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Original article

Perturbations in plasma amino acid and lipoprotein subfraction profiles in anorexia nervosa before and after refeeding: A metabolomic cross-sectional and longitudinal analysis

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SUMMARY

Background: Anorexia nervosa (AN) is a life-threatening eating disorder, which is increasingly being considered a metabo-psychiatric condition. We aimed to assess how the lipoprotein subfraction and plasma metabolome are altered in acutely underweight patients with AN (AcAN), if they change with short-term weight-restoration, and whether these changes point towards altered cardiometabolic risk.
Methods: Using nuclear magnetic resonance spectroscopy, we measured and compared the plasma concentrations of 132 metabolites, aminoacids and lipoprotein subfractions in young female patients with AcAN before (n = 72) versus after (n = 46) a short-term inpatient refeeding program resulting in weight-restoration (longitudinal analysis), as well as versus female healthy control (HC) participants of similar age (n = 74) (cross-sectional analysis).
Findings: Patients with AcAN showed elevated plasma cholesterol levels due to higher concentrations of small and dense Low Density Lipoprotein (LDL-6) and of large and less dense High Density Lipoprotein (HDL-1) subfractions compared to HC. Additionally, they had lower plasma concentrations of branched chain amino acids and glucose and higher concentrations of the gluconeogenic amino acids glutamine, alanine and methionine. Refeeding elevated the plasma cholesterol levels further, but with a different pattern compared to AcAN, by increasing the concentrations of the larger and less dense LDL (LDL-1, LDL-2, LDL-3) particles and of smaller and more dense HDL (HDL-2, HDL-3) subfractions. However, refeeding only partially restored the amino acid concentrations.
Conclusion: Lipoprotein profiles in AcAN point towards a potentially elevated risk for atherosclerosis; an altered lipoprotein profile was also detected after refeeding. Metabolite profiles in AcAN indicate an advanced catabolic state with only partial restoration after refeeding.

1. Introduction

Anorexia nervosa (AN) is a frequent mental disorder among female adolescents and young adults characterized by low body weight due to undernutrition [1]. AN is accompanied by multiple severe metabolic and endocrine abnormalities, that can be life-

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threatening or lead to permanent disabilities [2]. Refeeding through nutrition is aiming to increase body weight and thereby to improve the metabolic, endocrine and mental state [3]. It is considered a crucial part of AN treatment and seen as the basis for successful cognitive behavioral therapy [3]. Since a substantial proportion of individuals with AN take a chronic course or relapse after recovery, many patients with AN undergo refeeding treatment several times [4]. Refeeding strategies have changed in recent years, prioritizing higher caloric intake in order to achieve faster and more profound body weight gain [3]. Thus, it is important to fully understand the metabolic and endocrine changes that occur in AN and the changes that accompany rapid weight restoration in these patients.

We have previously shown that refeeding in patients with AN may increase the concentrations of certain ceramide species associated with obesity and insulin resistance, as well as the concentrations, of more saturated triglycerides associated with higher risk of type 2 diabetes (T2D) [5]. Additionally, evidence from previous studies suggest that AN may induce changes that lead to higher total cholesterol, High Density Lipoprotein (HDL)- and Low Density Lipoprotein (LDL)-cholesterol as well as triglycerides [6]. Apart from LDL- and HDL-cholesterol [7,8], which are known cardiovascular risk markers, lipoprotein subfractionation analysis has recently identified further subgroups within HDL and LDL classes that demonstrate distinct atherogenic properties according to their size and density [9,10]. Specifically, the smaller and more dense LDL subfractions (LDL-5 or LDL-6) are stronger associated with atherosclerosis and coronary artery disease than the less dense ones (LDL-1 to LDL-3), even when other classic markers are not elevated (LDL-cholesterol, triglycerides) or not decreased (HDL-cholesterol) [9,10]. Similarly, the larger and less dense HDL particles (HDL-1 and HDL-2) are often inversely associated with cardiovascular risk [11,12]. Interestingly, recent studies have also linked the concentrations of LDL- and HDL-subfractions with the development of insulin resistance, beta-cell dysfunction and diabetes mellitus [13–15]. How AN may affect lipoprotein subfractions and whether refeeding can restore or further aggravate the AN-induced changes in lipoprotein signatures remains largely unknown. However, this is important in order to assess the cardiometabolic risk of these patients both in acute state and during refeeding, as well as potentially consider modifications in our refeeding as well as other treatment strategies.

Reduced energy intake is also characterized by initiation of catabolic processes that include rapid exhaustion of carbohydrate stores and use of lipids initially and later of proteins for energy production. We have previously shown that complete fasting in normal weight individuals leads within 48h to profound increases in the concentrations of free fatty acids due to lipolysis, as well as in the concentrations of the branched chain amino acids (BCAAs) isoleucine, leucine, valine [16]. The increase in BCAAs concentrations has been suggested to signal the shift from carbohydrate to lipid utilization as main energy fuel and to subsequent increase of ketone bodies formation [16,17]. In contrast to acute fasting, normal − weight women with chronic relative energy deficiency due to sports (RED-S) do not demonstrate any alterations in their concentrations of amino acids, carbohydrates and ketone bodies [16]. In AN, heterogeneous results have been published so far. Studies have reported both increased, unchanged or decreased concentrations for several amino acids with partial or no restoration after recovery of AN but with no clear recognizable pattern in terms of fuel utilization and shift from catabolic to anabolic processes [18,19].

In the current study, we aimed first to assess whether acute AN and its treatment through refeeding induce changes in lipoprotein subfraction concentrations and composition that can indicate an altered risk for adverse cardiovascular outcomes in the long-term perspective. Furthermore, we aimed to investigate whether a distinct metabolic signature can be identified in the plasma of patients with AN, whether this signature reflects starvation compared to traits of the underlying disease, and whether it is restored after refeeding.

2. Materials & methods

2.1. Study population

A total of 146 female volunteers participated: 72 underweight patients with AcAN (aged 12–29 years) and 74 HC (aged 12–29 years). Patients with AcAN were admitted to intensive treatment of a specialized eating disorder program at the child and adolescent psychiatry and psychosomatic medicine department of a university hospital, and assessed within 96 h of admission to treatment (timepoint 1). We selected AcAN participants at timepoint 1 with successful venipuncture, which yielded n = 76 potential participants. Out of the selected AcAN participants, n = 1 was excluded because she was identified twice (for different episodes of the eating disorder), and n = 3 were excluded because not enough plasma for the analyses could be provided, yielding n = 72 AcAN participants for timepoint 1. All participants were administered a modified version of the expert form of the Structured Interview for Anorexia and Bulimia Nervosa (SIAB-EX) by trained clinical research staff assessing DSM-5 criteria for AN, bulimia nervosa and binge eating disorder (Supplement) [20,21]. AcAN patients had to meet the DSM-5 criteria for AN including a body mass index (BMI) below the 10th age percentile (if < 15.5 years old) or below 17.5 kg/m^2 (if > 15.5 years old). AcAN patients, who had suffered from bulimia nervosa or binge eating disorder in the past, were excluded from the study. In HC, the SIAB-EX was administered to ensure normal eating behavior. Eating disorder-specific psychopathology was additionally assessed in all participants with the Eating Disorder Inventory-2 (EDI-2), depressive symptoms with the Beck Depression Inventory Version 2 (BDI-II) and general levels of psychopathology with the revised Symptom Checklist 90 (SCL-90-R) [22–24].

