




# Metabolomics for Distinguishing Cardiovascular Risk in Rheumatoid Arthritis Across Different Disease-Modifying Antirheumatic Drug Therapies

Inga Claus<sup>a</sup>, Meike Hoffmeister<sup>b</sup>, Selina Strathmeyer<sup>c</sup> , Steffen Heelemann<sup>c</sup> ,  
Constantin Remus<sup>a</sup>, Werner Dammermann<sup>d</sup>, Oliver Ritter<sup>a</sup>,  
Daniel Patschan<sup>a, c</sup> , Susann Patschan<sup>a</sup>

## Abstract

**Background:** Rheumatoid arthritis (RA) significantly increases the overall risk of cardiovascular disease (CVD). In addition to conventional risk factors, the inflammatory activity of the disease itself and medications that promote atherosclerosis contribute to an even greater risk. In this study, we performed metabolomic analysis in RA patients, both on and off disease-modifying anti-rheumatic drug (DMARD) therapy, with the aim of identifying new candidates for more sophisticated cardiovascular risk (CVR) assessment.

**Methods:** This is an observational, cross-sectional investigation that included patients with established RA. DMARD therapy, if prescribed, consisted of methotrexate (MTX) alone or in combination with other conventional disease-modifying anti-rheumatic drugs (cDMARDs) or biologic disease-modifying anti-rheumatic drugs (bDMARDs), or other cDMARDs or bDMARDs without MTX, respectively. Metabolomic profiling was conducted using a Bruker AVANCE NEO 600 MHz nuclear magnetic resonance (NMR) spectrometer. The spectra obtained were Fourier transformed using TopSpin software (version 4.0, Bruker Biospin, Germany). All spectra were automatically phased and subjected to baseline correction. Subsequently, the spectra were analyzed using the proprietary Profiler software (version

1.4\_Blood, lifespin GmbH, Germany), and a quantitative metabolite list was generated.

**Results:** In total, 200 patients were included in the study, 54 subjects were not receiving any DMARDs (n = 47 untreated at the time of inclusion, n = 7 with established disease but not receiving DMARD therapy), and 146 were receiving DMARD treatment. No metabolic differences were found in relation to drug therapy or RA activity. The following CVR factors were associated with significant metabolic abnormalities: distress, arterial hypertension, diabetes mellitus and an average higher Framingham score. Distressed individuals showed abnormalities in histidine metabolism.

**Conclusions:** Our findings have aided in the identification of potential surrogate markers for assessing the burden of CVD in individuals with RA. Histidine may be of particular diagnostic importance in CVR assessment in RA.

**Keywords:** Metabolomics; RA; Cardiovascular risk; DMARDs; Disease activity

## Introduction

Rheumatoid arthritis (RA) affects approximately 1% of adults in Central Europe [1], making it the most commonly isolated disease within the inflammatory rheumatic diseases. The etiology and pathogenesis of RA remain unknown, but at the core of the chronic inflammatory process is a progressive inflammation of the synovial membrane, which can lead to disability if not adequately treated. Extra-articular organ manifestations are possible, especially in seropositive individuals [2]. RA patients are particularly at risk for cardiovascular diseases (CVD). Meanwhile, cardiovascular risk (CVR) assessment and adequate anti-atherogenic therapy for those affected have become integral components of RA management. The European League Against Rheumatism (EULAR) published recommendations for the detection and treatment of cardiovascular complications in RA as early as 2015 [3].

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<sup>a</sup>Department of Internal Medicine I - Cardiology, Nephrology and Internal Intensive Medicine, Brandenburg University Hospital, Brandenburg Medical School (Theodor Fontane), 14770 Brandenburg an der Havel, Germany

<sup>b</sup>Institute of Biochemistry, Brandenburg Medical School (Theodor Fontane), 14770 Brandenburg an der Havel, Germany

<sup>c</sup>Lifespin GmbH, 93053 Regensburg, Germany

<sup>d</sup>Department of Internal Medicine II - Gastroenterology and Endocrinology, Brandenburg University Hospital, Brandenburg Medical School (Theodor Fontane), 14770 Brandenburg an der Havel, Germany

<sup>e</sup>Corresponding Author: Daniel Patschan, Department of Internal Medicine I - Cardiology, Nephrology and Internal Intensive Medicine, University Hospital of Brandenburg, Brandenburg Medical School (Theodor Fontane), 14770 Brandenburg an der Havel, Germany. Email: danielp@me.com

