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Article

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Abstract

Lifestyle-related diseases, such as overweight/obesity, *diabetes mellitus* type 2 (T2DM), cardiovascular disease, or osteoarthritis, are a major health burden in Western societies. Due to common risk factors, most patients suffer from multimorbidity, i.e., have been diagnosed with more than one of these diseases. Physical activity (PA) is known to have a positive effect on all of these diseases, however, little is known on the effects of PA on patients with multimorbidity. In particular, so far, there are no reliable biomarkers to predict and monitor the effects of PA-based lifestyle intervention programs on these subjects. Here, employing a "mini metabolomics" approach with dried blood spots, we analyzed concentrations of different metabolites in subjects with multimorbidity in the course of the lifestyle intervention program MultiPill-Exercise. We found increasing concentrations of all tested amino acids (AAs) and acylcarnitines (ACs) after 12 weeks (t1) and 24 weeks (t2) of the intervention. When correlating baseline (t0) metabolite concentrations with changes in physiological and clinical parameters, we observed strong associations of acylcarnitine levels and changes in metabolic and cardiovascular parameters. When analyzing metabolite acute reactions in response to exhaustive exercise (ergometer test), however, there were little overall changes. Nevertheless, there was a significant negative correlation between mobilization of medium-chain acylcarnitines (MC-ACs) at t2 and changes in peak power output (PPO) between t0 and t2. Taken together, these data suggest that AAs and ACs might be promising biomarkers to predict and monitor the effects of PA-based lifestyle intervention programs in subjects with multimorbidity.

Keywords: lifestyle-related diseases; multimorbidity; exercise; metabolomics; dried blood spots

1. Introduction

Lifestyle-related diseases, specifically overweight/obesity, *diabetes mellitus* type 2 (T2DM), cardiovascular disease, or osteoarthritis, represent a major health threat in Western societies. Inactivity and an unhealthy diet, excess alcohol consumption, smoking and psychosocial factors such as stress, are important risk factors. Most patients with lifestyle-related disease suffer from multimorbidity, mainly due to similar risk factors. Physical exercise is considered "poly-" or "multipill" in the context of lifestyle-related diseases. While a range of studies could clearly demonstrate preventive, therapeutic and rehabilitative efficacy of sports and everyday-life physical activity in a broad variety of settings, studies focusing on the aspect of multimorbidity are still scarce (for review, see Nicholson et al., 2023).

The "MultiPill-Exercise" study aimed at filling this gap. Organized as a pilot study, followed by a large-scale randomized controlled trial, it focused on enhancing sport and physical activity commitment in subjects with more than one lifestyle-related disease (for details, see Schweda et al., 2022). Since a healthier lifestyle is known to yield metabolic benefits, we hypothesized that we might be able to detect changes in subjects' metabolomes throughout the intervention, which might be

associated with individuals' health benefits and which might be employed as biomarkers for training adaptation and/or tools for individual training management in the future.

The most central players in a person's metabolome are amino acids (AAs) and acylcarnitines (ACs). Indeed, literature data suggest effects of both acute exercise and exercise training on AA plasma profiles, since AA turnover adapts to physical activity in multiple ways and might reflect metabolic changes in response to sports (for review, see Sakaguchi et al., 2019; Schranner et al., 2020; Tian et al., 2021; Khoramipour et al., 2022). Consequently, AAs are interesting candidate biomarkers in the context of exercise, namely aromatic and branched-chain AAs (BCAAs). Nevertheless, little is known on AA patterns in subjects with multimorbidity. Interestingly, BCAAs, glucagon secretion and insulin sensitivity are mutually connected in a complex manner, suggesting associations with metabolic syndrome and T2DM pathogenesis (for review, see Flynn et al., 2020). Consistently, data by Hansen et al., 2015, suggest differential responses to exercise in people with T2DM with regard to their AA profiles. Similarly, ACs are intermediates of fatty acid metabolism, but can also be generated as by-products of BCAA metabolism or derivates of other organic acids (Rinaldo et al., 2008). Exercise and training are known to affect both AC and free carnitine concentrations in the circulation. Acute exercise is known to enhance concentrations of most ACs paralleled by decreases in free carnitine levels (Adeva-Andany et al., 2017; Schranner et al., 2020). In addition, several authors demonstrated increased AC concentrations in the context of overweight/obesity, insulin resistance and T2DM. Their pro-inflammatory activity is associated with decreased fatty acid oxidation and a low level of metabolic flexibility, rendering them important candidate biomarkers in subjects with multimorbidity (Rutkowsky et al., 2014; McCoin et al., 2015). Interestingly, Huffman et al., 2011, demonstrated decreased concentrations of circulating ACs in parallel to activity-induced improvements in insulin sensitivity. Consistently, data of a large study by the same group suggest that skeletal muscle metabolic signatures of subjects at risk of metabolic disease change during a six-months training intervention – and that they are associated with changes in cardiovascular parameters and might be indicators of metabolic flexibility and plasticity (Huffman et al., 2014).

These data suggest that metabolic parameters might be important biomarkers in the context of exercise-based lifestyle intervention programs for subjects with multimorbidity, such as MultiPill-Exercise. For routine applications, however, easy and cost-effective procedures are required, such as analysis of metabolites from dried spots of capillary blood via tandem mass spectrometry (MS/MS), a method that has been established for the detection of metabolic diseases in newborns for a long time (Skogvold et al., 2023). Here, we employed this method to detect changes in AA and AC profiles in selected MultiPill-Exercise subjects and correlated patterns with the development of physiological and clinical data throughout the intervention.

