Metafollow : one-year longitudinal follow-up to assess metabolites' variations in healthy subjects

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1 Introduction

In healthcare, almost all metabolomics' studies focus on pathologies by studying inter-individual variation of metabolites. But if we want to apply metabolomics to use this approach in clinical practice for personalized medicine, we must first understand normal intra-individual variations of metabolites before preventively detect a pathological deviation. Our knowledge of the intra-individual « normal » variations of the metabolome is currently very poor and to expand our expertise, we have first to explore healthy people's metabolome over a certain period. For this purpose, we selected healthy subjects that we followed for one year. Blood, urine and saliva samples' collection was performed to have short-term and long-term variability of metabolites. We firstly focus on serum analysis based on an NMR metabolomics approach in order to explore intra- and inter-individual metabolites' variation.

2 Cohort and methods

30 healthy volounteers (14 men and 16 women) aged from 20 to 40 were recruited and followed for one year. Serum, urine and saliva samples were first collected each week for ten weeks (short-term variability) and then each month for ten months (long-term variability). First, sera samples (with QC samples) were analyzed separately for the two samples sets (short and long-term) by ^{1}H-NMR after proteins removal by ultrafiltration. 45 metabolites were then quantified in each spectrum based on the maleic acid peak using ChenomX software (**Figure 1**).

Before determining intra- and inter-individual variations of metabolites, multivariate (PCA) analysis were performed on all samples, including QC to identify putative outliers and to exclude any analytical bias or batch effects.

Finally, several mixed-ANOVA analyses were performed on each cohort to obtain intra and inter-individual variabilities of metabolites (Coefficient of Variation : CV %).

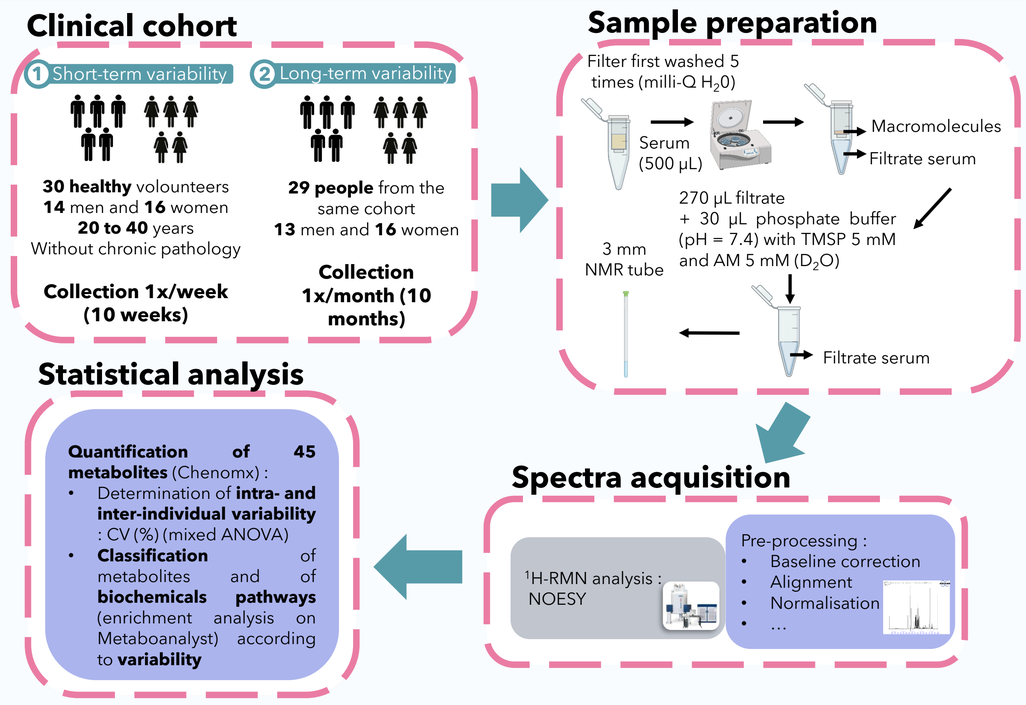

Figure 1. Clinical cohort and worflow of the ^{1}H-NMR metabolomics analysis.

3 Results

Intra-individual variability

Weekly samples

Mixed-ANOVA analyses on short-term samples show, **within-subject**, CV of each metabolite and reveal that metabolites can be classified according to their variability. Four classes can be defined: very low variability (0-10 %), low to moderate variability (10-20 %), high variability (20-40 %) and very high variability (> 40 %). For a more detailed analysis, we also wanted to separate the data for men and women. Only one difference in classification is found between these data : creatine which has a higher variability in men compared to women (**Figure 2**).

