Human Stool Metabolome Differs upon 24 h Blood Pressure Levels and Blood Pressure Dipping Status: A Prospective Longitudinal Study

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Metabolites **2021**, *11*(5), 282; https://doi.org/10.3390/metabo11050282

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Introduction

Dysbiosis of gut microbiota (GM) has been involved in the pathophysiology of arterial hypertension (HT), via a putative role of short chain fatty acids (SCFAs). Its role in the circadian regulation of blood pressure (BP), also called “the dipping profile” (defined as a drop of night-time BP compared with diurnal BP) has been poorly investigated. Sixteen male volunteers and 10 female partners were subjected to 24 h ambulatory BP monitoring and were categorized in normotensive (NT) versus HT, as well as in dippers versus non-dippers. In the present study, we have performed a 5-year longitudinal follow-up of initially non-hypertensive male volunteers with a focus on the association between GM, GM metabolome, fecal SCFA levels, and 24 h BP levels (including the BP dipping status) using untargeted nuclear magnetic resonance (NMR)-based metabolomics. Additionally, we have recruited the female partners (n = 10 participants) in order to test our hypothesis in both genders and to assess the influence of a similar environment on GM composition and stool metabolome.

Materials and methods

Patients. The non-hypertensive male volunteers of our princeps (i.e., 2015 male cohort, n = 16) and their female partners (n = 10) were contacted to form the “2020 male cohort” and “2020 female cohort”, respectively [1]. After signed informed consent, 24 h ABPM (Spacelabs 90,207 device) was performed. BP was measured every 20 min during the day and every 30 min during the night. Participants were divided into two groups: normotensive (NT) or HT, based on European Society of Hypertension (ESH) criteria [2]. A patient was categorized as dipper when his night–day systolic BP (SBP) ratio was ≤0.9 or non-dipper when his night–day SBP ratio was >0.9

Samples. Faeces were collected at home using stool collection tubes provided with the PSP Spin Stool DNA Plus Kit (ISOGEN Life Science) for GM composition analysis and Fecal Swab Collection® tubes for the anaerobic cultures and the metabolomics study. Samples in stool collection tubes were immerged with stool DNA stabilizer solution [3] and stored at −80 °C.

Microbiology. Anaerobic culture of stool samples., 16S Amplicon sequencing and microbiota profiling were performed

1H-NMR untargeted metabolomics Stool samples were centrifuged and prepared for the NMR analysis (**figure 1**). CPMG sequence was used and once obtained, the spectra were pre-processed by using MestReNova software (v14.1.1) in way to perform the multivariate statistical analysis through SIMCA (version 13.0.3, Umetrics AB, Umea, Sweden). PCA-X and OPLS-DA models were used for identify outliers and distinguish samples in defined classes respectively. Relevant features for the separation in clusters were highlighted (acetate, propionate and butyrate) and quantified by integration of their signal using maleic acid as internal standard. Univariate statistical analysis was performed.

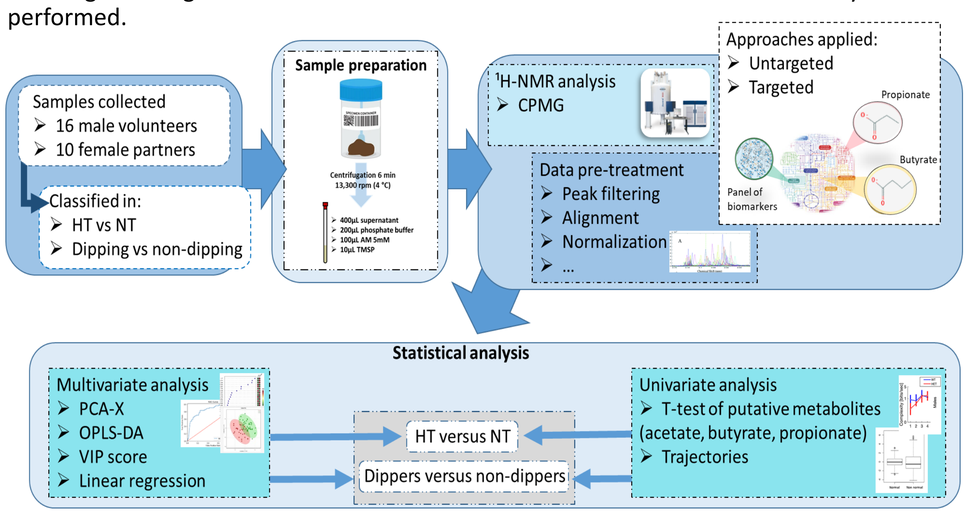

Figure 1. Workflow of 1H-NMR untargeted metabolomics analysis.