2. Materials and Methods

2.1. MultiPill-Exercise Pilot Study

The study was conducted in accordance with the Declaration of Helsinki, approved by the "Ethics Committee of the Medical Faculty, University of Tübingen" (reference number: 298/2019BO2, 04.05.2019) and registered at the German clinical trial register (DRKS00016702). Written informed consent was obtained from all subjects. The lifestyle intervention program was designed as a 24-week schedule, split up into two twelve-week blocks, the first of which was more closely supervised and the second more self-directed. Subjects were required to meet "multimorbidity characteristics", which meant that they had to have been diagnosed with at least two of four lifestyle diseases (T2DM/manifest or risk thereof, overweight/obesity, cardiovascular disease/ hypertension or osteoarthritis (OA)). Diagnostics, including blood sampling, was carried out at baseline (t0), after the first twelve-week block (t1) and at the end of the intervention (t2). (for details, see Schweda et al., 2022). Altogether, n=39 subjects were included in the study, which were analyzed in two "waves", one starting in late August of 2019 (n=20; MP1901-1925) and the other in mid-January of 2020 (n=19; MP1927-1953). Missing numbers were assigned to potential subjects that had to be excluded from the

study during diagnostics. Due to the COVID19 pandemic, t1 diagnostics for all subjects of "wave 2" had to be cancelled, in addition, for this group, the intervention program had to be significantly modified according to contact restrictions associated with the pandemic (for details, see Schweda et al., 2022). Finally, there were some dropouts from the study (for details, see Table 1). As a consequence, "at rest" data for all three time points (t0, t1 and t2) were only available for n=6 subjects, all of them female, with a mean age of 59.67 years, were available. For subject MP1902 (female, age 54 years), at least t0 and t1 data were available. At t2, metabolomics patterns were also assessed in response to acute exercise, i.e., dried blood spots were sampled before and after spiroergometry diagnostics. Here, data for n=26 subjects were available.

Table 1. Subjects and data.

		spiroergometry, clinical parameters, blood drawings			Guthrie cards (pre exercise)			Guthrie cards (post exercise)		
		t0	t1	t2	t0	t1	t2	t0	t1	t2
1	MP1901			dropout	no sample		dropout			dropout
2	MP1902			dropout			dropout			dropout
3	MP1903				no sample					
4	MP1905				no sample					no sample
5	MP1906		dropout		no sample	dropout				no sample
6	MP1907				no sample		no sample			no sample
7	MP1910				no sample					
8	MP1911				no sample	no sample				no sample
9	MP1912			dropout	no sample	no sample	dropout			dropout
10	MP1913		dropout	dropout	no sample	dropout	dropout		no sample	dropout
11	MP1914				no sample					
12	MP1915									
13	MP1916									
14	MP1917				no sample					no sample
15	MP1918				no sample					
16	MP1919			dropout	no sample		dropout			dropout
17	MP1922									
18	MP1923									
19	MP1924									
20	MP1925									
21	MP1927									
22	MP1928			dropout			dropout			dropout
23	MP1930									
24	MP1932									
25	MP1933			dropout			dropout			dropout
26	MP1934									
27	MP1937									
28	MP1939		no t1 diagnostics due to COVID19 lockdown	dropout		no t1 diagnostics due to COVID19 lockdown	dropout	no sample	no t1 diagnostics due to COVID19 lockdown	dropout
29	MP1940									
30	MP1942									
31	MP1944			dropout			dropout			dropout
32	MP1946									
33	MP1947									
34	MP1948									
35	MP1949									
36	MP1950									
37	MP1951									
38	MP1952									
39	MP1953									

(Subjects 1–20: wave 1; subjects 21–39: wave 2. In wave 1 "Guthrie cards (post exercise)" a large "no sample" region spans the t0/t1 columns.)

Table lists subjects of both waves and available data and samples (green shading). Red shading indicates that the respective piece of data or sample was not available.

2.2.". Mini Metabolomics": Metabolomics Analysis from Dried Blood Spots

Capillary blood was taken from subjects' earlobes, immediately spotted on "Guthrie" paper cards and allowed to dry. Metabolomics patterns were determined by the "Screening Labor Hannover" using MS/MS, for some metabolites, for example BCAAs, coupled to liquid chromatography (LC) (LC-MS/MS). Table 2 lists all metabolites analyzed and abbreviations used in this paper. Usually, ACs are classified as short-chain (SC-ACs, C2-C6), medium-chain (MC-ACs, C8-C12) and long-chain (LC-ACs, > C12).

Table 2. Metabolomics and abbreviations.

Abbreviations	Metabolites
Carnitine-u, C0	free / unconjugated carnitine
Cx	total carnitine
C2-u	acetylcarnitine
C3-DC+C4-OH-u	malonylcarnitine (C3-DC) + 3-hydroxybutyrylcarnitine (C4-OH)
C3-u	propionylcarnitine (C3)

C4-u	butyrylcarnitine + isobutyrylcarnitine (C4)
C4-DC+C5-OH-u	methylmalonylcarnitine (C4-DC) + 3-hydroxyisovalerylcarnitine (C5-OH)
C5-u	isovalerylcarnitine + methylbutyrylcarnitine (C5)
C5-DC+C6-OH-u	glutarylcarnitine (C5-DC) + 3-hydroxyhexanoylcarnitine (C6-OH)
C6-u	hexanoylcarnitine (C6)
C6-DC-u	methylglutarylcarnitine (C6-DC)
C8	octanoylcarnitine (C8)
C10-u	decanoylcarnitine (C10)
C10:1-u	decenoylcarnitine (C10:1)
C12-u	dodecanoylcarnitine (C12)
C12:1-u	dodecenoylcarnitine (C12:1)
C14-u	tetradecanoylcarnitine (C14)
C14-OH-u	3-hydroxytetradecanoylcarnitine (C14-OH)
C14:1-OH-u	3-hydroxytetradecenoylcarnitine (C14:1-OH)
C14:1-u	tetradecenoylcarnitine (C14:1)
C14:2-u	tetradecadienoylcarnitine (C14:2)
C16-u	palmitoylcarnitine (C16)
C18-u	stearoylcarnitine (C18)
C18:1-u	oleoylcarnitine (C18:1)
C18:2-u	linoleoylcarnitine (C18:2)
C16-OH-u	3-hydroxypalmitoylcarnitine (C16-OH)
C18:1-OH-u	3-hydroxyoleoylcarnitine (C18:1-OH)
C18:2-OH-u	3-hydroxylinoleoylcarnitine (C18:2-OH)
SC-ACs	short-chain acylcarnitines
MC-ACs	medium-chain acylcarnitines
LC-ACs	long-chain acylcarnitines
Cit	citrulline
Met	methionine
Phe	phenylalanine
Tyr	tyrosine
Val	valine
Xle	leucine + isoleucine

Table lists all metabolites analyzed in this study and their abbreviations.

