

# *5-(3',4'-Dihydroxyphenyl)- $\gamma$ -Valerolactone is a substrate for human paraoxonase: a novel pathway in flavan-3-ol metabolism*

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# 5-(3',4'-Dihydroxyphenyl)- $\gamma$ -Valerolactone Is a Substrate for Human Paraoxonase: A Novel Pathway in Flavan-3-ol Metabolism

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**Scope:** Dietary flavan-3-ols are known to mediate cardiovascular benefits. Currently, it is assumed that the levels of flavan-3-ol catabolites detected in humans, 5-(3',4'-dihydroxyphenyl)- $\gamma$ -valerolactone ( $\gamma$ VL) and 5-(3',4'-dihydroxyphenyl)- $\gamma$ -valeric acid ( $\gamma$ VA), and their corresponding phase II metabolites, are determined exclusively by the action of the gut microbiome. However, a family of human proteins, paraoxonase (PON), can theoretically hydrolyze  $\gamma$ VL metabolites into the corresponding  $\gamma$ VAs. This study aims to determine if PON is involved in  $\gamma$ VL and  $\gamma$ VA metabolism in humans.

**Methods and results:** A rapid conversion of  $\gamma$ VL into  $\gamma$ VA is detected in serum *ex vivo* (half-life =  $9.8 \pm 0.3$  min) that is catalyzed by PON1 and PON3 isoforms. Phase II metabolites of  $\gamma$ VL are also reacted with PON in serum. Following an intake of flavan-3-ol in healthy males ( $n = 13$ ), the profile of  $\gamma$ VA metabolites detected is consistent with that predicted from the reactivity of  $\gamma$ VL metabolites with PON in serum. Furthermore, common PON polymorphisms are evaluated to assess the use of  $\gamma$ VL metabolites as biomarkers of flavan-3-ol intake.

**Conclusion:** PONs are involved in flavan-3-ol metabolic pathway in humans. PON polymorphisms have a minor contribution to inter-individual differences in the levels of  $\gamma$ VL metabolites, without affecting their use as a nutritional biomarker.

## 1. Introduction

Flavan-3-ols, including (–)-epicatechin and (+)-catechin, as well as their oligomers, the procyanidins, are a class of polyphenolic bioactives found in foods and beverages such as tea, pome fruits, cocoa products, and berries.<sup>[1–3]</sup> Accumulating epidemiological evidence, most notably a recent large-scale randomized clinical study, have shown that flavan-3-ol intake can exert beneficial cardiovascular effects.<sup>[4–6]</sup> These reports supported the creation of dietary recommendations for the consumption of flavan-3-ols,<sup>[7]</sup> recognizing these bioactives as a valuable tool for primary prevention of cardiovascular disease and the promotion of healthy aging. After intake, flavan-3-ols are extensively transformed into a wide variety of metabolites that are thought to be the actual mediators of the beneficial effects observed after flavan-3-ol intake.<sup>[8,9]</sup> The most abundant group of flavan-3-ol metabolites in humans is represented by 5-(3',4'-dihydroxyphenyl)- $\gamma$ -valerolactones ( $\gamma$ VLs) and their

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phase II metabolites.<sup>[10,11]</sup> Although their potential biological role remains to be established, an increasing number of investigations in vitro provide some initial information about the possible biological activities of these compounds.<sup>[12–14]</sup> Furthermore, a particular set of  $\gamma$ VL metabolites have been validated as nutritional biomarkers to objectively assess flavan-3-ol intake.<sup>[4,15]</sup> Thus, to fully characterize the metabolic pathways modulating  $\gamma$ VL levels, it is necessary to understand their potential biological relevance and use as nutritional biomarkers of flavan-3-ol intake.

$\gamma$ VLs are products of gut microbiome-mediated catabolism of dietary flavan-3-ols.<sup>[16]</sup> After absorption,  $\gamma$ VLs are conjugated by phase II reactions, yielding sulfated, and to a lesser extent, glucuronidated and methylated metabolites.<sup>[10]</sup> Once these metabolites reach the systemic circulation, it is assumed that a substantial portion of the  $\gamma$ VL metabolite pool does not undergo any further metabolic transformation before being excreted in urine. However, a family of human enzymes, namely paraoxonases (PONs), could potentially metabolize circulating  $\gamma$ VL metabolites and thus modulate  $\gamma$ VLs levels. PONs are hydrolase enzymes with diverse catalytic activities, including that of lactonases, which catalyze the hydrolysis of various lactone-containing compounds.<sup>[17–19]</sup> As such, PONs could theoretically hydrolyze both,  $\gamma$ VL and  $\gamma$ VL metabolites, thus generating the corresponding 5-(3',4'-dihydroxyphenyl)- $\gamma$ -valeric acids ( $\gamma$ VAs), which are hitherto thought to be derived exclusively from the gut microbiome. PONs are calcium-dependent enzymes<sup>[20]</sup> present as three isoforms (PON1, PON2, and PON3).<sup>[21]</sup> PON1 and PON3 are expressed in the liver and are also found in the systemic circulation associated with high-density lipoproteins (HDL),<sup>[22,23]</sup> while PON2 is expressed intracellularly in many types of human cells, including gastric enterocytes and vascular endothelial cells.<sup>[24]</sup> Various polymorphisms have been described for all PON isoforms, with PON1 presenting polymorphisms known to contribute to inter-individual differences in activity and expression levels of the enzyme.<sup>[25–28]</sup> Taken together, PON has a potential to take part in the metabolism of  $\gamma$ VLs, which may affect the putative biological activities of  $\gamma$ VLs and the use of  $\gamma$ VL metabolites as nutritional biomarkers of flavan-3-ol intake.

This study aimed at determining whether or not dietary flavan-3-ol-derived  $\gamma$ VLs are metabolized into  $\gamma$ VAs by PON in humans. Additional studies were conducted to understand the impact of this metabolic pathway by exploring the influence of common PON polymorphisms on inter-individual differences and use of  $\gamma$ VLs metabolites as nutritional biomarkers of flavan-3-ol intake.

## 2. Experimental Section

### 2.1. Materials

Authentic analytical standards, including  $\gamma$ VL, 5-(4'-hydroxyphenyl)- $\gamma$ -valerolactone-3'-sulfate, 5-(3'-hydroxyphenyl)- $\gamma$ -valerolactone-4'-sulfate, 5-(4'-hydroxyphenyl)- $\gamma$ -valerolactone-3'-glucuronide, 5-(3'-hydroxyphenyl)- $\gamma$ -valerolactone-4'-glucuronide, 5-(3'-methoxyphenyl)- $\gamma$ -valerolactone-4'-glucuronide, 5-(4'-methoxyphenyl)- $\gamma$ -valerolactone-3'-glucuronide, 5-(3'-methoxyphenyl)- $\gamma$ -valerolactone-4'-sulfate and 5-(4'-methoxyphenyl)- $\gamma$ -valerolactone-3'-sulfate were provided by Mars, Inc. (McLean, VA, USA).  $\gamma$ VA was obtained from Toronto Research Chemicals, Inc. (Toronto, Canada). In the absence of authentic standards a ref-

erence material was created for the identification of  $\gamma$ VA metabolites (Figure S1 and Table S1, Supporting Information). Isomer-specific PON antibodies were purchased from ThermoFisher (Waltham, MA, USA; PON1), MilliporeSigma (St. Louis, MO, USA; PON3), and Santa Cruz Biotechnology (Dallas, TX, USA; PON2). Cloning vectors for PON1, PON2, and PON3 were purchased from GenScript (Piscataway, NJ, USA). All other chemicals were purchased from MilliporeSigma.

