Single and joined behaviour of circulating biomarkers in high-fit and low-fit healthy females

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Abstract

Background

Biomarkers are important in the assessment of health and disease but are poorly studied in healthy individuals. Especially responses of biomarkers in the systemic circulation to longer-term and short-term lifestyle interventions are incompletely understood, neither is their relative response to each other. This study investigated how single biomarkers, functional biomarker categories and total biomarker profiles respond to a difference in longer-term physical activity and recent exercise in healthy individuals.

Methods

A total of 102 biomarkers were analysed in serum or plasma samples from 30 young, healthy, female adults divided into a high-fit ($\dot{V}O_2$peak ≥ 47 mL/kg/min, N = 15) and low-fit ($\dot{V}O_2$peak ≤ 37 mL/kg/min, N = 15) group, at baseline and overnight after a single bout of exercise (60 min, 70% $\dot{V}O_2$peak).

Results

Total biomarker profiles were similar between high-fit and low-fit females, with only significantly lower leptin levels in high-fit females (adj.P_{group} = 0.076). Recent exercise significantly affected several single biomarkers related to inflammation and lipid metabolism, and adiponectin (all adj.$P_{exercise}$ < 0.01). Furthermore, functional biomarker categories corresponded to biomarker clusters generated via hierarchical clustering models.

Conclusion

This study provides insight in the single and joined behaviour of circulating biomarkers in healthy females and identified functional biomarker categories that may be used for characterization of human health physiology.

Trial registration:

Registered in the Dutch trial register (ref.no. NL7891) on 2019-07-23.

Background

Lifestyle factors play a dominant role in health maintenance and the prevention of chronic diseases, such as type 2 diabetes (1), cardiovascular disease (2), and cancer (3). Adopting a healthy lifestyle, including regular physical activity performance, is associated with a lower chronic disease risk (4). Biomarkers are

important in the assessment of the impact of lifestyles on health status (5). For monitoring disease risk and the progression from a healthy to an unhealthier state, the use of biomarkers that reflect key physiological processes, such as metabolism, inflammation, and oxidative stress, has been proposed (6). The dynamics of biomarkers has often been studied in disease conditions, but it is hardly understood how these biomarkers behave in healthy individuals adhering to different lifestyles and how they respond to a short-term lifestyle interventions. Moreover, many of these biomarkers have not been studied relatively to each other, especially not in healthy individuals.

Physical activity is one of the lifestyle factors that has been associated with systemic changes that are linked to a reduced chronic disease risk (7). High levels of physical activity has been shown to reduce insulin resistance (8), improve lipoprotein profiles (9), and lower interleukin (IL)6 levels on the long-term (10), which contributes to a lower chronic disease risk (11–14). However, short-term exercise, e.g., a single bout of exercise, also provokes acute systemic changes, which can last for up to 24 hours (15–17). These short-term exercise responses can differ between individuals with high and low levels of physical activity, due to the metabolic and physiological adaptations of the body to regular exercise (18,19). Hence, not only the basal levels of circulating biomarkers could differ between high- and low physically active individuals, but the biomarker response to a single exercise session might also be affected. This, however, has been studied only to a limited extent, with most studies focusing on male individuals (15), while physiological responses between males and females can be strikingly different (20).

This study investigated how serum and plasma biomarkers are affected by different longer-term physical activity levels and by recent exercise. Routine physiological biomarkers such as insulin, LDL cholesterol, and c-reactive protein (CRP) were included as well as markers that represent other metabolic, immunological, and oxidative stress-related processes. All biomarkers were allocated to three functional biomarker categories: 1) peptide hormones, 2) inflammation and oxidative stress-related markers, and 3) metabolism markers, the latter comprising protein, carbohydrate, and lipid metabolism. Biomarker analysis was performed in high aerobically fit (high-fit) and low aerobically fit (low-fit) females with a validated difference in $\dot{V}O_2$peak to reflect a difference in training status. Previously, we found a significant difference in skeletal muscle mitochondrial capacity (21) and mitochondrial function in peripheral blood mononuclear cells (PBMCs) (22) in our healthy study population. Biomarker analysis in this study will now show whether single biomarkers, functional biomarker categories and total biomarker profiles differ between these high-fit and low-fit females, and how these biomarkers respond to a recent bout of exercise. This information will improve our understanding on the effect of longer-term lifestyle differences and recent lifestyle interventions on biomarkers of health and disease and contribute to the application of preventative and health improvement interventions.

Methods

Study subjects

Healthy young females (18–28 years of age, BMI 18.5–25 kg/m^2) were recruited from the local university and community population. Exclusion criteria were as follows: history of cardiovascular, respiratory, hematological, or metabolic disease; use of prescribed chronic medication; anemia (hemoglobin concentration < 12 g/dL); blood donation within two months before the start of the study; smoking (> 5 cigarettes per week); recreational drug use or over the counter drug use during the study; use of performance-enhancing supplements; pregnancy or lactating. Subjects were selected if they had a $\dot{V}O_2$peak ≥ 47 mL/kg/min (high-fit group) or ≤ 37 mL/kg/min (low-fit group) determined using a maximal exercise test, measured using the validated screening protocol of Lagerwaard et al. (21,23), which minimized the risk for selective bias. Sixteen high-fit and sixteen low-fit subjects were included. The $\dot{V}O_2$peak data and results of skeletal muscle mitochondrial capacity of these subjects has been published previously by our group (21). A total of 111 maximal exercise tests were performed to end up with the desired sample size. One subject was excluded due to medication intake and one subject was excluded due to symptoms of illness directly after completion of the study protocol. The use of oral contraceptives (OC) was not excluded; only the use of monophasic OC containing low synthetic estradiol and progesterone was allowed and was controlled for (N = 7 in the high-fit and N = 6 in the low-fit group). The 17bèta-estradiol levels were measured using a chemiluminescent immunoassay on a Lumipulse G1200 analyzer (Fujirebio Incl) at the Erasmus Medical Centre (Rotterdam, the Netherlands) and were not significantly different between those high-fit (527.7 [353.1–610.0]) and low-fit females (217.4 [109.1–895.2]) that did not use oral OC (P = 0.321).