2.3. Statistical Analysis

Statistical analysis was carried out using software SPSS for Macintosh, Version 26.0 (IBM Corporation, Armonk, New York). After compiling all data in a comprehensive table, metabolite baseline concentrations (t0) as well as changes in physiological and clinical parameters after 12 weeks (t0-t1) were tested for normal distribution using Kolmogorov-Smirnov and Shapiro-Wilk statistical tests. In case of normal distribution, t-tests for paired samples were applied, otherwise, Wilcoxon testing for differences was carried out. Potential correlations were tested using Spearman rank correlation coefficients. Significance was defined as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). The MultiPill-Exercise pilot study represents an explorative, hypothesis-generating approach. Therefore, no correction factors for multiple testing were introduced.

3. Results

3.1. Individual Metabolite Kinetics Throughout the Intervention

To characterize individual metabolite patterns, we first determined time courses for individual subjects throughout the intervention. Unfortunately, for various reasons, mainly technical issues and dropout from the study, complete data sets consisting of t0, t1 and t2 data were only available for n=6

subjects (MP1915, MP1916, MP1922, MP1923, MP1924, and MP1925), for one additional participant (MP1902), at least t0 and t1 data could be obtained. Both t0 and t2 data were available for n=21 participants (MP1915, MP1916, MP1922, MP1923, MP1924, MP1925, MP1927, MP1930, MP1932, MP1934, MP1937, MP1940, MP1942, MP1946-MP1953) (Table 1). As shown in Figure 1 and Table 3, there was a general increase in all AAs and ACs throughout the study, whereas free carnitine showed a moderate decline. In addition, there were some individual variations, specifically with regard to Cit, Phe, free carnitine and MC-ACs.
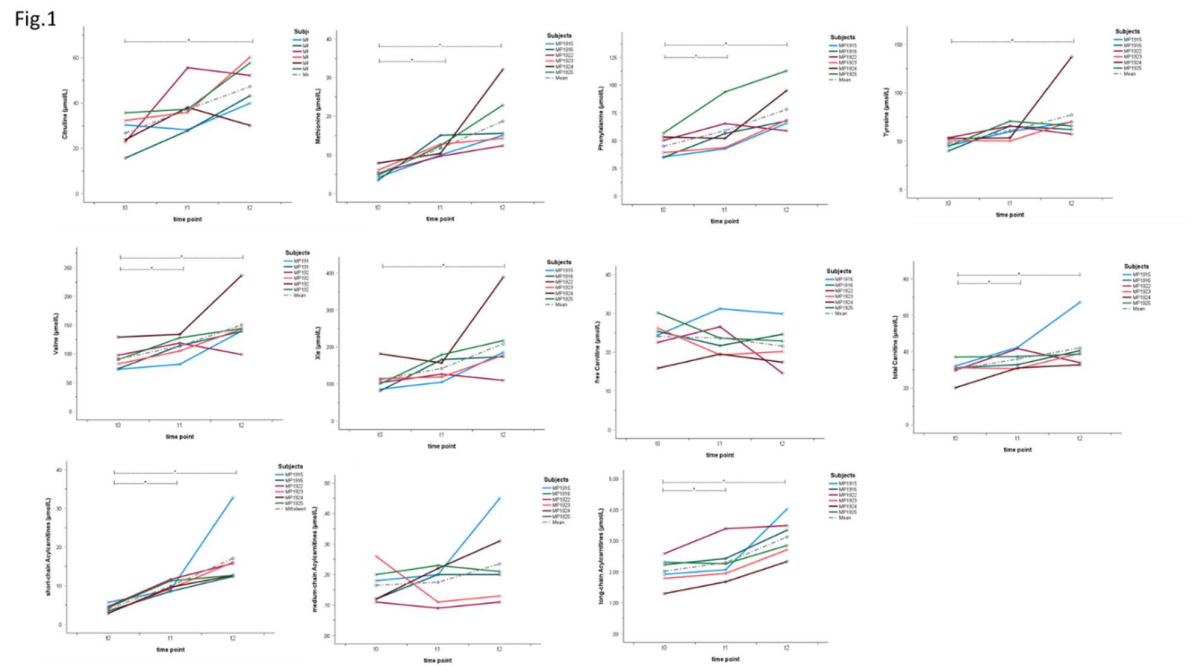
Figure 1. Spaghetti plots of individual metabolite concentrations. Graphs depict changes in individual metabolite concentrations at rest at t0, t1 and t2, as assessed for the six individual subjects for which data for each time point were available.