### 2.2. PON Lactonase Activity Assay

PON lactonase activity was assessed by adding 50  $\mu$ L of pooled male human serum purchased from MilliporeSigma to 50  $\mu$ L of PBS containing 2 mM CaCl<sub>2</sub> or 10 mM EGTA and 2  $\mu$ M of one of the  $\gamma$ VL metabolites as substrates. For assessing PON lactonase activity in heat inactivated serum, pooled human serum was incubated at 57 °C for 30 min and cooled to room temperature prior to its use in the assay. For the assessment of PON2 lactonase activity in HUVECs, 90  $\mu$ L of HUVEC cell samples (see below for collection method), pre-treated with 1 mM CaCl<sub>2</sub> or 5 mM EGTA, were mixed with 10  $\mu$ L of 10  $\mu$ M  $\gamma$ VL. For the assessment of PON isomer-specific lactonase activity in PON-transfected HEK293FT cells, 90  $\mu$ L of HEK293FT cell samples (see below for collection method) pre-treated with 1 mM CaCl<sub>2</sub> or 5 mM EGTA were mixed with 10  $\mu$ L of 10  $\mu$ M  $\gamma$ VL. Assay mixtures were incubated for 1 h at 37 °C. For a time response curve of serum PON lactonase activity, samples were collected at 0 (baseline), 10, 20, 30, 45, and 60 min following an incubation at 37 °C. For a  $\gamma$ VL dose-response curve of serum PON lactonase activity,  $\gamma$ VL concentrations ranging from 10 to 500  $\mu$ M were incubated for 15 min at 37 °C. Following the corresponding incubation period, 10  $\mu$ L of 10  $\mu$ M 4-methylumbelliferyl-sulfate was added as a recovery standard and the reaction was terminated by adding 400  $\mu$ L of 1% formic acid/acetonitrile solution. Samples were prepared for UPLC-MS analysis by filtering through Phree solid phase extraction plates (Phenomenex, Torrance, CA, USA). Eluted samples were concentrated by vacuum centrifugation to reduce the volume to approximately 100  $\mu$ L. Samples were stored at –20 °C prior to analysis by UPLC-MS. Standard curves using authentic standards were generated for  $\gamma$ VA,  $\gamma$ VL, and  $\gamma$ VL phase II metabolites. PON lactonase activity using  $\gamma$ VL metabolites as substrates was expressed as the percentage of initial substrate consumed during incubation. To ensure the disappearance of  $\gamma$ VL metabolites during the incubation was due to PON activity, experiments with each  $\gamma$ VL metabolites were repeated in the presence of 5 mM EGTA. In addition, the appearance of the respective  $\gamma$ VA metabolites was monitored using reference standards (Figure S1 and Table S1, Supporting Information).

### 2.3. Cell Culture

Pooled human umbilical vein endothelial cells (HUVEC) purchased from PromoCell (Heidelberg, Germany) were seeded onto 100 mm dishes at a density of 5000 cells cm<sup>-2</sup> per 230  $\mu$ L of growth media (PromoCell ECGM2) and cultured to confluence. At confluence, cells were rinsed twice with cold PBS solution and the wash solution was aspirated as much as possible.

Cells were then scraped with 1 mL of lysis buffer containing 25 mM Tris pH 7.5, 1 mM CaCl<sub>2</sub>, and protease inhibitor cocktail without EDTA (Roche, Mannheim, Germany), and transferred to microcentrifuge tubes. Cell samples were probe-sonicated on ice with multiple short bursts and centrifuged at 16 000 × g for 5 min. Protein content in supernatant samples was quantified using BCA protein assay (Pierce BCA gold) and samples were stored at −20 °C.

#### 2.4. HEK293FT Cell Expression of Isomer-Specific PON

HEK293FT cells were seeded onto 100 mm dishes at a density of 5000 cells cm<sup>−2</sup> per 230 μL of growth media (10% FBS/DMEM supplemented with non-essential amino acids) and cultured to approximately 75% confluence. HEK cells were transfected with human PON1 (NM\_000446.7), PON2 (NM\_000305.3), or PON3 (NM\_000940.3) inserted in a pcDNA3.1(+) vector, using Lipofectamine 3000 (ThermoFisher), according to the manufacturer's protocol. After 40 h of incubation, cells were harvested and stored at −20 °C as described above.

#### 2.5. Western Blot Analysis

For western blot analysis, cell samples prepared as described above were mixed with 2× concentrated Laemmli buffer at a 1:1 ratio. Pooled serum sample was mixed with 1× Laemmli buffer at 1/100 ratio. Samples were boiled at 95 °C for 15 min and quickly chilled on ice. Samples were separated on 8–16% gradient polyacrylamide gels and transferred onto PVDF membranes. PVDF membrane was incubated with PON1, PON2, or PON3 antibody followed by a secondary antibody conjugated to horseradish peroxidase. Protein bands were visualized by enhanced chemiluminescence (Genesee, San Diego, CA, USA) and digital images were captured on a Bio-Rad (Hercules, CA, USA) instrument.

#### 2.6. Dietary Intervention for the Quantification of γVA and γVL Metabolites after Flavan-3-ol Intake

The dietary intervention study consisted of a single acute intake, single arm, non-randomized study in which adult men were asked to consume a flavan-3-ol-containing drink. Test drink consisted of a fruit-flavored beverage mix prepared with a flavan-3-ol-containing Cocompro cocoa extract. Cocoa flavanols were defined as the flavan-3-ol monomers and procyanidins with a degree of oligomerization up to seven obtained from the seeds of *Theobroma cacao* L. and quantified using the official AOAC2020.05 method.<sup>[29]</sup> The amount of (−)-epicatechin and cocoa flavan-3-ols provided with the test drink was 160 and 829 mg 70 kg<sup>−1</sup> BW, respectively. Test drink was provided by Mars, Inc. Healthy men between 25 and 40 years of age were recruited by public advertisement in the city of Davis and surrounding areas (CA, USA). Exclusion criteria included a body mass index higher than 30 kg m<sup>−2</sup>, blood pressure (BP) higher than 140/90 mmHg, allergies to peanut or cocoa, avoidance of caffeinated food products and beverages, a history of cardiovascular disease, stroke, renal, hepatic, or thyroid disease, gastrointestinal tract disorders, previous gastrointestinal surgery (except appendectomy), the current