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In the general population, the risk of conditions such as heart attack or stroke is commonly assessed according to guidelines set by the European Society of Cardiology (ESC) [4]. This assessment focuses on numerous factors, including general characteristics such as age, gender, and family history. It also takes into account pro-atherogenic morbidities (arterial hypertension including severity, diabetes mellitus, hyperlipidemia). Additionally, hypertensive or atherogenic organ changes need to be identified and quantified. The limitations of this system become apparent in individuals with inflammatory rheumatic diseases. Not captured are the proatherogenic effects of chronic inflammatory processes themselves, as well as the potential side effects of medications such as glucocorticoids and non-steroidal anti-inflammatory drugs (NSAIDs) [5]. Similarly, not accounted for are the potential cardioprotective effects of disease-modifying anti-rheumatic drugs (DMARDs) like methotrexate (MTX) [6]. Due to the identified diagnostic limitations of established strategies for CVR risk stratification, biomarkers could potentially help to fill relevant diagnostic gaps.

Metabolomics involves the detection and quantification of low-molecular-weight molecules in biological specimens such as blood or tissue samples [7]. The concept of “omics” shall enable researchers to evaluate the complexity of biological processes in a more sophisticated manner. In this study, we conducted metabolomics analysis on RA patients undergoing different DMARD therapy regimens and exhibiting varying levels of CVD risk factors. The aim was to identify new candidate molecules that could potentially aid in CVD risk assessment.

## Materials and Methods

### Design

The study is an observational, cross-sectional, monocentric study conducted at the Health Center of the Brandenburg University Hospital (Brandenburg Medical School Theodor Fontane) from November 2022 to January 2023. The study was formally approved by the Ethics Committee of the Brandenburg Medical School (location: Neuruppin, Brandenburg, Germany, approval number: E-01-20200316, approval date: October 29, 2020; approval of amendment: November 11, 2022). All participants provided written informed consent by signing an informed consent form. The study was conducted in accordance with the tenets of the Helsinki Declaration of 1975 (revised 2013). All medical, patient-related data were de-identified, making it impossible to identify one or more individuals. The reporting of this study conforms to the STROBE guidelines [8].

### Patients

All patients included in the study were screened and finally recruited from the Health Center of the Brandenburg University Hospital, one of three university hospitals affiliated to the Brandenburg Medical School in Germany. To be eligible for inclusion, patients had to be 18 years of age or older and meet the ACR/EULAR 2010 rheumatoid arthritis classification

criteria [9]. In addition, their DMARD therapy had to include no DMARD (DMARD-native) or at least one conventional or biologic DMARD. The daily dose of prednisolone was variable and adjusted according to disease activity. Patient exclusion criteria included uncontrolled psychiatric disorders, additional autoimmune-mediated diseases, uncontrolled infectious diseases such as human immunodeficiency virus (HIV), hepatitis B or C, and tuberculosis, uncontrolled drug or alcohol dependence, and pregnancy. Patient characteristics collected included height, weight, comorbidities, medications, smoking status, and family history of CVD. Disease activity was assessed using the DAS28-CRP (28-joint Disease Activity Score-C-reactive protein) score, with remission, low, moderate and high disease activity defined by scores of < 2.6, 2.6 - 3.2, 3.2 - 5.1 and > 5.1, respectively. Additional disease activity assessment tools included the visual analogue scale (VAS) ranging from 0 (no pain) to 10 (maximum pain imaginable), swollen and painful joint assessment, and the Hannover Functional Questionnaire (HFQ) [10]. Therapy-related data collected included current DMARD therapy (agent), NSAID use (dose, and frequency of use), and daily prednisolone dose in milligram. CVR assessment included the following morbidities and laboratory parameters: arterial hypertension, diabetes mellitus including hemoglobin A1c (HbA1c, %), past and current smoking (at least 10 cigarettes per day), alcohol consumption (no alcohol, 1 - 3 times per month, 1 - 3 times per week, and daily), physical activity (‘no physical activity’ and ‘physical activity’, and: none, 2 - 3 times a week, daily), total cholesterol (mmol/L), low-density lipoprotein (LDL) (mmol/L), high-density lipoprotein (HDL) (mmol/L) and lipoprotein(a) (Lp(a)) (nmol/L). Other laboratory parameters measured included rheumatoid factor (RF) and anti-citrullinated protein antibody (ACPA) titer, CRP levels (mg/L), complete blood count, and serum creatinine (μmol/L).

