
UNIVERSITY OF BIRMINGHAM

# THE CLINICAL & METABOLIC CORRELATES OF FATIGUE IN INFLAMMATORY ARTHRITIS & CLINICALLY SUSPECT ARTHRALGIA

By

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# Abstract


Background:

The earliest stages of inflammatory arthritis are associated with systemic inflammation and a complex set of symptoms including fatigue. Fatigue is common and burdensome. The mechanisms underlying fatigue are not well understood and, as a result, it is not always easy to manage.

Systemic inflammation and metabolic perturbations may play a role in the development of fatigue. A correlation between systemic inflammation and metabolic perturbation has previously been observed in early inflammatory arthritis. While low-density lipoprotein lipids were some of the chief metabolites which contributed to the metabolic signature associated with systemic inflammation, technical limitations in those studies limited their ability to define more broadly other metabolic pathways involved.

Given the likely importance of changes in metabolism to the development of fatigue, I set out to further analyse metabolic disturbance in an early arthritis cohort, assessing both sera, filtered to remove interfering high molecular weight species, and urine to provide a broader and clearer picture of the metabolic status of the patients. I have assessed the relationships between metabolic profiles and [1] systemic inflammation and [2] fatigue to shed further light on the drivers of fatigue in early inflammatory arthritis.

Methods:

Patients were recruited via the Birmingham Early Arthritis Cohort (BEACON), a well-established prospective cohort of patients with newly presenting, DMARD naive inflammatory arthritis or clinically suspect arthralgia (CSA). Sociodemographic and clinical data were collected, and fatigue was assessed using the FACIT-F (Functional Assessment of Chronic Illness Therapy - Fatigue) and the VAS-F (Visual Analogue Scale Fatigue). Serum and urine samples were collected at initial presentation. Clinical data were also collected at follow up after at least 12 months.

$^{1}$H NMR spectra of 3000MWCO filtered serum and urine were acquired. The spectra were analysed using principal components analysis (PCA), partial least square regression (PLS-R) and orthogonal projections to latent structure discriminant analysis (OPLS-DA) of the lowest tertile versus the highest tertile in each patient group.

Pre-processing of complex data can have significant effects on subsequent data analyses and so a systematic approach to pre-processing was taken to assess the effects of varying pre-processing parameters. Analyses then focused on relating metabolite profiles of both serum and urine to [1] CRP, as a measure of systemic inflammation, and [2] fatigue.

Results:

Of the 739 patients (63.9% female, age 50.9 +/- 15.3 years (mean +/- SD)) with baseline clinical data, 582 (78.8%) had inflammatory arthritis and 157 (21.2%) had CSA. Fatigue was common. Multivariate analysis revealed that low mood and disability were independently associated with fatigue in both

patient groups; additionally, in inflammatory arthritis patients, tender joint count was independently associated with fatigue.

Serum and urine samples were available at baseline from 280 and 178 patients respectively. PQN normalisation of serum metabolomics data and greater generalised logarithmic (g-log) transformation of both urinary and serum metabolomics data resulted in better performance amongst OPLS-DA models ($p<0.001$). Using PLS-R analysis, a relationship between CRP and the serum ($r^2= 0.481$, $p<0.001$) and urinary ($r^2= 0.134$, $p=0.021$) metabolome was detectable. The most highly weighted metabolites in these models included glucose, amino acids, lactate, and citrate. These findings suggest increased glycolysis, permutations in the citrate cycle, oxidative stress, protein catabolism and upregulation of the urea cycle are key characteristics of newly presenting inflammatory arthritis and CSA patients with elevated CRP.

An inflammatory signal was isolated and removed from both the serum and urinary metabolome. Subsequent PLS-R analysis showed no relationship remained between CRP and the serum ($r^2=0.0356$, $p=0.059$) and urinary ($r^2=0.0187$, $p=0.241$) metabolome.

PLS-R analysis showed a relationship between FACIT-F and the entire serum metabolome ($r^2= 0.181$, $p<0.001$) and the serum metabolome devoid of the inflammatory signal ($r^2= 0.123$, $p<0.001$). Likewise, PLS-R analysis also showed a relationship between FACIT-F and the entire urinary metabolome ($r^2= 0.266$, $p<0.001$) and the urinary metabolome devoid of the inflammatory signal ($r^2= 0.197$, $p<0.001$). These models suggested that down regulation of the urea cycle, oxidative stress, collagen degradation, reduced glycolysis and skeletal muscle degradation are associated with fatigue.

Conclusions:

The study confirms the extent and magnitude of fatigue in early inflammatory arthritis. Fatigue was comparable in magnitude in patients with CSA compared with those with early arthritis.

This study demonstrates the effect of varying pre-processing parameters on multivariate metabolomics analysis and suggests an alternative method approach for pre-processing $^1$H NMR metabolomics data.

This study confirmed that there is a relationship between the serum metabolite profile and inflammation, and furthermore showed that this relationship extends to those metabolites seen in urine.

This study provides evidence of a relationship between fatigue and metabolism, which persists when controlling for the effect of inflammation. Identification of these metabolic permutations provides insights into the pathoaeitology of fatigue and provides the basis for future studies which may identify therapeutic targets.

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# Abbreviations

| | |
|---|---|
| ACPA | Anti-citrullinated protein antibodies |
| ACR | American College of Rheumatology |
| AMP | Adenosine monophosphate |
| AS | Ankylosing spondylitis |
| BASDAI | Bath ankylosing spondylitis disease activity index |
| BCAA | Branched chain amino acids |
| bDMARD | Biological disease modifying anti-rheumatic drugs |
| BEACON | Birmingham Early Arthritis Cohort |
| BMI | Body mass index |
| BRAF-MDQ | Bristol rheumatoid arthritis fatigue multidimensional questionnaire |
| BRAF-NRS | Bristol rheumatoid arthritis fatigue numerical rating scales |
| CFQ | Chalder fatigue questionnaire |
| CFS | Chronic fatigue syndrome |
| CIS8R | Checklist individual strength fatigue subscale |
| CoA | Coenzyme A |
| COVID | Coronavirus |
| CRP | C reactive protein |
| CSA | Clinically suspect arthralgia |
| csDMARD | Conventional synthetic disease modifying anti-rheumatic drugs |
| DAS28 | Disease activity score for 28 joints |
| DMARD | Disease modifying anti-rheumatic drugs |
| ESR | Erythrocyte sedementation rate |
| EULAR | European League Against Rheumatism |
| FACIT-F | Functional assessment of chronic illness therapy - fatigue |
| FSS | Fatigue severity score |
| FVAS | Visual Analogue Scale Fatigue |
| g-log | Generalised logarithmic |
| GSH | Glutathione |
| HAQ | Health assessment questionnaire |
| HIF | Hypoxia inducible factor |
| HLA | Human leukocyte antigen |
| IBD | Inflammatory bowel disease |

| | |
|---|---|
| IL | Interleukin |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| LDH | lactate dehydrogenase |
| LDHA | Lactate dehydrogenase-A isomer |
| LV | Latent variable |
| MAF | Multi-dimensional assessment of fatigue |
| MCID | Minimum clinically important difference |
| MFI-20 | Multi-dimensional fatigue inventory |
| MMP | Matrix metalloproteinases |
| MRI | Magnetic resonance imaging |
| MS | mass spectrometry |
| mTORC | Mammalian target of rapamycin complex |
| MWCO | Molecular weight cut-off |
| nf-κb | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NMR | Nuclear magnetic resonance |
| NO | Nitric oxide |
| OA | Osteoarthritis |
| OAT | Ornithine aminotransferase |
| ODC | Ornithine decarboxylase |
| OPLS-DA | Orthogonal projections to latent structure discriminant analysis |
| PC | Principal components |
| PCA | Principal components analysis |
| PDH | Pyruvate dehydrogenase |
| PDK1 | Pyruvate dehydrogenase kinase 1 |
| PET | Positron emission tomography |
| PHQ-9 | Patient health questionnaire 9 |
| PKM2 | Pyruvate kinase isozyme M2 |
| PLS-DA | Partial least square discriminant analysis |
| PLS-R | Partial least square regression |
| POMS | Profile of mood states |
| POMS-F | Profile of mood states fatigue subscale |
| PPAR | Peroxisome proliferator-activated receptor |
| PQN | Probabilistic quotient normalization |
| ProF | Profile of fatigue |
| PROM | Patient reported outcome measure |

| | |
|---|---|
| PsA | Psoriatic arthritis |
| PTPN22 | Protein tyrosine phosphate, non-receptor type 22 |
| RA | Rheumatoid arthritis |
| RF | Rheumatoid factor |
| ROS | Reactive oxygen species |
| SARS-CoV-2 | Severe acute respiratory syndrome coronavirus 2 |
| SF-36 | Short form-36 vitality score |
| SF-36 VT | Short form-36 vitality score vitality |
| SIMCA | Soft Independent Modeling of Class Analogy |
| SJC | Swollen joint count |
| SLE | Systemic lupus erythematosus |
| STAT | Signal transducer and activator of transcription |
| TGF | Tissue growth factor |
| TJC | Tender joint count |
| TNF | Tumour necrosis factor |
| TSA | Total spectrum area |
| tsDMARD | Targeted synthetic disease modifying anti-rheumatic drugs |
| TSP | 3-(trimethylsilyl)-2,2',3,3'-tetradeuteropropionic acid |
| VAS-G | Global visual analogue scale |
| VAS-P | Pain visual analogue scale |
| VIP | Variable importance for projection |

# Publications

- Jutley GS and Young SP. Metabolomics to identify biomarkers and as a predictive tool in inflammatory diseases. Best Pract Res Clin Rheumatol. 2015 Dec;29(6):770-82.

- Harrison RS, Jutley GS, Li D, Sahbudin I, Filer A, Hewison M, Raza K. Vitamin D and early rheumatoid arthritis. BMC Rheumatol. 2020 Jul 27;4:38.

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# 1 Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by swelling, tenderness and destruction of synovial joints (1). In addition to significant disability, it also leads to premature mortality (2). Although RA is characterised by articular disease, a systemic inflammatory response is also associated with changes in many organs and with a number of extraarticular symptoms. One such symptom is fatigue.

Fatigue can be defined as 'an overwhelming, sustained sense of exhaustion and decreased capacity for physical and mental work' (3). Fatigue is common, with 80% of RA patients reporting clinically relevant levels of fatigue, which is comparable to rates detected among other inflammatory rheumatic disease (4, 5). Fatigue directly impacts on the patient's functionality and can be a barrier to work which can greatly affect the patient's quality of life and contribute to societal fiscal burden (6, 7).

Fatigue is a multifaceted and debilitating symptom. As complex as the symptom, so too is its aetiology, with multiple factors thought to play a role. Measuring fatigue has proved to be challenging. Subjective measures may be subject to bias and some fatigue questionnaires may screen for symptoms that overlap with disease activity, disease related damage and mood disorders.

Metabolomics has been used to identify alterations in a comprehensive array of metabolites simultaneously. Metabolomics studies have provided insights into disease aetiology and potential biomarkers for diagnosis and prognostic evaluation including in relation to response to therapy (8, 9). In this project I have applied nuclear magnetic resonance (NMR) metabolomics to assess serum and urine metabolites in a cohort of patients with newly presenting inflammatory arthritis and clinically suspect arthralgia with the aim of providing novel insights into mechanisms of fatigue and to identify potential biomarkers of fatigue.

## 1.1 Inflammatory arthritis

RA is a chronic inflammatory condition which can cause damage to synovial joints. RA has a worldwide prevalence of 0.5-1% (10). RA chiefly affects the joints but should be thought of as a syndrome with associated extraarticular features such as rheumatoid nodules, interstitial lung disease, vasculitis and increased risk of systemic comorbidities. No diagnostic criteria exist for RA, however classification criteria have been developed and are used primarily in a research context. The 1987 American College of Rheumatology (ACR) criteria have limitations as they were developed to discriminate patients with established RA from those with a combination of other definite rheumatological diagnoses (11). These criteria are of limited use for identifying patients with very early RA, and, to overcome this limitation, the 2010 ACR/European League Against Rheumatism (EULAR) criteria were developed (12). RA disease activity can be measured in a number of ways. Several composite disease activity scores have been created, with those incorporating joint counts recommended for daily practice (13). For example a disease activity score (DAS28) is a composite score calculated by physician assessment of 28 joints for swelling and tenderness, ESR and patient global assessment of health using a visual analogue score (14). These measures can be used to classify disease activity states into high, moderate, low, and remission.

Disease modifying anti-rheumatic drugs (DMARDs) are used to reduce synovial inflammation and consequent structural damage. Glucocorticoids have long been used to treat inflammatory arthritis as they deliver prompt symptomatic relief and disease modifying effects, however they are associated with dose dependent morbidity and mortality (15). DMARDs are categorised to synthetic and biological (bDMARDs), where the synthetic DMARD category is further subdivided into conventional

synthetic (csDMARD) and targeted synthetic (tsDMARD) (16). First line treatment is typically monotherapy with a csDMARD with or without glucocorticoids. If despite dose escalation, disease activity remains elevated, another csDMARD is typically commenced followed, if necessary, by the addition of bDMARD or tsDMARD. Examples of DMARDs are found in Table 1-1 below:

**Table 1-1 Examples of Disease modifying antirheumatic drugs used in inflammatory arthritis.**
**csDMARD**- conventional synthetic disease modifying anti-rheumatic drug; **bDMARD**- biological disease modifying anti-rheumatic drug; **tsDMARD**- targeted synthetic disease modifying anti-rheumatic drug

| csDMARDs | bDMARDs | tsDMARDs |
|---|---|---|
| Methotrexate | Anti-tumour necrosis factor α (adalimumab, etanercept, infliximab, certolizumab pegol, golimumab) | Janus kinase inhibitors (tofacitinib, baricitinib, upadacitinib) |
| Leflunomide | Anti-IL6 (sarilumab, tocilizumab) | |
| Sulfasalazine | CTLA4-Ig (abatacept) | |
| Hydroxychloroquine | Anti-CD20 (rituximab) | |
| Azathioprine | | |
| Gold | | |
| Ciclosporin | | |

Pre-RA is a collection of distinct phases which precede clinically apparent RA (17). EULAR initiatives have laid down important consensus foundations that 'map' the 'pathogenic journey' from heath to disease in those who develop RA (Fig. 1). In some cases, individuals will move through phases sequentially from preclinical phases through clinical phases, and finally to a disease classified as RA (18), although patients may skip phases or may move “backwards”, for example, develop unclassified arthritis (UA) followed by spontaneous resolution of symptoms. Genetic and environmental risk factors predate the development of autoimmunity and similarly in seropositive patients, the development of autoantibodies, such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA), can be present for up to a decade before symptoms emerge (19-21). Individuals at risk of RA may progress to develop symptoms but without clinical arthritis. This phase has been termed ‘clinically suspect arthralgia’ (CSA) when a rheumatologist has a high index of suspicion for the development of future clinical joint swelling and subsequently RA. Table 1-2 below shows features that identify CSA patients who are at high risk of progressing to RA as outlined by a EULAR initiative (22). Individuals at risk of RA may also develop palindromic rheumatism or UA before eventually developing RA (23).

**Table 1-2 EULAR defined characteristics describing arthralgia at risk for RA**
**EULAR**- European League Against Rheumatism; **RA**- rheumatoid arthritis; **MCP**-metacarpophalangeal

| |
|---|
| History: |
| Joint symptoms of recent onset (duration <1 year) |
| Symptoms located in MCP joints |
| Duration of morning stiffness ≥60 min |
| Most severe symptoms present in the early morning |
| First-degree relative with RA |
| Physical examination: |
| Difficulty making a fist |
| Positive squeeze test of MCP joints |

Early treatment initiation in patients with clinically manifest rheumatoid arthritis (RA) is associated with improved disease outcomes. There also appear to be improved clinical outcomes associated with early initiation of disease modulating therapy in pre-RA patients. There is evidence to suggest early initiation of methotrexate into UA patients retarded radiographic progression in those who progressed to RA (24). B-cell modulatory therapy for CSA patients resulted in a delay of 12 months in the onset of RA versus placebo (25), whilst work to investigating the effectiveness of T-cell modulation in ACPA positive CSA patients is currently ongoing (24). The treatment challenge lies in reliably selecting which patients should have preventative treatment commenced. The EULAR definition of CSA patients is a good foundation for patient selection; however a combination of clinical, serological markers and imaging may avoid selecting patients for DMARDs who would have otherwise resolved spontaneously.