intake of herbal-, plant-, or botanical-containing dietary supplements, persons following vegan/vegetarian diet, and those adhering to an uncommon diet or a weight loss program. To determine eligibility, participants were asked to complete health and lifestyle questionnaires, have their height, weight, and in-office BP determined and to provide a blood sample for the assessment of different clinical laboratory parameters. Enrolled participants started the study protocol between 1 and 3 weeks after eligibility was determined and were asked to maintain their typical daily activities and diet for the duration of the study. To control for potential dietary flavan-3-ol intake during study days, volunteers were asked to follow a defined low-flavan-3-ol diet on the day prior to and during the study day. All volunteers were instructed on how to follow a low-flavan-3-ol diet, receiving foods containing low or negligible amounts of flavan-3-ols including the dinner for the night previous to the study day. Additionally, volunteers were asked to restrain from consuming alcohol, coffee or any other caffeine-containing beverages for 1 day prior and during the study day. Volunteers were required to fast overnight (12 h, water ad libitum) before the study day. Before test drink consumption, volunteers were asked to collect urine samples corresponding to −12 to 0 h. After test drink intake, urine was collected over 36 h in four collection periods (from 0 to 4 h post intake, 4 to 8 h post intake, 8 to 12 h post intake, and 12 to 36 h post intake). Urine was collected in a fresh container for each collection period, using 20 mL of 2 M sodium acetate (pH = 4.5) and 2 mL of 0.5% w/v thymol in isopropanol as preservatives. Volunteers returned the containers upon completion of sample collection, and urine aliquots were stored at −80 °C until analysis. Dietary intervention was conducted at the Ragle Human Nutrition Center, Department of Nutrition, UC Davis. The study protocol (#429275) was approved by UC Davis internal review board under its ethical guidelines. All of the participating volunteers reviewed, understood, and signed the informed consent form prior to the study. A total of 14 men were screened for eligibility and 13 volunteers were enrolled and completed the study. No adverse events were reported during the study. Characteristics of volunteers are shown in Table S2, Supporting Information.

#### 2.7. UPLC-MS Analysis

Serum and cell supernatants from PON lactonase activity assays were thawed at 4 °C and filtered through 0.2 μm syringe filters before injection. Urine samples derived from the dietary intervention studies were mixed with 10 μM 3-methyl-hippuric acid (internal standard) in 0.1% formic acid solution at 1:1 ratio and filtered through 0.2 μm syringe filters before injection. Samples were analyzed using a Dionex UltiMate 3000 UPLC system equipped with a Phenomenex Kinetex 2.6 μm F5 column (100 × 4.6 mm). The elution conditions at a flow rate of 0.5 mL per min were as follows: line A, 0.1% formic acid solution; line B, 90% acetonitrile and 10% methanol solution; line C, 5% 0–2 min, 5–18% linear gradient from 2 to 13 min, 18–95% from 13 to 13.1 min, held at 95% from 13.1 to 15 min, 95–5% from 15 to 15.1 min, held at 5% from 15.1 to 16 min (end of run). Detection was achieved with a Thermo Scientific Q Exactive Focus mass spectrometer (MS) system fitted with an electrospray interface (ESI; Thermo Fisher), with an ESI in negative ionization mode. Data

**Table 1.** PON1 single nucleotide polymorphisms investigated.

PON1 single nucleotide polymorphism		Minor allele frequency		
		Global population <sup>a)</sup>	European population <sup>a)</sup>	EPIC-Norfolk
rs662	Coding region, missense variant (Q192R)	0.31	0.29	0.29
rs854560	Coding region, missense variant (L55M)	0.35	0.37	0.36
rs705379	Promoter region (-108C/T)	0.41	0.45	0.47
rs705381	Promoter region (-162A/G)	0.24	0.24	0.25
rs854572	Promoter region (-909G/C)	0.45	0.49	0.47

<sup>a)</sup> Data were obtained from NCBI (<https://www.ncbi.nlm.nih.gov/snp/>)

processing was performed using Xcalibur software program version 4.4. MS conditions were set by tuning with 5-(4'-hydroxyphenyl)- $\gamma$ -valerolactone-3'-glucuronide dissolved in the initial UPLC mobile phase into the source at a flow rate of 0.5 mL min<sup>-1</sup>. Capillary temperature was 300 °C; sheath and auxiliary gasses were 35 and 5 units min<sup>-1</sup>, respectively; source voltage was 4 kV; S-lens radio frequency level was 50%. The mass spectrometer was operated in full scan mode from *m/z* 150 to 600. The identification and quantification of  $\gamma$ VA,  $\gamma$ VL, and conjugated  $\gamma$ VL metabolites were based on coelution and standard curves prepared with authentic standards. Since the authentic standards for conjugated  $\gamma$ VA metabolites were not available, a reference material was created for their identification based on retention time and fragmentation pattern (Figure S1 and Table S1, Supporting Information). Quantifications of  $\gamma$ VA metabolites were estimated with standard curves using the corresponding  $\gamma$ VL metabolites as surrogate standards and further correcting by the ratio of  $\gamma$ VA/ $\gamma$ VL signals to account for differences in signal between lactone and acid forms (Table S3, Supporting Information). This approach proved to perform better than other methods commonly used to quantify polyphenol metabolites with surrogate standards (Table S3, Supporting Information). Amount of  $\gamma$ VA and  $\gamma$ VL metabolites in 0–36 h urine were expressed in  $\mu$ moles.

## 2.8. PON Polymorphisms and Inter-Individual Differences in $\gamma$ VL Metabolite Levels and Use of $\gamma$ VL Metabolites as Biomarkers of Flavan-3-ol Intake

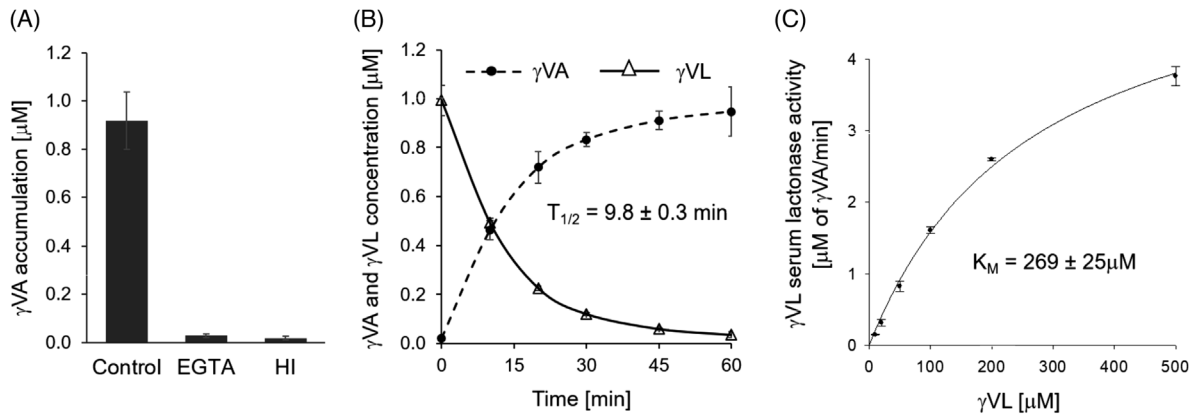
A series of genetic variants of PON1 (single nucleotide polymorphisms, SNP) with a wide distribution in the general population (minor allele frequency >20%) and known to modulate enzyme activity or expression level<sup>[30–33]</sup> (Table 1) were selected to test the impact on inter-individual differences and use of a specific set of  $\gamma$ VL metabolites, namely the sum of  $\gamma$ VL-3'-sulfate and  $\gamma$ VL-3'-glucuronide ( $\gamma$ VLM<sub>B</sub>), as a biomarker of flavan-3-ol intake. To accomplish this, urinary concentrations of  $\gamma$ VLM<sub>B</sub> were compared among the different variants of the selected PON1 polymorphisms in a sub-cohort of the EPIC-Norfolk study (*n* = 16 672). Data were adjusted by specific gravity as described elsewhere.<sup>[15]</sup> Data analysis was conducted after stratifying by sex and adjusting by age (Model 1), by age and biomarker-estimated (–)epicatechin intake (i.e., the sum of structurally related (–)epicatechin metabolites [SREMs]<sup>[34]</sup>; Model 2), and age