### Nuclear magnetic resonance (NMR) spectroscopy

After thawing for approximately 3 h at room temperature, 350 μL of serum was mixed with 350 μL of aqueous buffer. The buffer consisted of H<sub>2</sub>O *pro analysi*, 0.1 g/L NaN<sub>3</sub>, 0.067 mol/L Na<sub>2</sub>HPO<sub>4</sub>, 0.033 mol/L NaH<sub>2</sub>PO<sub>4</sub> (pH: 7.15 ± 0.05), 5% D<sub>2</sub>O as field-locking substance and an internal standard (6 mM pyrazine) for quantification. From this mixture, 600 μL were transferred into a 5 mm Bruker NMR tube and sealed with a barcoded lid. The subsequent NMR measurement was performed with the following parameters: spectrometer Bruker AVANCE NEO 600 MHz, measurement method 1D 1H noesygppr1d\_d20, NS = 16, T = 310 K, with a measurement time of 6.5 min per sample. All measured spectra passed the quality control routine and were released for data analysis.

### Data analysis

The spectra were Fourier transformed using TopSpin software (version 4.0, Bruker Biospin, Germany). All spectra were automatically phased and baseline corrected. Subsequently, the spectra were analyzed using the proprietary Lifespin Profiler

software (version 1.4\_Blood) to generate a quantitative metabolite list. All metabolites were quantified in mmol/L.

### Statistical analysis

Statistical significance in this analysis was determined by the Wilcoxon-Mann-Whitney test. The resulting P values were corrected for multiple testing (false discovery rate (FDR) correction) and converted to \* notation as follows:  $P \leq 0.001$  (\*\*\*),  $P \leq 0.01$  (\*\*),  $P \leq 0.05$  (\*), and  $P > 0.5$  (not significant (n.s.)). For effect sizes, fold change and Cohen's *d* were calculated. For two groups, fold change is calculated as the quotient of the means of both groups, where a fold change of 1 indicates that the means are equal, and a fold change of 0.5 indicates that the mean of group 2 is twice the mean of group 1. Cohen's *d* was calculated by dividing the difference in the means of the respective groups by their pooled standard deviation and taking the absolute value, with effects being interpreted as small for a Cohen's *d*  $\leq 0.5$ , moderate for a Cohen's *d* of 0.5 - 0.8, and large for a Cohen's *d*  $\geq 0.8$ .

For the multivariate analysis, we performed principal component analysis (PCA), a statistical technique used for dimensionality reduction, data compression, and exploratory data analysis. It transforms a large set of variables into a smaller set of uncorrelated variables, called principal components, which capture the most important information in the data. Principal components are linear combinations of the original variables that typically capture most of the variance in the data set. A scree plot visualizes the variance explained by each component to help determine the number to retain, while loadings indicate the contribution of each original variable to the components, and scores represent the coordinates of the data in reduced-dimensional space. We also used partial least squares discriminant analysis (PLS-DA), a supervised classification method that uses class information to identify variables maximizing the separation between classes. This method determines a set of components where each successive component captures the highest possible covariance between the data set X (measured data points) and their labels Y (group classifications). The quality of the PLS-DA model is evaluated using the cross-validation parameters R<sup>2</sup> (variance explained) and Q<sup>2</sup> (predictive ability). R<sup>2</sup><sub>X</sub> and R<sup>2</sup><sub>Y</sub> represent the variance explained by the X and Y matrices, respectively, while Q<sup>2</sup><sub>Y</sub> indicates the predictive accuracy. An effective model is indicated by values of the cumulative parameters R<sup>2</sup><sub>X</sub>, R<sup>2</sup><sub>Y</sub> and Q<sup>2</sup><sub>Y</sub> close to 1. For each model building (PCA, PLS-DA), the nearZeroVar function in R was used to remove predictors with zero variance, i.e., metabolites with very few unique values relative to the number of samples.