Psoriatic arthritis (PsA) is a heterogeneous condition with musculoskeletal involvement, including arthritis, enthesitis, dactylitis and axial involvement; as such it can mimic other inflammatory arthritides. As well as possible skin and nail disease, other extra-articular features include mucous membrane lesions, iritis, urethritis, diarrhoea and metabolic syndrome (26). Many parallels can be drawn between the management of RA and PsA. For instance, many that are effective for RA and also effective for PsA.

### 1.1.1 Summary

Inflammatory arthritides and CSA are conditions with both musculoskeletal and systemic features, such as fatigue. There are number of overlapping treatment options for both CSA and inflammatory arthritis. Whilst inflammatory arthritis patients require DMARDs to control their disease, there is no consensus treatment for CSA patients.

## 1.2 Fatigue in inflammatory arthritis & CSA

It has been suggested that fatigue may be associated with the systemic inflammatory response. Animal models have shown that pro-inflammatory cytokines act on the brain to generate a sickness behaviour response. This includes drowsiness, anorexia, decreased activity and social withdrawal (27,

28). When humans are given Interleukin (IL)-1 and IL-6 it leads to fever, headache and fatigue (29-31). Studies investigating the effect of immunomodulatory treatment on fatigue, however, have only shown partial benefit in treating fatigue (32-36). It is possible that metabolic pertubations as a result of a systemic proinflammatory response, could, in part, be responsible for fatigue experienced by inflammatory arthritis and CSA patients.

Several studies have found depression, pain, sleep disorders, smoking, alcohol consumption, low levels of physical activity, lack of social support, high disease activity, and arthritis treatment to be associated with fatigue in rheumatic diseases (37-52).

Most studies in inflammatory arthritis have assessed fatigue in patients with long standing established disease on DMARDs (38, 39, 44, 47-51). In contrast, studies in patients with early arthritis are limited. One multicentre French study of RA patients with a median symptom duration of 3 months (53) identified factors associated with fatigue at presentation and determinants of change in fatigue at 1 year follow up. Fatigue was measured using a FVAS and Short Form-36 Vitality (SF-36) score. At baseline, 813 patients' fatigue as assessed by FVAS or SF-36 was independently associated with young age, female sex, low education level, smoking, increased DAS28, waking up at night, and worse Arthritis Impact Measurement Scales 2 Short Form (AIMS2-SF) physical, affect, and symptom scores. A favourable change in fatigue scores correlated with increased baseline AIMS2-SF physical and affect scores, high baseline fatigue scores, and improved 1-year AIMS2-SF affect scores.

These findings were supported by a cross-sectional study in Sweden of 276 RA patients with a disease duration of less than one year (54). Fatigue, as measured by FVAS, was associated with female sex, higher disease activity, pain and activity limitation, poor mental health and sleep disturbance. Similarly, a French study of 708 patients with inflammatory back pain of recent onset (<3 years) suggestive of axial spondyloarthropathy identified factors associated with fatigue at baseline and follow up. Fatigue was assessed by a numerical rating scale between 0-10 using the first question of the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI). Factors related to fatigue in early and pre-axial spondyloarthopathy included female sex, low education, increased disability and disease activity (55).

Correlates of fatigue in early inflammatory arthritis appear to be similar across studies thus validating their findings. Recurring themes include associations with depression, pain, disability and disease activity.

Evidence suggests that the earliest clinically apparent stages of RA offer an important therapeutic window, where timely treatment can significantly alter disease progression and outcome (56-58). An understanding of which symptoms are associated with RA development allows identification of patients who may benefit from treatment.

Quantitative work has been undertaken to explore the symptoms of individuals at risk of RA. Survey questions are largely based on symptoms characteristic of established RA and are therefore assumed to be present in at-risk individuals too (59). Common clinical manifestations in symptomatic patients prior to the development of joint swelling include symmetrical pain affecting the upper and lower extremities (60-62), in particular the small joints of the hands (61, 62).

A qualitative study investigated symptom complexes in 11 newly presenting RA patients and 15 ACPA positive patients with arthralgia (63). Fatigue was perceived by both groups. The non-RA ACPA positive patients described extreme fatigue resulting in falling asleep "That fatigue; like I'm sitting in the couch ready to watch some TV and then I get so fatigued that I completely doze off, really extreme" (63). Another non-RA ACPA positive patient described fatigue that resulted in difficulty getting off the floor

"If I would fall now, I will just remain lying down, doesn't matter where I am. It's really extreme fatigue, not just a little tired" (63). RA and non-RA ACPA positive patients linked fatigue with disturbed sleep "Sometimes I wake up fatigued, I think I didn't sleep well that night" (63). This qualitative study provided insights into the degree of fatigue and its profound impact on CSA patients' life.

### 1.2.1 Summary

Fatigue is a burdensome symptom present in both inflammatory arthritis and CSA patients. Work investigating the associations of fatigue with other aspects of disease has focussed on patients with established inflammatory arthritis; by contrast these issues have not been as well studied in patients with CSA, early and newly presenting DMARD-naïve inflammatory arthritis. Evidence suggests that CSA and early inflammatory arthritis represent important distinct phases within the inflammatory arthritis continuum and thus further research in these phases may offer novel insights into the mechanisms and potential biomarkers of fatigue.

## 1.3 Aetiology of rheumatoid arthritis

The aetiology of RA is not fully understood but both genetic and environmental factors play a role. It is likely that a genetically susceptible host is exposed to an unknown pathogen or other environmental insult (e.g. cigarette smoke) and that leads to a persistent immunological response. RA is heterogenous condition which can be divided into two major subtypes according to the presence or absence of autoantibodies such as ACPA or rheumatoid factor (RF). ACPAs can be detected in approximately two thirds of RA patients (64).

Several genetic factors have been shown to influence predisposition to RA. Genome-wide association studies using single nucleotide polymorphisms have identified over a hundred loci associated with RA risk, most of which implicate altered immune function (65). The human leukocyte antigen (HLA) complex, especially HLA-DRB1, implicates peptide binding in pathogenesis. Some HLA genotypes associate with erosive disease and with higher mortality (66). Disease associated alleles share a mutual amino acid sequence in the peptide-binding groove which is referred to as the shared epitope, which is linked to development of ACPA and RF (67). Another genetic risk factor in RA is protein tyrosine phosphate, non-receptor type 22 (PTPN22), a gene encoding a protein tyrosine phosphatase that plays a role in T cell and B cell signalling (68). Both the shared epitope and PTPN22 predispose to ACPA positive RA, which suggests ACPA positive RA is a different disease compared to ACPA negative RA. Several other genetic loci contribute to risk of RA development such as modified co-stimulatory pathways, cytokine signalling, and innate immune activation (69).

Interaction between genes and environmental factors result in the precipitation and perpetuation of disease. One of the possible mechanisms of this is epigenetic change. Both genetic and epigenetic changes lead to aberrant gene expression. DNA methylation and modified histone acetylation can affect synovial fibroblasts and leucocytes and contribute to increased risk of developing RA (70, 71).

Multiple environmental factors have been implicated in the development of RA. One of the most consistently reported risk factors for developing RA is cigarette smoking (72, 73). Multiple potential explanations for this observation has been proposed. For instance, through citrullination of peptides in the lungs, smoking could result in breakage of tolerance and ACPA formation in genetically susceptible individuals (73). Smoking is also associated with increased oxidative stress which may

increase the systemic inflammatory burden. Cigarette smoke contains a high number of free radicals and can provoke depletion of glutathione (74), which can induce cell injury secondary to increased oxidative stress (75). Importantly cigarette smoking appears to be a risk factor for ACPA positive RA but not ACPA negative RA.

Periodontal disease has also been associated with RA (76). One of the proposed mechanisms for this association is the effect of *Porphyromonas gingivalis*, a gram-negative oral anaerobe that is involved in the pathogenesis of periodontitis. *P. gingivalis* is capable of citrullinating both its own and its host proteins and provokes a local breach of tolerance to citrullinated peptides via endogenous expression of its PADI4, which converts arginine to citrulline (77). Smoking is a well-established risk factor for periodontal disease (78) and thus there seems to be a four way relationship between smoking, periodontitis, the shared epitope and ACPA positive RA. Other infectious agents that have been proposed as potential triggers for RA include, *Proteus mirablis*, *Escherichia Coli*, and *Epstein-Barr*, with the underlying postulated mechanism to be molecular mimicry (79).

The intestinal microbiota may also play a role in development of RA. Gastrointestinal dysbiosis has been observed in RA patients (80). Intestinal microbiota populations undergo alterations which correlate with or are influenced by disease phase (e.g. early RA is associated with a higher prevalence of *Prevotella Copri* than longstanding arthritis), the extent of inflammation (e.g. as measured by CRP), ACPA status and treatment with DMARDs (81). It is likely that there is a genetic impact on the microbiome, which may contribute to RA via the host immune system (82).

### 1.3.1 Summary

Multiple factors have been postulated to contribute to the development of RA. Genetic factors, including the shared epitope and PTNP22 and smoking play important roles in the development of ACPA positive RA. Factors contributing to ACPA negative RA are less clearly defined.

## 1.4 Pathology of rheumatoid arthritis

The normal synovial membrane is relatively acellular structure consisting of an intimal lining layer of 1-2 cells thickness and a synovial sublining layer. The sublining layer is relatively acellular, containing scattered blood vessels, adipose cells, and fibroblasts, with few lymphocytes or macrophages (83).

In established RA, there is evidence of systemic immune activation and immune cell infiltration into synovial joints (84). Detectable joint swelling reflects synovial membrane inflammation secondary to immune activation and is characterised by leucocyte infiltration into the synovial compartment. The cellular composition of synovitis in RA includes innate immune cells (e.g. macrophages, dendritic cells, mast cells), innate lymphoid cells and adaptive immune cells (e.g. T-helper-1 and T-helper-17 cells, B cells, plasmablasts, and plasma cells) and fibroblasts. Synovial fibroblasts take on an aggressive inflammatory, matrix regulatory, and invasive phenotype. These fibroblasts, together with increased chondrocyte catabolism and synovial osteoclastogenesis, promotes articular destruction (85, 86).

Recent advances in RNA sequencing (seq) technologies, and the use of these in disaggregated synovial tissue, has allowed more precise definition of key cellular subpopulations in the RA synovium (87). Integrating single cell RNA-seq and bulk RNA seq data in a cross-sectional study of patients with established RA, 18 separate cellular subsets (four fibroblast, four monocyte/macrophage, six T cell and four B cell) have now been identified (87). Importantly, this approach allowed the assessment of whether inflammatory cytokine upregulation in RA synovium is driven by global upregulation within a

single synovial cell type or types or specific upregulation within a discrete cell subset defined by single cell RNA-seq. Thus, whilst TNF was produced at a high level by multiple monocyte, B cell and T cell populations, *IL6* expression was restricted to *HLA-DRA*$^{hi}$ sublining fibroblasts (SC-F2) and a subset of B cells (SC-B1). Interestingly, CD8+ T cells, rather than CD4+ T cells, were the dominant source of interferon gamma gene (*IFNG)* transcription (87).

Limited data are available regarding the rheumatoid synovium in very early RA though there may be some differences compared with established RA (88, 89). In pre-RA, there is minimal T-cell infiltration into the synovium and in early-RA, B-cells and macrophages too (90, 91). However, there is modified expression of IL-13 in the serum of both seropositive and seronegative arthralgia patients (92), as well as IL-4 expression in seropositive arthralgia patients (93). Furthermore, the same cytokines are found in the synovial fluid of early RA patients (94). These cytokines are important for the survival of inflammatory phenotype fibroblasts (95).

Although synovial inflammation characterises both RA and PsA, a number of differences have been identified between RA and PsA synovitis. PsA synovium may have less pronounced intimal lining layer hyperplasia and fewer synovial T cells and macrophages compared with RA (96). Furthermore, PsA is characterised by tortuous, bushy, elongated blood vessels, whereas RA synovitis is characterised by straight blood vessels (97). However, there are also many shared features e.g. there is overexpression of many proinflammatory cytokines in the synovial tissue of active RA and PsA, including tumour necrosis factor (TNF)-α and IL-6.

Hypoxia is a feature of the synovial membrane in both RA and PsA (98). The degree of hypoxia correlates with the intensity of the inflammatory process. One of the postulated reasons for this observation is cell proliferation and immune cell infiltration generates a high metabolic demand and increases the distance between the blood supply and the cells (99). The hypoxic microenvironment encourages anaerobic glycolysis and production of reactive oxygen species (ROS) (100).

### 1.4.1 Summary

These findings suggest that there is highly metabolically active immune cell infiltration in established inflammatory arthritis patients' synovium. Whilst this observation is not seen in CSA there is systemic immune cell activation. These immune cells are metabolically active, and the resulting metabolites can act as effector molecules and in addition to cytokine production may result in changes to systemic metabolism, which can be assessed by metabolomics.

## 1.5 Metabolism in chronic inflammation

The musculoskeletal system is very metabolically active, with skeletal muscle, bone and other structural components having high requirements for energy and substrates. Inflammation adds to these demands. For example, in RA resting energy expenditure is higher than healthy controls (101-103). Changes in metabolism may be seen in pre-RA, for example, decreases in blood lipids are an early feature (104), which may be modified following treatment (105, 106). The mechanism for the increased metabolic rate in RA is yet to be fully elucidated, though active local/systemic inflammation is likely to be a major contributor. The effect of inflammation on metabolism has been visualised using positron emission tomography (PET), which relies on the enhanced uptake of a labelled but nondegradable derivative of glucose (18-F-deoxyglucose) that accumulates in joints in pre-RA (107) and RA (108) patients, thus indicating enhanced glycolytic activity.

Inflammation also drives other processes such as the decrease in muscle mass and increased fat tissue, in a process known as rheumatoid cachexia, which involves significant alterations in systemic metabolism. PET imaging has also been reported to identify such enhanced metabolic activity in extra-articular sites, in particular subcutaneous nodules and lymph nodes (109), indicating increased systemic metabolism. The liver is another extraarticular site that has increased metabolism as the inflammatory response leads to the hepatic production of acute phase proteins and the likely enhancement of the Cori cycle to clear excess blood lactate produced in inflammatory sites (110). Broad changes in metabolism observed in inflammatory arthritis are considered in the following sections.

### 1.5.1 Cachexia

The strong relationship between metabolism and inflammation can be exemplified by cachexia, the loss of cellular mass associated with disease. TNF-α is a central cytokine in cachexia. RA is associated with systemic chronic inflammation and with rheumatoid cachexia. Muscle wasting in rheumatoid cachexia is a shared feature with classical cachexia, but low body mass index (BMI) is unusual as the fat mass is maintained or increased (111). Cytokines, such as TNF, IL-1 and IL-6, are hypothesised to be responsible for the muscle loss observed in rheumatoid cachexia. The ubiquitin–proteasome pathway is activated by TNF, which is responsible for proteolysis. Pro-inflammatory cytokines may inhibit protein synthesis in response to nutritional intake, the so-called anabolic resistance; hence, rheumatoid cachexia-induced muscle wasting is related to RA disease activity (112).

### 1.5.2 Glucose metabolism

Inflammation impacts systemic glucose metabolism and glucose metabolism locally at sites of inflammation, with a general trend to shift to glycolysis. Proinflammatory cytokines, such as TNF-α and IL-6, can result in insulin resistance. Furthermore, proinflammatory cytokines can reduce β-cell function and result in apoptosis (113, 114). These phenomena are potentially reversible, with both improved insulin sensitivity and β-cell function noted following anti-TNF therapy (115). Furthermore, proinflammatory cytokines can alter macrophages and neutrophils metabolism by favourably utilise aerobic glycolysis to supply ATP (116).

Furthermore, the metabolism of the cells within the inflamed synovium are also altered. Inflamed joints have a hypoxic microenvironment. Cells use oxygen recognition mechanisms, including those involving the transcription factor HIF (hypoxia inducible factor), to make necessary adjustments. HIF induces glycolytic enzymes, which drive glycolytic metabolism preferentially over oxidative phosphorylation (117). Thus, ATP production continues despite inadequate oxygen, albeit at the expense of efficiency per molecule of glucose (117). Hypoxia activates tissue-resident macrophages, and hypoxic sites of chronic inflammation collect macrophages (118) and upregulation of proinflammatory cytokines (119-122). HIF-induced upregulation of lactate dehydrogenase A compounds this accumulation (117). In proliferative tissues such as lymph nodes and inflamed synovium, there is a predisposition toward metabolising glucose to lactate, pyruvate and carbon dioxide, and glutamine to ammonia, glutamate and aspartate (123, 124).