and HDL (Model 3). Differences in biomarker concentration between genotypes were calculated using the age-adjusted model and anti-logged after calculation. Data were evaluated with the Wald-test and significance was considered when *P* < 0.05. Differences in the distribution of PON1 polymorphisms between quintiles of  $\gamma$ VLM<sub>B</sub> were investigated using chi-squared test. Results were not adjusted for multiple testing. The impact of PON1 polymorphisms on the suitability of  $\gamma$ VLM<sub>B</sub> as nutritional biomarker were investigated by including genotype data in a linear regression model used previously.<sup>[4]</sup> Briefly, in sex-stratified models, the study investigated the association between systolic blood pressure as dependent variable and  $\gamma$ VLM<sub>B</sub> (log<sub>2</sub> transformed) adjusted by age, BMI, and genotype. The Wald-test was used to establish the contribution of each variable. Significance was considered when *p* < 0.05.

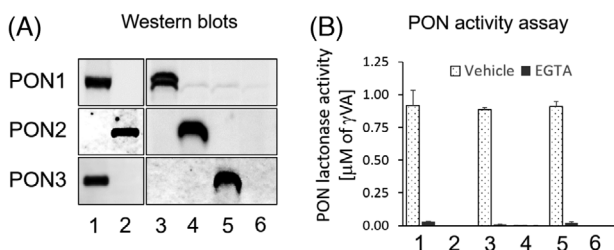
## 3. Results

### 3.1. $\gamma$ VL is Transformed into $\gamma$ VA in Serum

As the main objective of this study, we investigated if dietary flavan-3-ol-derived  $\gamma$ VL is a substrate of PON. To accomplish this, pooled human serum was incubated with 1  $\mu$ M of  $\gamma$ VL for 1 h at 37 °C and accumulation of  $\gamma$ VA, the expected PON-mediated product, was measured using UPLC-MS. The peak corresponding to  $\gamma$ VA was identified and quantified using an authentic standard. Since PON is a Ca<sup>2+</sup>-dependent enzyme, a subset of samples was co-incubated with 5 mM EGTA, a calcium chelator. In addition, a subset of serum samples were heat-inactivated to test if the reaction was enzyme-dependent. The results showed an increase in  $\gamma$ VA following an incubation with  $\gamma$ VL for 1 h, while the increase of  $\gamma$ VA was diminished markedly by serum pretreated with EGTA and heat-inactivation (Figure 1A). Next, we determined the time–response curve of serum lactonase activity by incubating 1  $\mu$ M of  $\gamma$ VL for 1 h. We observed a time-dependent decrease in the substrate  $\gamma$ VL and a concomitant increase in the product  $\gamma$ VA (Figure 1B). Based on the time response curve, the apparent half-life of  $\gamma$ VL in serum at 37 °C was 9.8  $\pm$  0.3 min. Finally, we determined the dose–response curve of  $\gamma$ VL serum lactonase activity by incubating  $\gamma$ VL in concentrations ranging from 10 to 500  $\mu$ M (Figure 1C). There was a dose-dependent increase in the rate of  $\gamma$ VL to  $\gamma$ VA conversion and the apparent *K<sub>M</sub>* for the substrate  $\gamma$ VL was 269  $\pm$  25  $\mu$ M.



**Figure 1.** Serum lactonase activity. A) Accumulation of 5-(3',4'-dihydroxyphenyl)- $\gamma$ -valeric acid ( $\gamma$ VA) in serum after incubating 1  $\mu$ M of 5-(3',4'-dihydroxyphenyl)- $\gamma$ -valerolactone ( $\gamma$ VL) for 1 h in pooled serum (control), serum pre-treated with 5 mM EGTA and in heat-inactivated serum (HI). Data represent mean  $\pm$  standard deviation ( $n = 6$ ). B) Time-response curve of  $\gamma$ VL disappearance and concomitant  $\gamma$ VA increase during a 1 h incubation in pooled serum at 37  $^{\circ}$ C. Data represent mean  $\pm$  standard deviation ( $n = 4$  per time point).  $\gamma$ VL concentrations were fitted to a first-order kinetic to then calculate half-life ( $T_{1/2}$ ). C)  $\gamma$ VL serum lactonase activity as a function of increasing concentrations of  $\gamma$ VL. Data represent mean  $\pm$  standard deviation ( $n = 4$  per concentration). Data were fitted to Michaelis–Menten equation to determine apparent  $K_M$  value.



**Figure 2.** Characterization of PON isoforms and activities. A) Presence of PON isoforms in transfected HEK cells, serum, and HUVEC cell lysate assessed by western blot analysis. The figure is a representation of four independent assays. B) PON lactonase activity assessed as an accumulation of 5-(3',4'-dihydroxyphenyl)- $\gamma$ -valeric acid ( $\gamma$ VA) in serum, PON-transfected HEK cells and in HUVEC cell lysate after incubating 1  $\mu$ M of 5-(3',4'-dihydroxyphenyl)- $\gamma$ -valerolactone ( $\gamma$ VL) with or without 5 mM EGTA for 1 h. Both PON1 and PON3 converted  $\gamma$ VL to  $\gamma$ VA and the reaction was  $Ca^{2+}$  dependent. PON2 did not convert  $\gamma$ VL to  $\gamma$ VA. Data represent mean  $\pm$  standard deviation ( $n = 6$ ). Numbers on x-axis: 1—pooled serum; 2—HUVECs; 3—PON1-transfected HEK cells; 4—PON2-transfected HEK cells; 5—PON3-transfected HEK cells; 6—non-transfected HEK cells.

### 3.2. $\gamma$ VL is a Substrate of PON1 and PON3, but not PON2

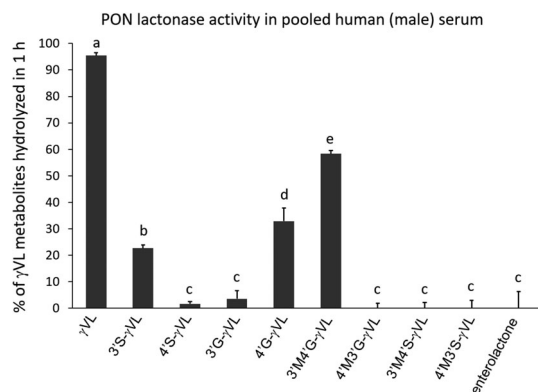
PON lactonase activity was assessed in HEK cells transfected with isomer-specific PON to assess if lactonase activity was due to PON rather than other enzymes in serum, and if so, to identify the PON isoforms involved in the hydrolysis of  $\gamma$ VL to  $\gamma$ VA. To accomplish this, western blot analysis with isomer-specific PON antibodies was used to confirm the presence or absence of PON isoforms in PON-transfected HEK cells (Figure 2A). We observed  $Ca^{2+}$ -dependent PON lactonase activity for the conversion of  $\gamma$ VL into  $\gamma$ VA in PON1- and PON3-transfected HEK cells as well as in pooled serum but not in PON2-transfected HEK cells (Figure 2B). Lack of lactonase activity for  $\gamma$ VL was replicated in HUVECs with PON2 expression (Figure 2B).