**Table 3.** Individual kinetics of metabolite concentrations (t0, t1, t2).

		t	n	min	max	MD	ME	SD	Δ (t0-t1) p	Δ (t0-t2) p
amino acids	Cit (µmol/L)	t0	6	15.7	35.7	27.05	26.78	7.34	10.37 .156	20.45 .031*
		t1	6	27.8	55.6	36.60	37.15	10.10		
		t2	6	30.1	60.3	47.70	47.23	11.56		
	Met (µmol/L)	t0	6	3.5	7.9	5.05	5.30	1.56	6.43 .016*	13.43 .031*
		t1	6	9.7	15.1	11.40	11.73	2.09		
		t2	6	12.4	32.1	15.35	18.73	7.47		
	Phe (µmol/L)	t0	6	34.8	56.6	44.65	44.83	9.63	14.10 .031*	33.27 .013*
		t1	6	42.6	93.8	54.15	58.93	19.04		
		t2	6	58.8	112.9	68.10	78.10	21.11		
	Tyr (µmol/L)	t0	6	39.9	53.5	47.95	47.83	5.36	13.07 .109	29.20 .031*
		t1	6	50.2	70.7	62.70	60.90	7.91		
		t2	6	57.2	137.2	67.75	77.03	29.88		
	Val (µmol/L)	t0	6	73.1	129.2	86.25	91.12	20.87	22.60 .016*	59.17 .031*
		t1	6	82.0	134.0	116.55	113.72	18.58		
		t2	6	99.0	236.4	141.55	150.28	45.54		
	Xle	t0	6	81.4	181.6	102.15	111.32	36.50	30.75	97.63

		t	n	min	max	MD	ME	SD	D(t0–t1)	D(t0–t2)
	(µmol/L)	t1	6	104.9	179.1	141.75	142.07	29.43	.109	.031*
		t2	6	109.9	389.1	181.80	208.95	94.96		
(acyl)carnitines	Carnitine-u (µmol/L)	t0	6	15.9	30.2	24.88	24.09	4.76	-0.43 .844	-2.50 .313
		t1	6	19.3	31.2	22.68	23.66	4.59		
		t2	6	14.6	29.9	21.53	21.59	5.44		
	Cx (µmol/L)	t0	6	20.2	37.1	31.20	30.28	5.54	5.75 .047*	11.78 .031*
		t1	6	30.7	42.4	35.15	36.03	5.24		
		t2	6	32.8	67.2	38.90	42.07	12.69		
	SC-ACs (µmol/L)	t0	6	2.9	5.7	3.93	4.01	1.09	5.88 .016*	13.07 .031*
		t1	6	8.6	11.7	9.47	9.89	1.27		
		t2	6	12.4	32.8	14.26	17.08	7.87		
	MC-ACs (µmol/L)	t0	6	0.1	0.3	0.15	0.17	0.06	0.01 .484	0.07 .313
		t1	6	0.1	0.2	0.20	0.18	0.06		
		t2	6	0.1	0.5	0.21	0.24	0.13		
	LC-ACs (µmol/L)	t0	6	1.3	2.6	2.08	2.02	0.45	0.27 .031*	1.10 .031*
		t1	6	1.7	3.4	2.17	2.30	0.59		
		t2	6	2.3	4.0	3.10	3.12	0.61		

Table shows concentrations of individual metabolites at the three different time points and their changes (D) between t0 and t1 and between t0 and t2. t: time point, n: number of subjects, min: minimum value, max: maximum value, MD: median, ME: mean, SD: standard deviation.

3.2. Correlation Analysis

Against this background, we analyzed potential correlations between metabolite patterns and physiological and clinical parameters. For this purpose, using Spearman correlation analysis, we first examined potential associations between t0 metabolite concentrations and changes in physiological and clinical parameters between t0 and t1, i.e., after 12 weeks of training intervention. As shown in Table 4 and Figure 2, we found a few significant correlations between AA t0 concentrations and clinical parameters. Specifically, we could detect positive correlations between Cit concentrations and changes in fasting blood glucose as well as between tyrosine concentrations and changes in systolic RR. However, when analyzing carnitine and AC concentrations, we found a range of significant correlations with changes in clinical parameters, specifically relating to inflammation, glucose as well as lipid metabolism and blood pressure / cardiovascular disease. In particular, there was a positive correlation between free carnitine and ΔCRP, as well as of SC- and MC-ACs with ΔHbA1c, ⊚fasting glucose and ⊚cholesterol. In addition, t0 concentrations of free and total carnitine negatively correlated with ΔdiastRR und ΔsysRR. These data indicate that baseline concentrations of carnitine and ACs might represent promising biomarkers in the context of training adaptation in this cohort.
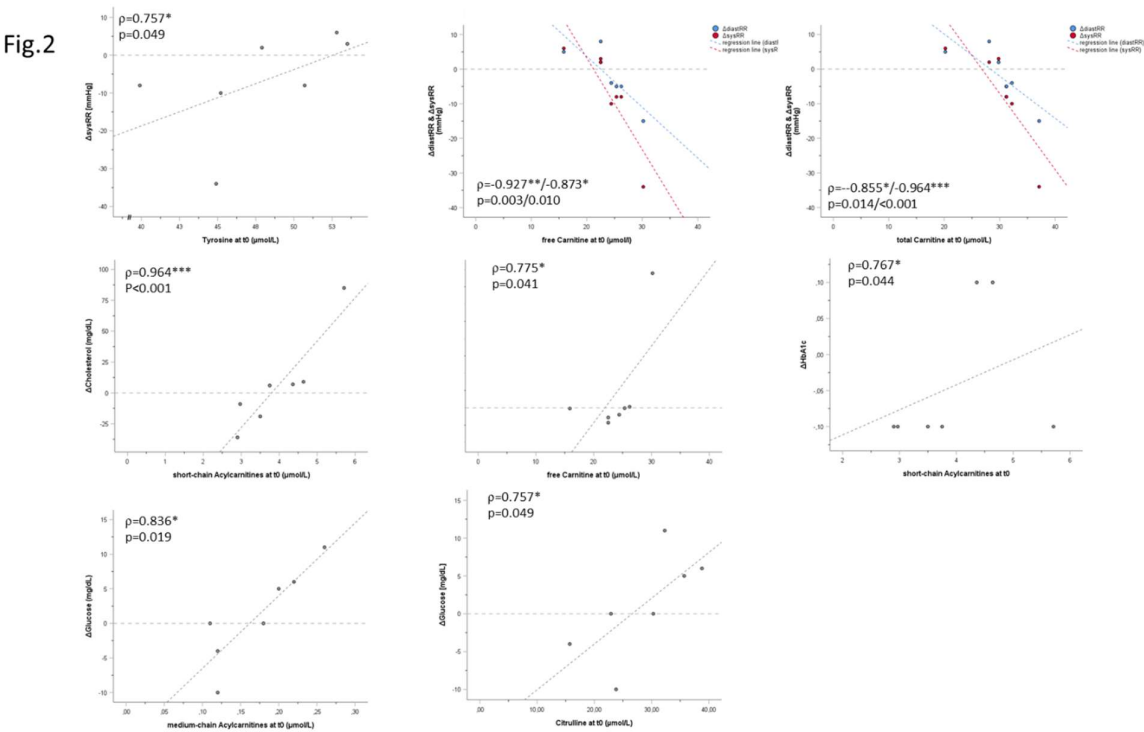
Figure 2. Correlations of baseline metabolite parameters and the development of physiological and clinical parameters between t0 and t1. Graphs depict significant correlations between baseline (t0) metabolite concentrations and changes between different physiological / clinical parameters between t0 and t1 as indicated.