### 1.5.3 Alternative energy sources

Inflammation is a highly energy-dependent process (125). Damage to the joint in inflammatory arthritis is associated with the presence of fatty acids into the synovium. Reduced chylomicron and triglycerides associated with very low-density lipoproteins in synovial fluid of RA patients, suggest an enhanced use of fats, an energy source in the joint despite the hypoxic environment. Blood plasma levels of lipids and acetylated glycoprotein were also elevated in RA (126).

The kidneys and liver release ketone bodies into the circulation after breaking down fatty acids. Ketone bodies such as acetoacetate and 3-hydroxybutyrate deliver an essential energy source for the heart and brain, by reconversion to acetyl-coenzyme A (acetyl-CoA) and re-entering the citric acid cycle in the tissues, while acetone is expelled as waste. Changes in ketone body metabolism have been detected in inflammatory diseases in the absence of glucose restriction. Chronic inflammation may cause metabolic changes, especially in the context of cachexia-associated catabolism and ketogenesis. The presence of the ketone body 3-hydroxybutyrate in urine has been linked to respiratory chain deficiency leading to impaired NADH oxidation (127).

In inflammatory states there appears to be increased lipolysis and utilisation of ketone bodies as an energy source. This may, in part, be a result of impaired glucose metabolism.

### 1.5.4 Tissue degradation and waste

Specific tissue-derived metabolites provide evidence of tissue destruction. For instance, hyaluronic acid is a major component of articular cartilage proteoglycan aggregates and correlates with joint destruction in RA patients (128). Essential amino acids cannot be synthesised de novo, assuming a stable diet, variations in concentration must be attributable to catabolism of current proteins derived from tissue destruction, apoptosis or cellular autophagy (129, 130). There appears to be a relationship between increased essential amino acids and increased inflammation (131). Interestingly, dietary supplementation with branched chain amino acids (BCAAs), a subset of essential amino acids, has been shown to drive an increase in Th1-like responses via IL-1, IL-2, TNF and interferon, which suggests the presence of essential amino acids may contribute to chronic inflammation (132).

### 1.5.5 The impact of the intestinal microbiome on metabolism

The microbiome influences the host immune system (133). Dysbiosis and impaired intestinal barrier function may result in systemic metabolic perturbations and chronic inflammation (134). RA patients appear to have a low diversity of gut microbiota and dysbiosis (135, 136). It is unknown whether this is a cause or consequence of RA. In addition to regulating local immune functions, gut bacteria are also responsible for metabolite production, which can be absorbed systemically. Fermentation of undigested carbohydrates and protein results in the production of several metabolites including short chain fatty acids, branched chain fatty acids, ammonia, amines, phenolic compounds, and gases, including hydrogen, methane, and hydrogen sulphide (137, 138). In addition to being used as a source of calories, short chain fatty acids act as a signalling molecule involved in both lipid and glucose metabolism and insulin regulation (139).

### 1.5.6 Summary

The widespread systemic effects mediated by pro-inflammatory cytokines in conditions such as RA, impact on systemic metabolism in addition to immunometabolism. Furthermore, factors playing an

aeitopathogenic role in RA influence metabolite levels. These changes in systemic metabolism could be responsible for symptoms such as fatigue.

## 1.6 Measuring metabolites

Metabolomics is a comprehensive analysis of metabolites in a biological specimen (140). Small molecules (<1500 Da) are quantified within compartments (e.g. synovial fluid, blood, urine, saliva, tears, cerebrospinal fluid and intact cells) and metabolite profiles are generated. The metabolites can be measured using a range of methods including mass spectrometry (MS) and NMR spectroscopy. The derived data take the form of spectra with individual peaks reflecting proton resonance in proton NMR or mass/charge (m/z) ratios in MS (141). Metabolites are recognised by referencing to metabolite databases or by direct metabolite assay. The current version of the Human Metabolome Database lists >41,000 metabolite entries; however, only 3000 have been associated with diseases (142). In common with genomic, proteomic and transcriptomic approaches, metabolomic experiments produce large bodies of data, necessitating the use of multivariate analysis to simplify and extract meaning.

Analysis of metabolomic data can conducted using either supervised or unsupervised approaches (141). Unsupervised analytic approaches include PCA, wherein the aim is to describe the maximum variation in the data set without supplying information to the model about the class groupings. The result is a series of orthogonal principal components describing the range of variations in the data through weighted peaks. If the source of greatest variation in the data is the difference between control and test samples, PCA alone is enough to distinguish class groupings (143). Confounders may conceal the true variances in the samples. Supervised analysis techniques may prove to be more suitable in such situations. PLS-R may be used to explain variation in one data set by referring to another, for example, using metabolite concentrations to predict outcome, progression or severity. Furthermore, partial least square discriminant analysis (PLS-DA) uses multivariate metabolomic peak data to describe the assignment of samples to binary cohorts. In essence, the technique focuses on pinpointing the variation that describes the differences between control and test samples (143). This makes it feasible to extract the clinically pertinent variation in the face of substantial unrelated noise. The outputs of these methods aim to produce statistical models from which it is possible to identify disease state or patient group from biological samples. However, the models in question are likely to be complex and include multiple correlated peaks. Forward selection regression analysis may therefore be utilised to iteratively remove peaks resulting in a small number of metabolites while preserving predictive proficiency, making it ideal for cheaper, more specific bioassays.

NMR has various advantages as an analytical tool to study metabolites. Aside from being more time-efficient and cost-effective than mass spectrometry, NMR spectroscopy can quantify metabolite concentrations, in contrast to mass spectrometry which is not quantitative. In NMR spectroscopy, the sample does not physically interact with the operating parts of the instrument and therefore instrument reproducibility is excellent. However, this is not the case with liquid or gas chromatography mass spectrometry as the sample inevitably interacts with the instrument. This leads to variations in measured analyte response over time both in terms of chromatography and mass spectrometry. Batch effect observed in NMR spectroscopy through magnetic drift is thus minimal compared to the batch effect associated with mass spectrometry (144).

Taking these factors into consideration, NMR spectroscopy is an ideal modality to use for an explorative experiment when the focus is biomarker identification. Although mass spectrometry

provides a more comprehensive assessment of the metabolome to assess for low abundant metabolites the advantages of NMR led us to choose this method (144).

### 1.6.1 Summary

The metabolic correlates of inflammation are being increasingly understood. Metabolites appear as both by-products and mediators of inflammation. Metabolomics presents a unique method of identifying the metabolic fingerprint of inflammation, which can identify novel therapeutic targets, providing the foundation stone for developing reliable downstream point-of-care tests and assessing disease activity and response to treatment.

## 1.7 How metabolomics has been applied to inflammatory rheumatic diseases

Metabolomics has been used to investigate a number of rheumatological conditions (145), providing novel insights into disease processes. A study examined NMR profiles in RA patient synovial fluid compared with serum. Synovial fluid contained higher lactate and lower glucose levels than the serum; both of which are consistent with metabolic changes associated with hypoxia. In addition to glycolysis, there is a tendency to utilise fatty acids as an energy source, even in a hypoxic environment (146, 147).

Furthermore, metabolomics has also shown diagnostic potential. Plasma metabolomics can distinguish RA patients from healthy controls and RA with active and controlled disease could also be distinguished. Cholesterol, lactate, acetylated glycoprotein and lipids were all identified in the RA cohort, with lactate being particularly elevated in those with active disease (126). Madsen et al. were able to diagnose RA patients with a sensitivity of 93% and specificity of 70% and showed increased glyceric acid, d-ribofuranose and hypoxanthine levels in RA patients' serum compared to healthy controls (148). Metabolomics have been used to identify axial spondyloarthropathy (149) and PsA (150) patients from healthy controls, respectively. In addition, metabolite profiles have been used to distinguish between different arthritides (151) and predicting those patients who will develop self-limiting and persistent arthritis in the early arthritis cohort (152).

In addition to diagnosis, metabolomics has successfully predicted those who would respond to certain treatments. A urinary metabolic fingerprint has been used to predict responses to anti-TNF (153). Urinary NMR metabolic profiles have been used to discriminate between RA patients who showed a good response to TNF therapy with a sensitivity of 88.9% and a specificity of 85.7% (153). Serum metabolomics have also been used to predict RA patients' response to methotrexate treatment (154).

### 1.7.1 Summary

Several studies have identified clinical variables associated with fatigue in patients with inflammatory arthritis. Most of these studies have been undertaken in patients with established arthritis with few undertaken in early arthritis. No quantitative data are available relating to fatigue in CSA patients. Growing evidence suggests there is a biological basis to fatigue. Metabolomics is a useful technique to investigate whether systemic metabolic disturbances relate to levels of fatigue and to gain insights into whether such disturbances play a role in the development of fatigue.

## 1.8 Aims and hypothesis

I will test the hypotheses that:

- The degree of fatigue reported by CSA patients is comparable to new presenting DMARD naïve inflammatory arthritis patients
- Inflammatory arthritis and CSA are associated with metabolic features which can be identified in both blood and urine and that correlate with the extent of systemic inflammation
- Metabolic features in the blood and urine correlate with the extent of fatigue, and there is a relationship between these which is independent of systemic inflammation.

My aims are:

- To investigate the relationship between clinical variables and fatigue in patients with newly presenting inflammatory arthritis and CSA.
- To investigate approaches to analysing NMR data from inflammatory arthritis bio fluids specifically the effect of the g-log transformation and normalisation techniques on the results of multivariate analyses.
- To identify whether there is a relationship between the metabolites found in sera and urine and the level of systemic inflammation as measured by serum CRP levels.
- To investigate the metabolic profiles of DMARD naïve inflammatory arthritis and CSA patients in relation to their levels of fatigue to explore potential biological mechanisms underlying fatigue.

# 2 Material & methods

## 2.1 Patients

The BEACON cohort consists of patients presenting with DMARD naive inflammatory arthritis or CSA; details have been reported previously (16). Patients were recruited between January 2013 and September 2015. All patients were aged 18 years or over and had inflammatory arthritis (as evidenced by the presence of at least one clinically swollen joint in which the swelling was attributed to synovitis) or CSA (defined as a history consistent with an inflammatory cause for the patient's musculoskeletal symptoms in the absence of a clinically swollen joint and with no other clear diagnosis e.g. soft tissue rheumatism, fibromyalgia). Follow up data including patients' final classified diagnosis were collected at the 12-month point, or the next visit following that if the patient did not attend for 12-month follow-up. A cross-sectional analysis identified factors associated with fatigue at initial presentation to secondary care and a longitudinal analysis identified factors that correlated with a change in fatigue over time.

The study was approved by the Black Country Research Ethics Committee and all patients gave written informed consent. The study was conducted over two sites: City Hospital NHS Trust, Dudley Road, Birmingham and Queen Elizabeth Hospital Birmingham NHS Trust. Patients were recruited from early arthritis clinic. Referral to the clinic was predominantly via the standard GP referral pathway, however some referrals was a result of rheumatology consultations conducted on inpatients. GP referral letters to all consultant rheumatologists across both sites were screened and those suggesting a diagnosis of early inflammatory arthritis led to the patient being assessed in the Early Arthritis Clinic.

The following data were collected at baseline and follow up: age, gender, family history, symptom duration, duration of early morning stiffness, past medical history, current medications, educational level, occupation, smoking status, tender (68), swollen (66) joint counts and extraarticular features (subcutaneous nodules, vasculitic rash, psoriasis, gouty tophi, circinate ballanitis, keratoderma blenorrhagicum, nail pitting, onycholysis, iritis, episcleritis, scleritis, conjunctivitis, rectal bleeding/diarrhoea, urethritis, lymphadenopathy).

The following patient reported outcome measures (PROMs) were also collected at the baseline and follow up visits: visual analogue scores for global assessment, pain and fatigue (VAS-G, VAS-P and FVAS) and the Health Assessment Questionnaire (HAQ), FACIT-F and Patient Health Questionnaire 9 (PHQ-9). HAQ measures functional status using Likert scales across 8 categories and generates a score between 0-3 (155). PHQ-9 is 9 item subscore of the PHQ (156), which measures depression and results in a score between 0-27. The greater HAQ and PHQ-9 score, the greater the severity of disability and depression experienced by the patient respectively.

Blood was collected at baseline and follow up and processed for metabolomics analyses as described in **section 2.2.1**, whilst urine was only collected at the baseline visit and was processed for metabolomics analyses as described in **section 2.2.2**. Laboratory variables collected at all visits included: full blood count, liver function test, kidney function, CRP and ESR. Laboratory values for the following were collected for the baseline visit only: RF, ACPA, thyroid stimulating hormone, 25OH vitamin D, anti-nuclear antibody, serum immunoglobulins. Established criteria were used after each visit to classify patients as having RA (28, 29), psoriatic arthritis (30), spondyloarthritis (31), reactive arthritis (32) and gout (33). Patients with clinically detectable synovitis who did not meet classification criteria were classified as having an UA. Furthermore, patients without synovitis whose musculoskeletal symptoms were thought to be inflammatory aetiology and at high risk of developing RA (22), were classified as CSA.

The BEACON cohort consists of patients presenting with DMARD naive inflammatory arthritis or clinically suspect arthralgia (CSA). The advantages of studying the metabolic permutations associated with inflammation and with fatigue in this patient population are as follows. Firstly, this patient population are naïve to immunomodulatory therapy at the time of recruitment and sample collection which minimises pharmacologically induced metabolic permutations. Secondly, as this patient population is newly presenting, at least some will have disease of short duration. Growing evidence suggests early RA (within the first 12 weeks of symptom onset) represents a unique phase of disease, associated with a synovial tissue and synovial fluid characteristics that are different from those in long standing disease and which may explain the clinically apparent therapeutic window of opportunity (58). Thus, studying metabolic permutations associated with systemic inflammation and fatigue at this part of the patient journey may provide unique insights into disease pathoaeitology. As this clinic recruit's patients across a range of diagnoses, it allows an assessment of whether metabolic permutations associated with inflammation and fatigue are similar in different diagnostic groups.

Although newly presenting inflammatory arthritis and CSA patients are likely to have a degree of systemic inflammation measurable by CRP and fatigue, this clinical population is not without its limitations when studying the relationships between the metabolome, inflammation and fatigue. It is likely that many patients will have low to normal CRP values; this skewing of CRP results may make it difficult to identify a correlation between metabolic permutation and inflammation if this exists. Furthermore, there is growing evidence to support different subtypes of fatigue (157). It is possible that early in their disease course that most patients have one particular type of fatigue e.g. an inflammatory type of fatigue, rather than fatigue that relates to pain or low mood. This could then therefore lead to incomplete assessment of fatigue.

## 2.2 Samples

### 2.2.1 Serum

Blood was collected in vacutainer tubes containing clotting accelerator (Greiner Bio-one) at the first visit to the early arthritis clinic at Sandwell and West Birmingham Hospitals NHS Trust or University Hospital Birmingham. Samples were then transferred to the University of Birmingham where they were centrifuged at 600g for 10 minutes, serum removed and then stored at minus $80^0$C until analysis. Samples were also collected at follow up visits.

### 2.2.2 Urine

Mid-stream urine samples were collected at the first visit to the clinic, centrifuged as above and stored at minus $80^0$C.

## 2.3 Sample preparation for NMR

### 2.3.1 Materials

Difluorotrimethylsilylmethylphosphonic acid (DFTMP, Manchester Organics, Manchester, UK),

3-(Trimethylsilyl)-1-propanesulfonic acid-d6 sodium salt (DSS-d6, all from Merck, Southampton, UK)

NMR tubes standard series 1.7mm, (Bruker).

Filters: Nanosep 3K Omega, (Pall).

Deuterium oxide ($D_2O$) 99.9%, (Sigma-Aldrich).

$NaH_2PO_42H_2O$ (Fisons Scientific Equipment)

Sodium azide (Sigma-Aldrich)

### 2.3.2 Serum samples

Serum samples were thawed at 4°C centrifuged at 15,000g at 4°C for 5 minutes. To remove proteins, 200µl from the middle of the sample was placed into a Nanosep® Omega 3000 Da (Pall Lifesciences, UK) molecular weight cut-off (MWCO) and centrifuged at 10,000g at 4°C for 15 minutes. Immediately prior to use, to remove the preservative glycerol, the filters were washed 6 times in distilled water at 37°C by centrifugation at 3000g for 15 minutes (158). The resulting filtrate was diluted in a 1+3 ratio with NMR buffer containing 1.6mM Difluorotrimethylsilylmethylphosphonic acid (DFTMP, Manchester Organics, Manchester, UK), 400mM phosphate, 40% $D_2O$, 0.4% azide and 2mM 3-(Trimethylsilyl)-1-propanesulfonic acid-d6 sodium salt (DSS-d6, all from Merck, Southampton, UK). An aliquot (60ul) was removed to a glass champagne vials (Cole-Parmer, Saint Neots, UK) and stored at -80°C until analysis.