Results

Microbiology

Analysis on anaerobic culture of fecal bacteria and by 16S amplicon sequencing show no significant difference in faeces between HT versus NT patients, as well as between dippers versus non-dippers.

Untargeted Metabolomics

HT *versus* NT

Multivariate analysis of stool metabolomes did not discriminate male from female groups in the 2020 cohort, even after using supervised methods (Q^2 at −0.0364; n = 18). Multivariate analysis (principal component analysis (PCA)-X and orthogonal partial least squares discriminant analysis (OPLS-DA)) of stool metabolomes data were not discriminant between HT versus NT individuals in the 2020 male cohort (Q^2 at 0.0354; n = 15), as well as in the entire cohort (Q^2 = at 0.0966; n = 25) (**figure 2A**). By using partial least square regression lines, significant correlations between stool metabolomes and 24 h MBP levels were found in 2020 male and female cohorts (R^2 = 0.7189; n = 14 and R^2 = 0.7921; n = 9, respectively) . However, less significant correlation was found after considering the entire 2020 cohort (R^2 = 0.4521; n = 23) (**figure 2B**).

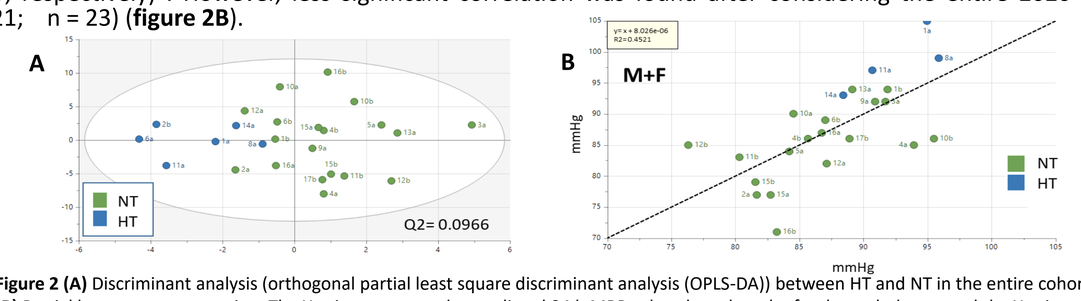

Figure 2 (A) Discriminant analysis (orthogonal partial least square discriminant analysis (OPLS-DA)) between HT and NT in the entire cohort (M+F). **(B)** Partial least square regression. The X axis represents the predicted 24 h MBP values based on the fecal metabolomes and the Y axis represents the actual 24 h MBP values. The units for both axes are expressed in mmHg. The significance level was set for R2 ≥ 0.4.

Even if OPLS-DA discriminant analyses of HT versus NT individuals was not significant a list of VIP was however generated. In this listing, acetate, propionate, and butyrate were identified, and a relative quantification was used to perform Mann–Whitney test for comparisons between two groups. Fecal amounts of acetate, propionate, and butyrate were higher in HT patients than in NT patients in the entire cohort (p = 0.0475, p = 0.038, and p = 0.038, respectively) **(figure3)** .

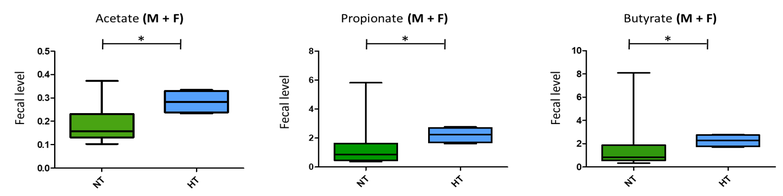

Figure 3. Levels of acetate, propionate, butyrate in the stools of HT versus NT patients in the entire cohort (Male (M) + Female (F)). The significance level was set at the 5% level. * 0.01 > *p* < 0.05.

The dietary survey showed fairly similar diets between members of the same couple, except for one couple, who were excluded from this analysis. By considering each couple as a group, the variation between the individuals belonging to the same cluster, named “inertia within group”, was measured. In this case, a higher inertia within the group was found in stool metabolomes of couples with a different BP status (51.4% of inertia; n = 8) compared with the couples in whom both individuals presented the same BP status (37.5% of inertia; n = 10).

Dippers *versus* Non-dippers

Multivariate analysis (OPLS-DA) of stool metabolomes data was discriminant between dippers versus non-dippers in both 2020 male and female cohorts (Q^2 = 0.809; n = 14 and 0.979; n = 9, respectively) and in the entire 2020 cohort (Q^2 = 0.678; n = 18) (**figure 4**).