A subgroup of 46 out of the 72 AcAN patients were not only assessed at admission (timepoint 1 for this subgroup, AcAN_TP1) but also after short-term weight restoration through oral refeeding (timepoint 2, AcAN_TP2 patients). Specifically, a number of n = 52 AcAN patients met the inclusion criterion of a BMI increase of at least 12 % for the follow-up assessment. Six participants were excluded because not enough plasma could be provided for the analyses, yielding n = 46 acAN participants for timepoint 2 (Flow diagram in supplement). Patients received higher calorie refeeding as described by Bargiacchi et al. (starting daily caloric intake 1500–2400 kcal/day, expected rates of body weight gain 0.5–2 kg/week) [25]. Refeeding was achieved according to a structured, nutritionally balanced meal plan (3 meals, 3 snacks per day) developed in collaboration with a nutritionist. The meal plan was adjusted for each participant based on the severity of undernutrition and on the food intake before admission to inpatient treatment. Body weight gain was monitored daily. Based on the achieved weight gain, caloric intake was typically increased every 2–3 days in the first weeks of inpatient treatment. Patients were monitored for signs of refeeding syndrome (see Supplement for details). The weight and height trajectories of patients were followed to assess whether they could be included for timepoint 2. HC had to be of normal weight, eumenorrheic and without any history of mental illness (as assessed by the German versions of the M.I.N.I. international neuropsychiatric interview or the M.I.N.I. KID interview for children and adolescents) and were recruited through advertisement among middle/high school and college students.

Information regarding exclusion criteria (Supplement) was obtained from all participants using the SIAB-EX supplemented by our own semi-structured interview and medical records as well as (in HC) the M.I.N.I. interview. Comorbid diagnoses in AcAN were taken from medical records and confirmed by an expert clinician. BMI standard deviation scores (BMI-SDS) were computed to provide an age- and gender-corrected index. Study data were managed using Research Electronic Data Capture.[S8] All protocols received ethical approval by the Ethics Commission at the Technische Universität Dresden (Protocol EK 536122015, approval in 2016, start of the study in 2017), and all participants (and, if underage, their guardians) gave written informed consent. The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments.

2.2. Sample preparation and NMR metabolomics analysis

Venous blood samples were collected between 7 and 9 a.m. after an overnight fast (for the AcAN_TP1 group within 96 h of admission to treatment. Aprotinin was added and after centrifugation plasma samples were stored at −80 °C until analysis, which was performed in a Bruker 600-MHz Avance III Neo NMR-spectrometer, as previously described [26]. Details about the sample preparation and NMR analysis are provided in the supplement.

2.3. Statistical analysis

Statistical analysis was performed with SPSS v19.0 (SPSS, Inc., Chicago, IL) for Windows, with GraphPad prism 7 (GraphPad Software Inc., La Jolla, CA), and with MetaboanalystR [27] and is described in detail in supplement. Briefly, an orthogonal partial-least squares discriminant analysis (OPLS-DA) was used to compare, a) HC vs AcAN, b) HC vs AcAN_TP2, c) AcAN_TP1 vs AcAN_TP2, followed by volcano plots based on Mann–Whitney-U-Test or Wilcoxon signed-rank test with false discovery (FDR) < 0.05 (Figs. 1 and 2 and Supplemental Tables 1–3). Missing values were replaced by 1/5 of min positive values of their corresponding variables and for normalization all data were mean-centered and divided by the standard deviation of each variable. Absolute concentrations of the significant parameters based on fold changes and for parameters strongly related to the significant ones (i.e. components of the same density lipoprotein particles) were also assessed (Tables 1–4 as mean ± SEM). The study had above 80 % power to detect differences between two groups of an effect size d > 0.5 (unadjusted) or d > 0.8 (adjusted for multiple comparisons) at two-tailed a = 0.05 (more details in the supplement).

3. Results

Demographic and clinical characteristics of the different groups are presented in Table 1. Patients with AcAN and HC were of similar age. Patients with AcAN had a lower BMI compared to HC and a robust BMI increase after refeeding (% BMI increase, Mean ± SEM = 28.2 ± 1.3; AcAN_TP2 vs AcAN_TP1). The mean duration of refeeding for restoration of body weight was almost 86 days. The mean age of onset of AN was 14.4 years.

3.1. Lipoprotein and metabolite profile in AcAN (cross-sectional analysis)

The NMR metabolomic analysis assessed the concentrations of 153 parameters including amino acids, carboxylic acids, keto acids, sugars and their derivatives, as well as lipoprotein composition of main fractions and subfractions (Supplemental Appendix). Features with more than 50 % of values below detection limit were excluded from further analysis. This accounted for 21 parameters in total (some amino acids, sugars and ketone bodies) (Supplemental Appendix).

In an orthogonal partial least-squares discriminant analysis (OPLS-DA) between AcAN and HC, component $1^{\text{HC vs AcAN}}$ consisting of fifteen parameters partially discriminated HC from patients with AcAN (green circles clustering at the right half of the score plot – in the area of positive values of component 1 and red circles clustering at the left half of the score plot) (Fig. 1a). Next, we identified the relevant variables of Component $1^{\text{HC vs AcAN}}$, contributing to the proposed OPLS-DA model by calculating their Variable Importance in Projection (VIP) scores (Fig. 1b). Among them, we primarily found cholesterol and free cholesterol parameters, particularly from HDL subfractions, from VLDL-2 and VLDL-3 and from LDL-6 (Fig. 1b). Additionally, it included all three BCAAs (leucine, isoleucine and valine). In contrast, component $2^{\text{HC vs AcAN}}$ did not discriminate HC from patients with AcAN (similar distribution of red and green circles along the y axis of the plot – Fig. 1a).