### 2.3.3 Urine samples

Samples were prepared using a standard protocol that has been used in other studies of urine (159). After thawing, urine samples (1ml) at 4°C were centrifuged at 15,000g for 5 minutes. A cleared sample (0.5ml) was mixed at 1:3 ratio with the 4x NMR buffer as for the serum above. The pH was adjusted (twice over a period of 30minutes) to pH 7.0. The samples were centrifuged at 15000g for 5 minutes and a sample (60ul) was removed to glass champagne vial and frozen at -80°C prior to NMR spectroscopy.

### 2.3.4 NMR Spectroscopy

Samples were defrosted and transferred to 1.7mm NMR tubes (Bruker Biospin, Coventry, UK) using an Anachem Autosampler. After capping the tibes and wiping with dust-free paper, one-dimensional $^1H$ spectra were acquired at 300K using a standard 1D-$^1H$-Nuclear Overhauser Effect spectroscopy (NOESY) pulse sequence with water saturation using pre-sat in a Bruker AVANCE II 600 MHz NMR spectrometer (Bruker Corp., USA) equipped with a 1.7 mm cryoprobe. Spectral width was set to 12 ppm and the scans were repeated 128 times. Samples were loaded into racks and held at 6°C in the SampleJet sample handing device until processed. Two-dimensional 1H J-resolved spectra were also acquired to aid metabolite identification (160).

### 2.3.5 Metabolomics analysis

All samples were analysed at the same time. Samples were processed and data were calibrated against the DSS-d6 signal. Spectra were read into ProMetab (160), custom written software in MatLab version 7 (The MathWorks), and were truncated to the chemical shift range of 0.8–10.0 parts per million. Pre-

processing as outlined in **section 3.3** below was applied which included baseline correction, probabilistic quotient normalization (PQN), noise reduction and a generalized log with a transform parameter of $1e^{-8}$ (160, 161). Binned data were then compiled into a matrix, with each row representing an individual sample.

## 2.3.6 Statistical analyses

### *2.3.6.1 Principal components analysis (PCA)*

The data bins from groups of spectra were mean centred and then assessed by PCA using Soft Independent Modeling of Class Analogy (SIMCA) version 14 (Umetrics) (162). PCA is an unsupervised multivariate mathematical analysis that produces patterns and relationships in multivariate datasets, enabling an understanding into the causes and effects behind these relationships. The basis of this method is the generation of a new coordinate system based upon hyperplanes of maximal variance to reduce dimensionality, so called principal components (PC), where the new variables are independent linear combinations of the original variables. The first PC accounts for as much of the variability in data as possible, with subsequent PC's placed in descending order with respect to the amount of variability accounted for in the data. The fundamental basis behind PCA is the relationship between the numerical values against each principal component (163). For PCA, class separations in scores are exposed only when within-class variation is less than between-class variation. Supervised multivariate analysis can be particularly useful in such instances, which are described below.

### *2.3.6.2 Supervised Multivariate analysis: Orthogonal partial least square discriminant analysis (OPLS-DA) & Partial least square regression (PLS-R) analysis*

Whilst PCA describes the relationship between possibly correlated variables in a single large multivariate matrix (matrix X) of data using PCs, partial least square is a multivariate analysis which attempts to describe the relationship between two different matrices of data using a latent variable (LV) approach to modelling the covariance in these two spaces. The second matrix of data (matrix Y) is introduced by the operator prior to analysis and is thus a form of supervised multivariate analysis. When the Y matrix contains categorical data a discriminant analysis is performed, however if the Y matrix is continuous a regression analysis is performed. The OPLS-DA method can separate the systematic variation in the X variable into two parts: (1) that which is linearly related to Y, and (2) that which is orthogonal to Y and thus do not contribute to discrimination between the groups (164). OPLS-DA was used to perform supervised clustering of samples using SIMCA version 14 (Umetrics) (162, 165). The OPLS-DA models were cross-validated using Venetian blinds (165), a method which reassigns randomly selected blocks of data to the OPLS-DA model to determine the accuracy of the model in correctly assigning class membership. The application of such methods to clinical studies is well established and guards against over fitting the model (166).

A PLS-R finds a linear regression model by projecting a predicted variable, which is created following application of an algorithm using latent variables to describe the covariance between the X and Y matrix, and the continuous variable in the Y matrix. Data bins were also subjected to PLS-R using the PLS Toolbox (version 5.8) (Eigenvector Research) in MatLab (release 2018b; MathWorks). This method identifies which metabolites can predict a continuous variable. This analysis yields an $r^2$, a measure of the cross-validated goodness-of-fit of the linear regression, while permutation testing performed by multiple analyses using random data subsets, was used to assess the significance of this prediction.

Models can be further optimised using a forward selection approach, which identifies a proportion of the metabolome that correlates with the continuous variable. This process ranks each bin in descending order of each individual bin's correlation coefficient. Multiple PLS-Rs are undertaken with the most highly predictive bins to give a mean $r^2$ for that model. This process is repeated in an iterative fashion, introducing bins, which have been sorted by regression coefficient, into the model to generate a mean $r^2$ value for each model with each model incorporating increasing number of bins. These mean $r^2$ values are then sorted into descending order to establish which number of bins sorted by regression coefficient generates the model with the highest $r^2$ value. A PLS-R analysis can be then undertaken on the proportion of the metabolome identified by forward selection and the continuous variable, as opposed the entire metabolome.

#### *2.3.6.3 Identification of metabolites & pathway analysis*

Bins of interest, which may represent biomarkers, were identified for each statistically significant analysis. Weightings for each bin in multivariate analysis models were assigned, known as variable importance for projection (VIP) scores in OPLS-DA models and regression coefficients for PLS-R analysis models. VIP indicate how important each variable was in the data for predicting the classification, with VIP score of greater than one considered to be a potential biomarkers (167). With respect to regression coefficients from PLS-R models, potential biomarkers were identified using +/- 2 standard deviations of the mean regression coefficient of the entire dataset (168). NMR spectra were labelled using Chenomx NMR suite (Chenomx, professional version 8.5) (169) programme. The Human Metabolome Database version 4.0 (142) and published lists of metabolites detectable by NMR spectroscopy of serum (170) and urine (171) were also used for labelling spectra. NMR bins meeting the requisite for potential biomarkers were translated into a list of metabolites using labelled spectra. Each NMR bin could potentially represent several metabolites due to overlapping of metabolites on NMR spectra. Due to the number of metabolites, overlapping of metabolites in NMR spectra occurs to a greater extent in urine than serum.

Functional interpretation of the biomarkers implicated by the models was undertaken using MetaboAnalyst version 4 (172). A combination of both enrichment analysis and pathway analysis was used. Both analyses rely upon the identification of a metabolite as a biomarker, however they do not account for the direction of change of the metabolite. The enrichment analysis is an "over-representation" analysis. This tests whether a group of compounds involved in a pathway is enriched compared by random hits, thus are represented more than would be expected by chance. This analysis looks for of certain metabolites which are over-represented with respect to a reference metabolome (173). A hypergeometric test is used to generate a p value, which represents the probability of observing at least a specific number of metabolites from a certain metabolite set in a compound list. The fold enrichment value is generated by the number of metabolites identified in the list divided by the number of metabolites expected to be present as per the reference metabolome. If the value is greater than 1 than it is over-represented, however if the value is less than one then it is underrepresented.

Pathway analysis incorporates both over representation analysis as discussed above and pathway topological analysis to determine which pathways are more likely to be involved by considering the pathway structure. This works on the premise that key metabolites within a pathway may have a greater impact on the pathway or greater network than minor or relatively isolated metabolites. This analysis uses betweenness centrality to assess the importance, known as centrality, of points where pathways intersect within a network, known as nodes. Betweenness centrality uses an algorithm to

assess the shortest paths between nodes (174). This takes into account number of paths (count), size of the paths (proportion) and finally an index of overall network centralisation (175, 176). The resulting centrality measure is normalised by the sum importance of the pathways implicated. MetaboAnalyst gives each pathway a “total importance” score of 1, the importance measure of each metabolite node is expressed as a percentage with regards to the total pathway importance, and the pathway impact score is the cumulative percentage from the matched metabolite nodes.

# 3 NMR metabolomics pre-processing

$^{1}$H NMR spectroscopy yields information rich data sets which, if processed incorrectly can be subjected to systematic bias which is true of many complex types of data. Thus, correct processing of NMR data prior to data analysis, called pre-processing, is essential to accurately obtain useful information in any NMR-based metabolomic study. The data flow of an NMR experiment can be summarised as in Figure 3-1 below.

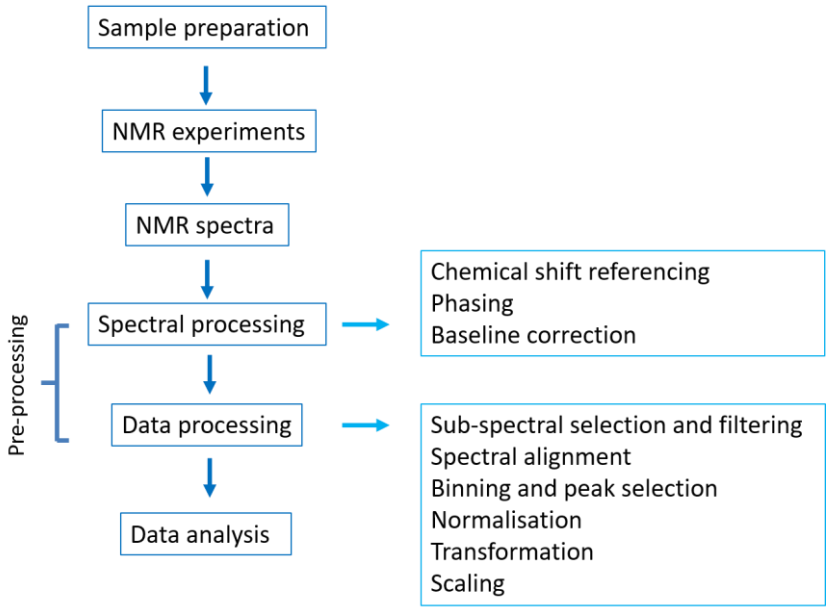



**Figure 3-1 Overview of NMR experiments**

## 3.1 Background

Pre-processing can be broadly separated into spectral processing and data processing. Spectral processing includes chemical shift referencing, phasing and baseline correction. Chemical shift referencing is important for peak alignment which is integral for multivariate analysis and compound identification (177). Typically, DSS-d6 and 3-(trimethylsilyl)-2,2',3,3'-tetradeuteropropionic acid (TSP) are used as chemical shift reference standards (178). Both have disadvantages to consider. Whilst TSP is pH sensitive and can thus result in problems with spectral alignment (179), DSS-d6 tends to bind to proteins and lipoproteins, which can result in inaccurate peak intensities (180).

Phasing is an NMR spectral alteration process that is intended to reveal the true absorptive amplitude and symmetry of all NMR peaks. An error in spectral phasing can result in inaccuracies that compound as further spectral processing proceeds, such as spectral malalignment, problems with binning and measured peak areas (179). Phasing can be done both automatically and manually. Automatic phasing is available on most NMR spectrometers/processing programmes and allows greater throughput and avoids operator bias, although it may result in distorting of smaller peaks. Whilst manual phasing is time consuming and may result in bias, it can maintain the integrity of smaller NMR peaks. It appears the most robust approach is combination of both techniques (177).

Baseline correction, as the name suggests, results in a semi-automated process carried out by software whereby spectral artefacts are removed to result in a flat baseline in signal free areas in the

spectra. Baseline correction in NMR spectra is normally a two-step process, which involves identifying areas of reliable baseline manually, followed by a computer-generated spline fit. These artefacts can be as a result of electronic distortions, inadequate digital filtering or incomplete digital sampling. Without baseline correction, it is possible that low abundance metabolites could have inaccuracies in their spectral peak quantification by a significant magnitude (177).

The data processing involves multiple steps which include sub-spectral selection and filtering, spectral alignment, binning and peak selection, normalisation, transformation and scaling. Sub-spectral selection is a filtering procedure which identifying and removing uninformative regions of an NMR spectra. Such regions include 0-0.6 ppm, which is the initial area of the spectra where no metabolites signals exist and 4.5-4.9 ppm, which is the water signal. After suppression of the water signal, there is a residual solvent signal which is not of interest and interferes with the analysis of other metabolites signals.

Spectral alignment is a process that iteratively move peak positions in multiple spectra so that the peaks relating to the same compounds can be overlaid or aligned. This allows comparison and quantification across multiple spectra and mitigates against a phenomenon called chemical shift drift. Chemical shift drift is an environmental effect which results in peaks corresponding to one metabolic signal can vary across multiple spectra, which may be as a result of sample pH, ionic strength, temperature, instrumental factors, degree of compound dilution and concentration of specific ions (181, 182). One approach to control for chemical shift is through standardising pH and salt concentration by adding a buffer to the sample. In addition, there are multiple software approaches to address chemical shift (183-186).

Binning NMR spectra is often done to decrease the complexity of datasets and involves dividing NMR spectra into small regions, which are sufficiently wide to include one or multiple peaks. The size of each bin is determined by calculating the area under the curve. Binning can be done in advance using prior knowledge of metabolite position or using an automated algorithm.

Normalisation is a process which controls for inherent concentration differences. This process is less important in biofluids where concentration is tightly regulated, whilst being critical in biofluids where volume is variable and thus subject to dilution effects, such as urine (187). Several methods to account for this variation have been proposed such as normalisation relative to the osmolality or creatinine, whilst other normalisation techniques utilise an algorithmic approach. Of the multiple numerical approaches for normalisation, total spectrum area (TSA) and probabilistic quotient normalization (PQN) are two commonly used for NMR metabolomics. In TSA, normalisation is undertaken by summing the entire spectral area excluding the regions comprising the water and urea resonances (188). Whereas PQN normalisation relies upon identification of a reference spectrum using median values. For each variable, the quotient of a given test spectrum and the reference spectrum is calculated and the median of all quotients is estimated. All variables of the test spectrum are then divided by the median quotient (189). The optimal normalisation technique is influenced by the type of biofluid and whether the experiment aims to discriminate two groups or to discover a biomarker. PQN has several advantages in that performs as robustly as other normalisation techniques (190), especially in biomarker discovery and it correlates with urinary osmolality and urine output and thus can control for dilution effects (177).

Scaling and transformation both aim to make the metabolomics data more normally distributed. Metabolite concentrations can range over several degrees of magnitude which can result in bias to multivariate analysis (191). Scaling includes dividing the data by a scaling factor, which is different for each variable that endeavours to attune for differences in concentration relative to the scaling factor.

This tends to inflate small values which could be the result of a measurement error. The scaling factor is commonly derived from either data dispersion, for instance data standard deviation or size measure such as the mean (192).

Transformation of data attempts to reduce heteroscedastic noise, which is generated as a result of different variables between subgroups, into homoscedastic information, that is where variables are similar across subgroups. This will enhance information contained in small peaks and assist in discriminant analysis (193). G-log is one of the several transformation techniques used in NMR metabolomics. The equation for g-log transformation is shown below, where y represents the untransformed data, λ is the transform parameter, and z is the transformed data.

$$z = In[y + \sqrt{(y^2 + \lambda)}]$$

Lambda (λ) is a parameter that affects the gradient of the function and thus the degree of the transformation. This parameter is specific to each type of biological sample and set of NMR conditions. One technique for elucidating the λ value is subjecting a single pooled biological sample to a maximum likelihood method (161, 194). However, this method was originally created for microarray data. Another disadvantage of calibrating the g-log parameters in this fashion is the reliance upon additional biological specimens to create the pooled sample. In those circumstances where biological specimens are limited, there appears to be no alternative method for calculating the λ value.

Pre-processing techniques are used to decrease bias and to enhance the value of the data in subsequent analysis. Many approaches have been used but it is unclear which methodology is most suitable to this dataset.

