**Title:** A METABOLOMIC EXPLORATION OF THE URINARY METABOLOME TO IDENTIFY NOVEL BIOMARKERS OF DIETARY INTAKE IN THE EUROPEAN PROSPECTIVE INVESTIGATION ON CANCER AND NUTRITION (EPIC) COHORT

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**Abstract:** Dietary biomarkers are increasingly used in studies on the validation of traditional dietary assessment instruments or to complement or substitute dietary measurements obtained through these instruments. Such biomarkers may limit errors and biases commonly met when using traditional tools and improve subject classification. This biomarker approach utilizes the huge diversity of constituents found in foods and their related metabolites measured in different human biofluids. Metabolomics applied to urine or plasma samples should allow identification of novel dietary markers.

This approach was developed within the European Prospective Investigation into Cancer and nutrition cohort (EPIC), which includes about half a million subjects from 23 centres in 10 European countries. A calibration study was designed on 37,000 subjects for whom detailed 24-hr dietary recalls (24HDRs), blood and urine samples were collected. The design of this study, along with the wealth of existing dietary and lifestyle information and ready availability of biological samples makes it an ideal framework for the conduct of methodological studies for the identification of novel dietary biomarkers.

24-Hour urine samples (n=481) from the EPIC calibration study for which both 24HDRs and Food Frequency Questionnaires existed were pre-acquisition normalised by dilution to specific gravity and acquired in one analytical batch on a high-resolution mass spectrometer (UPLC-QToF Agilent 6550 “Ion Funnel”) with pooled quality control (QC) urine samples every fifth injection. Acquired data were peak picked using the XCMS software package and QC based signal drift correction performed in R. Mass spectral features were then annotated based on monoisotopic mass data of dietary polyphenols and their metabolites extracted from the Phenol-Explorer database (www.phenol-explorer.eu) and the automatic generation of potential phase II enzyme conjugates and electrospray adducts. Annotations were then filtered according to correlations with acute and habitual intake of some main dietary sources of polyphenols. High confidence tentative assignments of mass spectral features were made based upon sub 10 ppm mass accuracy, <30% relative standard deviation in total pooled QC samples, correlation with dietary records (>0.3 Pearson correlation coefficient) and presence within food of interest. Hierarchical clustering was then used to examine metabolite-metabolite associations and to further aid identification.

The efficiency of the approach will be illustrated by the identification of markers for six main food sources of polyphenols (orange juice, coffee, tea, chocolate, red wine and apple). Over 300 unique mass spectral features matching these confidence criteria could be found representing parent polyphenols, their phase II conjugates and gut microbial metabolites. Final assignments are currently being confirmed using automated MS/MS fragmentation methods and by comparison to authentic standard compounds.

**Acknowledgments.** This research is supported by the European Union (NutriTech FP7-KBBE-2011-5 Grant #289511, EUROCAN FP7-KBBE-2010.2.4.1-2 Grant #260791).
Title: ACUTE CONSUMPTION OF FLAVAN-3-OL-ENRICHED DARK CHOCOLATE AFFECTS HUMAN ENDOGENOUS METABOLISM

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Abstract: Cardiovascular disease (CVD) is a primary cause of premature deaths worldwide, with incidence rates in the United Kingdom being amongst the highest in the world. Consumption of dietary polyphenols, secondary plant metabolites that are ubiquitously present in plant-derived foodstuffs and beverages, has been linked to improved cardiovascular health in humans. Plant-derived substances such as flavan-3-ols may beneficially affect atherosclerosis and impact on cardiovascular risk, but information on their bioavailability is limited. We performed a randomised controlled cross-over intervention trial to assess the acute effects of consumption of dark chocolate enriched in flavan-3-ols and procyanidins, compared with standard dark chocolate and white chocolate. NMR and MS-based metabolomics were used to profile urine and blood plasma samples collected at time 0, 2 and 6 hours post intake for each of 42 healthy volunteers. Multivariate (MV) statistics could readily separate the different time points. MV and univariate statistics showed that the largest differences between pre and post intake urines were due to exogenous metabolites originating from the chocolate intake (epicatechin derivatives, methylxanthines and microbial breakdown products). Interestingly a proportion of the variance was also associated with changes in the levels of endogenous compounds such as N1-methylnicotinamide, creatinine, lactate, and some amino acids. Urinary levels of these metabolites decreased 6 h after intake of both dark chocolates compared to white chocolate and all followed the same trend: mean level in flavan-3-ol-enriched dark chocolate < dark chocolate < white chocolate. These results demonstrate the power of untargeted NMR-based metabolite profiling to reveal the modulation of human metabolism following a controlled dietary challenge.
Title: DO WE PEE WHAT WE EAT? – ESTIMATING COMPLIANCE TO DIETARY PATTERNS FROM URINE FINGERPRINTS