Similarly, a non-parametric analysis adjusted for FDR identified 7 parameters (BCAAs, glucose, and VLDL-5 parameters) whose relative concentrations (fold changes) were lower in patients with AcAN (Fig. 1c – marked blue) compared to HC (Supplemental Table 1). On the contrary, the relative concentrations of 25 parameters (primarily cholesterol and several parameters in LDL-6

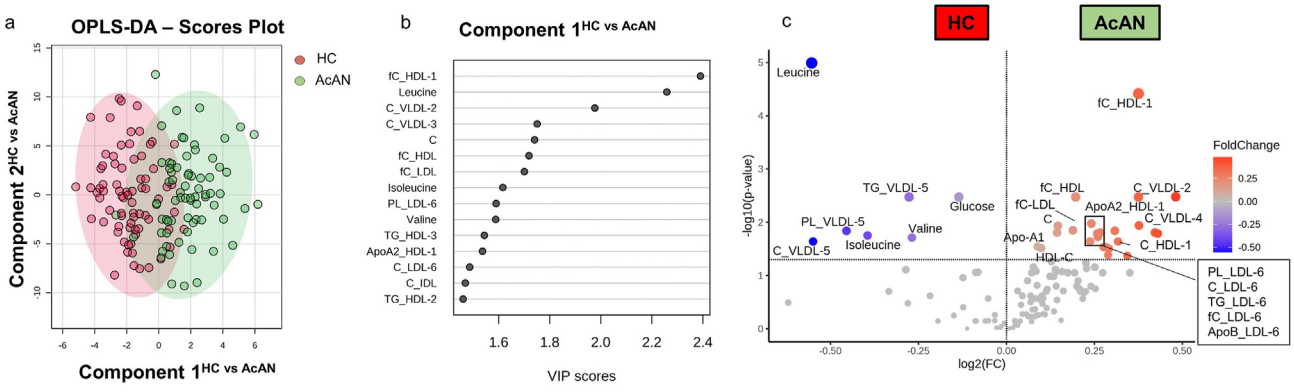

Fig. 1. NMR metabolomic differences between patients with AcAN and HC. a. OPLS-DA scores plot of components 1 and 2, b. Parameters contributing mostly to component 1 according to Variable Importance in Projection (VIP) scores, c. Volcano plot based on FDR-adjusted non-parametric tests (complete list of parameters in Supplemental Table 1). Blue dots indicate parameters that have significantly lower concentrations and red dots parameters that have significantly higher concentrations in AcAN compared to HC.

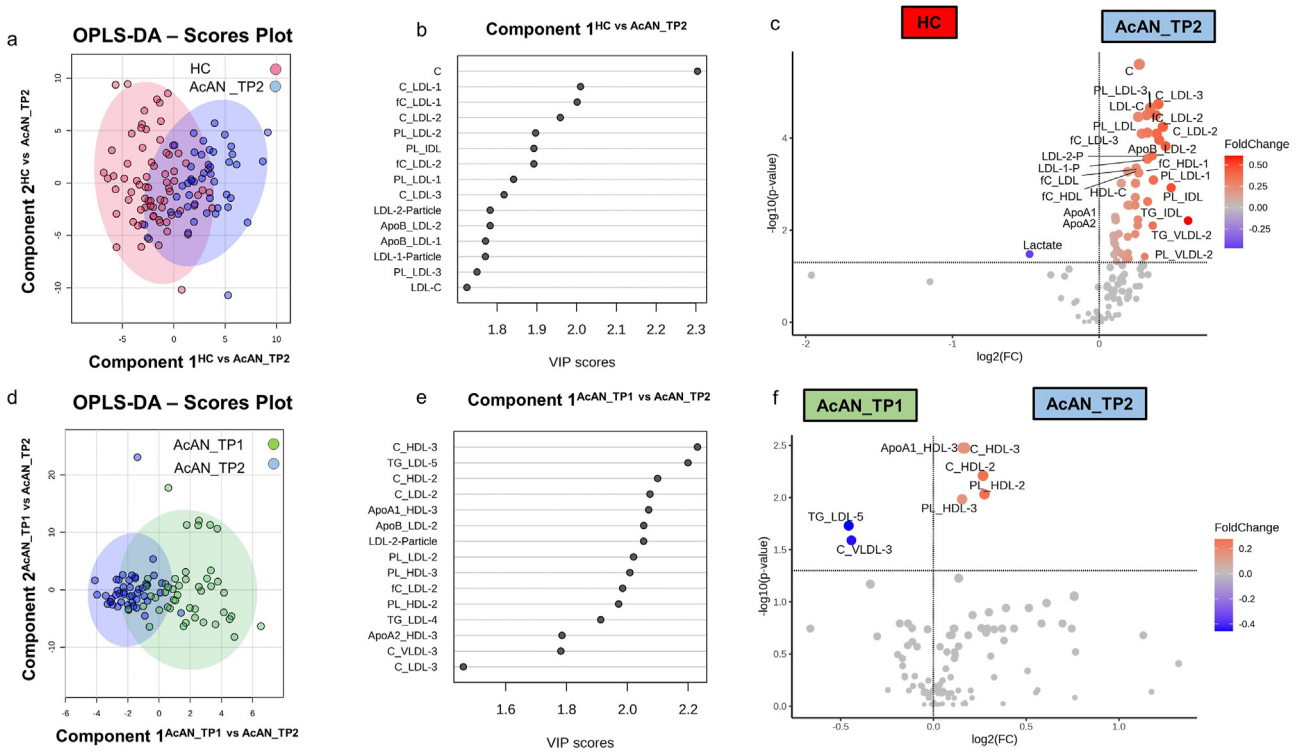

Fig. 2. NMR metabolomic differences between patients with AcAN after refeeding (AcAN_TP2) and HC or between patients with AcAN who have two measurements, both before (AcAN_TP1) and after refeeding (AcAN_TP2). a. OPLS-DA scores plots of components 1 and 2 for differentiating AcAN_TP2, from HC. b. Parameters contributing mostly to component 1 according to VIP scores for differentiating AcAN_TP2 from HC. c. Volcano plot based on FDR-adjusted non-parametric test. Blue dots indicate parameters that have significantly lower concentrations and red dots parameters that have significantly higher concentrations in AcAN_TP2 compared to HC (complete list of parameters in Supplemental Table 2). d. OPLS-DA scores plots of components 1 and 2 for differentiating AN before (AcAN_TP1) from after refeeding (AcAN_TP2). e. Parameters contributing mostly to component 1 according to VIP scores for differentiating AN before (AcAN_TP1) with after refeeding (AcAN_TP2). f. Volcano plot based on FDR-adjusted non-parametric paired test. Blue dots indicate parameters that have significantly lower concentrations and red dots parameters that have significantly higher concentrations in AN after feeding (AcAN_TP2) compared to before refeeding (AcAN_TP1) (complete list of parameters in Supplemental Table 3).

Table 1
Characteristics of study cohort.