### 3.3. Circulating $\gamma$ VL Metabolites are Substrates of PON

We investigated if the phase II  $\gamma$ VL metabolites, normally found in the circulation following an intake of dietary flavan-3-ols, are also substrates of PON. These  $\gamma$ VL metabolites included sulfated, glucuronidated, methylated, and combinations thereof that were tested as potential PON substrates in pooled serum samples. Since authentic standards for  $\gamma$ VA metabolites were not available, disappearance of  $\gamma$ VL metabolites were measured over time as a means to assess PON lactonase activity. The results obtained showed that the extent of hydrolysis of  $\gamma$ VL metabolites by serum PON was dependent on the structure of the metabolites. The order of metabolite reactivity with PON was  $\gamma$ VL-3'-methoxy-4'-glucuronide >  $\gamma$ VL-4'-glucuronide >  $\gamma$ VL-3'-sulfate ( $p < 0.01$ , ANOVA, Holm-Sidak post-hoc), while the rest of the metabolites, including  $\gamma$ VL metabolites sulfated at the 4' position, 3'-glucuronides, and their respective methylated forms, showed poor reactivity with PON (Figure 3). Further supporting the occurrence of lactonase reaction with these specific  $\gamma$ VL metabolites, the presence of  $\gamma$ VA metabolites resulting from hydrolysis of the corresponding  $\gamma$ VL metabolites in serum was confirmed using a  $\gamma$ VAs reference material (Figure S1 and Table S1, Supporting Information). Finally, another dietary polyphenol-derived lactone, enterolactone, was also tested but no lactonase activity was detected (Figure 3).

### 3.4. Profile of $\gamma$ VA and $\gamma$ VL Metabolites in Urine after Flavan-3-ol Intake is Consistent with the Reactivity of $\gamma$ VL Metabolites with PON

To explore the possibility of PON affecting the levels of  $\gamma$ VA and  $\gamma$ VL metabolites in vivo, we examined the amount and profile of these phase II metabolites in urine after an intake of dietary flavan-3-ols in men ( $n = 13$ ; Figure 4). Consistent with the observation that 3'-methoxy-4'-glucuronidated, 4'-glucuronidated, and 3'-sulfated  $\gamma$ VLS are substrates for PON (Figure 3), we



**Figure 3.** PON lactonase activity in serum with conjugated  $\gamma$ VL metabolites and enterolactone as substrates. Amount of  $\gamma$ VL metabolites hydrolyzed after incubation of 1  $\mu$ M of  $\gamma$ VL and its conjugated metabolites in pooled serum for 1 h at 37 °C. Data represent mean  $\pm$  standard deviation ( $n = 6$ ) and expressed as the percentage of the initial amount of  $\gamma$ VL metabolites hydrolyzed after 1 h. Different letters indicate groups that are statistically significant from others ( $p < 0.01$ , ANOVA, Holm-Sidak posthoc). 5-(3',4'-dihydroxyphenyl)- $\gamma$ -valerolactone ( $\gamma$ VL), 5-(4'-hydroxyphenyl)- $\gamma$ -valerolactone-3'-sulfate (3'S- $\gamma$ VL), 5-(3'-hydroxyphenyl)- $\gamma$ -valerolactone-4'-sulfate (4'S- $\gamma$ VL), 5-(4'-hydroxyphenyl)- $\gamma$ -valerolactone-3'-glucuronide (3'G- $\gamma$ VL), 5-(3'-hydroxyphenyl)- $\gamma$ -valerolactone-4'-glucuronide (4'G- $\gamma$ VL), 5-(3'-methoxyphenyl)- $\gamma$ -valerolactone-4'-glucuronide (3'M4'G- $\gamma$ VL), 5-(4'-methoxyphenyl)- $\gamma$ -valerolactone-3'-glucuronide (4'M3'G- $\gamma$ VL), 5-(3'-methoxyphenyl)- $\gamma$ -valerolactone-4'-sulfate (3'M4'S- $\gamma$ VL), 5-(4'-methoxyphenyl)- $\gamma$ -valerolactone-3'-sulfate (4'M3'S- $\gamma$ VL).

observed that the majority of  $\gamma$ VA metabolites detected in urine samples were 3'-methoxyl-4'-glucuronidated, 4'-glucuronidated and 3'-sulfated  $\gamma$ VAs (Figure 4A). In addition, as observed from serum PON activity assay, a very low level of 3'-glucuronidated  $\gamma$ VA was detected in urine (Figure 4A) even though there was a relatively high amount of 3'-glucuronidated  $\gamma$ VL (Figure 4B). Importantly, in the absence of authentic standards of  $\gamma$ VA metabolites, the profile of  $\gamma$ VA metabolites illustrated in Figure 4 did not change significantly when using different approaches for the quantification of these metabolites using surrogate standards (Table 3, Supporting Information).

### 3.5. PON Polymorphisms have a Minor Impact on the Use of Selected $\gamma$ VL Metabolites as Biomarkers of Flavanol Intake

Recent studies have validated the use of a specific set of  $\gamma$ VL metabolites, namely the sum of  $\gamma$ VL-3'-sulfate and  $\gamma$ VL-3'-glucuronide ( $\gamma$ VLM<sub>B</sub>), as a biomarker of flavan-3-ol intake.<sup>[4,15]</sup> Given that PON presents various polymorphisms that influence the activity and expression of the enzyme,<sup>[30–33]</sup> we investigated whether or not PON polymorphisms contribute to inter-individual differences in  $\gamma$ VLM<sub>B</sub> levels and as such, should be considered when using  $\gamma$ VLM<sub>B</sub> as a biomarker of intake. To accomplish this,  $\gamma$ VLM<sub>B</sub> levels in spot urines were compared among the different alleles of common, validated PON1 polymorphisms in a sub-cohort of the EPIC-Norfolk study ( $n = 16\ 672$ ). The results obtained showed no differences of  $\gamma$ VLM<sub>B</sub> levels in men (Table 2), and only small differences in  $\gamma$ VLM<sub>B</sub> levels among rs662 and rs705381 alleles in women (Table 2). Ad-

justing for interindividual differences in SREM-estimated (–)epicatechin intake, one of the precursors of  $\gamma$ VLs, did not change these results (Table 2). PON1 is bound to HDL in circulation and HDL level is, on average, higher in females than males. However, adjustments by HDL did not change the results obtained either (Table 2). As expected, no differences in SREM levels were observed among the different variants of PON polymorphisms tested in men and women (Table S4, Supporting Information).