## 3.2 Aim & objective

The aim is:

To identify the optimal normalisation and transformation parameters for both serum and urine NMR metabolomics multivariate discriminant analysis

The objectives are as follows:

1) A range of generalised logarithmic transformations using the following transform parameters (λ) 1.00E-06, 4.00E-07, 1.00E-08, a computer-generated $\lambda$ or no g-log shall be applied to assess the impact on PCA and OPLS-DA of serum and urine $^{1}$H NMR metabolomics.
2) The effects of TSA and PQN normalisation techniques on PCA and OPLS-DA of serum $^{1}$H NMR metabolomics will be evaluated.

## 3.3 Methods

Sera (n=270) and urine (n=178) samples were available from patients presenting with DMARD naive inflammatory arthritis or CSA to the BEACON cohort for metabolomics analysis. The details of these patients have been described in **section 5.3** Patients below.

NMR data were acquired as described above in **section 2.3.5**. Spectra were read into Prometab (160) which is custom written software in Matlab (version 7, The Math Works, Natick, MA), and were truncated to a 0.8-10.0 ppm range. DSS-d6 was used to provide a chemical shift reference standard.

All aspects of spectral pre-processing (see Figure 3-1) were standardised with the exception of normalisation, for serum, and transformation, for both serum and urine. The baseline correction was undertaken using a spline baseline correction. All spectra had automated phasing applied to them followed by visual assessment of each spectra and manual phasing. Sub-spectral filtering was undertaken truncating spectra to a 0.8-10.0 ppm range and the water peak was also removed. Noise filtration was undertaken by selecting a flat part of spectra between 9-10 ppm. The noise value is calculated by analysing 20% of spectra, in the flat region and is set at 3 SD of spectrum points in the selected noise region. Once the noise threshold is set, this noise can also be filtered away. Spectra were aligned to the DSS-d6 signal. Spectra were segmented into 0.005 ppm (2.5Hz) chemical shift bins and the spectral areas within each bin were integrated. Urine was normalised to PQN, which controlled for any dilution effects. However filtered serum was subjected to TSA and PQN normalisation. Finally, a range of transformations were applied to metabolomics data including the default 1.00E-06 transform parameter ($\lambda$) for g-log transformation, 4.00E-07, 1.00E-08, a computer-generated $\lambda$ or no g-log application.

Once the pre-processing was complete, binned data were then compiled into a matrix, with each row representing an individual sample and subsequently uploaded to SIMCA. Systemic inflammation, as measured by CRP, has previously been shown to have a relationship with the serum metabolome (152) and urinary metabolome (195). Young et al showed that following categorisation of CRP into tertiles, early inflammatory arthritis patients in the lowest CRP tertile and the highest CRP tertile were discriminated using OPLS-DA analysis (152). Thus for each biofluid in the present study, the cohort was split into tertiles based upon their CRP. OPLS-DA was then undertaken on each biofluid with one tertile group having low CRP and the other having high CRP.

## 3.4 Results

The patient cohort was split into tertiles based upon CRP levels. OPLS-DA models of metabolic data derived from all patients' serum and urine using the low CRP and high CRP tertile groups with a combination of different g-log transformations and normalisation techniques, the results of which are displayed in Table 3-1 and Table 3-2 respectively.

**Table 3-1 Normalisation and generalised logarithmic transformations techniques using serum metabolic data.**

PCA and OPLS-DA models of metabolic data derived from all patients' sera using the lowest CRP and highest CRP tertile groups with a combination of different g-log transformations and normalisation techniques.

***λ***- transform parameter for generalised logarithmic transformation; **PC**– principal components; **PCA**- principal components analysis; **LV**– latent variables; **OPLS-DA**- orthogonal partial least square discriminant analysis

* p value calculated by analysis of variance testing cross validated predictive residuals

| Normalisation | $\lambda$ | PCs | PCA model $r^2$ | LV | OPLS-DA $r^2$ | p value* |
|---|---|---|---|---|---|---|
| PQN | 0 | 26 | 0.958 | 1+1 | 0.0999 | 0.647 |
| TSA | 0 | 29 | 0.878 | 1+2 | 0.152 | 0.148 |
| PQN | Optimise | 38 | 0.892 | 1+2 | 0.368 | <0.001 |
| TSA | Optimise | 32 | 0.89 | 1+2 | 0.35 | <0.001 |
| PQN | $1e^{-6}$ | 34 | 0.922 | 1+3 | 0.373 | <0.001 |
| TSA | $1e^{-6}$ | 32 | 0.89 | 1+2 | 0.35 | <0.001 |
| PQN | $4e^{-7}$ | 34 | 0.922 | 1+3 | 0.373 | <0.001 |
| TSA | $4e^{-7}$ | 35 | 0.9 | 1+2 | 0.351 | <0.001 |
| ***PQN*** | ***$1e^{-8}$*** | ***41*** | ***0.915*** | ***1+3*** | ***0.453*** | ***<0.001*** |
| TSA | $1e^{-8}$ | 32 | 0.89 | 1+2 | 0.349 | <0.001 |

**Table 3-2 Normalisation and generalised logarithmic transformations techniques using urinary metabolic data.**

PCA and OPLS-DA models of metabolic data derived from all patients' urine using the lowest CRP and highest CRP tertile groups with a combination of different g-log transformations.
***λ***- transform parameter for generalised log transformation; **PC**– principal components; **PCA**- principal components analysis; **LV**– latent variables; **OPLS-DA**- orthogonal partial least square discriminant analysis
* p value calculated by analysis of variance testing cross validated predictive residuals

| Normalisation | $\lambda$ | PCs | PCA model $r^2$ | LV | OPLS-DA $r^2$ | p value* |
|---|---|---|---|---|---|---|
| PQN | 0 | 29 | 0.709 | 1+1 | 0.05 | 1 |
| PQN | Optimise | 29 | 0.709 | 1+1 | 0.104 | <0.001 |
| PQN | $1e^{-}6$ | 28 | 0.704 | 1+1 | 0.118 | <0.001 |
| PQN | $4e^{-}7$ | 27 | 0.699 | 1+1 | 0.121 | <0.001 |
| **PQN** | **$1e^{-}8$** | **28** | **0.714** | **1+1** | **0.126** | **<0.001** |

Table 3-1 shows that no g-log transformation with PQN and TSA normalisation results in no separation between the high CRP tertile and low CRP tertile group in the OPLS-DA models of metabolic data derived from all patients' sera. Similarly, Table 3-2 shows no g-log transformation with PQN normalisation results in no separation between the high CRP tertile and low CRP tertile group in the OPLS-DA models of metabolic data derived from all patients' urine. These findings underscore the importance of applying a transformation before subjecting metabolic data to multivariate discriminant analysis. PQN normalisation with a λ of $1e^{-}8$ for g-log resulted in a statistically significant models with the highest $r^2$ values for both biofluids of all the pre-processing combinations investigated in this experiment. Therefore PQN normalisation and a λ value of $1e^{-}8$ will be applied for metabolomics experiments undertaken in the following chapters with accompanying pre-processing parameters as described in **section 3.3**.

## 3.5 Discussion

For both urinary and serum pre-processing, low value λ for g-log and thus greater g-log resulted in greater separation on OPLS-DA multivariate analysis of the metabolomics data. PQN normalisation of sera metabolomics data results in OPLS-DA models of greater $r^2$ values compared to TSA normalisation in serum. Evidence suggests that the type of pre-processing applied to each experiment should be bespoke for that data type and experimental model (196). The present study demonstrates the impact of the magnitude of logarithmic transformation has on discriminant analysis. Independent of what method is used to determine pre-processing parameters, it is important that a visual inspection of spectra is undertaken following the application of pre-processing to assess its effects.

One of the main limitations to this study is the normalisation and transformation was assessed using discriminant multivariate analysis of CRP values, which is an area I will investigate in more detail (**chapter 5**). Although as stated above (196) discerning pre-processing parameters discerned specifically for a given experiment is common practice, this methodology runs the risk of introducing bias for the subsequent experiment.

# 4 Fatigue in rheumatic conditions

Fatigue is common in patients with inflammatory rheumatic diseases with 80% of RA patients reporting clinically relevant levels at some stage during their illness (4). Patients have described their fatigue to be a “massive weariness” that is “almost the biggest symptom” of RA (197).

There are several different self-report tools that can be used to measure fatigue. Such PROMs capture information on self-reported symptoms, feelings and difficulties experienced by patients. Some PROMs are designed to be disease specific while others are generic instruments, having been developed for use in and validated across numerous diseases. Many of the PROMs developed in relation to fatigue, were validated originally in non-rheumatic conditions, predominately in cancer patients and neurological conditions.

## 4.1 PROMs used to measure fatigue

Multiple PROMs have been developed to measure fatigue and have been validated for use across many rheumatic conditions. To select the most appropriate fatigue PROM for a cohort of newly presenting CSA and inflammatory arthritis patients the following literature review was conducted. The main purpose of this review was to identify which PROMs had been validated to assess fatigue in rheumatic conditions and then to determine which of these PROMs have 1) favourable psychometric properties, 2) low response burden, and 3) are able to assess multiple dimensions of fatigue.

The search strategy used for each database is outlined in the appendix. Scales were assessed against strict inclusion and exclusion criteria also outlined in the appendix. The search conducted on pubmed, EMBASE, CINAHL, psychinfo identified 20’639 abstracts in total. These were screened by one author (GJ) and 1120 were identified where the focus of the paper was on a tool to measure fatigue. This identified 63 fatigue measures, 51 of which did not meet our inclusion criteria. The specific reason why each scale was not included is outlined in appendix. This left 12 PROMs which met our criteria, and which are discussed below.

The **fatigue severity score (FSS)** was originally validated in multiple sclerosis and SLE populations (198), and has since been used in systemic lupus erythematosus (SLE), RA, osteoarthritis (OA), ankylosing spondylitis (AS), joint hypermobility, Ehlers Danlos, Marfan syndrome, primary Sjögren's syndrome, systemic sclerosis, and chronic fatigue syndrome (CFS) (41, 49, 199-207). The FSS is a nine-item scale capturing data related to the severity and impact of fatigue. The FSS has been shown to have good internal consistency, reliability, criterion and construct validity with good responsiveness to change.

The **SF-36 Vitality subscale (SF-36 VT)** was developed from a general population including patients with a range of medical conditions (208). SF-36 VT has been used in studies of patients with RA, PsA, axial spondylitis, systemic sclerosis, primary Sjögren's syndrome, SLE, fibromyalgia, and OA (209-216). The SF-36VT has good construct validity, internal consistency and sensitivity to change. However, criterion validity and test-retest reliability are variable. In addition, there are apprehensions over the postulation that fatigue represents a poverty of energy levels. Vitality may be thought of as energy and drive and not merely the opposite of fatigue.

The **Profile of mood states (POMS)** (217) was developed from a cohort of healthy individuals and patients with psychiatric conditions. It was used originally to measure workforce health. It has been used in SLE and RA (47, 197, 218, 219). The POMS fatigue subscale (POMS-F) has good criterion validity with multi-dimensional assessment of fatigue (217) in RA patients; but little information can be found on divergent validity in rheumatic conditions. It is not used as frequently in rheumatic conditions as

the other scales presented in this review, however enough psychometric data are available to validate its use in RA. It has been used more extensively in the oncology population and has strong psychometric data in this population.

The **checklist individual strength fatigue subscale (CIS8R)** was developed to assess patients with CFS, and is part of a larger 20-item assessment of CFS patients, CIS20R (220). It is mainly used in CFS but has been used in other rheumatic conditions such as RA, fibromyalgia and sarcoidosis (221-223). The CIS8R has good internal consistency and reliability, construct and criterion validity, and sensitivity to change.

The **functional assessment of chronic illness therapy of fatigue (FACIT-F)** is a subscale of FACIT, a scoring system originally validated in a study assessing quality of life indicators in oncology patients with anaemia (224). It has since been used for many rheumatological conditions including PsA, Sjögren's syndrome, RA, systemic sclerosis and SLE (40, 209, 210, 215, 225-230). The FACIT-F has good responsiveness to change, criterion and construct validity, internal consistency and reliability.

The **fatigue visual analogue score (FVAS)** is a 100-mm horizontal VAS, anchored by 2 statements representing extreme ends of a single fatigue continuum and assess fatigue severity. The FVAS has a low respondent and administrative burden. It also has good test-retest reliability, construct and criterion validity.

The **fatigue assessment scale** was developed in 2004 in Holland, and assesses physical and mental fatigue separately with 5 questions each (231). It has been used predominately in sarcoidosis but has also been used in PsA (230, 232). It has shown good internal consistency, test-retest reliability and convergent validity. Outside of sarcoidosis it has not been used extensively in rheumatological cohorts.

The **multi-dimensional assessment of fatigue (MAF)**, developed from the Piper fatigue score, was originally developed using a cohort of RA patients (233) and comprise four dimensions- severity, distress, interference with activities of daily living, and frequency of symptoms. Subsequently, the score has been used for a variety of other rheumatological conditions including ankylosing spondylitis, OA, SLE, fibromyalgia, RA, systemic sclerosis and Sjögren's syndrome (209, 210, 216, 225, 234-238). It has been shown to have a good reliability, construct and criterion validity, internal consistency and it is responsive to change. However, there is limited assessment of the impact of ADLs.

Although originally validated in a cancer population, the **Multi-dimensional fatigue inventory (MFI-20)** (239) is a 20 item questionnaire that has been used in RA, fibromyalgia, CFS, anylosing spondylitis, Sjögren's syndrome, SLE and antineutrophil cytoplasmic antibodies associated vasculitis (209-211, 240-243). The score has multiple elements including physical fatigue, activity, motivation and mental fatigue. Construct and criterion validity are good, but internal consistency and test-retest reliability have generated mix-results. Studies using the MFI-20 have shown that a significant proportion of patients score either minimum or maximum scores, suggesting that there may be a significant ceiling and floor effects. Sensitivity to change was poor for rheumatic conditions.

The **Bristol Rheumatoid Arthritis Fatigue Multidimensional Questionnaire (BRAF-MDQ)** was developed to assess the experience and impact of fatigue in RA patients (244-246). It assesses physical fatigue, living with fatigue, cognitive fatigue and emotional fatigue. It is a RA specific scoring system. Internal consistency, test-retest reliability, and construct and criterion validity are good. The same study group created the Bristol rheumatoid arthritis fatigue numerical rating scales (BRAF-NRS) for severity, effect and coping. Like the BRAF-MDQ, the BRAF-NRS is RA specific and has good construct

and criterion validity, and good test-retest reliability. The coping subscale, however, showed weaker test-retest reliability.

The **Profile of Fatigue (ProF)** was developed to quantify fatigue in Sjögren's syndrome (247). It includes physical and mental fatigue items. In addition to Sjögren's syndrome it has been used in RA (248). It has excellent criterion validity and internal consistency. Test-reliability is only moderate.

The **Chalder fatigue questionnaire (CFQ)** was originally developed to assess fatigue severity in hospital and community populations (249). The questionnaire assesses physical and mental fatigue. The CFQ has been used in SLE, Sjögren's syndrome, fibromyalgia and CFS (250-253). This fatigue questionnaire's strength lies in the fact that it measures fatigue severity as opposed to impact or consequences, and it also has both physical and mental themes. Construct and criterion validity are only moderate in rheumatological conditions and variability has been seen in discriminant validity.

### 4.1.1 Summary of fatigue scales

This review of PROM-based instruments identified 12 tools that have been used to measure fatigue in patients with a range of rheumatic conditions. The quality and extent of psychometric validation varied among the scales.

Some scales offer the advantage of assessing many aspects of fatigue, however this is at the expense of more questions and thus increased response burden. In contrast scales which assess predominantly one aspect of fatigue, often physical, are quick to administer but at the expense of measuring the extent of other dimensions of fatigue, such as social and cognitive. Many of the fatigue PROMs discussed above have been minimally validated in rheumatic conditions. Fatigue PROMs have been used in several rheumatological conditions without being validated for that particular condition. Many fatigue PROMs such as SF-36, the fatigue assessment scale, the MAF, the MFI-20 and the CFQ were created before the current methodical approach of development and without consultation with patients.