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Abstract: Human nutrition research is increasingly concerned with health effects of complex diets, since a complex dietary pattern better reflects real life. Dealing with complex diets is a challenge not only for studying dietary effects but also for estimating dietary exposure and compliance. Fingerprinting of the urine metabolome by untargeted metabolomics is a promising tool to discover discriminant metabolites for complex diets. Also, it may be possible to use untargeted metabolomics as a screening tool for compliance to a dietary pattern.

A six month parallel intervention study has been carried out in which 220 participants were randomized to follow either a New Nordic Diet (NND) or an Average Danish Diet (ADD). The two diets were defined by intake levels of fifteen food groups in g/10MJ, such as nuts, legumes, root vegetables and berries. Weighed dietary records made on the same day as 24 h urine collections were used to study how the dietary patterns were reflected in the urine metabolome. Samples were analyzed by UPLC-qTOF-MS.

A double cross-validated reduced partial least squares discriminant analysis (PLS-DA) model revealed 67 features as the best urinary discriminants for the diets. The features corresponded to 52 metabolites and were grouped in three main clusters (two characterizing ADD and one characterizing NND). Features in the ADD clusters were mainly citrus and chocolate metabolites, while the NND cluster mainly consisted of metabolites from nuts and fish. The misclassification rate for the model was 22 % in a test set of 139 samples. A principal component analysis (PCA) of the reported food intakes revealed oil, vinegar, natural yogurt, hazelnuts and apple as the most characteristic for NND, while tomato, wheat buns, wheat crispbread, filled chocolate and muesli were most characteristic for ADD.

In conclusion, the two dietary patterns were well discriminated in urine and untargeted metabolomics may be a potential tool to evaluate dietary compliance. Importantly, however, the food origin of the discriminating urine metabolites differed from the most discriminating foods in the dietary patterns and very few foods characterizing the dietary patterns were clearly reflected among the main discriminant metabolites in urine.

The study is part of the OPUS project ‘Optimal well-being, development and health for Danish children through a healthy New Nordic Diet’. Supported by a grant from the Nordea Foundation.
Title: DYNAMICS OF HUMAN POSTPRANDIAL METABOLISM IN LEAN AND OBESE SUBJECTS — THE BLOOD AS A SOURCE OF NUTRITIONAL HEALTH BIOMARKERS

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Presenting author: Guy Vergères

Abstract: A major challenge in nutritional science is the identification of biomarkers that are acutely and transiently influenced by diets in healthy persons and that are in a causal relationship to changes leading to - or preventing – the long-term development of chronic metabolic diseases. We have tackled this issue by investigating the postprandial kinetic response of healthy lean (n=19) and obese (n=18) subjects to three caloric doses of a high-fat meal (500, 1’000, 1’500 kcal). Serum markers of nutrient metabolism (glucose, insulin, triglycerides, total cholesterol, HDL cholesterol) and of low grade inflammation (C reactive protein, interleukin-6, endotoxin) as well as the blood cell transcriptome were measured under fasting conditions and for up to six hours after ingestion of the high-fat meals.