	HC (N = 74)	AcAN (N = 72)	AcAN_TP1 (N = 46)	AcAN_TP2 (N = 46)	HC vs AcAN	HC vs AcAN_TP2	AcAN_TP1 vs AcAN_TP2
					P		
Age (years)	16.7 ± 0.4	16.6 ± 0.4	16.2 ± 0.3	16.4 ± 0.3	0.86	0.72	**<0.0001**
Age (years) onset of AN		14.4 ± 0.4	14.4 ± 0.3				
Days of treatment			85.8 ± 3.4				
BMI (kg/m^2)	20.9 ± 0.3	14.7 ± 0.2	15.0 ± 0.1	19.2 ± 0.1	**<0.0001**	**<0.0001**	**<0.0001**
% BMI increase by refeeding			28.2 ± 1.3				
BMI − SDS	N/A	−2.9 ± 0.1	−2.8 ± 0.1	−0.5 ± 0.1	N/A	N/A	<0.0001
Minimum BMI	19.7 ± 0.2	14.4 ± 0.2	14.8 ± 0.2	N/A	**<0.0001**	N/A	N/A
EDI-2, total score	5.4 ± 0.7	22.7 ± 1.3	21.0 ± 1.5	13.1 ± 1.5	**<0.0001**	**<0.0001**	**<0.0001**
BDI-II, total score	140.1 ± 3.3	209.1 ± 5.9	202.9 ± 6.7	188.3 ± 8.1	**<0.0001**	**<0.0001**	0.044

HC, Healthy controls; AcAN, anorexia nervosa - whole population assessed within 96 h of admission to treatment - before feeding; AcAN-TP: Subgroup of the AcAN population with two measurements, i.e. TP1: within 96 h of admission to treatment – before feeding and TP2: after recovery of body weight by feeding. BMI-SDS, body mass index standard deviation score; EDI-2, Eating Disorder Inventory-2; BDI-II, Beck Depression Inventory; Unadjusted p-values depending on distribution from unpaired t-test, Mann–Whitney test or paired t-test and Wilcoxon-signed rank test. Bold numbers of p-values correspond to comparisons that remained significant after FDR-adjustment. N/A, not applicable.

and HDL-1 subfractions) were significantly increased (Fig. 1c – marked red and Supplemental Table 1).

For the significant parameters described above and for parameters strongly related to the significant ones, we additionally compared the absolute concentrations between groups in order to assess whether their concentrations fell within or outside the normal range and in order to identify biochemically relevant patterns. Total cholesterol was higher in AcAN (Table 2) compared to HC (p-adjusted = 0.012) due to elevated concentrations of both HDL-C (p-adjusted = 0.031), Intermediate Density Lipoprotein (IDL)-C (p-adjusted = 0.041) and a trend to elevated LDL-C (p-unadjusted = 0.027). Interestingly, patients with AcAN had higher concentrations especially of the most dense LDL particles, i.e. the LDL-6 particles (approx. 18 % higher in AN compared to HC, p-adjusted = 0.026). Cholesterol (p-adjusted = 0.016), free cholesterol (p-adjusted = 0.019), triglyceride (p-adjusted = 0.016), phospholipid (p-adjusted = 0.011) and apoB (p-adjusted = 0.023) concentrations deriving from LDL-6 particles were also approx. 20 % higher in AcAN compared to HC, corresponding to the 18 % higher concentrations of LDL-6 particles in AcAN and indicating no

Table 2
Plasma concentrations of lipoproteins and their subfractions which differ significantly in AcAN compared to HC.

	HC (N = 74)	AcAN (N = 72)	AcAN_TP1 (N = 46)	AcAN_TP2 (N = 46)	HC vs AcAN	HC vs AcAN_TP2	AcAN_TP1 vs AcAN_TP2
Cholesterol	**(mg/dl)**				***P***		
Total C	170.5 ± 3.2	188.7 ± 4.6	190.3 ± 5.1	206.1 ± 4.8	**0.001**	**<0.001**	0.018
VLDL-C	12.6 ± 0.7	14.4 ± 0.8	15.7 ± 0.8	14.4 ± 1.1	0.072	0.180	0.379
LDL-C	95.5 ± 3.1	105.3 ± 3.8	105.6 ± 4.2	119.4 ± 4.4	0.027	**<0.001**	**0.002**
IDL-C	8.0 ± 0.4	9.8 ± 0.52	10.4 ± 0.6	9.8 ± 0.6	**0.010**	0.014	0.412
HDL-C	55.3 ± 1.2	59.2 ± 1.3	58.3 ± 1.6	61.3 ± 1.3	**0.006**	**<0.001**	0.103
LDL particles	**(nmol/l)**						
LDL total	1077 ± 36	1180 ± 45	1170 ± 49	1278 ± 47	0.042	**<0.001**	0.091
LDL-1	165.8 ± 6.7	176.4 ± 8.5	176.9 ± 10.2	208.2 ± 8.6	0.326	**<0.001**	0.036
LDL-2	177.8 ± 8.0	175.9 ± 9.7	176.3 ± 13.1	228.4 ± 9.0	0.858	**<0.001**	0.002
LDL-3	191.7 ± 7.5	207.2 ± 8.7	204.5 ± 10.7	240.8 ± 8.7	0.110	**<0.001**	0.008
LDL-4	200.1 ± 11.2	219.9 ± 12.5	219.8 ± 14.8	212.2 ± 10.6	0.347	0.172	0.991
LDL-5	163.2 ± 9.8	190.8 ± 11.4	190.5 ± 14.3	175.3 ± 9.3	0.108	0.139	0.516
LDL-6	234 ± 9.2	275.9 ± 11.7	270.9 ± 14.1	268.1 ± 10.7	**0.004**	0.011	0.869
LDL-6	**(mg/dl)**						
C_LDL-6	15 ± 0.6	18.1 ± 0.8	17.7 ± 0.9	18.1 ± 0.7	**0.002**	**0.001**	0.755
fC_LDL-6	4.4 ± 0.3	5.3 ± 0.2	5.2 ± 0.3	5.2 ± 0.2	**0.003**	**<0.001**	0.889
TG_LDL-6	3.0 ± 0.1	3.6 ± 0.1	3.5 ± 0.2	3.2 ± 0.1	**0.002**	0.323	0.069
PL_LDL-6	8.9 ± 0.3	10.5 ± 0.4	10.2 ± 0.4	10.1 ± 0.3	**<0.001**	**0.005**	0.893
ApoB_LDL-6	12.9 ± 0.5	15.2 ± 0.6	14.9 ± 0.8	14.8 ± 0.6	**0.004**	0.011	0.869
HDL-1	**(mg/dl)**						
C_HDL-1	17.2 ± 1.1	21.4 ± 1.3	20.5 ± 1.6	21.6 ± 1.0	**0.012**	**0.005**	0.552
fC_HDL-1	5.9 ± 0.2	7.7 ± 0.3	7.6 ± 0.3	7.1 ± 0.2	**<0.001**	**<0.001**	0.851
PL_HDL-1	22.3 ± 1.3	27.4 ± 1.5	26.5 ± 1.8	26.7 ± 1.2	**0.010**	0.021	0.903
Apo-A1 HDL-1	32.6 ± 1.7	40.4 ± 2.0	39.4 ± 2.3	37.6 ± 1.8	**0.004**	0.063	0.456
VLDL	**(mg/dl)**						
TG_VLDL-5	2.61 ± 0.13	2.15 ± 0.13	2.21 ± 0.16	2.59 ± 0.18	**<0.001**	0.965	0.058
PL_VLDL-5	1.24 ± 0.10	0.91 ± 0.09	0.95 ± 0.10	1.21 ± 0.13	**0.002**	0.754	0.138
C_VLDL-5	0.75 ± 0.09	0.51 ± 0.08	0.52 ± 0.09	0.67 ± 0.13	**0.004**	0.306	0.231
C_VLDL-4	2.31 ± 0.18	2.99 ± 0.18	3.27 ± 0.21	2.66 ± 0.22	**<0.001**	0.199	0.048
C_VLDL-3	2.20 ± 0.13	2.95 ± 0.17	3.16 ± 0.20	2.38 ± 0.19	**<0.001**	0.428	**0.001**
C_VLDL-2	2.21 ± 0.13*	3.09 ± 0.17	3.29 ± 0.21	2.73 ± 0.20	**<0.001**	0.025	0.067