Table 4. Baseline metabolomics (t0): correlation analysis.

Δphysiological / clinical parameters (t0-t1)		statistics	AA concentration (t0)						AC concentration (t0)				
			Cit	Met	Phe	Tyr	Val	Xle	C0	Cx	SC-ACs	MC-ACs	LC-ACs
sports	ΔrelVO2max [mL/kgxmin]	correlation [ρ]	-0.624	-0.459	-0.532	-0.184	-0.716	-0.514	0.019	0.167	0.239	-0.278	0.184
		p (2-sided)	0.134	0.300	0.219	0.694	0.070	0.238	0.969	0.721	0.606	0.546	0.694
		n	7	7	7	7	7	7	7	7	7	7	7
	ΔPPO [W/kg]	correlation [ρ]	-0.144	0.180	-0.487	0.487	-0.054	0.018	-0.245	-0.373	0.108	-0.055	0.162
		p (2-sided)	0.758	0.699	0.268	0.268	0.908	0.969	0.596	0.41	0.818	0.908	0.728
		n	7	7	7	7	7	7	7	7	7	7	7
body weight	ΔBMI [kg/m2]	correlation [ρ]	-0.679	0.107	0.036	0.214	-0.036	0.071	-0.577	-0.360	-0.179	-0.577	-0.321
		p (2-sided)	0.094	0.819	0.939	0.645	0.939	0.879	0.175	0.427	0.702	0.175	0.482
		n	7	7	7	7	7	7	7	7	7	7	7
inflammation	ΔIL6 [ng/L]	correlation[ρ]	0.643	-0.250	0.071	-0.143	0.036	-0.214	0.342	0.414	0.643	0.306	0.464
		p (2-sided)	0.119	0.589	0.879	0.760	0.939	0.645	0.452	0.355	0.119	0.504	0.294
		n	7	7	7	7	7	7	7	7	7	7	7
	ΔCRP [mg/dL]	correlation[ρ]	0.000	-0.071	0.536	-0.429	-0.071	0.143	.775*	0.595	-0.321	0.270	0.179
		p (2-sided)	1.000	0.879	0.215	0.337	0.879	0.76	0.041	0.159	0.482	0.558	0.702
		n	7	7	7	7	7	7	7	7	7	7	7
	Δfibrinogen [mg/dL]	correlation[ρ]	0.257	0.086	-0.029	0.429	0.029	0.086	0.261	0.174	0.429	0.116	0.486
		p (2-sided)	0.623	0.872	0.957	0.397	0.957	0.872	0.618	0.742	0.397	0.827	0.329
		n	6	6	6	6	6	6	6	6	6	6	6
diabetes	Δinsulin [pmol/L]	correlation[ρ]	0.000	0.107	-0.250	0.321	0.000	0.036	0.144	-0.072	0.143	0.090	0.429

