**Investigation of endogenous and/or exogenous phenolic metabolites in humans using *(un)targeted* metabolomics (ENDOPHENOL)**

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This PhD research project aims at characterizing low-molecular weight (poly)phenols (LMWP) in biological samples, through metabolomics approaches, in a controlled, (poly)phenol-free diet, with or without coffee, as a known source of LMWP. The main parameters leading to interindividual variability will also be considered. LMWP are common metabolites of endogenous metabolic pathways and dietary proteins, as well as common colonic catabolites of dietary (poly)phenols. However, the contribution of each possible source to the circulating LMWP is poorly understood and a current gap in (poly)phenol research.

**Studio dei metaboliti fenolici endogeni e/o esogeni nell’uomo mediante metabolomica (un)targeted (ENDOPHENOL)**

Questo progetto di tesi di dottorato mira alla caratterizzazione dei (poli)fenoli a basso peso molecolare (LMWP) in campioni biologici, attraverso approcci metabolomici, in un contesto di dieta controllata priva di (poli)fenoli con o senza il consumo di caffè come fonte nota di LMWP. I principali parametri responsabili della variabilità interindividuale verranno presi in considerazione. I LMWP sono metaboliti comuni di alcuni pathway metabolici endogeni, così come possono derivare dal catabolismo colonico dei (poli)fenoli introdotti con la dieta. Tuttavia, il contributo di ciascuna delle possibili fonti di LMWP circolanti è poco conosciuto e rappresenta una lacuna nella ricerca sui (poli)fenoli.

**1. State-of-the-Art**

Due to their broad spectra of biological activities, plant (poly)phenols are considered important components of the human diet. They represent organic molecules that vary in size and complexity regarding their chemical structure (Vivarelli *et al.*, 2022). After ingestion, the greatest fraction of consumed (poly)phenols follows its path to the large intestine, where they are catabolized by gut microbiota enzymes before entering colonocytes (Rodriguez-Mateos *et al.*, 2014). This means that the route of (poly)phenols from ingestion to bloodstream and urine results in their transformation (75-99%) into a plethora of generally smaller and conjugated catabolites (Scalbert and Williamson, 2000). These metabolites, known as low-molecular weight (poly)phenols (LMWP), appear in plasma in higher concentrations than the parent substances, and are likely responsible for the reported biological activity of plant (poly)phenols.

Among the gaps and challenges in (poly)phenol research, metabolic convergence is a recognized issue in terms of tracking the sources and parent compounds of LMWP derived from the diet (Di Pede *et al.*, 2023). However, one aspect of metabolic convergence is rarely discussed and investigated: the production of certain LMWP species from catecholamines and amino acids and their actual contribution to the pool of bioavailable catabolites. For instance, hippuric acid is an abundant catabolite of the *in vivo* metabolism of many (poly)phenols. However, it is also produced by the glycine deportation system using benzoic acid as means to excrete glycine in urine. Benzoic acid is a common catabolite of numerous (poly)phenol classes, such as flavonoids, but it also derives from phenylalanine/tyrosine metabolism (Vong *et al.*, 2022). Another example, 3′,4′-Dihydroxyphenylacetic acid is originated by the microbial catabolism of several dietary (poly)phenols and is also a catabolite of dopamine (Eisenhofer *et al.*, 2004).

Besides the different possible sources of LMWP, there are factors of variability among individuals, such as age, sex, genetics, and the microbiota diversity, which could influence both concentration and nature of compounds in biological samples (Manach *et al.*, 2005). Accordingly, steps must be taken to understand and consistently report circulating LMWP species, concentrations, and possible sources, to further comprehend their role in nutrition and health, also advancing the field of personalized nutrition.

**2. PhD Thesis Objectives and Milestones**

During the 1st year of the PhD, the proposed activities were i) literature search and review for the sake of being up to date with the state of the art and ii) conduction of a randomized crossover clinical trial, in which 30 volunteers (adults, aged between 20-40 years old, BMI of 18-28 kg/m2) followed a personalized and standardized (poly)phenol-free diet for 5 consecutive days, and, on the morning of the third day, received a single dose of sweetened decaffeinated coffee or sweetened hot water (volume of 180 mL) and samples (urine, blood and faeces) were collected.

The foreseen activities for the 2nd and 3rd years are described below and subdivided according to the Gantt diagram given in Table 1:

A1) **Analyses contemplated within the primary outcome:** characterization of LMWP pool in urine with and without a source of dietary (poly)phenols, to discriminate their endogenous or exogenous origin; identification of potential (poly)phenol catabolites not previously characterized, providing a comprehensive picture of the impact of coffee-(poly)phenol consumption on the urinary metabolome in humans. These analyses will be done by LC-MS targeted and untargeted approach.

A2) **Analyses contemplated within the secondary outcome:** identification of those variables which could be associated with metabolic phenotype or different response to the intervention, specifically for (poly)phenol metabolism. i) Fecal microbiota profiling and characterization to assess the influence of colonic microbiota composition over the endogenous and/or exogenous LMWP production and evaluation of interindividual variability in metabolite production; ii) feces analysis to identify produced but not absorbed exogenous (controlled-diet + coffee) and endogenous (controlled-diet + water) LMWP; iii) genotyping following genome-wide single-nucleotide polymorphism (SNP) analysis.

A3) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

***Table 1*** Grantt diagram for PhD thesis project.

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Activity Months | | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** |
| A1) | ***Analyses primary outcome*** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | 1) Targeted metabolomics urine |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | 2) Untargeted metabolomics urine |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| A2) | ***Analyses secondary outcome*** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | 1) Microbiota profiling |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 2) Targeted metabolomics feces |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 3) Untargeted metabolomics feces |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 4) Gene SNP analysis |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| A3) | ***Thesis and Paper Preparation*** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |

**3. Selected references**

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