VLDL-C, LDL-C and LDL particles concentrations total and from LDL-1 to LDL-5 are not statistically different between AcAN vs HC but they are included for completion and comparison with the significant parameters. HC, Healthy controls; AcAN, anorexia nervosa - whole cohort assessed within 96 h of admission to treatment - before feeding; AcAN_TP1: Subgroup of the AcAN cohort with two measurements i.e. TP1: within 96 h of admission to treatment – before refeeding and TP2, i.e. after short-term recovery of body weight by refeeding. C, Cholesterol; fC, free cholesterol; PL, phospholipids; TG, triglycerides; *P* shows the unadjusted p-values depending on distribution from unpaired t-test, Mann–Whitney test (cross-sectional analysis: HC vs AcAN and HC vs AcAN_TP2) or paired t-test and Wilcoxon-signed rank test (longitudinal analysis: AcAN_TP1 vs AcAN_TP2). Bold numbers of p-values correspond to comparisons that remained significant after FDR-adjustment (s. Supplemental Tables 1–3 for the FDR-adjusted p values).

Table 3
Plasma concentrations of amino acids and of glucose which differ significantly in AcAN before and/or after refeeding (AcAN-TP2) compared to HC.

	HC (N = 74)	AcAN (N = 72)	AcAN_TP1 (N = 46)	AcAN_TP2 (N = 46)	HC vs AcAN	HC vs AcAN_TP2	AcAN_TP1 vs AcAN_TP2
Aminoacids	**(μmol/l)**				***P***		
Leucine	75 ± 4	50 ± 3	51 ± 4	65 ± 4	**<0.001**	0.032	0.016
Isoleucine	39 ± 3	28 ± 2	31 ± 7	33 ± 6	**0.002**	0.052	0.813
Valine	190 ± 7	157 ± 7	157 ± 9	181 ± 6	**0.003**	0.550	0.041
Glutamine	421 ± 29	519 ± 32	527 ± 43	514 ± 28	**0.004**	0.081	0.822
Alanine	310 ± 12	363 ± 17	386 ± 23	320 ± 10	0.011	0.581	0.010
Methionine	35 ± 4	48 ± 4	49 ± 5	47 ± 5	0.033	0.044	0.947
Glucose (mmol/l)	5.1 ± 0.2	4.6 ± 0.1	4.5 ± 0.2	5.0 ± 0.1	**<0.001**	0.106	0.025

HC, Healthy controls; AcAN, anorexia nervosa - whole cohort assessed within 96 h of admission to treatment - before feeding; AcAN_TP1: Subgroup of the AcAN cohort with two measurements i.e. TP1: within 96 h of admission to treatment – before refeeding and TP2, i.e. after short-term recovery of body weight by refeeding. *P* shows the unadjusted p-values depending on distribution from unpaired t-test, Mann–Whitney test (cross-sectional analysis: HC vs AcAN and HC vs AcAN_TP2) or paired t-test and Wilcoxon-signed rank test (longitudinal analysis: AcAN_TP1 vs AcAN_TP2). Bold numbers of p-values correspond to comparisons that remained significant after FDR-adjustment (s. Supplemental Tables 1–3 for the FDR-adjusted p values).

major differences in the relative LDL-6 composition between AcAN and HC (Table 2). The higher HDL-C in AcAN derived almost exclusively from the elevated cholesterol concentrations in the HDL-1 subfraction (p-adjusted = 0.023). Free cholesterol (p-adjusted<0.001), phospholipids (p-adjusted = 0.029) and Apo-A1 (p-adjusted = 0.015) concentrations were also higher in HDL-1 subfraction, thus indicating possibly higher concentrations of the HDL-1 particles, which were not directly measured in our study. Furthermore, cholesterol was lower in VLDL-5 (p-adjusted = 0.023) but higher in VLDL-4 (p-adjusted = 0.012), VLDL-3 (p-adjusted = 0.016) and VLDL-2 (p-adjusted = 0.003) subfractions in AcAN, thus resulting in no significant differences in total VLDL-C between AcAN and HC.

Leucine, isoleucine and valine concentrations were reduced in AcAN in comparison to HC by 34 % (p-adjusted<0.001), 29 % (p-adjusted = 0.018) and 18 % (p-adjusted = 0.019), respectively (Table 3 and Supplemental Table 1). Fasting glucose levels were also lower in AcAN compared to HC (p-adjusted = 0.003). The concentrations of glutamine were higher (p-adjusted = 0.029) and of alanine (p-unadjusted = 0.011) and methionine (p-unadjusted = 0.033) tended to be higher in AcAN. No significant differences were observed in the concentrations of the other amino

Table 4
Plasma concentrations of lipoproteins and their subfractions which differ significantly in AcAN-TP2 after refeeding compared to HC and to pre-feeding AcAN or AcAN-TP1 state.