Study design

Subjects refrained from heavy physical exercise 48 hours prior to the first study day and from any physical exercise and alcohol consumption 24 hours prior to the first study day. Subjects adhered to dietary guidelines 24 hours prior to each study day, which included the consumption of a standardized evening meal (73% carbohydrates/16% protein/11% fat, 1818 kJ) before 8:00PM and dietary guidelines for the consumption of breakfast, lunch, drinks, and snacks. After an overnight fast, blood was collected in the morning of the first study day (= baseline timepoint) and on the morning of the second study day, i.e., 21 hours after a single bout of exercise (= post-exercise timepoint). Blood samples (3 x 6 mL) were collected by venipuncture in vacutainers containing dipotassium dipotassium (K2-) ethylenediaminetetraacetic acid (EDTA) (K2-EDTA) as anticoagulant for plasma collection (2 x 6 mL, BD Biosciences, Vianen, the Netherlands, 367525) and a vacutainer containing silica as a clot activator for serum collection (1 x 6 mL, BD Biosciences, Vianen, the Netherlands, 367837). Blood tubes for plasma collection were kept on ice-water and processed within 30 minutes after blood collection. Blood tubes for serum collection were kept at room temperature (RT) for 60 minutes to allow clotting and immediately processed afterwards. Body fat percentage was measured according to the four-site method by Durnin-Womersley using the skinfold measurements of the triceps, biceps, sub scapula and supra iliac, measured using a skinfold calliper (Harpenden, UK). Subjects received breakfast and after two hours, subjects completed an individualized exercise protocol consisting of 60 minutes cycling on an electrically braked bicycle ergometer (Corival CPET, Lode, the Netherlands) at a workload aiming to equal 70% of their $\dot{V}O_2$peak. Oxygen consumption, carbon dioxide production, and air flow were measured using MAX-II

metabolic cart (AEI technologies, Landivisiau, France). Exhaled air was continuously sampled from a mixing chamber and averaged over 15-second time windows. Oxygen consumption was measured in the first and last 15 minutes of the exercise test and used to confirm the relative oxygen consumption. If needed, small adjustments in workload were made to reach 70% of the $\dot{V}O_2$ peak of the individual. After the exercise protocol subjects went home, refrained from moderate to heavy physical activity, were instructed to keep low levels of light physical activity, and refrained alcohol consumption until blood collection on the second study day. The habitual dietary intake of the study subjects was determined via a validated food frequency questionnaire (FFQ) that assessed dietary intake in the past four weeks (24). The self-reported diets of the high-fit and low-fit subjects were similar with no significant differences in total daily energy intake, carbohydrate intake, protein intake or fat intake (**Supplementary Fig. 1, Additional File 1**).

Plasma and serum isolation

Plasma tubes were centrifuged for 10 minutes at 1200*g* at 4°C, and the supernatant (plasma) was collected, transferred to a new tube, and mixed. In case of turbid plasma, samples were centrifuged again for 10 minutes at 1200*g* at 4°C to remove any insoluble matter. Plasma samples were snap-frozen in liquid nitrogen and afterwards cryopreserved at -80°C. Serum tubes were centrifuged for 10 minutes at 1300*g* at RT, and the supernatant (serum) was collected, transferred to a new tube, and mixed. In case of turbid serum, samples were centrifuged again for 10 minutes at 1300*g* at RT to remove any insoluble matter. Serum samples were snap-frozen in liquid nitrogen and afterwards cryopreserved at -80°C. For biomarker analysis, plasma and serum samples were thawed on ice and individually mixed until a clear solution was reached.

ELISAs in serum and plasma

Commercially available enzyme-linked immunoassay (ELISA) kits were used to analyse serum levels of the peptide hormones leptin, insulin, and adiponectin and the plasma levels of inflammatory and oxidative stress-related markers (tumour necrosis factor (TNF), IL6, IL10, CRP, the soluble monocyte differentiation antigen cluster of differentiation 14 (CD14), monocyte chemoattractant protein 1 (CCL2, better known as MCP1), soluble intercellular adhesion molecule 1 (ICAM1), lipopolysaccharide binding protein (LBP), and oxidized low density lipoprotein (oxidized LDL) according to the manufacturers' instructions (Table 1).

Table 1
ELISA kits used for serum and plasma biomarker analysis

Biomarker	Matrix	Company	Catalogue number
Leptin	Serum	R&D Systems[a]	DLP00
Insulin	Serum	Mercodia[b]	10-1113-01
Adiponectin	Serum	R&D Systems	DRP300
TNF	Plasma	R&D Systems	HSTA00E
IL6	Plasma	R&D Systems	HS600C
IL10	Plasma	Invitrogen[c]	BMS215HS
CRP	Plasma	R&D Systems	DCRP00
CD14	Plasma	R&D Systems	DC140
MCP1	Plasma	R&D Systems	DCP00
Soluble ICAM-1	Plasma	R&D Systems	BBE1B
LBP	Plasma	Hycult Biotech[d]	HK315-01
Oxidized LDL	Plasma	Mercodia	10-1143-01
[a]R&D systems Inc., Minneapolis, MN, Canada			
[b]Mercodia, Uppsala, Sweden			
[c]Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA			
[d]Hycult Biotech, Uden, the Netherlands			

Proton NMR (^{1}H NMR) in plasma

EDTA-plasma samples were measured using the standardized, targeted high-throughput proton NMR (^{1}H NMR) metabolomics from Nightingale Health (Nightingale Health Ltd., Helsinki, Finland, nightingalehealth.com/biomarkers). This platform provides simultaneous quantification of 162 individual metabolites and 87 metabolite ratios or sizes. For analysis of this study, all individual metabolites were selected, except for metabolite concentrations within lipoproteins or lipoprotein subclasses (e.g., 'total lipids in VLDL'), and concentrations of clinical LDL cholesterol, remnant cholesterol, total cholesterol minus HDL cholesterol and total branched-chain amino acids (BCAAs). All metabolite ratios or sizes were also excluded from analysis. A complete list of the selected metabolites included in the analysis can be found in **Supplementary Table 1, Additional File 2.**