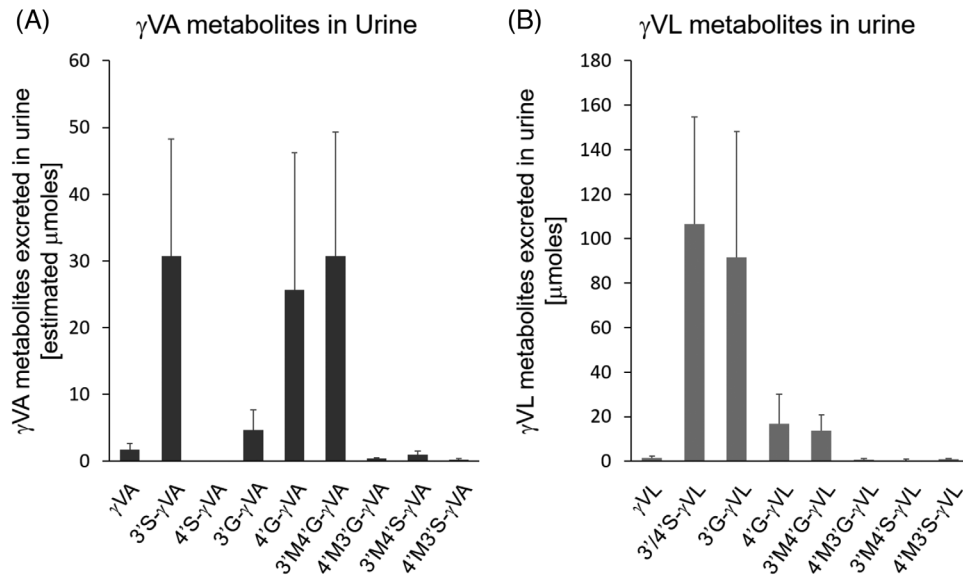
To evaluate if these interindividual differences in  $\gamma$ VLM<sub>B</sub> levels associated to rs662 and rs705381 polymorphisms should be considered when using  $\gamma$ VLM<sub>B</sub> as a biomarker, participants were ranked according to  $\gamma$ VLM<sub>B</sub>-estimated flavan-3-ol intake and separated into quintiles. Subsequently, the distribution of rs662 and rs705381 alleles was compared among these quintiles (Figure 5). The results obtained showed no significant differences in the distribution of rs705381 alleles among the different  $\gamma$ VLM<sub>B</sub> quintiles in men and women ( $p = 0.4$  for women;  $p = 0.6$  for men; chi-squared-test). While there were statistically significant differences in the distribution of rs662 alleles among different quintiles in women ( $p = 0.013$ ; chi-squared test; Figure 5), the highest difference in the distribution of alleles was only 6 percentage points between the top quintile and bottom quintile (Figure 5). Finally, we evaluated whether or not considering rs662 and rs705381 influences the previously shown inverse association between  $\gamma$ VLM<sub>B</sub>-estimated flavan-3-ol intake and systolic blood pressure.<sup>[4,5]</sup> However, considering rs662 and rs705381 did not influence this association neither in men nor in women ( $p = 0.81$  for rs662 and  $p = 0.19$  for rs705381, Wald-test; Figure S2, Supporting Information).

## 4. Discussion

This investigation demonstrates the presence of a lactonase activity in human serum that converts dietary flavan-3-ol-derived  $\gamma$ VL and some of its conjugated phase II metabolites into their corresponding  $\gamma$ VA metabolites, and that this conversion is catalyzed by PON1 and PON3 enzymes present in serum (Figures 2 and 3). Furthermore, the profile of  $\gamma$ VA metabolites excreted in urine after flavan-3-ol intake (Figure 4) was consistent with the different reactivity of  $\gamma$ VL metabolites with serum PON (Figure 3). In this regard, 3'-methoxy-4'-glucuronidated, 4'-glucuronidated, and 3'-sulfated  $\gamma$ VAs, that accounted for the majority (>90%) of  $\gamma$ VA metabolites detected in urine following flavan-3-ol intake (Figure 4), also corresponded to the product of the  $\gamma$ VL metabolites hydrolyzed by serum PON (Figure 3). Finally, amply validated PON polymorphisms known to modulate PON activity and expression showed, albeit small in magnitude, the modulation of certain  $\gamma$ VL metabolites in humans (Table 2). Taken together, these results show that PONs could metabolize  $\gamma$ VLs in humans and that the  $\gamma$ VA metabolites detected after flavan-3-ol intake are, potentially to a large extent, the product of PON lactonase activity. Thus, this novel finding not only updates the current view that  $\gamma$ VAs detected in humans are solely derived from the action of the gut microbiome, but also questions to what extent the gut microbiome directly contributes to the systemic presence of  $\gamma$ VA metabolites in humans.

PONs are hydrolase enzymes with three recognized catalytic activities, including paraoxonase (hydrolysis of