## Results

### Patients

A total of 200 patients were included in the study, 54 subjects were not receiving any DMARDs (*n* = 47 patients with newly diagnosed, untreated RA at the time of inclusion, *n* = 7 with

established disease but not receiving DMARD therapy), and 146 were receiving DMARD treatment. One hundred thirty-four (67%) subjects were female and 66 (33%) were male. The mean age of all subjects was  $62.5 \pm 12.4$  years. RF was positive in 64.1% of cases, and anti-cyclic citrullinated peptide (anti-cyclic citrullinated peptide (CCP)) in 55.6%. The mean VAS at baseline was  $4 \pm 2.5$  and the mean DAS28 was  $3.7 \pm 1.4$ . Table 1 summarizes all baseline characteristics. In addition to epidemiological data, these include information on drug therapy, CVR factors and laboratory findings.

### Variables of RA activity and treatment

First, variables of disease activity and RA therapy were considered. Seropositive RA patients did not differ from seronegative individuals. In addition, three ranges of RF elevation were defined: normal ( $< 14$  IU/mL), slightly elevated ( $14 - 25$  IU/mL), and significantly elevated ( $> 25$  IU/mL). Metabolic profiling also showed no differences between these ranges. According to DAS28, three activity categories (low, medium and high disease activity) were defined:  $0 - 3.2$ ,  $3.2 - 5.1$ , and  $> 5.1$ . There were no significant differences in metabolite concentrations between the three categories. Visual analog scale scores were also assigned to one of three categories:  $0 - 3$ ,  $4 - 7$ , and  $8 - 10$ . None of the analyzed metabolites differed between patients in the respective categories. Regarding DMARD therapy, five groups were distinguished: early RA without previous DMARD use (*n* = 47), MTX monotherapy (*n* = 67), MTX in combination with other DMARDs (*n* = 49), other DMARDs without MTX (*n* = 28), and established RA without DMARD use (*n* = 7). There were no differences in metabolic profile between the groups. Since glucocorticoids are used in the majority of RA individuals (in variable doses), patients were assigned to one of four dose groups: no glucocorticoid (*n* = 47), daily dose  $< 5$  mg prednisolone equivalent (*n* = 53), daily dose  $5 - 10$  mg prednisolone equivalent (*n* = 94) and daily dose  $> 10$  mg prednisolone equivalent (*n* = 3) (data missing: *n* = 1). Only one metabolite, HDL, differed between the groups, with the concentration being lowest in the " $< 5$  mg prednisolone equivalent" group. With regard to the use of NSAIDs in general and coxibs in particular, two analyses were always performed. First, NSAID/coxib native patients were compared with patients who regularly used at least one substance from either group. Second, the frequency of use was defined for both substance groups: none, once a day, 2 - 3 times a day, 2 - 3 times a week, and 2 - 3 times a month. Again, none of the analyses showed significant differences in the metabolome. A similar analysis was carried out for the variable "analgesics". This term refers to all analgesics except NSAIDs/coxibs. Again, analgesic users were distinguished from analgesic non-users, and the same procedure was used for frequency of use as for NSAIDs/coxibs. Both subgroup analyses also showed no differences in the metabolic profile.

### Variables of CVR

Cardiovascular analysis was based on both medical history and clinical findings. The variables considered separately were

**Table 1.** Baseline Characteristics of all Included Patients

Variables	Result
Gender females/males, n (%)	134 (67%)/66 (33%)
Age, years (mean $\pm$ SD)	62.5 $\pm$ 12.4
BMI, kg/m <sup>2</sup>	28.7 $\pm$ 5.6
VAS (mean $\pm$ SD)	4 $\pm$ 2.5
HFQ, % (mean $\pm$ SD)	75.2 $\pm$ 22.2
DAS28 (mean $\pm$ SD)	3.7 $\pm$ 1.4
Swollen small joints (mean n $\pm$ SD)	3.1 $\pm$ 7.8
Swollen large joints (mean n $\pm$ SD)	0.7 $\pm$ 1.3
Painful small joints (mean n $\pm$ SD)	5.8 $\pm$ 9.8
Painful large joints (mean n $\pm$ SD)	2.1 $\pm$ 2.4
RF, %	64.1
Anti-CCP, %	55.6
Medication	
NSAID therapy, %	23.4
Daily prednisolone, mg (mean $\pm$ SD)	3.4 $\pm$ 2.9
Sulfasalazine, %	12.5
Leflunomide, %	7.5
Anti-TNF-alpha, %	11
Anti-IL6, %	3
Anti-CD80/86, %	0
Anti-CD20, %	1.5
CV risk factors/morbidities	
Smoking, %	32.3
Physical exercise, %	42.3
Distress, %	38.9
Arterial hypertension, %	65
CAD, %	7
HF, %	4.1
Family history of CAD, %	23
Diabetes mellitus, %	14
Obesity, %	35.5
Laboratory findings	
CRP, mg/L (mean $\pm$ SD)	4.2 $\pm$ 7.3
Hb, g/L (mean $\pm$ SD)	134.8 $\pm$ 22.2
Platelets, $\times$ 100,000/ $\mu$ L (mean $\pm$ SD)	273.6 $\pm$ 73.1
Serum creatinine, $\mu$ mol/L (mean $\pm$ SD)	71.2 $\pm$ 15.9
HbA1c, % (mean $\pm$ SD)	5.6 $\pm$ 0.7
Triglycerides, mmol/L (mean $\pm$ SD)	1.5 $\pm$ 0.9
Total cholesterol, mmol/L (mean $\pm$ SD)	5.4 $\pm$ 1
LDL, mmol/L (mean $\pm$ SD)	3.1 $\pm$ 0.9
HDL, mmol/L (mean $\pm$ SD)	1.7 $\pm$ 0.5