Proton NMR (^{1}H NMR) in serum

Serum samples were measured using targeted high throughput ^{1}H NMR metabolomics at the EURECAT Technology Centre (Barcelona, Spain). For metabolite extraction, samples were placed in 2 mL 96 deep well plates using 200 mL methanol:water (8:1, for aqueous extraction) or 100 mL methyl-tert-butylether (MTBE):methanol:water (3:10:2, for lipidic extraction) in an automated fashion in the Bravo liquid handler (Agilent Technologies Santa Clara, California, USA). Methanol and MTBE were purchased at Merck (Darmstadt, Germany). After extraction, solvents from the samples were removed using a speed vacuum concentrator and samples were stored at -80°C until analysis. Some samples were lyophilized before ^{1}H NMR analysis. For ^{1}H NMR measurements, the hydrophilic extracts were reconstituted in 600 µL deuterium oxide (D_2O, Deutero, Kastellaun, Germany) PBS (Sigma-Aldrich, St. Louis, MO, USA), 0.05 mM, pH 7.4, 99.5% D_2O) containing 0.73 mM 3-(Trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt (TSP, Sigma-Aldrich), and the dried lipophilic extracts were reconstituted with a solution of deuterated chloroform ($CDCL_3$)/deuterated methanol (CD_3OD) (2:1, chloroform d-1 and methanol d-4 from Deutero) containing 1.18 mM tetramethylsilane (TMS, Sigma-Aldrich) and then vortexed. Both extracts were transferred into 5 mm NMR glass tube for ^{1}H NMR analysis. ^{1}H NMR spectra were recorded at 300 K on an Avance III 600 spectrometer (Bruker, Billerica, Massachusetts, MA, USA) operating at a proton frequency of 600.20 MHz using a 5 mm PABBO gradient probe. Aqueous extracted samples were measured and recorded in processing number (procno) 11. For aqueous extracts one-dimensional ^{1}H pulse experiments were carried out using the nuclear Overhauser effect spectroscopy (NOESY) presaturation sequence (RD–90°–t1–90°–tm–90° ACQ) to suppress the residual water peak, and the mixing time was set at 100 milliseconds. Solvent presaturation with irradiation power of 160 mW was applied during recycling delay (RD = 5 seconds) and mixing time. The 90° pulse length was calibrated for each sample and varied from 9.72 to 10.06 µs. The spectral width was 12 kHz (20 ppm), and a total of 256 transients were collected into 64 k data points for each ^{1}H spectrum. Lipidic extracted samples were measured and recorded in procno 22. In the case of lipophilic extracts, a 90º pulse with presaturation sequence (zgpr) was used to suppress water residual signal of methanol. A RD of 5.0 seconds with acquisition time of 2.94 seconds were used. The 90º pulse length was calibrated for each sample and varied from 9.92 to 10.04 µs. After 4 dummy scans, a total of 128 scans were collected into 64K data points with a spectral with of 18.6 ppm. The exponential line broadening applied before Fourier transformation was of 0.3 Hz. The frequency domain spectra were phased, baseline-corrected and referenced to TSP or TMS signal (d = 0 ppm) using TopSpin software (version 3.6, Bruker). All acquired ^{1}H NMR were compared to standards of the pure selected compounds from AMIX spectra database (Bruker®), HMDB, and Chenomx databases for metabolite identification. In addition, we assigned metabolites by ^{1}H–^{1}H homonuclear correlation (COSY and TOCSY) and ^{1}H–^{13}C heteronuclear (HSQC) 2D NMR experiments and by correlation with pure compounds run in-house when were needed. After pre-processing, specific ^{1}H NMR regions identified in the spectra were integrated using the AMIX 3.9 software package. Curated identified regions across the spectra that were integrated using the same AMIX 3.9 software package were exported to Excel to evaluate the robustness of the different ^{1}H NMR signals and to calculate the concentrations.

LC-MS/MS in plasma

Plasma acylcarnitines were quantified or semi-quantitated in plasma by liquid chromatography with tandem mass spectrometry (LC-MS/MS). Plasma samples were thawed at 4ºC and 30 µL sample were mixed with 270 µL 100% methanol containing the set of labelled internal standards (see **Supplementary Table 2, Additional File 2**). The mixture was vortexed for 15 seconds and centrifuged for 10 minutes at 3800*g* at 4ºC. The supernatant was transferred into a new plate and injected onto a Kinetex 2.6 µm Polar C18 column, 100 Å, 150 x 2.1 mm (Phenomenex, Torrance, CA, USA) using a UHPLC 1290 Infinity II Series system coupled to a QqQ/MS 6470 Series system (Agilent Technologies, Santa Clara, CA, USA). Metabolite extraction was carried out with a semi-automated process using Agilent Bravo Automated Liquid Handling Platform (Agilent Technologies, Santa Clara, CA, USA).

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics for Windows (Version 25.0, IBM Corp, Armonk, NY, USA), and R (Version 4.1.2. R Core Team, Vienna, Austria). Graphs were created using GraphPad Prism (Version 9.0, Graphpad Software, CA, USA) and R. In total 102 variables were included in the main analyses (RM-ANOVA, main effect analysis, PCA, heatmaps, correlation matrices). In the comparative analysis between identical metabolites in serum and plasma, 16 variables per platform (Nightingale or EURECAT) were included.

Data representation and transformation

Normality was checked using Shapiro-Wilk normality tests and tests for skewness and kurtosis. Normally distributed data is presented as mean ± standard deviation (SD) and not normally distributed data is presented as median [interquartile range (IQR)]. For univariate analyses (repeated-measures analysis of variance (RM-ANOVA) and main effect analysis), not normally distributed data was transformed (log, inverse, square, inverse square root). For multivariate analyses (principal component analysis (PCA), hierarchical clustering and heatmap plotting and correlation matrix analyses) all data was range scaled using the formula $(x - min(x)) / (max(x) - min(x))$ (25) because all biomarkers were measured in different units. Scaling resulted in a value ranging from 0–1 for every variable but preserved the dynamic range within each biomarker. One sample on the EURECAT platform did not pass the quality assurance tests during ^{1}H NMR analysis and was excluded from the analysis, resulting in N = 14 samples for the low-fit and N = 15 samples for the high-fit group for some analytes.

Bivariate tests, RM-ANOVA and main effects analysis

Subject characteristics were compared using a Student's unpaired t-test or Mann-Whitney U test. RM-ANOVA was used to study the effect of fitness level (between-subjects factor) and the effect of a recent bout of exercise (within-subjects factor) on single biomarker levels and the interaction between these two factors. All assumptions for RM-ANOVA were met. Partial eta square (η^2) is given per effect as measure for effect size. Since our study includes two repeated measures and the non-parametric alternative for a RM-ANOVA (Friedman ANOVA) requires three repeated measures, the six variables that did not achieve normality after data transformation were analysed using non-parametric bivariate analyses. Mann-

Whitney U tests on the ranked baseline values were used to study the fitness level effect and on the ranked difference between baseline and post-exercise values to study the interaction effect. Wilcoxon-Signed rank tests on the ranked baseline and post-exercise values were used to study the exercise effect. No partial effect size measure could be calculated for these non-parametric tests. Raw P-values were corrected for multiple testing using Benjamini-Hochberg correction in the R package 'FSA' (26) and a false discovery rate (FDR) set at 10%. FDR-corrected P-values < 0.10 (adjusted P ($P_{adj.}$)) were considered statistically significant. None of the interactions between fitness level and the recent bout of exercise ($P_{group*exercise}$) were < 0.10 and the main effects of fitness level and the recent bout of exercise were therefore also analysed in a model without the interaction term (**Supplementary Tables 6, 7, 8, Additional File 2**).

PCA, hierarchical clustering and heatmap plotting

For PCA, the covariance matrix was computed, eigenvector decomposition was performed for principal component identification, and the first and second largest principal components were plotted in a projection matrix, using the R packages 'ggplot2' (27), 'tidyverse' (28), 'factoextra' (29) and 'FactoMineR' (30). Hierarchical clustering was performed using Euclidean distance as the dissimilarity measure and complete linkage as the similarity measure between the clusters using the *hclust* function from R (31). Heatmaps were generated using the R package 'ComplexHeatmap' (32).