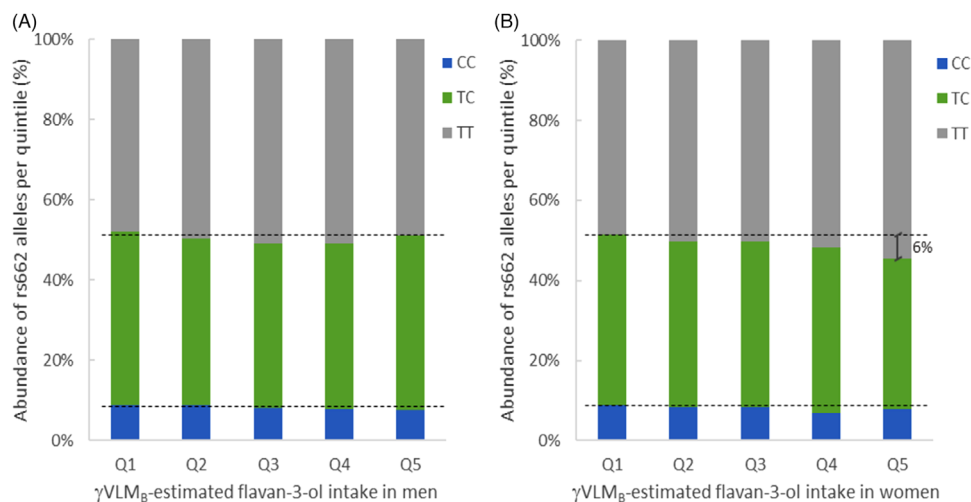


**Figure 4.**  $\gamma$ VA and  $\gamma$ VL metabolites detected in urine following an intake of dietary flavan-3-ols. Amount of  $\gamma$ VA (A) and  $\gamma$ VL (B) metabolites in urine collected over 36 h after an intake of 829 mg cocoa flavanols per 70 kg BW in men. Data represent mean  $\pm$  standard deviation ( $n = 13$ ). 5-(3',4'-dihydroxyphenyl)- $\gamma$ -valerolactone ( $\gamma$ VL), 5-(4'-hydroxyphenyl)- $\gamma$ -valerolactone-3'-sulfate (3'S- $\gamma$ VL), 5-(3'-hydroxyphenyl)- $\gamma$ -valerolactone-4'-sulfate (4'S- $\gamma$ VL), 5-(4'-hydroxyphenyl)- $\gamma$ -valerolactone-3'-glucuronide (3'G- $\gamma$ VL), 5-(3'-hydroxyphenyl)- $\gamma$ -valerolactone-4'-glucuronide (4'G- $\gamma$ VL), 5-(3'-methoxyphenyl)- $\gamma$ -valerolactone-4'-glucuronide (3'M4'G- $\gamma$ VL), 5-(4'-methoxyphenyl)- $\gamma$ -valerolactone-3'-glucuronide (4'M3'G- $\gamma$ VL), 5-(3'-methoxyphenyl)- $\gamma$ -valerolactone-4'-sulfate (3'M4'S- $\gamma$ VL), 5-(4'-methoxyphenyl)- $\gamma$ -valerolactone-3'-sulfate (4'M3'S- $\gamma$ VL), 5-(3',4'-dihydroxyphenyl)- $\gamma$ -valeric acid ( $\gamma$ VA), 5-(4'-hydroxyphenyl)- $\gamma$ -valeric acid-3'-sulfate (3'S- $\gamma$ VA), 5-(3'-hydroxyphenyl)- $\gamma$ -valeric acid-4'-sulfate (4'S- $\gamma$ VA), 5-(4'-hydroxyphenyl)- $\gamma$ -valeric acid-3'-glucuronide (3'G- $\gamma$ VA), 5-(3'-hydroxyphenyl)- $\gamma$ -valeric acid-4'-glucuronide (4'G- $\gamma$ VA), 5-(3'-methoxyphenyl)- $\gamma$ -valeric acid-4'-glucuronide (3'M4'G- $\gamma$ VA), 5-(4'-methoxyphenyl)- $\gamma$ -valeric acid-3'-glucuronide (4'M3'G- $\gamma$ VA), 5-(3'-methoxyphenyl)- $\gamma$ -valeric acid-4'-sulfate (3'M4'S- $\gamma$ VA) and 5-(4'-methoxyphenyl)- $\gamma$ -valeric acid-3'-sulfate (4'M3'S- $\gamma$ VA).

**Table 2.** Urinary concentrations of a specific set of 5-(3',4'-dihydroxyphenyl)- $\gamma$ -valerolactone metabolites used as a biomarker of flavan-3-ol intake ( $\gamma$ VLM<sub>B</sub>) among common paraoxonase 1 genotypes in EPIC-Norfolk.

	Women ( $n = 9020$ )								
	Alleles			$\gamma$ VLM <sub>B</sub> concentration in urine [ $\mu$ M]			Model 1	Model 2	Model 3
	A	B	C	A	B	C	( $p$ )	( $p$ )	( $p$ )
rs662	CC	TC	TT	2.56 (0.60–8.47)	2.45 (0.62–8.43)	2.90 (0.72–9.70)	<b>0.002</b>	<b>0.001</b>	<b>0.002</b>
rs854560	AA	AT	TT	2.64 (0.65–9.09)	2.67 (0.68–9.15)	2.73 (0.71–9.66)	0.403	0.699	0.412
rs705379	AA	GA	GG	2.55 (0.69–9.03)	2.73 (0.69–9.48)	2.62 (0.62–8.77)	0.173	0.096	0.171
rs705381	CC	TC	TT	2.49 (0.66–8.99)	2.92 (0.69–9.60)	2.68 (0.66–9.18)	<b>0.035</b>	<b>0.004</b>	<b>0.029</b>
rs854572	CC	CG	GG	2.62 (0.59–8.91)	2.73 (0.70–9.38)	2.53 (0.67–9.04)	0.111	0.075	0.105
Men ( $n = 7652$ )									
	Men ( $n = 7652$ )								
	Alleles			$\gamma$ VLM <sub>B</sub> concentration in urine [ $\mu$ M]			Model 1	Model 2	Model 3
	A	B	C	A	B	C	( $p$ )	( $p$ )	( $p$ )
rs662	CC	TC	TT	3.35 (0.97–10.59)	3.67 (0.97–11.91)	3.87 (1.05–11.56)	0.472	0.434	0.472
rs854560	AA	AT	TT	3.76 (1.02–11.68)	3.65 (0.97–11.83)	4.00 (1.17–11.05)	0.410	0.167	0.392
rs705379	AA	GA	GG	3.78 (1.02–11.83)	3.79 (1.06–11.87)	3.64 (0.93–11.18)	0.352	0.160	0.364
rs705381	CC	TC	TT	3.67 (1.02–11.70)	3.86 (1.00–11.68)	3.70 (0.97–11.15)	0.928	0.969	0.922
rs854572	CC	CG	GG	3.51 (0.90–11.12)	3.96 (1.07–11.83)	3.65 (1.00–11.82)	0.205	0.201	0.224

$\gamma$ VLM<sub>B</sub> concentrations were adjusted by specific gravity and shown as median (IQR). Significance ( $p$  values) for Wald-Test are shown after adjusting for age (Model 1), age and concentration of structurally related (–)-epicatechin metabolites (SREMs) as a surrogate marker of (–)-epicatechin intake (Model 2), and age and HDL (Model 3). <sup>a)</sup>  $\gamma$ VLM<sub>B</sub> corresponds to the sum of 5-(4'-hydroxyphenyl)- $\gamma$ -valerolactone-3'-sulfate and 5-(4'-hydroxyphenyl)- $\gamma$ -valerolactone-3'-glucuronide.



**Figure 5.** Distribution of rs662 alleles in the different quintiles of urinary concentration of a specific set of 5-(3',4'-dihydroxyphenyl)- $\gamma$ -valerolactone metabolites used as a biomarker of flavan-3-ol intake ( $\gamma$ VLM<sub>B</sub>) in men (A) and women (B). Data represent relative number of rs662 alleles in different quintiles of urinary concentration of  $\gamma$ VLM<sub>B</sub> in men ( $n = 7652$ ) and women ( $n = 9020$ ).  $\gamma$ VLM<sub>B</sub> corresponds to the sum of 5-(4'-hydroxyphenyl)- $\gamma$ -valerolactone-3'-sulfate and 5-(4'-hydroxyphenyl)- $\gamma$ -valerolactone-3'-glucuronide.