SD: standard deviation; BMI: body mass index; VAS: visual analogue scale; HFQ: Hannover Functional Questionnaire; RF: rheumatoid factor; DAS28: 28-joint Disease Activity Score; NSAID: non-steroidal anti-inflammatory drug; CV: cardiovascular; CAD: coronary artery disease; HF: heart failure; CCP: cyclic citrullinated peptide; TNF: tumor necrosis factor; IL: interleukin; CRP: C-reactive protein; Hb: hemoglobin; HbA1c: hemoglobin A1c; LDL: low-density lipoproteins; HDL: high-density lipoproteins.



**Table 2.** Significantly Different Metabolites Between Individuals With Elevated Versus Non-Elevated Stress Levels (Distress)

Metabolite	Notation (correlation)	Fold change	Cohen's <i>d</i>
Glucose	**	0.81	-0.49
Histidine	**	1.11	0.57

\*\*P ≤ 0.01. Glucose level was reduced, whereas histidine was increased.

obesity, alcohol consumption, arterial hypertension, diabetes mellitus, family history, physical activity, stress and smoking. The Framingham score was also taken into account. Further variable analyses (NSAID, glucocorticoids) have already been summarized in the previous section.

Obesity was classified according to severity [11]: first degree (n = 46), second degree (n = 15), third degree (n = 9). The metabolic profile showed no significant differences between the three degrees of obesity. The next variable, alcohol consumption, was surveyed in principle on the one hand and quantified on the other. The following categories were defined for quantification: no alcohol (n = 100), 1 - 3 times per month (n = 13), 1 - 3 times per week (n = 50) and daily (n = 6). Alcohol consumption had no influence on the metabolome of the RA patients, neither in principle nor in degree. Of the 200 patients included, the question of whether or not arterial hypertension was present could be answered with certainty in 197 patients. The prevalence of arterial hypertension was found to be 65%. Hypertensive RA patients showed significantly higher serum levels of the following three substances: glucose 1.29-fold increase, mannose 1.33-fold increase, and urea 1.22-fold increase. Diabetes mellitus was diagnosed in 14% of patients. The metabolic profile differed significantly from non-diabetics. A total of six metabolites were significantly increased in diabetics (glucose 1.76-fold increase, mannose 1.63-fold increase, glycoprotein acetyls B and A 1.16- and 1.15-fold increase, creatinine 1.37-fold increase, and pyruvic acid 1.42-fold increase), one metabolite was reduced (LDL 0.86-fold increase). There were no differences in any of the metabolites between those with a positive family history of CVD and those without. The analysis of the variable “physical activity” was first performed as a comparison between “physical activity” and “no physical activity”. In a second step, the intensity of the activity was graded (none, daily, 2 - 3 times a week). No significant differences were found in either case. Surprisingly, the variable “stress” was definitely associated with different findings. A total of 73 individuals reported a permanently elevated stress level. Affected individuals showed significantly lower glucose levels (0.81-fold decrease) and higher serum histidine (1.11-fold increase) (Table 2). The levels of other amino acids were consistent across the two stress levels (isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine). The variable “smoking” on the other hand (n = 62) was in no way associated with abnormal findings, neither in absolute terms (yes versus no), nor graduated (no, smoking cessation < 1 year, 1 - 5 years before, 5 - 10 years before, > 10 years before). The analysis of the Framingham data was categorized into one of three categories: < 10, 10 - 19, > 20. There were numerous significant differ-