		p (2-sided)	1.000	0.819	0.589	0.482	1.000	0.939	0.758	0.878	0.76	0.848	0.337
		n	7	7	7	7	7	7	7	7	7	7	7
	ΔHbA1c [%]	correlation[ρ]	0.356	-0.225	0.337	-0.019	0.206	-0.131	0.198	0.415	0.767*	-0.076	0.636
		p (2-sided)	0.434	0.628	0.460	0.968	0.658	0.78	0.67	0.354	0.044	0.872	0.125
		n	7	7	7	7	7	7	7	7	7	7	7
	Δglucose [mg/dL]	correlation[ρ]	0.757*	0.090	-0.180	0.000	-0.234	0.054	0.500	0.291	0.126	0.836*	0.018
		p (2-sided)	0.049	0.848	0.699	1.000	0.613	0.908	0.253	0.527	0.788	0.019	0.969
		n	7	7	7	7	7	7	7	7	7	7	7
lipoprotein and fatty acid metabolism	Δcholesterol [mg/dL]	correlation[ρ]	0.214	-0.357	0.000	0.000	-0.286	-0.321	0.180	0.523	0.964***	-0.018	0.571
		p (2-sided)	0.645	0.432	1.000	1.000	0.535	0.482	0.699	0.229	<.001	0.969	0.18
		n	7	7	7	7	7	7	7	7	7	7	7
	Δtriglycerides [mg/dL]	correlation[ρ]	-0.018	0.270	-0.450	0.468	-0.198	0.126	-0.073	-0.245	0.000	0.209	0.018
		p (2-sided)	0.969	0.558	0.31	0.289	0.67	0.788	0.877	0.596	1	0.653	0.969
		n	7	7	7	7	7	7	7	7	7	7	7
	ΔLDL [mg/dL]	correlation[ρ]	0.679	-0.214	-0.143	-0.071	0.000	-0.250	0.180	0.234	0.607	0.342	0.321
		p (2-sided)	0.094	0.645	0.76	0.879	1	0.589	0.699	0.613	0.148	0.452	0.482
		n	7	7	7	7	7	7	7	7	7	7	7
	ΔHDL [mg/dL]	correlation[ρ]	0.144	-0.108	0.360	-0.018	0.559	-0.036	-0.018	0.027	0.324	-0.309	0.505
		p (2-sided)	0.758	0.818	0.427	0.969	0.192	0.939	0.969	0.954	0.478	0.5	0.248
		n	7	7	7	7	7	7	7	7	7	7	7
cardiovascular parameters	ΔdiastRR [mmHg]	correlation[ρ]	0.072	0.414	-0.360	0.577	0.342	0.198	-0.927**	-0.855*	-0.090	-0.191	-0.541
		p (2-sided)	0.878	0.355	0.427	0.175	0.452	0.67	0.003	0.014	0.848	0.682	0.21
		n	7	7	7	7	7	7	7	7	7	7	7
	ΔsysRR [mmHg]	correlation[ρ]	-0.324	0.667	-0.036	0.757*	0.631	0.523	-0.873*	-0.964***	-0.450	-0.445	-0.396
		p (2-sided)	0.478	0.102	0.939	0.049	0.129	0.229	0.01	<.001	0.31	0.317	0.379
		n	7	7	7	7	7	7	7	7	7	7	7
	Δresting HR [bpm]	correlation[ρ]	-0.074	-0.296	-0.111	-0.037	0.111	-0.296	0.187	0.112	0.408	-0.224	0.741
		p (2-sided)	0.875	0.518	0.812	0.937	0.812	0.518	0.688	0.811	0.364	0.629	0.057
		n	7	7	7	7	7	7	7	7	7	7	7

Correlation of baseline (t0) metabolite concentrations and changes in physiological and clinical parameters between t0 and t1 as indicated. ρ: correlation coefficient (Spearman).

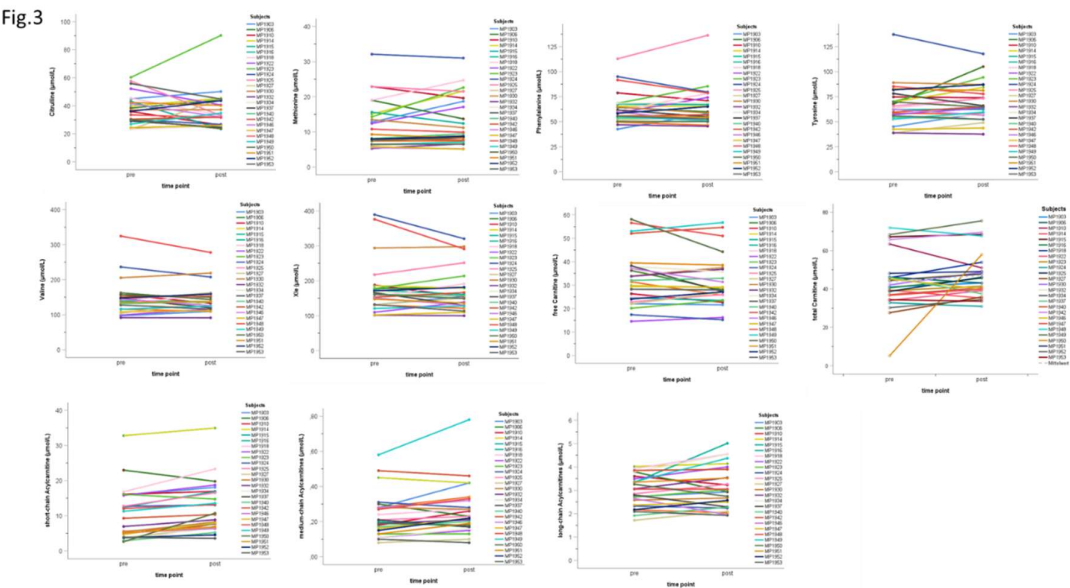### 3.3. Individual Acute Response at t2

In addition, we studied individual acute responses at t2 (n=26). As shown in Table 5 and Figure 3, on average, there were significant increases in SC- and MC-ACs in responses to exercise. All other metabolites did not show significant changes. However, still, there were large individual differences, suggesting that metabolite patterns might be suitable markers in the context of exercise adaptation. When assessing potential correlations between metabolite mobilization and changes in physiological or clinical parameters between t0 and t2, we found a significant negative correlation between MC-AC acute response and changes in PPO (peak power output), indicating that subjects with lower rates of MC-AC mobilization had higher gains in PPO (Table 6).

Table 5. Acute response at t2.

		t2	n	min	max	MD	ME	SD	p
amino acids	Cit (μmol/L)	pre	26	23.3	60.3	36.00	37.20	10.76	
		post	26	23.5	90.2	32.60	36.03	13.70	
		Δ	26	-24.6	29.9	-0.85	-1.17	11.40	.464
	Met (μmol/L)	pre	26	5.3	32.1	8.70	11.89	6.73	
		post	26	5.1	31.0	9.20	12.57	7.13	