According to one review of PROMs measuring fatigue in RA, the most comprehensive assessment of fatigue is by the FACIT-F (254), including an assessment of the impact on both cognition and behaviour. Furthermore, the original authors of the FACIT-F have been able to derive change in scale scores that correspond to minimum clinically significant differences (255). This application makes it an especially useful measure for intervention studies and the longitudinal assessment of fatigue. In addition to RA, FACIT-F has been used in a variety of other rheumatological conditions. This makes it an ideal PROM to assess fatigue in a diagnostically diverse cohort at multiple time points. The FVAS has the least respondent and administrative burden of all the PROMs of fatigue discussed and has been used to assess fatigue in several studies of rheumatic conditions. It may be beneficial to use two PROMs to assess fatigue, not only does this allow for cross validation of findings but the two PROMs may offer complementary advantages.

## 4.2 Fatigue in patients with newly presenting inflammatory arthritis and CSA

Many clinical parameters including depression, pain, sleep disturbance, smoking, alcohol consumption, low levels of physical activity, lack of social support, high disease activity and arthritis treatment have been linked to fatigue (38, 39, 44, 47-51).

Most studies relevant to inflammatory arthritis have assessed fatigue in patients with long standing disease on csDMARDs or bDMARDs (38, 39, 44, 47-51). In contrast, studies in patients with DMARD naive early arthritis are limited. One multicentre study of DMARD naive RA patients of a median symptom duration of 3 months (53), observed an association between fatigue, as assessed by SF-36 and FVAS, and young age, female gender, low education level, smoking, increased disease activity and disturbed sleep at baseline assessment. Those findings were supported by a study of patients with an RA duration of less than one year - the majority of whom had been exposed to DMARDs (54). Furthermore, a French study of early and pre-axial spondyloarthopathy showed that female gender, low education, disability scores and disease activity were all associated with fatigue as assessed by a numeric rating scale between 0 and 10 (55).

### 4.2.1 Aims & objectives

Unlike previous studies to date, we have included patients with a range of newly presenting peripheral inflammatory arthritides, with a range of different diagnoses and patients with CSA in an inception cohort and have assessed factors which associate with fatigue at presentation and with a change in fatigue over time. The specific aims are:

The aims are as follows:

1) To investigate the relationship between clinical parameters and fatigue in patients with newly presenting inflammatory arthritis and CSA.
2) To identify factors that correlate with changes in fatigue over time in these patients.

The objectives are as follows:

1) Clinical and demographic data will be collected from newly presenting CSA and inflammatory arthritis patients at baseline and then at a follow up of at least 12 months after the baseline visit.
2) Data collected from baseline attendance will be subjected to univariate and multivariate analyses to establish whether correlations exists between clinical parameters and fatigue.
3) Follow up data will be analysed using multinomial logistic regression techniques to determine which clinical parameters are associated with changes in fatigue.

### 4.2.2 Patients and Methods

We recruited patients presenting with DMARD naive inflammatory arthritis or CSA as outlined in **section 2.1**. Patients were recruited between January 2013 and June 2018. Follow up data were collected at the 12-month point, or the next visit following that if the patient did not attend for 12-month follow-up. A cross-sectional analysis identified factors associated with fatigue at initial presentation to secondary care and a longitudinal analysis identified factors that correlated with a change in fatigue over time.

The study was approved by the Black Country Research Ethics Committee and all patients gave written informed consent.

Various demographic and clinical data were collected as outlined in **section 2.1**, with FACIT-F and FVAS used as PROMs to assess for fatigue. The FVAS is a 100mm unidimensional visual analogue scale with 100 being the most fatigue experienced by the patient (256). The FACIT-F questionnaire was originally

formulated to assess fatigue in oncology patients with anaemia. It covers physical, functional, emotional and social consequences of fatigue using 13 items (224). There are 5 potential responses for each item from “not at all” to “very much” resulting in a score between 0 and 52; 0 represents the most severe fatigue experienced by the individual. Both the FVAS and FACIT-F scales have been used across a range of rheumatological conditions including spondyloarthropathies, Sjogren’s syndrome and RA (50, 257, 258). Fatigue assessment by a single visual analogue score has comparable sensitivity to change when compared with other fatigue scales (259) and has the advantage of being quick to administer. The FACIT-F has good responsiveness to change, criterion and construct validity, reliability and internal consistency (225).

### 4.2.3 Statistical Analysis

All continuous data were summarised as means and standard deviations (SD) or medians and interquartile ranges as appropriate. Normality was assessed using Q-Q plots. Changes in fatigue were also evaluated, calculating the proportion of patients with improved, unchanged, or worsened fatigue according to the minimum clinically important difference (MCID) in RA as -4 to +4 for the FACIT-F (225).

Factors associated with fatigue at baseline were analysed by analysis of variance or Pearson’s correlation coefficient if the data were normally distributed. Non-parametric data were analysed using the Kruskal-Wallis test or Spearman’s rank correlation. A multinomial logistic regression model was used to analyse factors associated with change in fatigue at follow up. Statistical significance was set at a p value less than 0.05. Data were analysed using IBM SPSS Statistics 20.0 (SPSS, Chicago, IL).

### 4.2.4 Results

739 patients were recruited with baseline diagnoses shown in Table 4-1. Patients’ demographic and clinical data are shown in Table 4-2.

**Table 4-1 Diagnoses of the 739 patients at time of recruitment**

*Seropositive is defined as positive for either rheumatoid factor or anti-citrullinated protein antibody

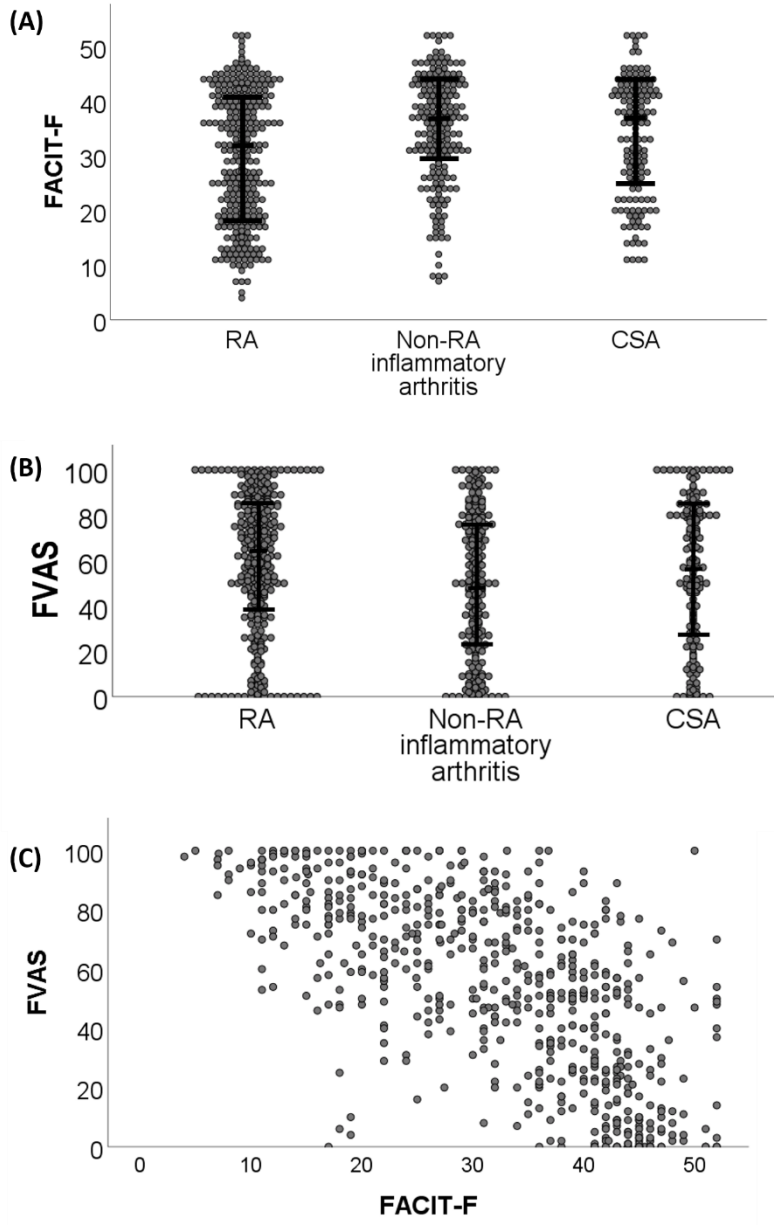
| ***Diagnosis*** | ***Number (percentage)*** |
|---|---|
| **Rheumatoid arthritis** | **352 (47.6)** |
| Seropositive* | 222 (30) |
| Seronegative | 130 (17.6) |
| **Other arthritis** | **230 (31.1)** |
| Unclassified arthritis | 132 (17.9) |
| Psoriatic arthritis | 64 (8.7) |
| Drug-induced arthropathy | 11 (1.5) |
| Gout | 8 (1.1) |
| Peripheral spondyloarthopathy | 7 (0.9) |
| Post infectious arthritis | 6 (0.8) |
| Sarcoidosis related arthritis | 1 (0.1) |
| Remitting seronegative symmetrical synovitis with pitting oedema | 1 (0.1) |
| **Clinically suspect arthralgia** | **157 (21.3)** |
| Seropositive* | 60 (8.1) |
| Seronegative | 97 (13.2) |

**Table 4-2 Demographic and clinical data of patients by diagnosis**

**RA**- rheumatoid arthritis; **CSA**- clinically suspect arthralgia; **SD**- standard deviation; **IQR**- interquartile range; **DAS28ESR**- disease activity score 28 with erythrocyte sedimentation rate; **DAS28CRP**- disease activity score 28 with C-reactive protein; **FVAS**- fatigue visual analogue scale; **FACIT-F**- functional assessment of chronic illness therapy fatigue subscale; **VAS-P**- visual analogue scale pain; **PHQ-9**- patient health questionnaire-9; **HAQ**- health assessment questionnaire; **ACPA**- anti-citrullinated protein antibody.

| | All patients (n=739) | RA (n=352) | Non-RA inflammatory arthritis (n=230) | CSA (n=157) |
|---|---|---|---|---|
| Age, mean +/- SD (years) | 50.9 +/- 15.3 | 53.5 +/- 15 | 50.4 +/- 16 | 45.7 +/- 13.5 |
| Missing (%) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Sex, female | 472 (63.9) | 240 (68.2) | 117 (50.9) | 115 (73.2) |
| Missing (%) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Highest education level | | | | |
| Primary | 90 (12.2) | 52 (14.8) | 28 (12.2) | 10 (6.4) |
| Secondary | 371 (50.2) | 177 (50.3) | 109 (47.4) | 85 (54.1) |
| Tertiary | 202 (27.3) | 83 (23.6) | 69 (30) | 50 (31.8) |
| Missing (%) | 76 (10.3) | 40 (11.4) | 24 (10.4) | 12 (7.6) |
| Employment status | | | | |
| Full time | 353 (47.8) | 146 (41.5) | 120 (52.2) | 87 (55.4) |
| Part time | 49 (6.6) | 19 (5.4) | 14 (6.1) | 16 (10.2) |
| None | 311 (42.1) | 171 (48.6) | 92 (40) | 48 (30.6) |
| Missing (%) | 26 (3.5) | 16 (4.5) | 4 (1.7) | 6 (3.8) |
| Smoking | | | | |
| Never | 351 (47.5) | 172 (48.9) | 102 (44.3) | 77 (49) |
| Ever | 235 (31.8) | 106 (30.1) | 82 (35.7) | 47 (29.9) |
| Current | 147 (19.9) | 72 (20.5) | 43 (18.7) | 32 (20.4) |
| Missing (%) | 6 (0.8) | 2 (0.6) | 3 (1.3) | 1 (0.6) |
| DAS28ESR, mean+/-SD | 4.4 +/- 1.6 | 5.3+/- 1.3 | 3.8 +/- 1.4 | 3.3 +/- 1.2 |
| Missing (%) | 41 (5.5) | 18 (5.1) | 18 (7.8) | 5 (3.2) |
| DAS28CRP,mean+/-SD | 4.2 +/- 1.4 | 5 +/- 1.2 | 3.8 +/- 1.2 | 3.2+/- 1 |
| Missing (%) | 68 (9.2) | 34 (9.7) | 21 (9.1) | 13 (8.3) |
| Symptom Duration, median (IQR) (weeks) | 26 (13-55) | 25 (13-52) | 24.5 (13-57) | 32 (18-74.5) |
| Missing (%) | 6 (0.8) | 3 (0.9) | 2 (0.9) | 1 (0.6) |
| FVAS, median (IQR) | 58 (29-80.5) | 63 (41-84.3) | 50 (23-76) | 57 (28-81) |
| Missing (%) | 18 (2.4) | 14 (4) | 2 (0.9) | 2 (1.3) |
| FACIT-F, median (IQR) | 33.3 (23-42) | 31 (19-41) | 36 (28-43) | 35 (25-42) |
| Missing (%) | 85 (11.5) | 43 (12.2) | 32 (13.9) | 10 (6.4) |
| VAS-P, median (IQR) | 58 (31-78) | 60 (35-79) | 59 (29-77) | 54 (25.5-74.5) |
| Missing (%) | 57 (7.7) | 38 (10.8) | 15 (6.5) | 4 (2.5) |
| PHQ-9, median (IQR) | 7 (3-13) | 9 (4-15) | 6 (2-12) | 6.5 (2-10) |
| Missing (%) | 76 (10.3) | 34 (9.7) | 31 (13.5) | 11 (7) |
| HAQ, median (IQR) | 0.9 (0.4-1.5) | 1.3 (0.6-1.9) | 0.8 (0.3-1.3) | 0.6 (0.2-1.3) |
| Missing (%) | 86 (11.6) | 44 (12.5) | 31 (13.5) | 11 (7) |
| Vitamin D (total) | | | | |
| Deficient: <30nmol/L | 155 (21) | 67 (19) | 47 (20.4) | 41 (26.1) |
| Insufficient:30-50nmol/L | 163 (22.1) | 77 (21.9) | 53 (23) | 33 (21) |
| Sufficient: >50nmol/L | 236 (31.9) | 112 (31.8) | 74 (32.2) | 50 (31.8) |

| | | | | |
|---|---|---|---|---|
| Missing (%) | 185 (25) | 96 (27.3) | 56 (24.3) | 33 (21) |
| Haemoglobin, mean +/- SD (gm/dl) | 132.5 +/- 15.3 | 130.2 +/- 14.4 | 134.4 +/- 16.2 | 135 +/- 15.1 |
| Missing (%) | 11 (1.5) | 8 (2.3) | 2 (0.9) | 1 (0.6) |
| C-reactive protein, median (IQR)(mg/litre) | 6 (3-14) | 8 (3-17.8) | 6 (2-14) | 4 (1-7) |
| Missing (%) | 7 (0.9) | 4 (1.1) | 3 (1.3) | 0 (0) |
| Erythrocyte sedimentation rate, median (IQR) (mm/hr) | 18 (9-32.3) | 23 (12-38) | 14 (7-29.5) | 15 (6-26) |
| Missing (%) | 37 (5) | 16 (4.5) | 17 (7.4) | 4 (2.5) |
| Rheumatoid factor positive | 270 (36.5) | 199 (56.5) | 25 (10.9) | 46 (29.3) |
| Missing (%) | 12 (1.6) | 5 (1.4) | 4 (1.7) | 3 (1.9) |
| ACPA positive | 249 (33.7) | 195 (55.4) | 7 (3) | 47 (29.9) |
| Missing (%) | 8 (1.1) | 3 (0.9) | 4 (1.7) | 1 (0.6) |




| FACIT-F median (IQR) | | p value* |
|---|---|---|
| RA | Non-RA IA | |
| 31 (19-41) | 36 (28-43) | <0.001 |
| RA | CSA | |
| 31 (19-41) | 35 (25-42) | 0.023 |
| CSA | Non-RA IA | |
| 35 (25-42) | 36 (28-43) | 0.195 |

| FVAS median (IQR) | | p value* |
|---|---|---|
| RA | Non-RA IA | |
| 63 (41-84) | 50 (23-76) | <0.001 |
| RA | CSA | |
| 63 (41-84) | 57 (28-81) | 0.183 |
| CSA | Non-RA IA | |
| 57 (28-81) | 50 (23-76) | 0.066 |

**Figure 4-1 Distribution of fatigue scores**

Scatter plots showing the distributions of (A) FACIT-F scores across diagnoses with IQR and median, and (B) FVAS scores across diagnoses with IQR and median. (C) Scatter plot showing the relationship between the FACIT-F and the FVAS across the entire cohort (Spearman's rank correlation coefficient: -0.684, p<0.001)

**FACIT-F**- functional assessment of chronic illness therapy fatigue subscale; **RA**- rheumatoid arthritis; **Non-RA IA**- non rheumatoid arthritis inflammatory arthritis; **CSA**- clinically suspect arthralgia; **IQR**- interquartile range; **FVAS**- fatigue visual analogue scale

* p value calculated using Kruskal-Wallis test

Figure 4-1A & B shows the distribution of both fatigue scores in patients with RA, non-RA inflammatory arthritis and CSA. Figure 4-1C showed a statistically significant correlation between the FACIT-F and FVAS (Spearman's rank correlation coefficient -0.684; p <0.001). The median FACIT-F scores were 31, 36 and 35 for the RA, non-RA inflammatory arthritis and CSA patients, respectively. The median FVAS scores were 63, 50 and 57 for the RA, non-RA inflammatory arthritis and CSA patients, respectively. A

diagnosis of RA was associated with greater fatigue than non-RA inflammatory arthritis ($p < 0.001$) and CSA ($p=0.023$) measured by FACIT-F, whilst similar results were found using FVAS. Fatigue levels in patients with CSA did not differ from patients with non-RA inflammatory arthritis patients as measured by FACIT-F ($p=0.195$) or FVAS ($p=0.066$).