**Table 3.** Metabolic Differences Between RA Patients With a Framingham Score of Under 10 Compared to Those With a Score of 20+

Metabolite	Notation (correlation)	Fold change	Cohen's <i>d</i>
Triglycerides	**	0.69	-0.96
Sphingomyelin	**	0.68	-0.98
Creatinine	**	0.66	-1.11
Untargeted signal 33	**	0.85	-0.85
Untargeted signal 35	*	0.68	-0.81
HDL	*	1.18	0.65
Glucose	*	0.85	-0.46
Untargeted signal 21	*	0.46	-0.78
Untargeted signal 34	*	0.86	-0.70
Mannose	*	0.75	-0.67

\*P ≤ 0.05. \*\*P ≤ 0.01. The majority of metabolites were significantly reduced (exception: HDL). HDL: high-density lipoproteins; RA: rheumatoid arthritis.

ences between the < 10 and > 20 categories, with the majority of metabolites being reduced in the “< 10” category: triglycerides 0.69-fold decrease, sphingomyelins 0.68-fold decrease, creatinine 0.66-fold decrease, untargeted signals 21, 33, 34 and 35: 0.46-, 0.85-, 0.86- and 0.68-fold decrease, glucose 0.85-fold decrease and mannose 0.75-fold decrease (Table 3). Untargeted signals refer to distinct signals observed in the NMR spectra, originating from an unidentified substance. They are of potential value for statistical analysis, providing valuable insights despite their uncertain identity. Only HDL was increased in patients in the “< 10” category (1.18-fold increase). Surprisingly, the separate evaluation of the Framingham data did not reveal any differences when women and men were considered separately.

Discussion

The aim of the present study was to perform metabolomic profiling of RA patients at increased CVR. Ultimately, the study should help optimize CVR screening in RA, as current risk stratification systems [4] hardly take into account the influence of the activity of the underlying disease and of known pro-atherogenic drugs (NSAIDs, glucocorticoids) on the risk of heart attack and stroke. Initially, the study did not show any relevant abnormalities in the metabolic profile, taking into account disease activity and treatment variables. Nevertheless, the literature available to date suggests that metabolomics is indeed suitable for assessing disease activity in RA. A recent review article from 2022 [12] summarized metabolic studies in RA. It stated, for example, that fatty acid metabolites (free fatty acids, polyunsaturated fatty acids) in particular were regularly measured at elevated concentrations in different compartments (e.g., serum, synovial fluid). The combined concentration (added individual concentrations) of aspartic acid, threonine, tryptophan, histidine and phenylalanine was named

as a very specific indicator of increased disease activity [13]. In 2022, Koh et al [14] published an analysis of synovial fluid and serum from RA patients with variable disease activity. The focus was on changes in lipid metabolism (lipidome). Synovial analysis revealed significant abnormalities in the lipid profile (e.g., lysophosphatidylcholine, phosphatidylcholine, ether-linked phosphatidylethanolamine), correlating with the severity of sonographic synovitis. The serum findings were roughly comparable, although less pronounced. The crucial difference from our study, aside from the materials analyzed, was the horizontal design, in which patients with RA in development were followed up. A study published in 2021 [15] involving 64 RA patients and 12 controls also followed a horizontal design. All patients were analyzed twice (plasma), the periods between the examinations were 6 to 12 months. Numerous metabolites (fatty acid metabolites, amino acid intermediates, carbohydrates) showed significant associations with DAS28-CRP. Our survey does not allow for comparable statements due to the single time point of the decrease. Irrespective of this, we did not identify any differences with different activity levels according to DAS28 at this time point. However, our study did not carry out correlation analyses but comparisons of metabolites between three activity severity levels.