With the exception of cholesterol, all metabolic and inflammatory parameters differed between lean and obese fasting subjects and a statistically significant postprandial change was also observed in the lean subjects in at least one time point and one caloric dose. The robustness of the postprandial effect differed among these parameters, insulin and triglycerides being the most responsive to the caloric dose. The panel of parameters was then extended to gene expression in blood cells. An analysis of the dynamics of the blood cell transcriptome revealed differences between lean and obese subjects that highlighted a deregulation in the ability of the obese subjects to cope with increased caloric intake. Remarkably, a set of genes and pathways (e.g. oxidative phosphorylation) were identified that not only differentiated lean from obese subjects under fasting conditions but that also changed postprandially in lean subjects in a direction that was predictive of the fasting status in the obese subjects. These genes and pathways point to the concept of nutritional health biomarkers and could be of value to both the food industry and to medicine as they characterize the quality of the interaction between food and the human organism.
Title: FOOD METABOLOMICS APPLIED IN COHORTS TO ACCELERATE THE DISCOVERY OF NUTRITIONAL BIOMARKERS


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Presenting author: Claudine Manach

Abstract: The purpose of dietary assessment is to estimate usual and recent intake of foods, nutrients, bioactive compounds and food contaminants for exploration of associations with health outcomes and monitoring of population nutritional status. These data are still extremely difficult to obtain. Methods currently used are based on dietary questionnaires which have inherent limitations linked to self-reporting. A complementary approach to questionnaires is the use of biomarkers. However, only a few biomarkers have been properly validated, which do not cover the wide range of foods consumed. Metabolomics has emerged as a promising approach to discover nutritional biomarkers. Typically, plasma or urine samples collected before and after acute intake of a specific food are profiled using NMR or high resolution Mass Spectrometry (MS) and compared using multivariate statistics to pinpoint the signals reflecting the consumption of the target food. In a proof-of-concept study on citrus, we showed that urine profiling of cohort subjects stratified by consumption could be a more effective strategy for discovery of sensitive biomarkers of intake.

As part of the ANR PhenoMeNEp project, we tested the approach on 20 selected plant foods. Using dietary questionnaire data (1994-2009), 144 high and 66 low consumers of fruit and vegetables (F&V) were selected from the SU.VI.MAX2 cohort. Morning spot urine samples were analyzed in positive and negative ion mode by LC-QToF MS. Subgroups of low and high consumers were selected for each of the 20 foods on the basis of questionnaire data, excluding from each selection any subject declaring high intake of other foods. For ten target foods, the urine metabolomes from low and high consumers were strongly discriminated by both univariate and multivariate statistics. The number of significant ions ranged from 133 for coffee to 428 for apple (p-value<0.05 ANOVA BH). Study of marker specificity showed that some, although highly discriminant for the target food, were not specific enough to make good candidate biomarkers. The long-term low and high consumption of F&V were also clearly reflected in the urine metabolomes, mainly through endogenous metabolites variations. Of the 39 exogenous markers having a higher intensity in the urine of high F&V consumers, 69% were recognized to be potential biomarkers of apple, tea, citrus and root vegetable intake. The study provided useful insights on the conditions for success and the limitations of the approach of applying metabolomics to cohort samples for rapid discovery of a wide range of nutritional biomarkers.

1Pujos-Guillot et al., J Proteome Res. 2013, DOI: 10.1021/pr300997c
Title: FROM MAN TO WORMS: SYSTEMS APPROACHES TESTED

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Presenting author: Hannelore Daniel

Abstract: I shall be using diabetes to challenge the systems approach with examples from humans, mice and *C. elegans*. Various biomarker discovery studies employing metabolomics have identified a small set of plasma metabolites with altered concentrations in humans with insulin resistance (IR) or those suffering from diabetes. Amongst the metabolites, amino acids (branched chain, aromatic AA) are over-represented with elevated levels in the obese state and in early phases of IR. In addition, ketone bodies such as α- and β-hydroxybutyrate as well as free fatty acids, selected lysosphatidids and phospholipids are recognized as marker metabolites. Although these findings from various studies are fairly robust, the origin of these changes in IR and the diabetic state are currently unknown. To be able to assess the underlying changes in the organs we screened various mouse models for corresponding changes in the plasma metabolome and identified the streptocotozin (STZ) model as closest to the human condition. In these animals we profiled in addition to plasma samples also muscle and liver for changes in the metabolome and combined these findings with microarray data obtained from STZ mice models. We also utilize *C. elegans* as a model for identification of the molecular mechanisms that are associated with the condition of impaired insulin receptor signalling. A combined approach using metabolomics and microarray profiling was used to determine the metabolic changes in insulin-receptor mutant (daf-2) animals and animals lacking the forkhead transcription factor daf-16 that is under control of daf-2.