Correlation analyses

Levels of identical metabolites measured in serum (EURECAT) and plasma (Nightingale) were compared using Spearman rank (for not normally distributed data) or Pearson (for normally distributed data) correlations on the raw data (16 variables per platform) to compare relative as well as absolute values. Spearman rho (ρ) or Pearson r (r) are given as effect size measures and P-values < 0.05 were considered statistically significant. The correlation matrix was generated by performing Spearman rank correlation analyses for all biomarker pairs. All scaled biomarker data (102 variables) of high-fit and low-fit subjects at baseline as well as at post-exercise (Fig. 5) or at baseline only (**Supplementary Fig. 4, Additional File 1**) was included. The correlation analysis used all scaled biomarker values without considering the fitness level or recent exercise effect. The correlation matrix was generated using the *hclust* function from R (31) and the R packages 'corrplot' (33) and 'Hmisc' (34), returning a correlation plot based on hierarchically clustered biomarkers. Significant correlations (P < 0.05) are depicted by coloured wells and non-significant correlations (P > 0.05) are left blank.

Results

All 102 biomarkers were analysed in samples from a well characterized study (21,22,35) of healthy females. This study population represents trained, physically active (high-fit; $\dot{V}O_2$peak ≥ 47 mL/kg/min, N = 15) and untrained, low physically active (low-fit; $\dot{V}O_2$peak ≤ 37 mL/kg/min, N = 15) females (Table 2), which was supported by a significantly higher skeletal muscle mitochondrial capacity (21) and a better mitochondrial function in PBMCs (22) in the high-fit compared to the low-fit females. Both groups were

assessed at baseline and 21 hours after a single bout of exercise. To establish the reproducibility of the biomarker determination, a random subset of 16 biomarkers involved in protein and lipid metabolism, was also determined using a similar ^{1}H NMR technology, but with different matrices and laboratories. Significant correlations were observed for all 16 markers, with correlation coefficients between 0.59–0.90 for the amino acids (all eight $P < 0.0001$), 0.42–0.65 for the six fatty acids (one $P < 0.01$, three $P < 0.001$, two $P < 0.0001$) and 0.63 and 0.86 for the two ketone bodies (both $P < 0.0001$, **Supplementary Fig. 2, Additional File 1**). This supports the validity of these biomarker measurements and demonstrates the robustness of our approach.

Table 2
Subject characteristics

	Low-fit (N = 15)	**High-fit (N = 15)**
Age (years)	24.5 [22.9–25.6]	21.8 [21.6–23.7]
Ethnicity	Caucasian (11), Asian (1), Indo-pacific (4)	All Caucasian
Weight (kg)	59.7 ± 7.1	61.2 ± 7.0
Height (m)	1.63 ± 0.08	1.68 ± 0.05
BMI (kg/m^2)	22.4 ± 1.4	21.7 ± 1.9
Fat mass (% of weight)	28.7 ± 3.9	25.1 ± 4.4 *
Hemoglobin (mM)	8.4 ± 0.6	8.5 ± 0.6
Use of birth control pill	6 / 15	7 / 15
$\dot{V}O_2$peak (mL ·kg ⁻¹ · min ⁻¹)	35.0 [31.6–35.6]	50.4 [49.0–54.0] ****
Baecke total score	7.3 ± 1.0	9.5 ± 0.8 ****
m$\dot{V}O_2$ recovery constant (% · min ⁻¹)	1.53 ± 0.46$^{\#}$	2.06 ± 0.57 *

BMI = body mass index, $\dot{V}O_2$peak = maximal oxygen consumption values, m$\dot{V}O_2$ = maximal oxygenation recovery constant in the *gastrocnemius* as proxy for skeletal muscle mitochondrial capacity. $^{\#}$N = 11. Values are mean ± SD for normally distributed data, and median [IQR] for not normally distributed data. *P < 0.05, *** P < 0.001, **** P < 0.0001.

Single biomarker analysis demonstrates a similar biomarker profile between high-fit and low-fit females

To better evaluate which functional processes are affected upon alterations in biomarker levels, we first linked each biomarker to one of the following physiological processes: hormone signaling, inflammation

and oxidative stress responses, and metabolism. This resulted in three overarching, functional biomarker categories: 1) peptide hormones (**Supplementary Table 3, Additional File 2**), 2) inflammation and oxidative stress responses (**Supplementary Table 4, Additional File 2**), and 3) metabolism (which was further divided in protein, carbohydrate, and lipid metabolites, **Supplementary Table 5, Additional File 2**). Since many biomarkers were related to lipid metabolism, this subcategory was further subdivided into fatty acids, cholines, ketone bodies, acylcarnitines, cholesterol metabolites and lipoproteins. All mean or median biomarker values and ranges for high-fit and low-fit females at baseline and after recent exercise are in **Supplementary Table 3, Additional File 2**. To assess the effect of fitness level and the recent bout of exercise on the individual biomarker responses, RM-ANOVA on the raw data (for normally distributed biomarkers) or transformed data (for not normally distributed biomarkers) was performed. This resulted in a fitness level effect ($rawP_{group}$), a recent exercise effect ($rawP_{exercise}$), and an interaction effect ($rawP_{group*exercise}$) for each biomarker, and these raw P-values were corrected for multiple testing, with a significance cut-off of < 0.10 for $adj.P_{group}$, $adj.P_{exercise}$ and $adj.P_{group*exercise}$. The detailed results of these analyses and the measure of effect size (η^2) are in **Supplementary Tables 3–5, Additional File 2**. None of the individual biomarkers was significantly impacted by fitness level, except for the 'peptide hormone' leptin (Fig. 1), which was significantly higher in low-fit females compared to high-fit females ($adj.P_{group}$= 0.076, Fig. 2A). Thus, none of the markers related to inflammation, oxidative stress, or metabolism was significantly impacted by fitness level in our healthy females, indicating that high-fit and low-fit females have similar biomarker profiles.

Recent exercise regulates single biomarkers related to inflammation, lipid metabolism and hormone signaling

Next, we assessed whether recent exercise altered individual biomarker levels and examined whether high-fit and low-fit females responded differently to recent exercise. Recent exercise significantly regulated 35 of the 102 biomarkers, related to hormone signaling, inflammation and oxidative stress, lipid metabolism, and protein metabolism (Fig. 1). The peptide hormone adiponectin was significantly increased after exercise in both groups ($adj.P_{exercise}$ = 0.001, Fig. 2B). Of the 10 biomarkers that are related to inflammation and oxidative stress, 7 were significantly regulated by exercise, the top-5 being N-acetylglycoproteins (up; $adj.P_{exercise} = 4.16x10^{-6}$), MCP1 (down, $adj.P_{exercise} = 4.16x10^{-6}$), TNF (down, $adj.P_{exercise} = 3.09x10^{-4}$), CRP (up, $adj.P_{exercise}$ = 0.003), and IL10 (down, $adj.P_{exercise}$ = 0.003, Fig. 2C – **G**). In total 27 metabolic markers were significantly regulated by exercise, with the top-5 all linked to lipid metabolism, with increased levels of lysophosphatidylcholine ($adj.P_{exercise} = 3.51x10^{-6}$, Fig. 2H) and increased levels of apolipoprotein A1, total esterified cholesterol, total cholesterol, and HDL cholesterol (Fig. 2I – **L**, all $adj.P_{exercise}$ = 0.001). Importantly, for none of the 102 biomarkers, the exercise response significantly differed between high-fit and low-fit females (all $adj.P_{group*exercise}$ > 0.10). We therefore performed an additional main effect analysis without the interaction term, which resulted in the same significantly regulated biomarkers as compared to the full interaction model, except for MUFA ($adj.P_{exercise}$ = 0.101, **Supplementary Tables 4, 5, 6, Additional File 2**). In summary, this single biomarker

analysis demonstrated that various biomarkers linked to inflammation, lipid and protein metabolism, and adiponectin were significantly regulated by recent exercise, while only leptin was affected by fitness level in these healthy females (Fig. 3).