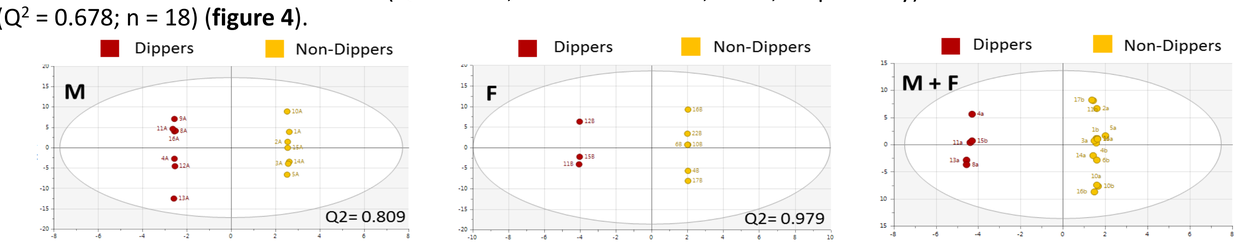

Figure 4. Discriminant analysis (orthogonal partial least squares discriminant analysis (OPLS-DA)) between dippers and non-dippers in the 2020 male cohort (M), the 2020 female cohort (F) and in the entire cohort (M + F). Significance level was set for Q^2 ≥ 0.5.

As well as for the previous analysis, for the OPLS-DA model a list of a list of VIP was generated. In this listing, acetate, propionate, and butyrate were identified, in contrast to the other features that were not identified as belonging to the matrix. After identification of the relevant features by means of correlation spectroscopy (COSY), heteronuclear single quantum coherence spectroscopy (HSQC) and HMDB, Chenomx, integration of acetate, propionate, and butyrate signals was done using maleic acid as internal standard. Fecal amounts of acetate, propionate, and butyrate were significantly higher in non-dippers versus dippers in the entire cohort (p = 0.033, p = 0.038, and p = 0.036, respectively) (**figure 5**).

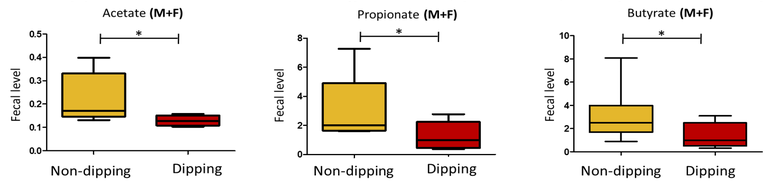

Figure 5. Levels of acetate, propionate, butyrate in the stools of dippers versus non-dippers versus NT patients in the entire cohort (Male (M) + Female (F)). The significance level was set at the 5% level. * 0.01 > *p* < 0.05.

In complement to these analysis, a trajectory of SCFAs according to dipping status between 2015 and 2020 was performed. From 2015 to 2020, six patients changed their dipping status as well. Significant changes were noted in the acetate, propionate, and butyrate levels between the two periods (p ≤ 0.0001, p = 0.032, and p = 0.032; n = 10), with higher levels of these SCFAs when the patients were non-dippers compared with when they were classified as dippers (**figure 6**).

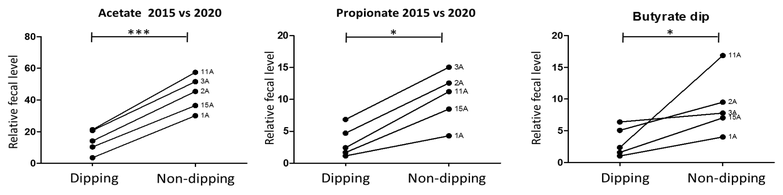

Figure 6. Trajectory of SCFAs according to dipping status between 2015 and 2020. Significant changes were noticed in the acetate, propionate, and butyrate levels in patients who changed their dipping status between the two periods (*p* ≤ 0.0001, *p* = 0.0317, and *p* = 0.0317; *n* = 10), with higher levels of SCFAs when the patients were non-dippers compared with when they were dippers. The significance level was set at the 5% level. *** *p* < 0.001; * 0.01 > *p* < 0.05.

Conclusion

In conclusion, our longitudinal 26-patient cohort with a mean follow-up of ~5 years confirms that the fecal metabolome is associated with 24 h MBP levels in both genders, with higher SCFA levels in the faeces of HT patients. Even if no significant change in GM composition was found, our data highlight a novel putative link between the stool metabolome (and specifically SCFAs) and the non-dipping BP profile as recently reported on our study cohort of 44 patients [4]. Moreover, our longitudinal study demonstrated that a modification of fecal SCFA levels is associated with a modulation of the dipping status of patients. Further investigations, including interventional trials aiming at evaluating the hypotensive effect of GM modifications and/or modulation of the fecal metabolome by pre-biotics, pro-biotics, or post-biotics (such as SCFAs per se), are needed to confirm our exploratory data.

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