In the STZ model plasma metabolites changed as in humans. It needs to mentioned that essentially the same metabolites have also been identified in human type 1 diabetes as markers and the STZ model seems therefore suitable. In muscle tissue of diabetic mice a large number of genes related to fatty acid handling and β-oxidation showed major increases in mRNA levels associated with elevated levels of a large number of acyl-carnitines that also increase in plasma. Intracellular concentrations of branched chain (BCAA) and aromatic amino acids (AA) but also aminobutyrate increased significantly although for most enzymes of the respective metabolic pathways that utilize these amino acids no significant changes in transcript levels could be observed. In *C. elegans* with impaired insulin signalling, amino acid levels (BCAA, AA) increased and mRNA profiling here suggested that enzymes in BCAA metabolism even increase in levels – except for that of the BCAA transaminase. Most interestingly, recent studies in mice and humans have demonstrated a down-regulation of this enzyme in adipose tissue and a deletion of the gene in mice was shown to increase plasma BCAA levels markedly. This strongly suggests that in the obese state and more so in IR and in diabetes, BCAA-transaminase levels decrease in adipose tissue leading to the characteristic changes in plasma BCAA levels; these effects are antagonised by bariatric surgery that restores the enzyme activity rapidly. Taken together, these findings shed light on a neglected area of adipose tissue biology and that is amino acid metabolism and demonstrate that a systems approach that takes biology from the holistic, descriptive level down to cell systems or model organisms can reveal new causative conditions.
**Title:** IN SEARCH OF EFFECTIVE THERAPY FOR COPD CACHEXIA

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**Presenting author:** Richard Casaburi

**Abstract:** For the practicing clinician, the diagnosis of cachexia is often easy. A patient, known to have severe organ disease, presents for a regular appointment and the change in appearance is profound: the patient appears gaunt, weak and unwell; weight loss is apparent. However, nothing else about cachexia is easy. Key questions that cry out for intensive research. This lecture focuses on COPD cachexia; cachexia in other conditions (e.g., renal or heart failure, AIDS) likely has similar manifestations and mechanisms.

**How can we best define cachexia?** Cachexia definitions include “wasting, loss of body weight, loss of muscle mass”. These definitions require reliable historical information, unlikely to be available to the researcher. Consequently, entry into cachexia studies has often been based only on measures made at the time of presentation. But patients with low muscle mass and/or low body weight may well not be cachectic. This difficulty has yielded misleading results regarding cachexia prevalence, mechanisms and therapies. Helpfully, recent consensus definitions include the presence of anorexia, fatigue, inflammation and weakness.

**What causes cachexia?** No unifying hypothesis has emerged. A central mystery concerns factors triggering the downward cachexia spiral. The following have been suggested:

- **Appetite suppression.** Clearly many patients have reduced caloric intake. Central (hypothalamic?) factors may be involved; in COPD, dyspnea may interfere with eating.
- **Increased metabolic rate.** Some studies suggest that cachectic patients are hypermetabolic. Increased work of breathing may contribute in COPD.
- **Altered hormonal balance.** Some studies have demonstrated low growth hormone and testosterone levels. Local tissue resistance to these hormones is also a possibility. These may contribute to muscle wasting and weakness.
- **Inflammatory mediators and/or oxidative stress.** In some (but not all) studies, inflammation is more prevalent in patients classified as cachectic. Studies in animal models have clearly shown that administration of high levels of inflammatory or oxidative stress mediators can produce changes similar to those seen in cachexia.

**How can we treat cachexia?** To the frustration of the practitioner who has identified cachexia and understands its dire prognosis, no effective therapy exists.