	HC (N = 74)	AcAN (N = 72)	AcAN_TP1 (N = 46)	AcAN_TP2 (N = 46)	HC vs AcAN	HC vs AcAN_TP2	AcAN_TP1 vs AcAN_TP2
LDL-1	**mg/dl**				***P***		
C_LDL-1	16 ± 0.8	17.7 ± 1.0	18 ± 1.2	21.9 ± 1.1	0.191	**<0.001**	0.023
fC_LDL-1	5.3 ± 0.2	6.1 ± 0.3	6.2 ± 0.4	7.0 ± 0.3	0.042	**<0.001**	0.099
TG_LDL-1	4.2 ± 0.2	4.3 ± 0.2	4.3 ± 0.2	4.2 ± 0.2	0.602	0.474	0.938
PL_LDL-1	9.3 ± 0.4	10.1 ± 0.5	10.3 ± 0.6	12.2 ± 0.5	0.265	**<0.001**	0.037
ApoB_LDL-1	9.1 ± 0.4	9.7 ± 0.5	9.7 ± 0.6	11.5 ± 0.5	0.324	**<0.001**	0.035
LDL-2	**mg/dl**						
C_LDL-2	17.2 ± 0.8	17.6 ± 1.1	17.7 ± 1.4	23.3 ± 1.0	0.811	**<0.001**	0.002
fC_LDL-2	5.9 ± 0.3	6.2 ± 0.3	6.2 ± 0.4	7.7 ± 0.3	0.482	**<0.001**	0.003
TG_LDL-2	1.86 ± 0.09	1.95 ± 0.11	1.89 ± 0.14	2.11 ± 0.08	0.316	0.017	0.296
PL_LDL-2	9.4 ± 0.4	9.5 ± 0.5	9.6 ± 0.7	12.3 ± 0.5	0.710	**<0.001**	0.006
ApoB_LDL-2	9.8 ± 0.4	9.7 ± 0.5	9.7 ± 0.7	12.6 ± 0.5	0.8552	**<0.001**	0.002
LDL-3	**mg/dl**						
C_LDL-3	18 ± 0.7	19.8 ± 0.9	19.7 ± 1.1	23.7 ± 0.9	0.073	**<0.001**	0.004
fC_LDL-3	6.4 ± 0.2	7 ± 0.2	6.9 ± 0.3	7.8 ± 0.3	0.022	**<0.001**	0.024
TG_LDL-3	2.04 ± 0.07	2.22 ± 0.07	2.23 ± 0.09	2.33 ± 0.07	0.042	**<0.001**	0.378
PL_LDL-3	10.0 ± 0.4	10.7 ± 0.4	10.6 ± 0.5	12.6 ± 0.4	0.117	**<0.001**	0.005
ApoB_LDL-3	10.5 ± 0.4	11.4 ± 0.5	11.3 ± 0.6	13.2 ± 0.5	0.110	**<0.001**	0.008
HDL-2	**mg/dl**						
C_HDL-2	7.8 ± 0.3	7.4 ± 0.3	7.1 ± 0.3	8.6 ± 0.3	0.633	**0.003**	**<0.001**
fC_HDL-2	2.59 ± 0.07	2.86 ± 0.06	2.83 ± 0.08	2.81 ± 0.06	0.002	**0.009**	0.831
TG_HDL-2	1.58 ± 0.11	1.25 ± 0.09	1.28 ± 0.11	1.57 ± 0.11	0.111	0.601	0.059
PL_HDL-2	12.1 ± 0.5	11.2 ± 0.4	10.8 ± 0.5	13 ± 0.4	0.167	0.032	**<0.001**
HDL-3	**mg/dl**						
C_HDL-3	10.2 ± 0.2	9.8 ± 0.2	9.7 ± 0.2	10.9 ± 0.2	0.168	0.012	**<0.001**
fC_HDL-3	2.8 ± 0.10	3.13 ± 0.11	3.16 ± 0.14	3.32 ± 0.12	0.026	**<0.001**	0.339
TG_HDL-3	1.9 ± 0.1	1.56 ± 0.08	1.64 ± 0.09	1.91 ± 0.11	0.010	0.935	0.076
PL_HDL-3	15.8 ± 0.3	14.9 ± 0.3	14.7 ± 0.3	16.3 ± 0.3	0.041	0.240	**<0.001**

HC, Healthy controls; AcAN, anorexia nervosa - whole cohort assessed within 96 h of admission to treatment - before feeding; AcAN_TP1: Subgroup of the AcAN cohort with two measurements i.e. TP1: within 96 h of admission to treatment – before refeeding and TP2, i.e. after short-term recovery of body weight by refeeding. C, Cholesterol; fC, free cholesterol; PL, phospholipids; TG, triglycerides; *P* shows the unadjusted p-values depending on distribution from unpaired t-test, Mann–Whitney test (cross-sectional analysis: HC vs AcAN and HC vs AcAN_TP2) or paired t-test and Wilcoxon-signed rank test (longitudinal analysis: AcAN_TP1 vs AcAN_TP2). Bold numbers of p-values correspond to comparisons that remained significant after FDR-adjustment (s. Supplemental Tables 1–3 for the FDR-adjusted p values).

acids, of ketoacids/ketone bodies and of other sugar derivatives (Supplemental Table 4).

3.2. Lipoprotein and metabolite profile in AN after refeeding (AcAN_TP2) vs HC

To examine to what extent the concentration of metabolites and lipoproteins in plasma of AN patients after refeeding and short-term weight restoration (AcAN_TP2) may already approach to or still differ from the concentrations of healthy controls, we performed an OPLS-DA of the obtained NMR data (Fig. 2a). Interestingly, component $1^{HC\ vs\ AcAN\_TP2}$ (but not component 2) could partially differentiate between the two groups (Fig. 2a). Component $1^{HC\ vs\ AcAN\_TP2}$ consisted primarily of cholesterol and of parameters from LDL-1, LDL-2 and LDL-3 subfractions (Fig. 2b).

Similarly, a non-parametric analysis adjusted for FDR identified 55 parameters (mainly LDL-1, LDL-2, LDL-3 components and also HDL, HDL-1, HDL-2 components) whose relative concentrations (fold changes) were higher in patients with AN after refeeding (AcAN_TP2), as compared to HC (Fig. 2c – marked red and also Supplemental Table 2). When comparing the absolute concentrations, the higher total cholesterol levels in AcAN_TP2 (vs HC; p-adjusted<0.001) were mainly due to higher LDL-C (p-adjusted<0.001) and to a lesser extent due to higher HDL-C (p-adjusted<0.001) (Table 2 and Supplemental Table 2). Specifically, the concentrations of LDL-1 (p-adjusted<0.001), LDL-2 (p-adjusted<0.001) and LDL-3 (p-adjusted<0.001) particles were 25–28 % higher in AcAN_TP2 compared to HC. Similarly, higher concentrations were also observed in cholesterol, free cholesterol, phospholipids and ApoB in these subfractions (LDL-1, LDL-2 and LDL-3) (all p-adjusted values < 0.001), but not in triglyceride concentrations (Table 4 and Fig. 2c).

3.3. Longitudinal changes in lipoprotein and metabolite profile with refeeding (AcAN_TP1 vs AcAN_TP2)

To study longitudinal changes of the plasma metabolome and lipoprotein fractions from admission (AcAN_TP1) till the end of the intense inpatient refeeding (AcAN_TP2), plasma samples of 46 patients were analyzed in a pairwise comparison. OPLS-DA could also partially discriminate AcAN_TP1 from AcAN_TP2 through component $1^{AcAN\_TP1/AcAN\_TP2}$ (in patients with paired measurements (n = 46); Fig. 2d). Component $1^{AcAN\_TP1/AcAN\_TP2}$ consisted primarily of parameters of HDL-2 and HDL-3, of LDL-2 subfraction as well as triglycerides from LDL-5 and LDL-4 fractions (Fig. 2e). The non-parametric analysis adjusted for FDR identified 5 parameters related to HDL-2 and HDL-3, which their concentrations increased with refeeding (higher concentrations in AcAN_TP2 vs AcAN_TP1) (Fig. 2f and Supplemental Table 3).