organophosphates such as paraoxon that gives the name to these enzymes), arylesterase, and lactonase activity.<sup>[19,35]</sup> However, lactonase is considered the native activity common to all PON isoforms.<sup>[36]</sup> PONs show a broad specificity for lactone substrates with  $K_M$  values ranging from 0.13 to 21 mM,<sup>[19,35]</sup> so it is not surprising that unconjugated  $\gamma$ VL, with a  $K_M$  value of 0.27 mM, and  $\gamma$ VL metabolites can act as substrates of these enzymes. There exists, however, a degree of specificity in the interaction between PON and  $\gamma$ VL as another polyphenol-derived lactone, enterolactone, was not a substrate of this enzyme (Figure 3). To assess the likelihood of  $\gamma$ VL and  $\gamma$ VL metabolites reacting with PON in vivo, physiologically relevant concentrations of these metabolites were incubated in serum, which is a representative compartment where such reaction can take place in vivo. The results obtained showed that conjugation of  $\gamma$ VL with sulfates and glucuronides in position 3' and 4' differentially affected the hydrolysis of  $\gamma$ VL metabolites by PON (Figure 3), with 4'-glucuronidation resulting in better substrates compared to other  $\gamma$ VL metabolites. Further conjugations in  $\gamma$ VL significantly reduced and, in some cases, completely prevented the hydrolysis of  $\gamma$ VL metabolites by PON (Figure 3). These findings are consistent with PON1 preferentially catalyzing the hydrolysis of lipophilic lactones.<sup>[19,35,36]</sup> It is worth noting that PON3 has the capacity to accommodate bulkier substrates such as statin lactones and spironolactone,<sup>[19,32,37]</sup> for which eventual differences in PON1 and PON3 isoforms could be expected regarding their interaction with the different  $\gamma$ VL metabolites. The lack of lactonase activity of PON2 with  $\gamma$ VL as a substrate is also consistent with previous findings that PON2 has a more restrictive set of substrates, primarily long-chain *N*-acylhomoserine lactones.<sup>[19]</sup> Overall, the results obtained in the current investigation demonstrate that PON1 and PON3 react with  $\gamma$ VL metabolites in humans, thus becoming a novel set of enzymes capable of interacting with flavan-3-ol metabolites in vivo distinct from the well-known human enzymes that are part of phase II metabolism. Further studies will be needed to

confirm and expand these findings and to explore the differential contribution of PON and gut microbiome to the levels and profile of  $\gamma$ VA metabolites in humans.

The recognition of PON being involved in the metabolism of flavan-3-ols poses questions about the meaning and implications of this finding. One important practical consideration relates to studies aiming at investigating the bioavailability of flavan-3-ols and of  $\gamma$ VL and  $\gamma$ VA metabolites in particular. Explicitly, the use of serum or plasma collected with any anticoagulant other than EDTA may result in artifacts in  $\gamma$ VL and  $\gamma$ VA metabolite quantification. This is because of the rapid degradation of  $\gamma$ VLs by PON in circulation (Figure 1B). As PON is a calcium-dependent enzyme, the use of EDTA as anticoagulant will result in the inactivation of PON and stop this fairly rapid degradation of  $\gamma$ VLs to occur after sample collection. Other possible implications of PON involvement in flavan-3-ol metabolism are related to the use of a particular set of urinary  $\gamma$ VL metabolites as objective biomarkers of flavan-3-ol intake ( $\gamma$ VLM<sub>B</sub>).<sup>[4,5,15]</sup> Given that PON presents common polymorphisms that affect activity and expression level,<sup>[30–33]</sup> it is worth considering whether or not these genetic variants should be taken into account when using  $\gamma$ VLM<sub>B</sub> as biomarkers of flavan-3-ol intake. The results obtained here showed that two of the five PON polymorphisms tested contributed to inter-individual differences of  $\gamma$ VLM<sub>B</sub> levels (Table 2). However, the magnitude of PON polymorphism-specific effects on interindividual differences in  $\gamma$ VLM<sub>B</sub> levels was not detectable in men and deemed small in women (Table 2). These findings are in line with the fact that  $\gamma$ VL-3'-sulfate and  $\gamma$ VL-3'-glucuronide, the specific  $\gamma$ VL metabolites that constitute  $\gamma$ VLM<sub>B</sub>, are not among the preferred  $\gamma$ VL metabolites reacting with PON (Figure 3). Furthermore, PON polymorphisms did not have a significant effect on the ranking of participants based on  $\gamma$ VLM<sub>B</sub>-estimated flavan-3-ol intake (Figure 5) and in the previously shown inverse association between  $\gamma$ VLM<sub>B</sub> and systolic blood pressure (Figure S2, Supporting Information).<sup>[4,5]</sup> Taken together, these findings indicate that PON polymorphisms play

only a minor role in the interindividual differences of  $\gamma$ VLM<sub>B</sub>. Thus, the benefits of considering PON polymorphisms when using  $\gamma$ VLM<sub>B</sub> as biomarkers of intake will be minor, and do not significantly impact the performance of  $\gamma$ VLM<sub>B</sub> as a biomarker.

An increasing number of in vitro studies are starting to suggest putative biological activities associated with  $\gamma$ VL metabolites.<sup>[12–14]</sup> If these findings were to be verified in vivo, it would be tenable for PON to potentially modulate these biological effects by hydrolyzing selective  $\gamma$ VL metabolites. Such a role for PON was shown with other compounds, including the inactivation of a series of active lactone-containing compounds, such as homocysteine thiolactone, which mediates protein damage by homocysteinylation of lysine residues,<sup>[38]</sup> and *N*-acylhomoserines, which act as quorum-sensing signals coordinating population density and pathogenesis in bacteria.<sup>[39]</sup> It has also been postulated that PON activity is involved in the generation of active molecules from inactive precursor substrates.<sup>[40,41]</sup> That is the case of the  $\gamma$ -butyrolactone that is converted to the active form  $\gamma$ -hydroxybutyrate (GHB).<sup>[18,42]</sup> GHB is an endogenous neuroactive compound that, at physiological concentrations, acts via specific GHB-receptors recently identified as the Ca-Calmodulin Kinase type II  $\alpha$ .<sup>[43]</sup> It is of note that GHB and the GHB receptor agonist NCS-435 present striking structural similarities with dietary flavan-3-ol-derived  $\gamma$ VA (Figure S3, Supporting Information), thereby making the GHB receptor a potential molecular target for flavan-3-ol metabolites. However, further studies will be required to test this hypothesis.

## 5. Conclusions

This work reports a novel pathway for the metabolism of flavan-3-ol in humans. There are noteworthy implications of this finding for biosampling and the quantification of circulatory  $\gamma$ VL and  $\gamma$ VA metabolites, although interindividual differences due to PON polymorphisms proved to play a minor role in the use of  $\gamma$ VLM<sub>B</sub> as biomarkers of flavan-3-ol intake. Further studies will be needed to expand these initial findings and to better understand the role of PON in flavanol metabolism. In addition, These findings highlight the importance of further characterizing the precise molecular pathways involved in the absorption, distribution, metabolism, and excretion of flavan-3-ols in humans, and provide new insights into the possible mechanisms of action that underpin the beneficial effects of dietary flavan-3-ol intake.

## Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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## Conflict of Interest

J.I.O. and H.S. are employed by Mars Inc., a company engaged in flavan-3-ol research and flavan-3-ol-related commercial activities. G.G.C.K. received an unrestricted grant from Mars, Inc. T.Y.M, R.Y.F, A.C, and J.L.E have no conflict of interest to declare.

## Author Contributions

T.Y.M. and J.I.O. designed the study with input from H.S., G.C.C.K., and A.C.; T.Y.M. conducted all ex vivo and in vitro work and analyzed data derived from these assays, J.L.E. recruited volunteers and conducted the dietary intervention study with flavan-3-ols. T.Y.M. and R.Y.F. created reference materials for  $\gamma$ VA identification and analyzed the samples derived from human study and analyzed the data from this study, G.C.C.K. and J.I.O. analyzed genotype data from EPIC; T.Y.M. and J.I.O. drafted the first version of the manuscript and monitored edits from co-authors.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Keywords

absorption, cocoa flavan-3-ols, epicatechin, excretion, flavan-3-ol bioavailability, paraoxonase

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