- **Nutritional supplementation.** Unlike in starvation, giving cachectic patients access to nutritional supplementation generally fails to increase body weight. Specific micronutrients may yet prove to be of value.
- **Appetite stimulation.** Both megestrol acetate and the cannabinoids have proven efficacy in stimulating appetite. Clinical trials of ghrelin are being organized.
- **Anabolic hormones.** Muscle building hormones seem rational therapy to combat muscle wasting. Both growth hormone and testosterone (and its analogs) have been shown capable of inducing muscle growth in some patient models. A theoretical concern is that these agents decrease fat mass.
- **Anti-inflammatory agents.** Although much attention has been focused on inflammation, systemic inflammation is apparently modest in most patients. Inflammatory cascade complexities make it difficult to predict effects of inhibition of one or multiple pathways.
- **Rehabilitative therapy.** In general, cachectic patients are considered candidates for rehabilitation, though there is little specific information regarding its effectiveness. Exercise is pro-inflammatory; is this of concern for the cachectic patient? It seems unlikely that major progress will be made in defining effective cachexia therapies until its mechanisms are clearly understood. Until then, these unfortunate patients will be sources of frustration to the practitioner.
Title: Metabolomic Strategies in Clinical Nutrition Research: From Diet to Uncovering Disease Risk Biomarkers

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Abstract: In nutrition research, metabolomics has the potential to unveil the metabolic signature that differentiates healthy from subjects with metabolic perturbations, access unexplored metabolic pathways impacted by diet, and help clinical measurements in explaining the inter-individual differences in responsiveness to dietary interventions. Application of nutrimetabolomic strategies in the discovery of new biomarkers in human nutritional research, suggesting three main categories: (1) assessment of nutritional and dietary interventions; (2) diet exposure and food consumption monitoring; and (3) health phenotype and metabolic impact of diet. For this purpose, several examples of these applications will be used to provide evidence and to discuss the advantages and drawbacks of these nutrimetabolomic strategies.
Title: NUTRITIONAL SYSTEMS BIOLOGY – A METABOLOMICS PERSPECTIVE

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Presenting author: Matej Orešič

Abstract: Systems biology views and studies the biological systems in the context of complex interactions between their building blocks and processes. Given its multi-level complexity, metabolic syndrome (MetS) makes a strong case for adopting the systems biology approach. Despite many MetS traits being highly heritable, it is becoming evident that the genetic contribution to these traits is mediated via gene–gene and gene–environment interactions across several spatial and temporal scales, and that some of these traits such as lipotoxicity may even be a product of long-term dynamic changes of the underlying genetic and molecular networks. This presents several conceptual as well as methodological challenges and may demand a paradigm shift in how we study the undeniably strong genetic component of complex disorders such as MetS, and how we developed innovative prevention strategies, e.g. by using a nutritional systems biology approach. Metabolome is sensitive to genetic and environmental factors contributing to complex diseases such as MetS. Metabolomics is therefore one of the key platforms for discovery and study of pathophysiological phenomena leading to MetS as well as to development of MetS-associated complications; and therefore a key platform for nutritional systems biology. The argument will be made that for adopting nutritional systems biology approaches to MetS an integrative framework is needed which glues the biological processes of MetS with specific physiological mechanisms and principles. The metabolic phenotypes, environmental/lifestyle factors as well as molecular and genetic networks can be modeled within the context of such integrative framework and the underlying physiology.
Title: "Omics" approaches to understand the complex nature of human obesity

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Presenting author: Karine Clément