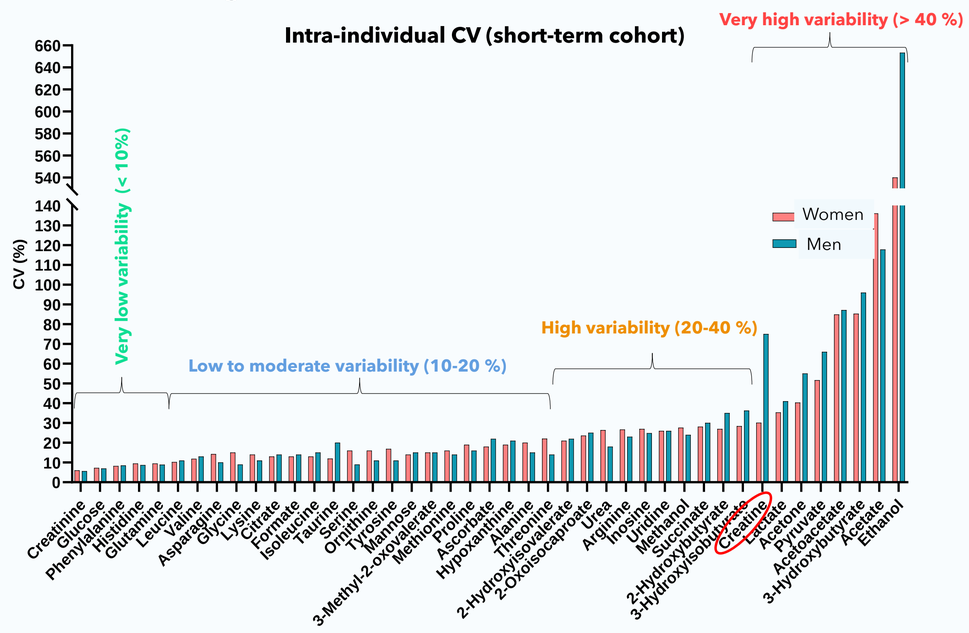

Figure 2. mixed-ANOVA analyses (men and women separately) : intra-individual CV (%) of metabolites.

Monthly samples

Mixed-ANOVA analyses were also performed on long-term set to compare results to those obtained with the weekly samples. **Table 1**, comparing these 2 analyses mixing both men and women, illustrates that metabolites' variations are in the same ranges for weekly and monthly data, except for the methanol which is classified in « very high variability » in the long-term set.

	Creatinine	Glucose	Phenylalanine	Glutamine	Histidine	Leucine	Asparagine	Valine	Glycine	Lysine	Ornithine
CV (weeks)	5.80	7.11	8.53	9.03	9.11	10.52	12.59	12.62	12.92	13.08	13.35
CV (months)	5.53	5.58	8.62	8.56	9.02	13.36	11.80	12.63	15.65	14.63	18.92
	Serine	Citrate	Formate	Isoleucine	Tyrosine	3-Methyl-2-oxovalerate	Mannose	Methionine	Taurine	Proline	Alanine
CV (weeks)	13.43	13.44	13.79	14.13	14.63	14.88	14.93	15.24	16.55	17.66	18.36
CV (months)	12.77	16.71	14.43	17.09	15.62	17.63	14.47	16.02	14.33	20.75	17.32
	Threonine	Ascorbate	Hypoxanthine	2-Hydroxyisovalerate	Urea	2-Oxoisocaproate	Inosine	Methanol	Uridine	Succinate	
CV (weeks)	18.84	20.14	20.25	22.34	22.38	24.31	25.81	26.00	26.43	28.92	
CV (months)	19.61	20.77	24.25	32.75	18.70	24.71	31.21	41.07	23.67	23.80	
	2-Hydroxybutyrate	3-Hydroxyisobutyrate	Lactate	Creatine	Acetone	Pyruvate	Acetoacetate	3-Hydroxybutyrate	Acetate	Ethanol	
CV (weeks)	31.92	33.35	38.01	49.69	53.07	58.18	89.13	93.72	128.86	592.62	
CV (months)	35.05	32.99	41.02	41.52	66.81	65.87	72.91	87.00	98.77	911.51	

Table 1. Comparison of short and long-term intra-individual metabolites' variabilities.

Variability of biochemichal pathways

Enrichment analysis (Metaboanalyst 6.0) was used to highlight pathways according to their within-subject variability. All metabolites with CV < 20% were first used to determine the least variable pathways and metabolites with CV > 20% were then used to have the most variable paths (**Figure 3**).

CV < 20 % : AA metabolism essentially

Arginine and proline metabolism
Glycine and serine metabolism
Methionine metabolism
Phenylalanine and tyrosine metabolism
Urea cycle
Carnitine synthesis
Spermine and spermidine biosynthesis
Ammoniac recycling
Glutathione metabolism

CV > 20 % : Glucidic metabolism essentially

Ketone bodies metabolism
Butyrate metabolism
Valine, leucine, isoleucine degradation
Pyruvate metabolism
Krebs cycle
Neoglucogenesis
Warburg effect
Ethanol degradation
Fatty acids biosynthesis

Figure 3. Biochemichal pathways : less (in green) and most (in red) variable ones.

Inter-individual variability

Weekly samples

Mixed-ANOVA analyses on short-term samples show, **between-subject,** CV of each metabolite. Compared to the intra-individual analysis, inter-individual CV are quite different according to gender. Results show that most of the metabolites have a within-subject variation higher than the between-subject variation, except 2-hydroxyisovalerate, creatine, creatinine, glutamine, glycine, inosine, ornithine and also proline, which is highligted only in the men's analysis (**Figure 4**).