However, there were some notable findings when variables or risk factors for increased cardiovascular morbidity were taken into account. These included arterial hypertension, diabetes mellitus, stress (or distress) and the Framingham score. Both arterial hypertension and diabetes mellitus were associated with significantly higher glucose and mannose concentrations. In addition, diabetics showed higher levels of pyruvic acid and glycoprotein acetyls. The latter were already described in 2022 as inflammatory biomarkers and “early” indicators of increased CVR in comparatively younger people [16]. Surprisingly, increased stress or distress was associated with reduced glucose levels, in contrast to hypertension and diabetes. Histidine, on the other hand, was found to be elevated. Elevated concentrations of histidine have already been reported in RA in several studies [17]. In the context of an increased CVR *per se*, i.e., independent of an inflammatory rheumatic disease, interesting findings regarding histidine have been published. Du Toit et al [18], for example, showed a negative correlation between urinary histidine and pulse wave velocity, an established marker of increased CVD burden. Streese et al [19] also identified an inverse association between histidine and CVR, but they examined blood samples. Finally, a study published in 2019 [20] had shown inverse correlations between central systolic blood pressure/central pulse pressure and various metabolites, including histidine. Reduced histidine availability with increased CVR thus appears to be a characteristic, if perhaps not specific, finding. Histidine could thus prove to be a potential indicator of increased CVR in RA patients with an accumulation of CVR factors (here: distress). It is important to acknowledge the inherent limitations of the variable “distress”, which is inherently susceptible to measurement error. The comparability of outcomes across subjects is hindered by the potential for individuals to overestimate their stress levels. This aspect of the study can be regarded as a constraint. In general, a prospective design over a longer period of time would certainly be needed to determine more precisely

the significance of the amino acid in predicting CVR in RA, for example. This would answer the question of whether RA patients with manifest cardiovascular events have different histidine concentrations than those who do not develop such events. In any case, histidine appears to be a promising candidate for CVR assessment in RA.

In principle, consistent with the results of the hypertension and diabetes analyses, the lowest Framingham category (< 10) was associated with lower glucose and mannose concentrations, but at the same time with significantly higher HDL than in patients in the > 20 category. Several fatty acid metabolites that increased with increasing Framingham scores had already been identified in 2021 [21]. Our data could support these findings: patients in the lowest Framingham category showed lower levels of various lipid metabolites including sphingomyelins and triglycerides. The increased availability of distinct lipids in individuals with higher CVR according to the Framingham score is in principle compatible with the increasing development of a pro-oxidative environment in patients with higher cardiovascular morbidity. However, these findings are by no means typical of RA.

## Conclusions

Our study potentially suggests a diagnostic role for histidine in CVD burden in RA. While reduced histidine concentrations in various compartments have been regularly identified in non-rheumatics with increased CVR, the situation is the opposite in RA. The extent to which histidine metabolism in this disease is modulated by the underlying inflammatory disease itself or, independently of this, can improve cardiovascular event prediction, can only be determined by follow-up studies.

## Limitations

The main limitation is the lack of follow-up data. These could provide information on cardiovascular events (heart attack, stroke, etc.) and thus provide a more precise statement on the prognostic value of histidine and other metabolites, for example. The lack of a healthy control group could be seen as a limitation, but the aim was to detect metabolic abnormalities in RA patients with increased CVR compared to those without increased risk. Finally, the comparatively small number of patients is a potential limitation.

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None to declare.

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## Conflict of Interest

Steffen Heelemann and Selina Strathmeyer are employees at lifespın.

## Informed Consent

All participants provided written informed consent.

## Author Contributions

Inga Claus collected blood samples and clinical data from all included patients. Meike Hoffmeister substantially helped with sample collection and storage. Selina Strathmeyer and Steffen Heelemann performed all metabolomic analyses. Constantin Remus assisted in patient recruitment and data collection. Werner Dammermann helped in table preparation. Oliver Ritter aided in data analysis. Daniel Patschan analyzed data, and assisted in writing. Susann Patschan designed the study, analyzed data, and wrote the article. All authors approved the final version of the article.

## Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

## Abbreviations

ACPA: anti-citrullinated protein antibody; ACR: American College of Rheumatology; CRP: C-reactive protein; CVD: cardiovascular disease; CVR: cardiovascular risk; DMARDs: disease-modifying anti-rheumatic drugs; bDMARDs: biologic disease-modifying anti-rheumatic drugs; cDMARDs: conventional disease-modifying anti-rheumatic drugs; ESC: European Society of Cardiology; EULAR: European League Against Rheumatism; HDL: high-density lipoprotein; HFQ: Hannover Functional Questionnaire; LDL: low-density lipoprotein; Lp(a): lipoprotein (a); NMR: nuclear magnetic resonance; MTX: methotrexate; NSAIDs: non-steroidal anti-inflammatory drugs; PLS-DA: partial least squares discriminant analysis; RA: rheumatoid arthritis; RF: rheumatoid factor; VAS: visual analogue scale

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