Abstract: Human obesity can be viewed as a set of phenotypes which evolve over time in a sequence of stages that need to be precisely measured. Environmental, behavioral, genetic and biological factors interact to cause obesity. Depending on an individual’s genetics, obesity reaches a plateau characterized in part by resistance to weight loss, propensity to weight regain and the appearance of obesity comorbidities. While experts raise the point that the “obesogenic” environment rather than the biology is the major player in obesity progression, these drivers are not mutually exclusive. More research is needed to examine the environmental stimuli promoting weight gain and/or weight loss resistance, and the interaction with biological systems that promote fat storage and associated biological perturbations. In this complex landscape, “omics” approaches have been conducted to identify relevant new targets and biomarkers associated with obesity comorbidities as well as resistance to weight loss. Programs using “omics” tools aim at identifying new candidate pathways at the tissue, cellular, fluid and molecular levels using human bioresources. In this presentation, example of successful approaches (transcriptomics, metabolomics, lipidomics) will be described. For example the evaluation of transcriptomic interactions characterizing human adipose tissue in different depots and conditions has demonstrated the strong relationship linking inflammatory processes to extra cellular matrix (ECM) remodelling components and contributed to the discovery of pathological alterations in human adipose tissues. The intricate relationships between metabolism and inflammation, referred to as ‘metaflammation’, emerges as a key feature of the metabolic syndrome and its components obesity, insulin resistance, and type 2-diabetes. The omics approaches concern not only the human biology but also the role of gut microbiome in close interaction with it. In this context, metagenomic approaches in the past eight years created a major burst in the number of scientific publications enlightening the role of microbiota dysbiosis (i.e. imbalanced microbiota compared to the one of healthy individuals) in the complex facet of obesity. Example of interaction between gut microbiota metagenomics and human adipose tissue transcriptomics will be discussed. Ambitious projects are now conducted to integrate bioclinical and environmental phenotyping together with personalized omics (metagenomics, metabolomics, transcriptomics) with the objective of developing new strategies for personalized medicine in metabolic and related diseases.

Grant acknowledgments for your research. National Agency of Research (OBCAT, RIOMA), European Community Frameworks (ADAPT, FLIP, HEPADIP, DIOGENES METACARDIS), French foundation for medical research (FRM), Fondacoeur
Title: **USE OF NUTRIGENOMICS IN UNDERSTANDING MECHANISMS UNDERLYING THE VASCULAR PROTECTIVE EFFECTS OF DIETARY POLYPHENOLS**

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Presenting author: Dragana Milenkovic

Abstract: Epidemiological, human and animal studies suggest a protective role of dietary polyphenols against cardiovascular diseases. Their capacity to modulate genes expression and signaling pathways may be involved in their cardiovascular protective effects but the mechanisms remain still unknown. The aim of our research is to investigate the cardiovascular protective property of polyphenols and decipher underlying molecular mechanisms using holistic nutrigenomics approach. In murin models of atherosclerosis, 16-week supplementation with nutritionally-relevant doses of polyphenols (catechin, bilberry anthocyanins, naringin or curcumin) decreased progression of atherosclerosis. Nutrigenomic studies of aorta indicated that polyphenols modified the expression of hundreds genes and functional analysis of these data identified a cluster of common pathways related to cell-cell adhesion, cell junctions, focal adhesion, and cell cytoskeleton. These processes regulate adhesion and transendothelial migration of monocytes into the intima of blood vessels, the initial step of atherosclerosis development. Immunofluorescence analysis of the aortic roots showed a reduction of the number of macrophages in intima. Additional in-vitro experiments revealed reduction of monocyte adhesion to endothelial cells, as well as modulation in expression of atherogenesis-related genes, when cells were pre-exposed to polyphenol metabolites at physiologically-relevant concentrations.

To deepen molecular mechanisms of polyphenols, we investigated the impact of plasma metabolites of polyphenols on cell signaling pathways and miRNA expression in endothelial cells. The nutrigenomic effect in endothelial cells was associated with modulation of the phosphorylation level of several transcription factors and signaling pathway proteins, such as p38, p65 or Akt. We also observed changes in expression of miRNA in endothelial cells and bioinformatic analysis suggests that miRNA-target genes are also involved in processes of adhesion, transendothelial migration, focal adhesion or cytoskeleton organization.

The role and impact of dietary polyphenols on endothelial function and gene expression in humans was investigated. We showed that orange juice decreases diastolic blood pressure and significantly improves postprandial microvascular endothelial reactivity and that hesperidin could be causally linked to the observed beneficial effect of orange juice. Nutrigenomics study revealed that orange juice and hesperidin consumption commonly modulated expression of 1,582 genes. Many of these genes were involved in chemotaxis, adhesion, infiltration and lipid transport, which is suggestive of a lower recruitment and infiltration of circulating cells to vascular wall and lower lipid accumulation.

In conclusion, these results provide both evidence for cardiovascular protective effect of polyphenols and a global integrated view of the potent mechanisms by which plasma metabolites of polyphenols works at the endothelial level.