Data-driven biomarker clusters link with functional biomarker categories

Next, we studied the joined dynamics of these biomarkers. Hierarchical clustering was applied on the scaled biomarker levels and visualized in a heatmap (Fig. 4). The heatmap generated multiple biomarker clusters that corresponded to our predefined functional biomarker categories, indicated by clustering of inflammation and oxidative stress related markers, amino acids, fatty acids, ketone bodies, acylcarnitines, lipoproteins and cholesterol metabolites along the y-axis (Fig. 4). Although some of these functional biomarker categories also displayed x-axis clustering (e.g., the lipoproteins and fatty acids), the overall heatmap pattern was only slightly related to fitness level and not related to recent exercise. Instead, the intra-individual biomarker response, i.e., baseline and post-exercise values within one subject, accounted for most of the x-axis clustering. The notion that biomarker levels were primarily affected by interindividual differences, rather than fitness level or the recent bout of exercise, was confirmed by PCA, where no clear separation was observed between our experimental conditions (**Supplementary Fig. 3, Additional File 1**). To obtain a more detailed understanding on data-driven relationships between biomarkers, a hierarchically clustered ($P < 0.05$, Fig. 5) correlation matrix was generated, with significant Spearman $\rho > 0.6$ or < 0.6 correlations indicated as potential physiological relevant links (**Additional File 3**). As above, these data-driven correlations corresponded to functional categories, such as amino acids (especially the branched-chain amino acids (BCAAs)), fatty acids, ketone bodies, acylcarnitines, cholesterol metabolites and lipoproteins (Fig. 5). However, some data-driven correlated biomarkers were not in line with our predefined functional biomarker categories, such as CRP and glycine ($r = -0.72$), glutamine and hydroxyisovalerylcarnitine (C5:0-OH, $r = 0.60$), tyrosine and hydroxyisovalerylcarnitine (C5:0-OH, $r = 0.64$), tyrosine and methylcrotonylcarnitine (C5:1, $r = 0.66$), betaine and octadecadienylcarnitine (C18:2, $r = 0.65$), and N-acetylglycoproteins and lysophosphatidylcholine ($r = 0.65$), all having a $P < 1.0 \times 10^{-7}$. Of note, similar patterns were observed when only the baseline levels from high-fit and low-fit females were included (**Supplementary Fig. 4, Additional File 1**). Overall, this integrated biomarker analysis demonstrated that data-driven biomarker clusters include biomarkers that are also functionally linked, and that various of these clusters correspond with our predefined functional biomarker categories.

Discussion

We performed an elaborate analysis of 102 circulating biomarkers, previously studied in disease conditions such as type 2 diabetes, obesity, and cardiovascular disease (36–39), but hardly in healthy individuals with different lifestyles. Analysis of a selection of these biomarkers across two platforms showed similar results, underpinning their reliability, and indicating the robustness of these platforms.

Except for leptin, individual biomarker levels were not significantly different between high and low aerobically fit females. Since leptin levels have been positively correlated to body fat percentage (40,41), the difference in leptin presumably results from a significant difference in body fat percentage between high-fit and low-fit females, further underpinning the validity of our data. Our observation that all other biomarkers were similar between the two groups, while previous studies in high and low aerobically fit individuals found significant differences in e.g., lipid and protein metabolites (42–47), is likely related to our standardized experimental set-up, as compared to other studies. We studied healthy, young-adult females of similar age and body mass index (BMI) in a highly controlled setting, while previous studies were performed with metabolically impaired individuals (38) and individuals with different BMI (42–44,47) or wider age ranges (42,47), in experimental conditions that were less standardized (42–45,47), and especially these factors impact circulating metabolite levels (38,42,43). Given that the levels of the analysed biomarkers were similar among the healthy females in our study, and multiple of these biomarkers showed dysregulation during disease, our findings imply that this biomarker set could be used to monitor progress from a healthy to an unhealthier state and may be use in health improvement interventions.

Studies that focus on recent exercise effects, i.e., effects on the day after exercise completion, are scarce compared to studies on acute or chronic exercise (15,18). Yet, recent exercise is especially relevant for biomarkers, as they can indicate whether physical activity of study subjects should be controlled prior to sampling. Here, we demonstrated that adiponectin, lipid metabolites, and inflammatory markers were most responsive to recent exercise, which is line with data from other studies (48–51). These findings suggest that future biomarker studies should consider standardization of study subjects' physical activity 24 hours prior to blood sampling, especially when they include hormones, and markers related to lipid metabolism and inflammation.

Multiple separate clusters that were obtained in the heatmap and correlation matrix included biomarkers that corresponded to biomarkers embedded in our predefined, functional biomarker categories. Examples are the BCAAs, fatty acids, ketone bodies, short-chain acylcarnitines, long-chain acylcarnitines, cholesterol metabolites, and lipoproteins, which suggests that the response of biomarkers within these (sub)categories are interdependent. This has two important implications. First, one biomarker within a cluster could be considered as representative of the total cluster (e.g., isoleucine for the BCAAs), which could be of relevance for studies that measure only one or a limited number of biomarkers from one correlated cluster. Second, it provides opportunities for future studies to compute one total, standardized score for all biomarkers within a cluster that are strongly correlated (e.g., a total BCAA score). From a disease risk assessment point of view, such an integrated score will likely have a larger power and stronger predictive value as compared to individual biomarker levels. Previously, Wang et al. have found that BCAA levels could predict type 2 diabetes risk (36). Integrated BCAA analysis is therefore promising as health-status biomarker. Not all biomarkers from functional categories can be integrated because of differences in the individual responses (e.g., peptide hormones, inflammation markers, and short- vs. longer-chain acylcarnitines). Clustering outside the functional category was also observed. The inverse association between CRP and the amino acid glycine has also been demonstrated previously (52,53) and

likely results from the inflammation modulating capacity of glycine (54,55). The positive association between N-acetylglycoprotein and lysophosphatidylcholine is also likely mediated via inflammation, since N-acetyglycoproteins plasma levels correlate with lipoprotein-associated phospholipase A2 levels (56), which generates lysophosphatidylcholine to promote inflammation (57,58). Direct positive links between glutamine, tyrosine, C5:0-OH and C5:1 acylcarnitines have not yet been described, but could be mediated by BCAA breakdown (59,60). The positive link between betaine and C18:2 acylcarnitine has not yet been demonstrated in humans, but may be related to fatty acid incorporation, as previously demonstrated in pigs (61). The observed correlations imply some revision of our a-priory functional categorization and, importantly, provide leads for biomarker integration and functional interpretation of changes in biomarker levels.