When assessing the absolute concentrations (AcAN_TP1 vs AcAN_TP2), HDL-2 and HDL-3 cholesterol and phospholipid concentrations after refeeding were elevated (Table 4, Supplemental Table 3), but this did not result in a significant increase of total HDL-C (Table 2). Interestingly, refeeding had also no impact on LDL-6 and HDL-1 subfractions, whose concentrations were altered in AcAN (Table 2) compared to HC. Refeeding though seems to increase LDL-1, LDL-2 and LDL-3 particle concentrations (Table 2), a result which was significant after FDR-adjustment when comparing AcAN_TP2 vs HC (all p-adjusted values < 0.001), and was also significant only at an unadjusted level when comparing AcAN_TP2 vs AcAN_TP1 (for LDL-1 p-unadjusted = 0.036, LDL-2 p-unadjusted = 0.002, LDL-3 p-unadjusted = 0.008), most probably due to smaller sample size of AcAN_TP1 compared to the sample size of HC as well as due to slightly non-significantly higher LDL-1 and LDL-3 in AcAN_TP1 compared to HC (Table 2).

Regarding amino acids, there was a partial restoration of the low concentrations of BCAAs and a complete restoration of the fasting glucose levels after refeeding (Table 3). In line with terminated starvation, the concentration of the gluconeogenic amino acid alanine, which has been higher in AcAN, tended to be reduce after refeeding (p-unadjusted = 0.010), whereas the elevated glutamine and methionine concentrations in AcAN remained unaffected (Table 3). No other profound alterations were observed in the other amino acids, keto acids/ketones and sugar derivatives.

4. Discussion

In this study, we demonstrate that lipoprotein profiles in AcAN point towards a potentially elevated risk for atherosclerosis due to increased concentrations of smaller, -more dense LDL particles (e.g. LDL-6), which are considered atherogenic and have been strongly associated with cardiovascular disease (CVD) risk [9,10]. A concomitant increase of larger, -less dense HDL particles (e.g. HDL-1) in AcAN might mitigate part of this elevated risk [11,12], albeit this finding should be interpreted cautiously, given the complex and less established (compared to LDL) role of HDL subfractions in cardiovascular and metabolic diseases. Short-term weight restoration by oral refeeding further increases the plasma cholesterol levels, but with an inverse pattern compared to AcAN, i.e. by increasing the concentrations of the larger, -less dense and less atherogenic LDL particles (e.g. LDL-1, LDL-2, LDL-3), and by increasing the concentrations of smaller, -more dense HDL (e.g. HDL-2, HDL-3) subfractions. Moreover, we show that AcAN is characterized by lower plasma concentrations of branched chain amino acids and glucose and higher concentrations of the gluconeogenic amino acids glutamine, alanine and methionine. Refeeding only partially restores the amino acid concentrations of patients with AcAN. These findings point towards long-term adaptations prioritizing proteins as main energy fuel in AcAN, which are partially maintained even after short-weight restoration due to refeeding.

4.1. Impact of AcAN and of refeeding on LDL-C and LDL - particles

High total cholesterol levels due to elevated LDL-C and HDL-C have been reported in several previous studies in patients with AN [6]. The causes of elevated cholesterol in AN remain largely unknown, with previous findings suggesting increased cholesterol absorption or increased cholesterol efflux due to starvation-induced lipolysis [6]. Hypercholesterinemia per se is not considered a relevant cardiovascular risk factor, and current guidelines do not suggest the reduction of cholesterol levels to protect from cardiovascular events. On the contrary, LDL-C levels are strong predictors of cardiovascular disease and mortality [28]. In our study, LDL-C levels were elevated in patients with AN up to a mean of 105 mg/dl and were further increased up to 119 mg/dl during refeeding. LDL-C concentrations between 100 and 130 mg/dl have been associated with a 30 % increased risk of cardiovascular disease mortality in a very low risk population which was followed for up to 28 years [29]. Thus, the higher LDL-C levels that we observe in AN both before and after refeeding might have clinical relevance if they are maintained in the long-term. The increase of LDL-C in AN demonstrates also similarities with the increase of LDL-C observed among lean adults with high triglyceride to HDL cholesterol ratio when they are on a carbohydrate-restricted diet (characterized as “lean mass hyperresponders”) [30]. Importantly, the increase observed in LDL-C in acutely underweight AN was due to higher concentrations of small dense LDL particles. These particles have been particularly associated with atherosclerosis, cardiovascular and metabolic diseases even under normal total LDL-C [9,10]. Denser LDL particles are also positively associated with carotid intima media thickness, an established marker of atherosclerotic disease, in adolescents [31]. Our findings are partly in line with a recent study in a smaller population that reported a shift in LDL distribution from large to small fractions in AN, assessed by relative quantification with gel electrophoresis [32]. In our study with NMR, that provides absolute concentrations of the different subfractions, we did not observe lower but similar or trends to higher levels in all LDL subfractions in AN compared to HC, with the difference being though more profound and significant for the smallest most dense and most atherogenic LDL-6 particles.

Current recommendations for the treatment of AN advocate high-calorie renutrition diets, which have been deemed safe in the short term [3,33]. Nevertheless, existing studies on renourishment have predominantly concentrated on aspects like electrolyte balance and the prevention of refeeding syndrome [3] leaving metabolic alterations, specifically long-term impacts on lipid profiles, underexplored. It is plausible to consider that while the high-calorie renourishment approach demonstrates safety concerning the development of refeeding syndrome, it may have potential adverse effects on metabolism in the long run [5]. In our study we observed that refeeding does not affect the concentrations of the small dense LDL fractions (e.g. LDL-6, LDL-5 and LDL-4), but it increases solely the concentrations of the larger and less dense LDL-particles (e.g. LDL-1, LDL-2 and LDL-3). Why there is a preferential formation of small dense LDL-particles in AN and of large, less dense LDL-particles in AN during or after refeeding remains unclear. It has been suggested that the synthesis of LDL-fractions depends on the activity of lipoprotein lipase (LPL) and hepatic lipase, which can progressively lipolyze IDL to large and then to small LDL [34,35]. Another mechanism implicates the cholesterol ester transfer protein (CETP) in LDL remodeling. According to this mechanism, CETP may modulate LDL size by affecting the exchange of cholesterol esters between VLDL, LDL and HDL [34]. The composition of the high-caloric renutrition diet might also play an important role both in LDL-C as well as in lipoprotein particles profile. In our study, the participants received nutritionally balanced meal plans, based on the severity of undernutrition and on their caloric intake before admission to inpatient treatment. In other studies, moderate re-introduction of carbohydrates among “lean mass hyperresponders” reduced LDL-cholesterol [30]. On the contrary, increased consumption of saturated fatty acids has been shown to increase LDL-C and particularly the concentration of large LDL particles [36], as observed in our study. Similarly, exercise is able to increase large LDL particles concentrations even without modulating LPL activity [37,38]. Independently of the mechanisms involved, the refeeding-induced upregulation of LDL-C concentrations derives exclusively from increases in the concentrations of the less pro-atherogenic subfractions, pointing towards a milder, if any, increase of the long-term cardiovascular risk by refeeding. Specifically, large LDL particles compared to small LDL particles have a lower affinity for the LDL receptor, thus remaining in the circulation for a shorter time [39]. Additionally, they are less capable of penetrating the arterial wall to promote endothelial injury [39,40]. Finally, in contrast to small LDL particles, large LDL particles have not been linked with inflammatory processes and oxidation or activation of fibrinolytic cascades for thrombus formation [39,40]. However, it is mandatory to evaluate lipoprotein (subfraction) concentrations also at later timepoints after refeeding, in order to understand whether the increase in total and LDL-cholesterol persists or rather displays a snapshot, exclusively limited to the early refeeding response. Moreover, it would be necessary to compare different refeeding protocols regarding their impact on total cholesterol and lipoprotein subfractions, preferably by methods allowing for absolute quantification such as NMR.

