF grade, Laboratory Animal Production Permit (Shaan) SCXK-2018-001) weighing between 250-300 g, were purchased from the Experimental Animal Centre of Xi'an Jiaotong University (Shaanxi, China). The animals were acclimatized for 1 week after arrival and maintained under specific pathogen-free conditions. After sacrificing, different organs such as brain, heart, liver, spleen, lung, kidney and other tissues were quickly collected on ice. Pig hearts were purchased from supermarket. 1 g of small pieces of organs/tissues was taken and homogenized with physiological saline (2 mL/g), then (1) 5mg DHPLA and 20 μL isopropanol were added to the homogenate; or (2) 5mg 3,4-Dihydroxyphenylpyruvate (DHPPA) was added to the homogenate; or (3) 5mg DOPA was added to the homogenate. The different homogenates were incubated in a water bath shaker at 37°C (rotating speed 100 r/min) for 6 hours. Then 0.5 mL of the homogenate was taken and centrifuged for 10 min at 9000 × *g*. 200 μL of the supernatant was taken and 3 times amount of methanol were added for precipitation of proteins, subsequently the sample was vortexed for 60 s, and centrifuge for 10 min at 9000 × *g*. The supernatant was taken and dried by nitrogen, the dried material was dissolved in mobile 10 % acetonitrile vortexed for 120 s, sonicated for 5 min, centrifuged for 10 min at 9000 × *g*, and finally filtered through a 0.22 μm organic filter. The samples were analyzed by HPLC-MS. Ethical approval for this study was granted by the Experimental Animal Management and

Ethics Committee of Northwest University (Approval number: NWU-AWC-20190306R).

Identification of microorganisms. All clinical samples including urine, feces and peripheral venous blood samples were collected from the recruited patients and healthy volunteers in the First Affiliated Hospital of Xi'an Jiaotong University in China from November to December 2015. Peripheral venous blood samples were injected into aerobic and anaerobic blood culture flasks, then were put into an automatic mycobacteria culture monitoring system (BacT/ALERT® 3D) for bacterial amplification until the growth of bacteria was observed. The amplified bacteria were aspirated and inoculated on Columbia blood agar medium and cultured at 35°C for 24 h. One microliter urine was drawn using one-off sterile inoculation ring and inoculated on Columbia blood agar medium and MacConkey agar medium at 35°C for 24 h. The same operation was performed on a small amount of fecal samples with size as large as sesame seed. Two or three bacterial clones cultured from blood or urine or feces were picked out and mixed with 0.45% normal saline to 0.5 McFarland turbidity, respectively. The strains were identified by automatic microbial identification and a drug sensitivity analysis system. Gram-positive cocci and Gram-negative bacilli were identified by GP identification cards (batch number: 241336510) and GN identification cards (batch number: 242344040), respectively. The reference standard bacterial strains were *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25913, *Enterobacter cloacae* ATCC 700323 and *Enterococcus casselifavus* ATCC 700327. The identification results were checked by an automatic rapid microbial mass spectrometry detection system (VITEK MS). The isolated microorganisms were systematically identified and classified by the 16S rRNA gene method. The strains were placed in a microbial strain storage tube and stored at −80°C for preservation.

Reversible interconversion between DOPA and DHPLA by Proteus species isolated from patients' urine and fecal samples. Five dominant species of bacteria were observed in the DHPLA-positive patients' fecal samples: *Proteus vulgaris*, *Proteus*

mirabilis, *Enterococcus faecium*, *Enterococcus faecalis* and *Escherichia coli*. They were incubated in LB medium (10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl) for 24 h, then bacterial cells were obtained by centrifuging the fermentation broth at 8000 × *g* for 15 min. The cells were washed with distilled water twice to remove the residual medium and diluted to an OD600 of 5 with 50mM Na_2HPO_3-NaH_2PO_3 buffer (pH 6.5). For biotransformation experiments, DOPA or DHPLA (0.5 mg/mL) was added to each of the bacterial suspension and incubated at 37°C for 24 h. The transformed products were detected by LC-MS, as reported by Zhang *et al.*(43)

Statistical analysis. Statistical analysis was performed with Statistical Product and Service Solutions software version 19.0.0.329. Comparisons between pairs of groups were carried out with one-way ANOVA, and $*p < 0.05$ was considered statistically significant.

Data Availability. All study data are included in the article and/or supporting information.

Author contributions

X.H.Z., X.P.Z. and T.P.F. conceptualized and designed the project. X.H.Z., X.P.Z., T.P.F., M.W., Y.J.C., Y.Z. and Y.J.B. planned and supervised the research and analyzed the data. Y.J.B., Y.Z., Y.J.C., M.W. and T.P.F. wrote the manuscript. X.X.W., Y.Y.C., J.W. and Y.J.B. optimized the HPLC-FLD detection procedures. Y.J.B., X.X.W., X.K.G. and X.X.A. developed the HPLC-UV purification procedure, purified DHPLA from urine, performed the trials and collecting/optimized the sample preparation conditions. L.J.Y., P.J., S.M.G., Y.S. and J.X. performed DHPLA detection and analysis by LC-Q-TOF-MS. X.M., K.L. and J.L. validation and confirmation of DHPLA by LC-Q-TOF-MS. P.J., X.W., Q.N.L. and Y.Z. optimized the GC-MS detection procedures. X.W., X.X.W., J.C.F., P.L., F.G.Q., Q.Y., X.R.H., Q.X.Y. and R.G. helped with the collection of urine samples from hospitals in China. W.B.Z. synthesized DHPLA and helped to analyse structures. X.L.Z., Q.Z.Z. and Y.F.N. validated the synthesis of DHPLA. J.E.L., L.X.D., Y.B.X. and N.E.Z. separated and analyzed the microorganisms from blood, urine and feces collected

from patients. Y.J.C., J.W., H.X.D., J.J.X., T.Z.X. and J.B.L. cultured *Proteus* and used it in the transformation experiment for DOPA and DHPLA. E.A., A.M.E.M. and M.W. were responsible for the collection of urine samples in Europe. H.K.K., S.J.H., Y.S.J., J.R., P.J. and Y.Z. tested urine samples from Europe by GC-MS. Y.J.Z., S.L., S.X.W., X.F.Z., C.N.X., J.Y., P.Y.Y., Q.S.L., R.W., N.P., X.W.W., X.L., H.J.L., Y.B., Z.J.L., Y.Y.Z., and J.B.Z. provided guidance, specialized techniques and expertise. Y.F and Y.Y carried out the transformation experiment of DOPA, DHPPA and DHPLA in mammalian tissues. All authors discussed the results, commented on the studies and contributed to aspects of manuscript preparation.

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Notes

The authors declare no competing financial interest.

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