Next to the functional links between biomarker pairs, the hierarchical clustering models also showed that the degree of clustering for the intraindividual biomarker response i.e., the baseline and post-exercise biomarker values of one subject, was higher than the degree of clustering of the group (high-fit vs. low-fit) and the timepoint (baseline vs. post-exercise) biomarker responses. This finding suggests a considerable level of interindividual variation in our study population, which might also explain our observation that ~ 35% of the biomarkers was significantly impacted by recent exercise, but that clustering did not separate total baseline and post-exercise biomarker profiles. Since Krug et al., also showed that the interindividual variability was increased by using challenge tests (62), one could speculate that the challenged biomarker responses within one individual over time might act as a better predictor of health status, as compared to a singular analysis of the average biomarker levels of a larger group during basal homeostasis.

Our study included some strengths and limitations. One of the strengths is the integrated approach to analyze single as well as joined biomarker behavior in a healthy, homogenous study population at basal as well as challenged conditions, which provides us better insight in the behavior of biomarkers relatively to each other. An understanding of biomarkers in the healthy individuals is a prerequisite for their use in preventive health, for example biomarker guided dietary advise for health improvement. Another strength of our study is the focus on female individuals, since sex can affect metabolic responses (20,42), and females are often underrepresented in biomarker studies (15). One of the limitations of our study is that we could not determine the contribution of intraindividual variation, i.e., the day-to-day variation within an individual, as we sampled only twice in a relatively short time span. Although previous studies have demonstrated that the intraindividual variation for circulating adipokines (63), inflammatory markers (63,64), and metabolites (65,66) is smaller than the interindividual variation, we cannot exclude this source of error in our study. Second, we did not include additional post-exercise sampling timepoints, e.g., immediately post-exercise or a few hours post-exercise. Since the levels of most inflammatory markers, oxidative stress related markers and metabolites change acutely or in the first few hours after exercise, with each marker having its own kinetic profile (15,18) and the fact that biomarker kinetics can also differ between individuals as a result of interindividual variation (62), sampling at multiple timepoints after the exercise bout would have given insight in the exercise-induced biomarker behavior over time. Third, our study focused on a total of 102 biomarkers related to hormone signaling, inflammation and oxidative

stress, and metabolism, while fitness level and single exercise stimulation have been associated with alterations in markers that were not included in our study, such as vitamins (44,45), ceramides (38), and individual lysophosphatidylcholines (38,42), which could possibly have provided additional insights in these biomarkers in view of the homogeneity of our study subjects characteristics and high level of study standardization.

Conclusions

In conclusion, we showed that the overall circulating biomarker profiles were similar between high-fit and low-fit young, healthy, adult females. Although recent exercise had a limited impact on the overall biomarker profiles, it significantly affected a selected number of individual biomarkers. This study provides insight in the single and joined behaviour of circulating biomarkers in healthy females and identified functional biomarker categories that may be used for characterization of human health physiology.

Abbreviations

BCAA = branched-chain amino acid

CD14 = soluble monocyte differentiation antigen cluster of differentiation 14

CRP = c-reactive protein

FDR = false discovery rate

FFQ = food frequency questionnaire

ICAM1 = soluble intercellular adhesion molecule 1

IQR = interquartile range

LBP = LPS-binding protein

MCP1 = monocyte chemoattractant protein 1

PBMC = peripheral blood mononuclear cell

PCA = principal component analysis

RM-ANOVA = repeated measures analysis of variance

RT = room temperature

TNF = tumour necrosis factor

$\dot{V}O_2$peak = peak oxygen uptake

Declarations

Ethical approval and consent to participate

The protocol for collection and handling of human samples was ethically approved by the medical ethical committee (METC) of Wageningen University (since January 2021 replaced by METC Oost-Nederland) with reference number NL70136.081.19 and registered in the Dutch trial register (NL7891) on 2019-07-23. All procedures performed were in accordance with institutional ethical standards, national law (WMO, The Hague, 1998) and with the 1964 Helsinki declaration and its amendments. Written informed consent was obtained from all individual subjects included in the study.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its additional files.

Competing interest

All authors declare no conflict of interest.

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Authors' contributions

Study: J.J.E.J., B.L.; Analysis: J.J.E.J., X.E., N.C.; Data interpretation: J.J.E.J., X.E., V.C.J.d.B., J.K.; Writing: original draft preparation: J.J.E.J.; review and editing: J.J.E.J., B.L., A.G.N., X.E., J.M.d.B., V.C.J.d.B., J.K.; Funding: J.J.E.J., V.C.J.d.B, J.K.