			n	min	max	MD	ME	SD	p
		Δ	26	-5.3	8.3	0.00	0.67	3.20	.579
	Phe (µmol/L)	pre	26	42.6	112.9	59.30	63.22	15.97	
		post	26	45.5	136.3	58.45	64.23	18.47	
		Δ	26	-15.4	23.4	-0.30	1.00	9.67	.886
	Tyr (µmol/L)	pre	26	38.9	137.2	62.90	65.99	20.07	
		post	26	37.6	117.8	64.85	69.34	18.76	
		Δ	26	-19.4	37.5	0.75	3.35	11.32	.210
	Val (µmol/L)	pre	26	91.7	324.5	139.35	145.65	48.15	
		post	26	91.4	277.7	137.15	144.50	39.17	
		Δ	26	-46.8	32.7	0.45	-1.14	18.37	.920
	Xle (µmol/L)	pre	26	101.8	389.1	161.00	175.87	72.28	
		post	26	100.0	320.3	155.85	171.62	58.62	
		Δ	26	-84.8	37.5	-2.80	-4.26	29.30	.745
(acyl-)carnitines	C0 (µmol/L)	pre	26	14.6	58.1	30.64	32.57	11.77	
		post	26	15.3	56.7	27.98	31.35	10.67	
		Δ	26	-13.8	4.9	-0.40	-1.22	4.76	.437
	Cx (µmol/L)	pre	26	5.1	71.9	41.55	43.66	14.52	
		post	26	30.9	75.5	44.35	47.12	12.03	
		Δ	26	-12.4	52.8	3.20	3.46	11.35	.070
	SC-ACs (µmol/L)	pre	26	2.6	32.8	8.12	10.16	7.29	
		post	26	3.6	35.0	10.55	12.36	7.06	
		Δ	26	-3.2	8.1	2.19	2.20	2.27	<.001**
	MC-ACs (µmol/L)	pre	26	0.1	0.6	0.21	0.23	0.12	
		post	26	0.1	0.8	0.23	0.26	0.14	
		Δ	26	-0.1	0.2	0.03	0.03	0.06	.027*
	LC-ACs (µmol/L)	pre	26	1.7	4.0	2.95	2.96	0.67	
		post	26	1.9	5.0	2.98	3.13	0.90	
		Δ	26	-0.9	1.7	0.15	0.17	0.54	.147

Table shows changes in concentrations of individual metabolites during spiroergometry at t2. pre: before spiroergometry, post: after spiroergometry, ◎: difference, n: number of subjects, min: minimum value, max: maximum value, MD: median, ME: mean, SD: standard deviation.

Fig.3

Figure 3. Spaghetti plots of individual acute responses at t2. Plots depict changes in individual metabolite concentrations during spiroergometry at t2 (acute response) as indicated.

Table 6. Correlation between acute response and changes in physiological / clinical parameters.

Δphysiological / clinical parameters (t0-t2)		statistics	amino acid mobilization during acute bout of exercise at t2						(acyl-)carnitine mobilization during acute bout of exercise at t2				
			Cit	Met	Phe	Tyr	Val	Xle	C0	Cx	SC-ACs	MC-ACs	LC-ACs
sports	ΔrelVO2max [mL/kgxmin]	correlation [ρ]	-0.047	0.012	-0.002	-0.034	0.014	-0.087	-0.079	-0.040	0.230	-0.170	0.124
		p (2-sided)	0.819	0.955	0.993	0.867	0.945	0.672	0.703	0.846	0.258	0.407	0.547
		n	26	26	26	26	26	26	26	26	26	26	26
	ΔPPO [W/kg]	correlation [ρ]	0.024	-0.073	-0.170	-0.077	-0.121	-0.158	-0.001	-0.011	0.044	-0.480*	0.113
		p (2-sided)	0.910	0.735	0.427	0.719	0.573	0.460	0.997	0.960	0.838	0.018	0.599
		n	24	24	24	24	24	24	24	24	24	24	24
body weight	ΔBMI [kg/m2]	correlation [ρ]	-0.090	-0.324	-0.080	0.015	-0.169	-0.032	0.017	-0.028	-0.266	-0.124	-0.240
		p (2-sided)	0.663	0.107	0.699	0.941	0.409	0.875	0.935	0.893	0.188	0.545	0.238
		n	26	26	26	26	26	26	26	26	26	26	26

Correlation of acute changes in metabolite concentrations during spiroergometry at the t2 and changes in physiological and clinical parameters between t0 and t2 as indicated. ρ: correlation coefficient (Spearman).

4. Discussion

Physical exercise is known to modulate AA plasma profiles, reflecting metabolic adaptations (for review, see Kelly et al., 2020). Our data indicate that the 24-week exercise-based lifestyle intervention program "MultiPill-Exercise" continuously upregulated concentrations of all tested AAs in the circulation. In 2010, Kamaura et al. carried out a similar study on subjects with metabolic syndrome undergoing a six-week lifestyle intervention program. Our results are in part consistent with theirs, specifically, they could also detect increased concentrations of valine, tyrosine and citrulline after the intervention. In contrast to our data, however, decreased concentrations of leucine and phenylalanine were observed. These discrepancies might be due to differences with regard to study population, exact nature of the intervention and also methodological differences, since Kamaura et al., 2010, analyzed plasma samples, whereas in this study, dried whole-blood samples were employed. Interestingly, high AA concentrations in plasma can stimulate muscle protein synthesis, which might be a mechanism of training adaptation (Wolfe et al., 1999).

When considering AAs as potential biomarkers in the context of training adaptation, particularly associations between AA patterns and changes in clinical and physiological parameters are interesting. Thus, we correlated baseline AA concentrations with changes in such parameters between t0 and t1. Interestingly, we could detect positive correlations between valine, leucine / isoleucine, tyrosine and phenylalanine and changes in clinical parameters such as BMI, waist circumference, systolic and diastolic blood pressure, fasting glucose and LDL, as well as negative correlations with changes in HDL. In addition, we observed strong negative correlations between baseline citrulline and valine concentrations and changes in VO2max, indicating that high concentrations of these AAs might be predictors of a weaker response to the lifestyle intervention program. These data confirm the results published by Kamaura et al., 2010, who had also demonstrated several correlations between low baseline concentrations of various AAs and beneficial training outcomes.