4.2. Impact of AcAN and of refeeding on total HDL-C and HDL-subfractions

There is a U-shaped relationship between HDL-C and mortality, with increased all-cause mortality risk by HDL-C below 50 mg/dl or above 68 mg/dl [41,42]. In our study, HDL-C levels in AN were mildly elevated (59 mg/dl) compared to HC group (55 mg/dl), with both groups being within low-risk range. Thus, it is questionable whether the higher HDL-C levels in AN provide any cardiovascular benefit. Some studies have reported a strong inverse association of large, -less dense HDL subfractions with risk of cardiovascular events and of type 2 diabetes [14,43], while others suggested that the small HDL-fractions are the ones that are stronger negatively associated with atherosclerosis or cardiovascular risk [44,45]. In our study, we observed higher concentrations of large HDL-subfractions in AN and an increase of the concentrations of small HDL-subfractions by refeeding. Whether these increases are able to mitigate, at least partially, the elevated risk due to higher LDL-C or due to higher concentrations of ceramides and short saturated triglycerides previously observed in AN and refeeding [5,46] seems likely, but it requires further investigation in mechanistic studies.

4.3. Impact of AcAN and of refeeding on amino acid profiles

The concentrations of all three BCAAs were significantly reduced in AN. This is most likely attributed to the gradual reduction in muscle tissue, diminished turnover of muscle proteins, and a subsequent decline in the appearance of BCAAs. Importantly, during the protein-sparing stage of starvation, there is a decrease in the activity of branched-chain keto acid dehydrogenase (BCKAD) in muscles, responsible for the deamination of all three BCAAs, often resulting in increased BCAA concentration during short-term starvation [16,17]. However, in the ultimate stage of starvation, characterized by a reduction in fatty acid oxidation due to the loss of adipose tissue, there is a notable increase in BCKAD activity in both muscles and the heart. This shift occurs as amino acids become the primary energy substrate [47,48]. Moreover, increased physical activity often present in anorexic patients may further aggravate the BCAA deficiency. Thus, we assume that the lower BCAA concentrations are probably of multifactorial origin. In contrast to BCAAs, we observed higher concentrations in glutamine, methionine and alanine. These three amino acids have an important role in protein synthesis, and their elevated concentrations may reflect increased protein breakdown and/or reduced protein synthesis occurring in AN and possibly in skeletal muscle. Importantly, BCAAs in muscle act as a source for synthesis of alanine and glutamine which are released into circulation to be used as gluconeogenic substrates [47,48]. Refeeding in our study restored partially but not completely the changes in amino acid concentrations. This finding indicates that the AN-induced differences in amino acid concentrations reflect long-term adaptations prioritizing protein utilization for energy production and not short-term alimentary deficits.

4.4. Limitations of the study

The current study has strengths and limitations. A major strength is its sample size, which is one of the largest used so far to address metabolite and lipoprotein-changes in AN both before and after refeeding and compared to healthy controls. A limitation is the lack of additional measurements in individuals with a history of AN who have fully recovered. This is important since several factors may influence the long-term measurement of LDL and HDL levels. Specifically, initial weight restoration may not fully normalize lipid metabolism, as prolonged undernutrition may disrupt in the long-term hepatic function and lipid metabolism [49]. Additionally, the normalization of hormonal state after weight restoration (e.g. recovery of hypothalamic-pituitary-gonadal, -thyroid and -somatotropic axis), which can have a major impact on lipid/lipoprotein metabolism, occurs rather slowly in most patients [2]. Behavioral and lifestyle factors combined with relational and social influences might also in the long-term affect lipid profiles through modifications in post-refeeding diet quality and in physical activity levels or by recurrence of restrictive behaviors. Another limitation of the study is the lack of information about the exact composition of the nutritional diet during refeeding, which was however tailored to individual patient needs. Finally, as all participants were female and most of them identified as White, our findings cannot be generalized to all individuals with AN.

4.5. Conclusions and clinical implications

Our study demonstrates alterations in LDL subfractions in AN before and after rapid refeeding that might be congruent with altered cardiometabolic risk. Concomitant increases in specific HDL subfractions upon refeeding may mitigate part of the cardiovascular risk, albeit they should be interpreted cautiously due to the complex U-shaped association of HDL-C levels with mortality. Furthermore, AN is characterized by distinct metabolite patterns pointing towards increased protein utilization as energy fuel with only partial restoration after short-term refeeding. It is known that a number of cardiovascular abnormalities are associated with AN [50]. Regarding the presence of atherosclerotic disease in AN, evidence is limited and inconclusive [50]. Development of cardiovascular complications due to atherosclerosis is a slow process demanding exposure to cardiovascular risk factors, such as hyperlipidemia, for a long time period. Nevertheless, it has been suggested that short-term exposure to severe undernutrition may have long-term consequences in cardiovascular risk. For example, women between 10 and 17 years old who experienced the Dutch famine between October 1944 to April 1945 demonstrated a 38 % increased risk for coronary heart disease in adulthood [51]. Experimental evidence explaining this elevated risk is currently limited and many factors may play an import role (from early impairments in cardiovascular structure and function to increased preference for high caloric food and high prevalence of dyslipidemia and metabolic syndrome later in life). Moreover, our study shows that after short-term weight-restoration, the LDL-C seems to reflect a somewhat less atherogenic pattern compared to the predominance of small LDL particles in the acutely underweight phase of AN. Thus, an important question that should be addressed in future studies is whether and when the lipoprotein and metabolite profile of patients with AN normalize after weight-restoration and whether patients with AN have an elevated risk for development of cardiovascular and metabolic diseases in the long-term, even after successful treatment of AN. If this is the case, it would be important to consider how these patients should be monitored for cardiovascular and metabolic diseases in the long-term, as well as how refeeding strategies, which are the cornerstone of AN treatment, can be further improved, possibly by tailoring the subcomponents of the diet.

Author contribution

NP: data analysis, data interpretation and drafting of the manuscript; AMF: data acquisition, data analysis, revising the manuscript; SJ: data acquisition and data analysis; TK: data analysis, revising the manuscript; IH and FIT: data acquisition, revising the manuscript; SRB, TC, PM: data interpretation, revising the manuscript, SE: design of the study, data interpretation, revising the manuscript. All authors approved the final version of the

manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Data sharing statement

The data that support the findings of this study are available from SE, upon reasonable request.

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Conflict of interest

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Appendix A. Supplementary data

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