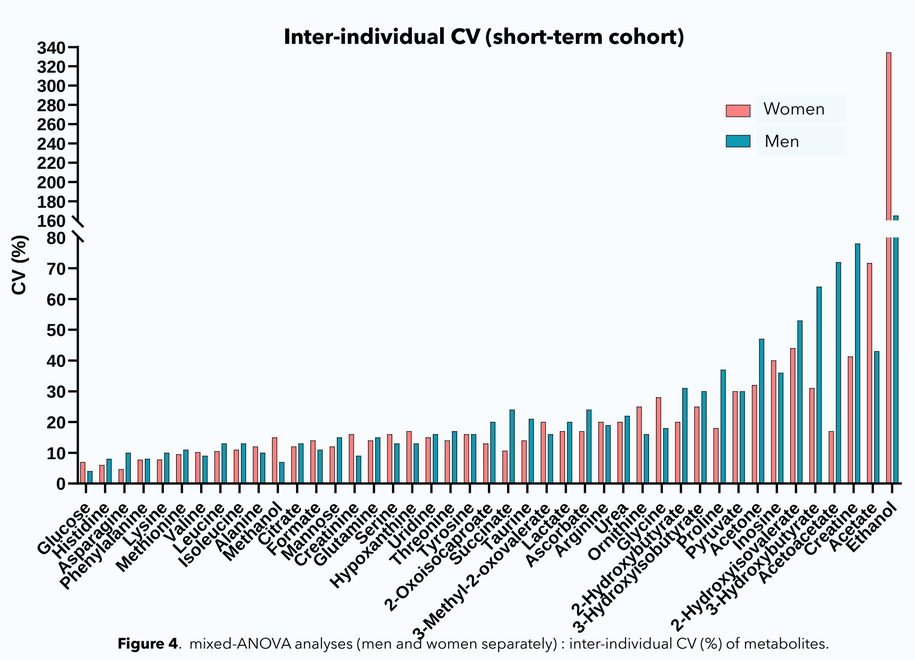

Figure 4. mixed-ANOVA analyses (men and women separately) : inter-individual CV (%) of metabolites.

Monthly samples

Mixed-ANOVA analyses were also performed on long-term set to compare results to those obtained with the weekly samples. **Table 2**, comparing these 2 analyses mixing both men and women, illustrates that metabolites' variations are in the same ranges for weekly and monthly data, except for the formate, uridine, 3-hydroxyisobutyrate, pyruvate, acetone, 3-hydroxybutyrate, acetate and acetoacetate.

	Glucose	Histidine	Asparagine	Phenylalanine	Lysine	Methionine	Alanine	Methanol	Citrate	Formate	Valine
CV (weeks)	5.74	7.69	7.89	7.96	9.66	10.70	10.83	11.75	12.17	12.42	12.83
CV (months)	6.47	8.39	7.68	6.69	10.39	11.20	13.67	14.80	13.78	27.01	14.60
	Isoleucine	Glutamine	Mannose	Serine	Uridine	Tyrosine	Threonine	Leucine	Hypoxanthine	Taurine	Lactate
CV (weeks)	14.38	14.50	14.61	14.84	15.04	15.21	15.28	15.30	16.48	17.49	17.67
CV (months)	18.37	14.28	16.06	13.08	9.82	12.93	14.52	17.03	19.02	15.64	24.18
	3-Methyl-2-oxovalerate	Succinate	Creatinine	2-Oxoisocaproate	Ornithine	Urea	Ascorbate	Glycine	2-Hydroxybutyrate	3-Hydroxyisobutyrate	
CV (weeks)	17.72	17.88	17.99	18.03	22.12	22.25	22.63	23.82	24.86	28.76	
CV (months)	12.30	17.00	17.47	21.35	25.04	18.70	22.64	22.77	20.79	18.57	
	Pyruvate	Proline	Inosine	Acetone	2-Hydroxyisovalerate	3-Hydroxybutyrate	Creatine	Acetate	Acetoacetate	Ethanol	
CV (weeks)	29.97	32.89	37.87	50.20	51.45	56.69	57.28	60.26	66.05	325.53	
CV (months)	43.09	36.05	33.67	33.95	46.88	21.81	59.21	23.32	25.75	166.83	

Table 2. Comparison of short and long-term inter-individual metabolites' variabilities.

4 Conclusion and perspectives

The first results obtained in this work highligth a stratification of blood metabolites according to their short and long-term intra- and inter-individual variations. For the within-subject CV, metabolites were also linked to metabolic pathways to identify the less and most variable ones. All the informations about intra-individual variations of metabolites are essential if we want to follow patients with a more personalized approach in a clinical context. Also, given that the majority of metabolites have higher intra-individual than inter-individual variability, it seems important that this within-subject variation shoulb be considered when biomarkers are identified in metabolomic studies investigating a pathology. In the future, it will be essential to analyse blood samples with other analytical techniques as MS in order to increase number of studied metabolites. Moreover, urine and saliva analyses will complete our data and are expected to give a more complete overview of the normal human intra-individual metabolome's variation, which is very important for the application of metabolomics in the context of personalized medicine.