By contrast, when analyzing AA mobilization in the context of acute exercise, there was almost no overall effect, despite partially huge inter-individual difference. A possible explanation might be

that AA mobilization might be less pronounced in subjects with low muscle mass, such as MultiPill-Exercise participants. In addition, it is possible that timing might have played a role: Spiroergometry tests in MultiPill-Exercise subjects, which were organized as incremental tests till exhaustion, might, due to subjects' comparatively low fitness, not have been long enough to detect significant AA mobilization.

When analyzing carnitine and ACs levels throughout the intervention, we found a moderate increase in total carnitine and a non-significant decrease in free carnitine. Levels of all classes of ACs also increased, despite the fact that data for medium-chain ACs did not reach significance.

Interestingly, there was a strong negative correlation between baseline concentrations of free and total carnitine and RR changes – both systolic and diastolic – suggesting that subjects with high levels of these metabolites at the start of the study had a higher cardiovascular benefit from the intervention.

Several studies could demonstrate distinct AC profiles in subjects with obesity, insulin resistance and non-alcoholic fatty liver disease, when compared to healthy controls (Mihalik et al., 2010; Zelijkovic et al., 2023). In this paper, we show that subjects with high concentrations of MC- and SC-ACs at inclusion had less positive effects with regard to fasting blood glucose and HbA1c amelioration when compared to subjects with lower baseline AC concentrations. In addition, high t0 concentrations of SC-ACs positively correlated with changes in cholesterol levels after 12 weeks. These data suggest that high AC concentrations might be negative predictors of training adaptation. Data presented by Carrard et al., 2022, who, in a systematic review, analyzed correlations of different metabolic markers with regard to their association with individual cardiorespiratory fitness, support this assumption: The authors clearly demonstrate that high AC concentrations, independently of chain length, are negatively associated with cardiorespiratory fitness, supporting the hypothesis that accumulation of circulating ACs reflects a state of compromised mitochondrial fatty acid oxidation, indicating poor metabolic health (for review, see McCoin et al., 2015). On the other hand, skeletal muscle ACs appear to be associated with a high degree of cardiovascular fitness, probably because they are linked to efficient fatty acid oxidation (Purdom et al., 2018). This is supported by results indicating that exercise can enhance mitochondrial fatty acid oxidation in skeletal muscle tissue (Lundsgaard et al., 2018).

Interestingly, we observed a significant increase in SC- and MC-ACs during acute exercise. A similar effect was previously described by Lehmann et al., 2010, who demonstrated increases particularly in MC-ACs during 60 min runs, suggesting that these metabolites are released as byproducts / intermediates of partial β oxidation. Since there was a negative correlation between medium-chain AC mobilization and ⊚PPO after 24 weeks, suggesting that subjects with a high degree of medium-chain AC mobilization were characterized by poor training effects. By contrast, short- and long-chain AC mobilization were positively related to VO2max, suggesting that these subjects showed efficient training adaptation.

Finally, despite the fact that a light breakfast, consisting of pretzels, apples and cereal bars, was offered to subjects on testing days, their diets were not completely standardized, which might have influenced our results. However, this was an intended effect, since, when installing metabolomics diagnostics in clinical routine in the future, it will also not be possible to do this in the context of fully standardized diets, i.e., useful biomarkers will be required to function in a real-world scenario of heterogeneous diets.

Taken together, our data data demonstrate that both AA and AC profiles are responsive to the lifestyle intervention program "MultiPill-Exercise" and might serve as biomarkers for clinical and physiological adaptation reactions in subjects with multimorbidity in the context of lifestyle intervention programs.

5. Conclusions

Our data indicate that "mini metabolomics", employing dried blood spots, might be an easy-to-perform, cost-effective and minimally invasive strategy to assess basic metabolomics patterns in

subjects with multimorbidity. The fact that this method has been well standardized and extensively validated in the context of newborn screening guarantees a high degree of reliability and reproducibility of the data. In addition, our results indicate that metabolomics patterns might indeed be linked to changes in clinical and/or physiological data in the context of lifestyle intervention programs in subjects with multimorbidity. Further studies should focus on validation of the data in larger cohorts.

6. Study Limitations

A major limitation of our study is the fact that due to restrictions in the context of the COVID 19 pandemic, complete sets of t0, t1 and t2 data were only available for n=6 subjects of wave 1, all of which were female. In addition, also due to COVID 19, the MultiPill-Exercise intervention design had to be substantially modified for subjects of wave 2, limiting comparability of results obtained of data obtained for the two waves. Furthermore, due to technical limitations, acute response data could only be collected at t2 (and not at t0), limiting the predictive value of these results. Finally, to maximize the number of subjects for which data were available, correlations of baseline metabolomics data with changes in clinical and physiological parameters were carried out only for deltas between t0 and t1, i.e., at 12 weeks into the intervention. By contrast and also aiming at maximizing the number of analyzable data, acute response data were studied in the context of changes between t0 and t2, despite the fact that, as mentioned above, the second phase of the intervention had to be substantially modified for subjects of wave 2.

This was an exploratory, hypothesis-generated study, thus, no correction for multiple testing was introduced. Future studies should focus on validation of metabolomics data in larger cohorts, potentially also considering differences between male and female subjects and also different (co)morbidities. In addition, long-term longitudinal studies and multidisciplinary approaches, encompassing for example genetic or endocrinological data, would be interesting – and also mechanistic approaches, allowing a deeper understanding of metabolic pathways reflected in metabolomics patterns. Eventually, metabolomics screening in subjects with multimorbidity could constitute a tool in personalized sports medicine, allowing the design of efficient, individual training regimens and lifestyle intervention programs. Finally, an important point is that in this study, only metabolites that are part of the standard newborn screening program, were quantified. In the future, it will also be interesting to establish procedures also allowing analysis of more specific metabolites, such as N-lactoylphenylalanine, which has recently been demonstrated to be an important modulator of exercise-induced weight loss in subjects with obesity (for review, see Naja et al., 2025), from dried blood spots.

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