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Figures

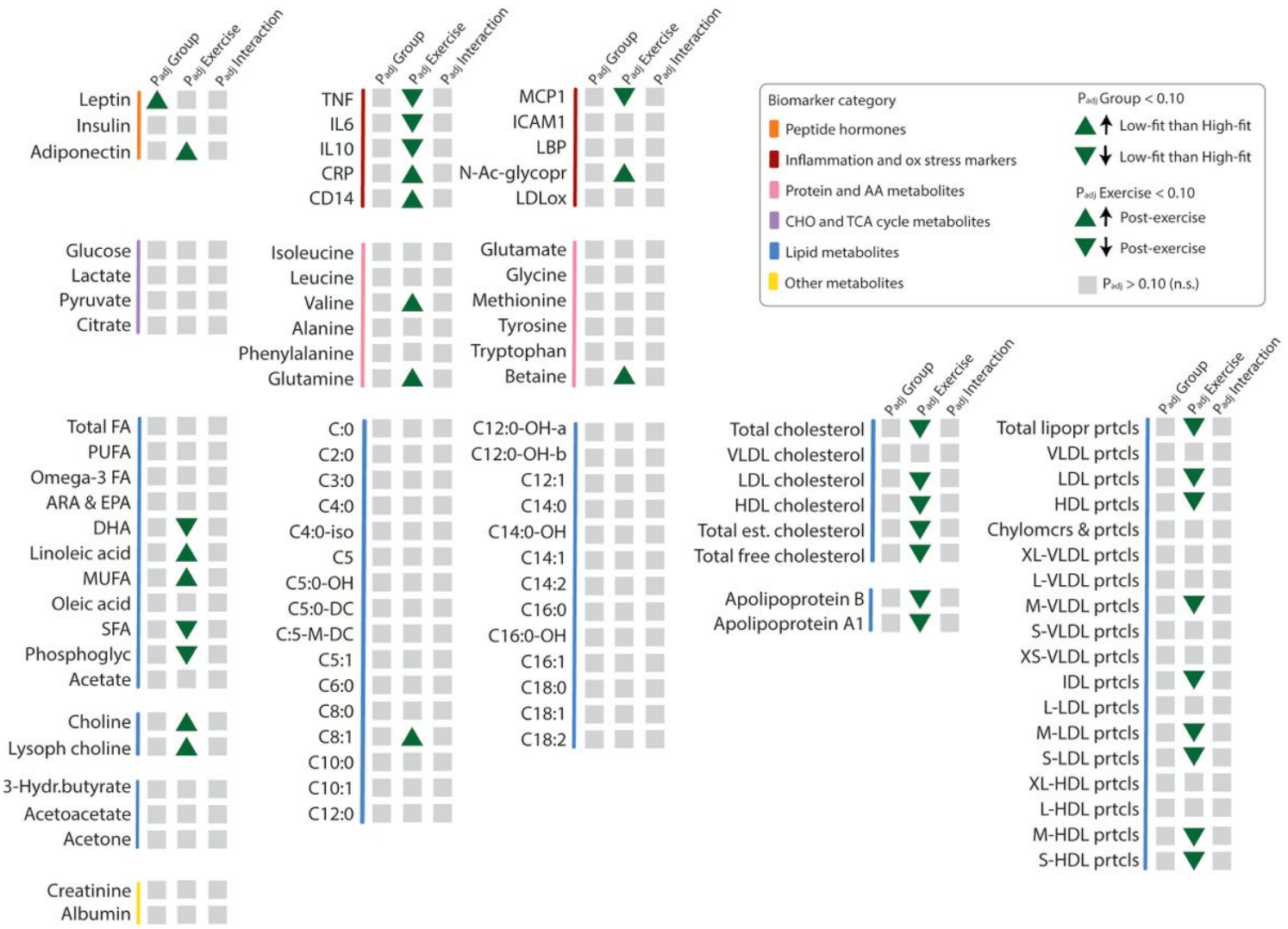

Figure 1

The effect of fitness level and a recent exercise bout on individual biomarker levels. Graphical summary representing the fitness level effect (P_{adj}Group), recent exercise effect (P_{adj}Exercise) and interaction effect (P_{adj}Group*Exercise, shown as P_{adj}Interaction) on individual biomarker levels within each functional biomarker category (indicated by colour). Significant fitness level effects (adj.P_{group} < 0.10) or recent exercise effects (adj.$P_{exercise}$ < 0.10) are depicted by upward and downward green triangles that indicate the direction of the effect. Non-significant effects (adj.P_{group}, adj.$P_{exercise}$ or adj.$P_{group*exercise}$ > 0.10) are depicted in grey squares (all interaction effects were not significant). Main effects (fitness level and recent exercise) and interaction effects were analysed using RM-ANOVA.

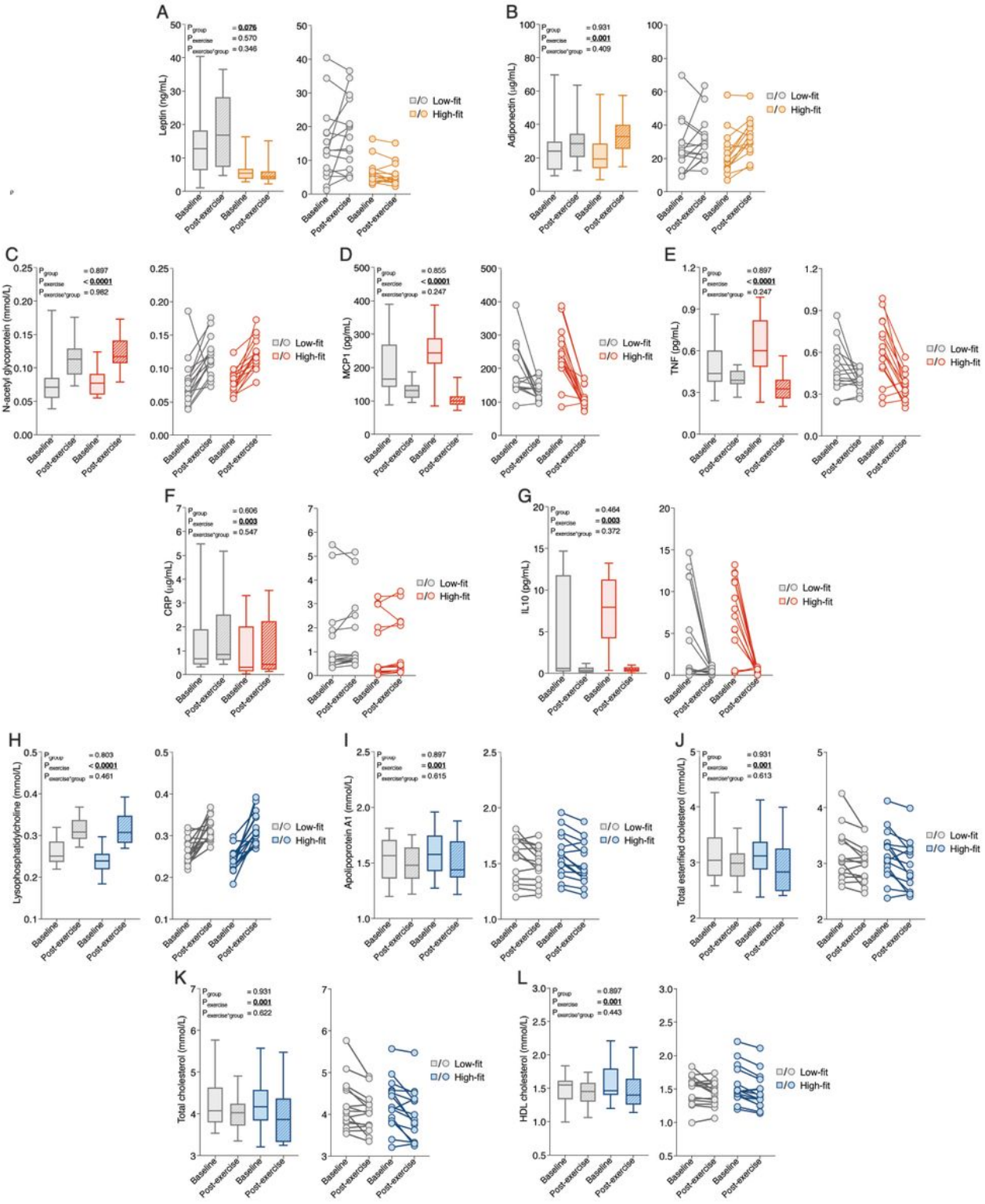

Figure 2

The response of the top-5 significantly regulated biomarkers within each biomarker category by fitness level or a recent bout of exercise. (**A**, **B**) Median group levels (box plots, left) and individual levels (scatter plots, right) of leptin (**A**) and adiponectin (**B**) in low-fit (N = 15, grey) and high-fit (N = 15, orange) females at baseline (transparent bars and dots) and after recent exercise (post-exercise ; dashed bars and transparent dots). (**C** – **G**) Median group levels (box plots, left) and individual levels (scatter plots, right)