Table 4-3 shows univariate analysis of data from inflammatory arthritis and CSA patients. There were positive correlations between measures of greater fatigue (for both FACIT-F and FVAS) and female sex, PHQ-9, VAS-P, HAQ, haemoglobin, ESR, DAS28ESR, DAS28CRP, swollen joint count (SJC) 66 and 28 and tender joint count (TJC) 68 and 28 in inflammatory arthritis patients. In CSA patients, female sex, seronegative antibody status, PHQ-9, VAS-P, HAQ, DAS28ESR, DAS28CRP, TJC 68 and 28 all correlated with fatigue as measured by FACIT-F and FVAS.

**Table 4-3 Associations with fatigue at baseline for patients with inflammatory arthritis and CSA: Univariate analysis**

**N**- number; **RA**- rheumatoid arthritis; **CSA**-clinically suspect arthralgia; **SD**- standard deviation; **IQR**- interquartile range; **DAS28ESR**- disease activity score 28 with erythrocyte sedimentation rate; **DAS28CRP**- disease activity score 28 with C-reactive protein; **FVAS**- fatigue visual analogue scale; **FACIT-F**- functional assessment of chronic illness therapy fatigue subscale; **SJC66**- swollen joint count 66, **SJC28**- swollen joint count 28, **TJC68**- tender joint count 68, **TJC28-** tender joint count 28; **VAS-P**- visual analogue scale pain; **PHQ-9**- patient health questionnaire-9; **HAQ**- health assessment questionnaire; **CRP**- C reactive protein; **ESR**- erythrocyte sedimentation rate; **ACPA**- anti-citrullinated protein antibody; **RF**- rheumatoid factor

* p value calculated using Spearman's rank correlation, ¶ p value calculated using Mann-Whitney U test, † p value calculated using Kendall's Tau-b, ↕ p value calculated using Kruskal-Wallis test

| | Inflammatory arthritis (n=582) | | | | | | CSA (n=157) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | FACIT-F (n=507; 75 missing) | | | FVAS (n=566; 16 missing) | | | FACIT-F (n=147; 10 missing) | | | FVAS (n=155; 2 missing) | | |
| | N. | Correlation coefficient OR median (IQR) | P value | N. | Correlation coefficient OR median (IQR) | P value | N. | Correlation coefficient OR median (IQR) | P value | N. | Correlation coefficient OR median (IQR) | P value |
| Age (years) | 506 | 0.151 | **0.001*** | 565 | -0.057 | 0.176* | 147 | 0.074 | 0.372* | 157 | 0.017 | 0.831* |
| Sex | 507 | | **<0.001¶** | 566 | | **<0.001¶** | 147 | | **0.018¶** | 155 | | **0.012¶** |
| Female | 306 | 31 (20.75-41) | | 347 | 65 (37-84) | | 105 | 33 (22-41) | | 115 | 61 (37-81) | |
| Male | 201 | 36 (28.5-43) | | 219 | 51 (22-75) | | 42 | 41 (29-44) | | 40 | 40.5 (13-80) | |
| Highest education level | 451 | | 0.203† | 513 | | **0.032†** | 135 | | 0.147† | 144 | | 0.392† |
| Primary | 68 | 33 (21.25-43) | | 80 | 62 (36-82) | | 10 | 26.5 (21-42) | | 10 | 71.5 (58-81) | |
| Secondary | 254 | 32 (21-41) | | 284 | 62(37.25-84) | | 80 | 33 (22.5-41) | | 84 | 56.5 (31-81) | |
| Tertiary | 129 | 36 (25-42) | | 149 | 57 (21-77.5) | | 45 | 38 (25.5-43) | | 50 | 57 (20-87) | |
| Employment status | 490 | | **0.08↕** | 556 | | 0.45↕ | 141 | | 0.948↕ | 150 | | 0.847↕ |
| None | 224 | 32 (21-42) | | 262 | 63.5 (36-84) | | 47 | 36 (22-43) | | 47 | 57 (21-81) | |
| Part time | 30 | 38 (30.8-43) | | 33 | 54 (25-78) | | 15 | 31.4 (28-42) | | 16 | 69 (36-81) | |
| Full time | 236 | 34 (24-41) | | 261 | 57 (25-77) | | 79 | 34 (26-41) | | 87 | 57 (29-82) | |
| Smoking | 502 | | 0.084↕ | 561 | | 0.379↕ | 146 | | 0.713↕ | 154 | | 0.841↕ |
| Current | 100 | 31.21 (21-39) | | 110 | 61 (42-86) | | 30 | 32 (24-42) | | 32 | 61 (41.5-82) | |
| Ever | 161 | 35 (25-42.63) | | 185 | 58 (29-81) | | 46 | 33 (23.5-41) | | 45 | 60 (28-80) | |
| Never | 241 | 33 (22-41.5) | | 266 | 59 (27-80) | | 70 | 37 (27-42) | | 77 | 52 (25-82) | |
| Diagnosis | 507 | | **<0.001↕** | 566 | | **<0.001↕** | -- | -- | -- | -- | -- | -- |
| RA | 309 | 31 (19-41) | | 338 | 63 (41-84) | | | | | | | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Other arthritis | 198 | 36 (28-43) | | 228 | 50 (23-76) | | | | | | | |
| DAS28 ESR | 477 | -0.427 | **<0.001*** | 531 | 0.421 | **<0.001*** | 142 | -0.339 | **<0.001*** | 151 | 0.359 | **<0.001*** |
| DAS28 CRP | 455 | -0.401 | **<0.001*** | 513 | 0.381 | **<0.001*** | 134 | -0.411 | **<0.001*** | 143 | 0.41 | **<0.001*** |
| Swollen joint count (SJC) | | | | | | | -- | -- | -- | -- | -- | -- |
| SJC66 | 507 | -0.229 | **<0.001*** | 566 | 0.204 | **<0.001*** | | | | | | |
| SJC28 | 507 | -0.183 | **<0.001*** | 566 | 0.174 | **<0.001*** | | | | | | |
| Tender joint count (TJC) | | | | | | | | | | | | |
| TJC68 | 507 | -0.409 | **<0.001*** | 566 | 0.346 | **<0.001*** | 147 | -0.397 | **<0.001*** | 155 | 0.342 | **<0.001*** |
| TJC28 | 507 | -0.385 | **<0.001*** | 566 | 0.318 | **<0.001*** | 147 | -0.359 | **<0.001*** | 155 | 0.342 | **<0.001*** |
| Symptom Duration (weeks) | 503 | -0.049 | 0.268* | 561 | 0.087 | **0.039*** | 146 | -0.150 | 0.071* | 154 | 0.125 | 0.122* |
| VAS-P | 459 | -0.247 | **<0.001*** | 518 | 0.254 | **<0.001*** | 144 | -0.334 | **<0.001*** | 152 | 0.382 | **<0.001*** |
| PHQ-9 | 503 | -0.806 | **<0.001*** | 502 | 0.623 | **<0.001*** | 146 | -0.737 | **<0.001*** | 144 | 0.524 | **<0.001*** |
| HAQ | 492 | -0.559 | **<0.001*** | 492 | 0.451 | **<0.001*** | 145 | -0.524 | **<0.001*** | 144 | 0.382 | **<0.001*** |
| Haemoglobin (gm/dl) | 498 | 0.09 | **0.045*** | 559 | -0.109 | **0.01*** | 146 | -0.005 | 0.951* | 154 | -0.05 | 0.538* |
| CRP (mg/L) | 501 | -0.045 | 0.316* | 559 | 0.076 | 0.072* | 147 | -0.064 | 0.438* | 155 | 0.125 | 0.122* |
| ESR (mm/hr) | 480 | -0.109 | **0.017*** | 533 | 0.146 | **0.001*** | 143 | -0.036 | 0.666* | 151 | 0.097 | 0.237* |
| RF or ACPA | 506 | | 0.447¶ | 565 | | 0.46¶ | 147 | | **0.048¶** | 155 | | **0.019¶** |
| positive | 222 | 32.75 (22-42) | | 242 | 58.5 (34-83) | | 57 | 38 (27.5-43) | | 58 | 48.5 (21-78) | |
| negative | 284 | 33.79 (24-42) | | 323 | 59 (27-79) | | 90 | 32.5 (22-41) | | 97 | 66 (38.5-84) | |
| Vitamin D (nmol/L) | 368 | 0 | 1* | 422 | -0.009 | 0.853* | 115 | 0.064 | 0.494* | 123 | -0.031 | 0.732* |

Multivariate analysis (Table 4-4) showed that elevated PHQ-9 and HAQ scores were independently associated with low FACIT-F (ie high fatigue) scores in both the inflammatory arthritis patients and the CSA patients. In addition, the TJC 68 was also independently associated with low FACIT-F scores in inflammatory arthritis patients. In the inflammatory arthritis patients, a general linear model built using HAQ, PHQ-9 and TJC 68 generated an $r^2$ value of 0.666 whilst in CSA patients a general linear model built using HAQ and PHQ-9 generated an $r^2$ value of 0.539. Therefore, whilst these general linear models explain over half of the variability of observed fatigue, a sizeable proportion of fatigue remains unexplained. Iteratively adding parameters to this model did not appreciably optimise the $r^2$ value.

**Table 4-4 Factors associated with fatigue at baseline as measured by FACIT-F: multivariate analysis**

**CSA**- clinically suspect arthralgia; **FACIT-F**- functional assessment of chronic illness therapy fatigue subscale; **PHQ-9**- patient health questionnaire-9; **HAQ**- health assessment questionnaire; **TJC68**- tender joint count score 68; **β**- regression coefficient; **SE**- standard error

*p-value calculated using the F-test

| | ***Inflammatory arthritis*** | | ***CSA*** | |
|---|---|---|---|---|
| | *β (SE)* | *P value** | *β (SE)* | *P value** |
| ***PHQ9*** | -1.153 (0.057) | **<0.001** | -1.118 (0.120) | **<0.001** |
| ***HAQ*** | -2.026 (0.495) | **<0.001** | -2.743 (1.102) | **0.014** |
| ***TJC68*** | -0.092 (0.024) | **<0.001** | N/A | N/A |
| ***$r^2$value for model*** | 0.666 | | 0.539 | |

Further analysis was undertaken on patients with baseline diagnoses of UA and with CSA to investigate the relationship between baseline fatigue and final diagnostic outcome (Table 4-5). A one-way analysis of variance showed no difference in the baseline fatigue in patients with UA who persisted with UA (n=44) at follow up compared with those who developed RA (n=16) or resolved (n=13). Furthermore, an independent t-test showed no statistically significant difference (p=0.055) between fatigue in patients with CSA who did not develop inflammatory arthritis at follow-up (n=84) compared with those who did (n=20). However, of the 84 patients with CSA who did not develop arthritis during follow up, 23 had been commenced on csDMARDs subsequent to their baseline visit (15 hydroxychloroquine, 2 methotrexate, 1 on prednisolone and 5 on combination therapies) which may have reduced the chances of the development of clinically apparent inflammatory arthritis in these patients.

**Table 4-5 Analysis of baseline FACIT-F score according to diagnoses at follow up**
**FACIT-F**- functional assessment of chronic illness therapy fatigue subscale; **IQR**- Interquartile range; **UA**- unclassified arthritis; **RA**- rheumatoid arthritis
* p value calculated using Kruskal-Wallis test

| Baseline diagnosis | Final diagnosis | Number | Median baseline FACIT-F (IQR) | P value* |
|---|---|---|---|---|
| Unclassified arthritis | Persistent UA | 44 | 37.5 (27.8-42.3) | 0.966 |
| | RA | 16 | 36 (26-46) | |
| | Resolved | 13 | 34 (30-43) | |
| Clinically suspect arthralgia | No inflammatory arthritis | 84 | 32 (22-41) | 0.055 |
| | Inflammatory arthritis | 20 | 38.5 (33.4-43) | |

Of the 739 patients recruited at baseline, 440 patients had complete data collected at follow up, whilst 299 patients had incomplete or no data collected at follow up. Supplementary table 1 (located in the appendix) shows baseline clinical and demographic data of patients with complete follow up data compared with patients without follow up data. There were no statistically significant differences between these two subgroups.

At 1 year follow up, analysis of FACIT-F scores revealed that 42.8% of patients had less fatigue (n=145), 30.1% (n=102) had the same level of fatigue whilst 27.1% (n=92) reported worse fatigue. Table 4-6 shows the univariate analysis to assess the relationship between the change in clinical parameters at follow up and the change in FACIT-F at follow up. A negative correlation was seen between change in DAS28 CRP and change in FACIT-F at follow up, thus increased disease activity as measured by DAS28 CRP at follow up is associated with increased fatigue at follow up as measured by FACIT-F. Likewise, negative correlations were also seen with change in FACIT-F and change in TJC 68, TJC 28, VAS-P, PHQ-9 and HAQ, thus an increase in the number of tender joints worsening pain, mood and function at follow up were associated with increased fatigue at follow up.

**Table 4-6 Univariate analysis of the relationship between the change in follow-up parameters and the change in fatigue measured using FACIT-F at follow up in inflammatory arthritis patients**

**FACIT-F**- functional assessment of chronic illness therapy fatigue subscale; **IQR**- interquartile range; **SD**- standard deviation; **DAS28ESR**- disease activity score 28 with erythrocyte sedimentation rate; **DAS28CRP**- disease activity score 28 with C-reactive protein; **SJC66**- swollen joint count 66; **SJC28**- swollen joint count 28; **TJC68**- tender joint count 68; **TJC28-** tender joint count 28; **VAS-P**- visual analogue scale pain; **PHQ-9**- patient health questionnaire-9; **HAQ**- health assessment questionnaire; **CRP**- C reactive protein; **ESR**- erythrocyte sedimentation rate

* p value calculated using Spearman's rank correlation.

| | Number | Baseline mean +/- SD OR median (IQR) | Follow up mean +/-SD OR median (IQR) | Correlation coefficient | P value* |
|---|---|---|---|---|---|
| DAS28ESR,<br>Missing | 199 | 4.7 +/- 1.5<br>36 (6.2%) | 3.5 +/- 1.6<br>97 (26.5%) | -0.122 | 0.086 |
| DAS28CRP<br>Missing | 209 | 4.5 +/- 1.3<br>55 (9.5%) | 3.3 +/- 1.4<br>86 (23.5%) | -0.153 | **0.027** |
| Swollen joint count | | | | | |
| SJC66<br>Missing | 259 | 4 (2-8)<br>0 | 1 (0-3)<br>2 (0.5%) | -0.062 | 0.322 |
| SJC28<br>Missing | 257 | 3 (1-7)<br>0 | 1 (0-3)<br>2 (0.5%) | -0.067 | 0.284 |
| Tender joint count | | | | | |
| TJC68<br>Missing | 259 | 12 (4-22)<br>0 | 5 (1-17)<br>2 (0.5%) | -0.140 | **0.025** |
| TJC28<br>Missing | 258 | 7 (3-13)<br>0 | 3 (0-10)<br>2 (0.5%) | -0.142 | **0.022** |
| VAS-P<br>Missing | 252 | 60 (33.5-78)<br>53 (9.1%) | 34 (13-63)<br>16 (4.4%) | -0.175 | **0.005** |
| PHQ-9<br>Missing | 260 | 8 (3-14)<br>65 (11.2%) | 5.8 (2-11)<br>80 (21.9%) | -0.293 | **<0.001** |
| HAQ<br>Missing | 260 | 1 (0.5-1.6)<br>75 (12.9%) | 0.6 (0.1-1.4)<br>66 (18%) | -0.176 | **0.004** |
| CRP (mg/litre)<br>Missing | 231 | 7 (3-17)<br>7 (1.2%) | 3 (0-6.8)<br>54 (14.8%) | 0.075 | 0.254 |
| ESR (mm/hr)<br>Missing | 217 | 19 (10-34)<br>33 (5.7%) | 11 (5-21)<br>71 (19.4%) | 0.075 | 0.269 |

Multinomial logistic regression analysis was undertaken to identify factors associated with changes in fatigue over time in the inflammatory arthritis patients. Specifically, comparisons were made between patients in a reference group (with no change in fatigue) and patients whose fatigue either worsened or improved. A multinomial logistic regression analysis showing the relationship between baseline clinical parameters and the change in FACIT-F is shown in Table 4-7. The change in FACIT-F was calculated by subtracting the baseline FACIT-F ($FACIT\text{-}F_{baseline}$) from the follow up FACIT-F ($FACIT\text{-}F_{follow\text{-}up}$). A MCID of 4 was used for the FACIT-F (225), so an increase in FACIT-F of greater than 4 was

categorised as an “improved FACIT-F” and a decrease in FACIT-F of greater than 4 was categorised as “worse FACIT-F”.