of N-acetylglycoproteins (**C**), MCP1 (**D**), TNF (**E**), CRP (**F**), and IL10 (**G**) in low-fit (N = 15, grey) and high-fit (N = 15, red) females at baseline (transparent bars and dots) and post-exercise (dashed bars and transparent dots). (**H** – **L**). Median group levels (box plots, left) and individual levels (scatter plots, right) of lysophosphatidylcholine (**H**), apolipoprotein A1 (**I**), total esterified cholesterol (**J**), total cholesterol (**K**) and HDL cholesterol (**L**) in low-fit (N = 15, grey (N = 14 for lysophosphatidylcholine)) and high-fit (N = 15, blue) females at baseline (transparent bars and dots) and post-exercise (dashed bars and transparent dots). Main effects (fitness level and recent exercise) and interaction effects were analysed using RM-ANOVA. Significant adj.P-values (< 0.10) are indicated in underlined bold.

Figure 3

The effect of fitness level and a recent exercise bout on biomarker category responses. Graphical summary representing the number of significantly regulated biomarkers between high-fit and low-fit females (fitness level effect, left bars) and the number of significantly regulated biomarkers between baseline and post-exercise (recent exercise effect, right bars). Non-significant effects (adj.P_{group} or adj.$P_{exercise}$ > 0.10) are depicted in light coloured bars and significant effects (adj.P_{group} or adj.$P_{exercise}$ < 0.10) are depicted in dark coloured, dashed bars. The filled area is calculated relatively to the number of biomarkers within the corresponding functional category.

Figure 4

Heatmap of hierarchical clustered biomarkers and the association with fitness level and recent exercise. Heatmap based on hierarchical clustering of all 102 biomarkers based on Euclidean distance and complete linkage clustering. Biomarkers are clustered along the y-axis and individual subjects are clustered along the x-axis. Subject ID (S0x) and timepoint (T0 for baseline, T1 for post-exercise) are given for each subject. Subject IDs are coupled to fitness level (dark grey for high-fit and light grey for low-fit subjects) and timepoint (dark brown for baseline and beige for post-exercise). The colour scale represents low (dark orange) to medium (dark blue) biomarker values.

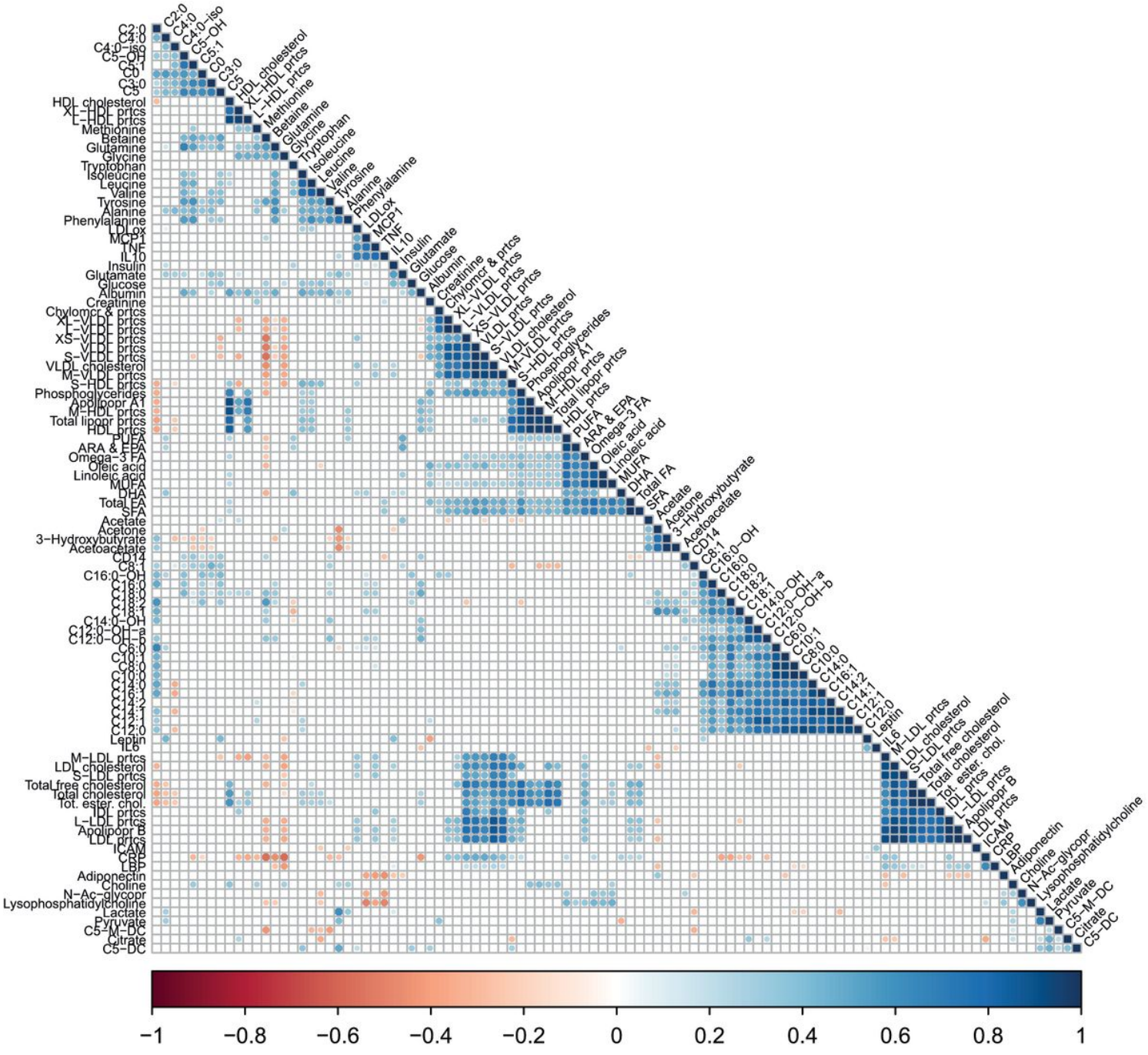

Figure 5

Correlation matrix showing the relationships between biomarker pairs. The correlation matrix based on Spearman correlation coefficients between biomarker pairs. Spearman rank correlation analysis was performed on the scaled biomarker values for all biomarker pairs using the combined data of high-fit and low-fit females at baseline and post-exercise. Relationships were considered statistically significant when $P < 0.05$. Significant relationships are indicated in red (negative correlation) or blue (positive correlation). Non-significant relationships ($P > 0.05$) are left blank.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFile1SupplementaryFigures.pdf
- AdditionalFile2SupplementaryTables.docx
- AdditionalFile3Dataincludedincorrelationmatrices.xlsx