Table 4-7 shows two multinomial logistic regression analyses showing the relationship between change in FACIT-F at follow up and i) change in clinical parameters at follow up (model A) and ii) improvement of DAS28 CRP of ≥1.2 (model B). To adjust for “change score and baseline score effect”, baseline FACIT-F was incorporated into both models (260). This effect occurs due to a statistical phenomenon known as regression to the mean (261). If an extreme measure is observed at baseline thus there is an increased likelihood of a decrease of FACIT-F score at follow up (262). Increased PHQ9 at follow up was associated with a worse FACIT-F score at follow up (p<0.001). Finally, those patients who showed an improvement of DAS28 CRP were less likely to have worse fatigue at follow up (p=0.018).

**Table 4-7 Multinomial logistic regression model of follow up parameters and their impact on change in fatigue in inflammatory arthritis patients.**

Model A assesses the association between VAS-P, HAQ and PHQ-9 measured at follow up (F/U) and the change in FACIT-F. Whilst model B assesses the association between change in DAS28 CRP and the change in FACIT-F.

**FACIT-F**- functional assessment of chronic illness therapy fatigue subscale; **PROM**-patient reported outcome measure; **Δ**- the change in; **F/U-** follow up; **VAS-P**- visual analogue scale pain; **HAQ**- health assessment questionnaire; **PHQ-9**- patient health questionnaire-9; **DAS28CRP**- disease activity score 28 with C-reactive protein

* p value calculated using Wald test. ¶ Change in FACIT-F is calculated using the formula FACIT-Ffollow-up– FACIT-Fbaseline. Using a minimum clinically important difference as -4 to +4 for the FACIT-F (226), an “improved FACIT-F” is defined as an increase in FACIT-F of greater than 4 and a “worse FACIT-F” is defined as a decrease in FACIT-F of greater than 4. ↕ calculated using the formula VAS-Pfollow-up– VAS-Pbaseline. † calculated using the formula HAQfollow-up– HAQbaseline. ¥ calculated using the formula PHQ-9follow-up– PHQ-9baseline. ǂ “Improvement in DAS28CRP” is defined as an increase of DAS28CRP of ≥1.2 from baseline.

| FACIT-F Change (improved/worse) | | Odds ratio | P value* | Confidence interval (95%) | |
|---|---|---|---|---|---|
| | | | | Lower Bound | Upper Bound |
| Model A Follow up PROMs | | | | | |
| Improved FACIT-F¶ | Δ VAS-P↕ | 0.998 | 0.727 | 0.987 | 1.009 |
| | Δ HAQ† | 0.678 | 0.203 | 0.373 | 1.233 |
| | Δ PHQ-9¥ | 0.858 | **<0.001** | 0.788 | 0.935 |
| | Baseline FACIT-F | 0.947 | **0.002** | 0.915 | 0.980 |
| Worse FACIT-F¶ | Δ VAS-P↕ | 1.002 | 0.647 | 0.993 | 1.012 |
| | Δ HAQ† | 1.562 | 0.141 | 0.862 | 2.829 |
| | Δ PHQ-9¥ | 1.161 | **<0.001** | 1.074 | 1.254 |
| | Baseline FACIT-F | 1.036 | 0.076 | 0.996 | 1.077 |
| Model B Change in DAS28 CRP | | | | | |
| Improved FACIT-F¶ | Improvement in DAS28CRPǂ | 1.563 | 0.237 | 0.746 | 3.274 |
| | Baseline FACIT-F | 0.936 | **<0.001** | 0.905 | 0.969 |
| Worse FACIT-F¶ | Improvement in DAS28CRPǂ | 0.395 | **0.018** | 0.183 | 0.853 |
| | Baseline FACIT-F | 1.035 | 0.097 | 0.994 | 1.078 |

### 4.2.5 Discussion

To our knowledge, to date there has been no cross-sectional analysis of fatigue in patients presenting with new onset inflammatory arthritides or arthralgia across a range of diagnostic categories. Furthermore, most studies assessing fatigue in patients with inflammatory arthritis have involved patients with longstanding disease. Fatigue in patients with early inflammatory arthritis and in those with CSA remains a poorly explored area. In addition to representing a pathologically distinct entity during disease evolution (263), early RA is also an important time for patients as they come to terms with their symptoms and the chronic nature of the disorder and its treatment. Whilst our cohort included patients with newly presenting RA and those at risk of RA, we have also captured data from patients with other forms of newly presenting inflammatory arthritis, providing a more complete view of fatigue in patients with inflammatory arthritis as they first present to rheumatologists (264).

The median FACIT-F score of 33 (23-42) (IQR) for inflammatory arthritis patients and 35 (25-42) for CSA patients, indicated greater fatigue than that seen in the general population in the United States (43.6 +/- 9.4) (265), but similar levels to that seen in established active RA patients with inadequate response to methotrexate, with two studies reporting mean FACIT-F scores of 28.3 +/- 10.1 (266) and 25.7 +/- 10.5 (267) respectively. Similarly, the median FVAS score of 59 (29.8-80.3) for inflammatory arthritis patients and 57 (28-81) for CSA patients were higher than the mean FVAS in healthy individuals of 31.6 +/- 23.2 (256) and comparable to established RA patients (50.2 +/- 25.5) (268).

The results of this study suggest a greater magnitude of fatigue, at least early in the disease course, in those with RA compared to other forms of inflammatory arthritis and CSA patients. Interestingly, CSA patients appear to have significant fatigue that is comparable to those with non-RA inflammatory arthritides, despite the absence of clinically apparent synovitis. These data are consistent with those from qualitative studies which highlight fatigue as a clinically important symptom in patients with CSA (63) and provides a novel quantitative insight into the extent of fatigue in CSA. Not only does this represent an area of interest for researchers, but clinicians should be aware of the extent of fatigue despite the lack of clinical findings in CSA patients.

In all patients, fatigue was associated with female gender, high disease activity, high pain, high disability, low mood and high tender joint count. In addition, in inflammatory arthritis patients, fatigue was associated with high swollen joint count, a diagnosis of RA compared to other inflammatory arthritis and CSA patients.

The multivariate analysis showed that PHQ-9, HAQ and TJC 68 were independently associated with a low FACIT-F score in the inflammatory arthritis patients. Multivariate analysis in the CSA patients revealed PHQ9 and HAQ to be independently associated with a low FACIT-F score. The resulting $r^2$ values were 0.666 and 0.539 in the arthritis and CSA patients, respectively. Other multivariate analyses assessing factors associated with fatigue in studies of early arthritis patients have $r^2$ values of 0.43 (53) and 0.46 (54) respectively. Multinomial logistic regression models of the follow up data underscored the importance of mood and disease activity. This highlights at risk groups who may benefit from nonpharmacological interventions such as patient education regarding modifying physical activity (269) and psychosocial interventions (270).

Previous studies have provided conflicting results in terms of the relationship between disease activity and fatigue (49, 271, 272). Data from the British Society for Rheumatology Biologics Registry showed that fatigue did not correlate with disease activity in patients with established and treated RA (39). However, other studies of patients with earlier disease, including ours, demonstrate a correlation

between measures of disease activity and fatigue (53, 54), suggesting a relationship, which may diminish over time and as csDMARDs and bDMARDs are introduced.

Our study showed that low mood had the strongest correlation with fatigue. This relationship is also seen in studies of early (53, 54) and established arthritis (273, 274). Fatigue appears to be linked to coping strategies (50, 51) and a history of an affective disorder (51, 275). Pain and disability have also been linked to fatigue in early (53, 54) and established arthritis (4, 38, 48, 49, 259, 272). In addition, several inflammatory arthritis studies, including ours, show a relationship between female sex and increased fatigue (44, 48, 54, 276-279) although this is not a consistent finding (4, 38, 50, 271, 280-283).

### 4.2.6 Limitations

Our study has several limitations. Firstly, there are limitations in using a PROM to assess fatigue. For instance, dimensions of fatigue such as cognitive, social and motivational fatigue are not fully captured by the scales used. PROMs are highly subjective measures, which are prone to substantial bias and could inadvertently assess for symptoms which could co-exist with fatigue such as damage secondary to the underlying rheumatic condition. On site PROMS completion may yield suboptimal results as the patients may not be in a physical or psychological state to give accurate opinions of their experience of health status. Patients may also be apprehensive about a perceived negative impact of their answers on the care given by health care providers and alter their responses accordingly (284). Secondly, whilst our results suggest that low mood and disability are closely related to levels of fatigue, our study design does not allow us to address the issue of causality. Thirdly, 299 patients had incomplete data at follow up. This reduction in follow up data means that our multinomial logistic regression model had to be split into two models (model A and model B) due to the reduction of sample size, which could introduce bias in the estimation of parameters and a reduction in the representativeness of the samples. Finally, comorbidities and medications which can impact on fatigue and diagnostic outcome were not controlled for; to do so would have required a significantly enhanced sample size.

### 4.2.7 Conclusion

Fatigue is a clinically important symptom to measure in patients with early arthritis and CSA. In the early stages of inflammatory arthritis and CSA it appears to be related to disease activity, disability, pain and low mood. Whether fatigue and low mood are causally related remains unclear, however the results appear to advocate a holistic approach when managing patients' fatigue. A significant proportion of variability of fatigue is not explained by clinical variables assessed this study and in other previous studies (53-55). Other correlates, such as changes in systemic metabolism, could account for the remaining variation in fatigue in patients with inflammatory arthritis and CSA. Subsequent chapters in this thesis address this issue.

The early arthritis and CSA population we have studied includes patients with a wide range of levels of fatigue and the distribution of that fatigue appears similar to that in other early arthritis populations. It is thus an appropriate population in which to explore the relationship between fatigue and levels of metabolites in the peripheral compartment to gain insights into mechanism underlying fatigue in patients with inflammatory rheumatic diseases.

# 5 Relationship between CRP & metabolism

## 5.1 Introduction

RA is a systemic inflammatory autoimmune disease characterised by inflamed synovium and bone damage. Pre-RA encompasses several components including genetic risk, environmental risk, systemic autoimmunity characterised by autoantibodies, CSA and UA. Prior and subsequent to the development of RA there is significant immune activation, evidenced in part by elevated levels of circulating cytokines and chemokines. The profile of these proteins vary dependent upon autoantibody status (92) and can change as the patient transitions into RA from pre-RA (93).

The inflammatory response is a complex series of events. Immune cells recognize pathogen-associated and damage-associated molecular patterns. Inflammatory cytokines, such as TNF-α, IL-1 and IL-6, are released. One, amongst many, function of these cytokines is to increase permeability of endothelium to facilitate the egress of immune cells (285). At sites of inflammation, these immune cells release enzymes which can result in the accumulation of ROS (286). Furthermore, circulating pro-inflammatory cytokines result in a systemic response known as the acute-phase reaction. During this, hepatocytes produce acute phase reactants, including CRP (287).

In addition to their effects on hepatocytes, cytokines also act on the brain, leading to symptoms associated with "sickness behaviour" such as fatigue, anorexia and fever (288). Numerous neuroendocrine changes also ensue including growth hormone resistance, thyroid dysfunction, a reduction in testosterone and an increase in prolactin and cortisol (289, 290). The net effect of these changes is the induction of lipolysis, glycolysis, glycogenolysis, proteolysis and gluconeogenesis. These catabolic effects can generate ROS which can perpetuate inflammation (291). Glutathione (GSH), synthesised from glutamine, is consumed as it acts as an antioxidant against ROS (292).

Lactate also increases during inflammation (293). Typically, lactate is regarded as an indicator of insufficient oxygen supply. Tissue hypoxia can occur during inflammatory states as metabolic demand increases coupled with microthrombi of platelets and leucocytes obstructing blood supply (294). If these microvascular obstructions resolve then ischaemia-reperfusion injury can occur which leads to further ROS production and increasing inflammatory burden (295). In addition to their responses to oxygen insufficiency, active immune cells can switch to glycolysis as their main source of ATP production even in the absence of hypoxia, a process known as aerobic glycolysis, which can result in increased lactate production (293).

While changes in individual metabolites have been reported in inflammatory conditions, in recent years, metabolomics has been used to provide a generalised outline of broader metabolite profiles. Small molecules (<1500 Da) are quantified within compartments (synovial fluid, blood, urine, saliva, tears, cerebrospinal fluids and intact cells) and metabolite profiles are generated, which may contain thousands of metabolites. The metabolites can be measured using MS or NMR spectroscopy. The derived data are represented by mass/charge (m/z) ratios in MS or spectra containing a number of peaks, signifying proton resonance in proton NMR (141). Metabolites are recognised by referencing to a metabolite databases or by direct metabolite assay. The Human Metabolome Database lists >41,000 metabolite entries in the latest version; however, only 3000 have been associated with diseases to date (296). In common with genomic, proteomic and transcriptomic approaches, metabolomic experiments produce large amounts of data often from multiple samples, necessitating the use of multivariate analysis to simplify and extract meaning from the resulting data.

Cells involved in inflammation are metabolically active and release cytokines which have distant effects, so effects on metabolism are both directly due to and also a consequence of inflammation. Several studies have performed metabolomics on the serum and urine of patients with rheumatic

diseases to study these changes. A study of early inflammatory arthritis patients ≤3 months duration showed a relationship between the serum metabolome and CRP using NMR metabolomics with lactate and lipids as discriminators of inflammation as measured by CRP (152). PLS-R models assessing the relationship between serum metabolome and CRP showed an $r^2$ value of 0.671 (n=89, p<0.001) and 0.4157 (n=127, p<0.001) in two separate groups of early arthritis patients. The association between the serum metabolome and CRP may, therefore, be a potential confounder when examining the relationship between the serum metabolome and other variables, such as fatigue, especially as fatigue appears to have a relationship with CRP (297, 298).

Metabolomics has also been used to show a relationship between inflammation and both the serum and the urinary metabolome in a cohort of 925 healthy individuals (195). In that study, the serum metabolome was assessed by mass spectroscopy metabolomics, whilst the urine metabolome was assessed by mass spectroscopy and NMR metabolomics. Inflammation as measured by highly sensitive CRP was associated with multiple changes in metabolome associated with oxidative stress and the urea cycle (195). Although urinary metabolomics has been shown to distinguish elevated disease activity in those with rheumatic diseases (299) and to predict responses to anti-TNF therapy in RA patients (153), urinary metabolomics has not been used to study the effect of inflammation as measured by CRP on metabolism in a cohort of patients with rheumatic diseases before. However, findings from the serum metabolome are typically applicable to the urinary metabolome for instance the prediction of response to anti-TNF therapy in RA patients (153, 300, 301) and distinguishing healthy individuals with elevated inflammatory markers (195). Nevertheless, the relationship between the urinary metabolome and inflammation in patients with inflammatory arthritis and arthralgia remains an understudied area.

The purpose of this study is to assess the relationship between systemic inflammation and metabolism. I chose CRP as a measure of systemic inflammation for the following reasons. Firstly, it is a readily available test reflecting the extent of IL-6 mediated inflammation. The fact that anti-IL6 therapies are routinely used in clinical practice means that the impact of anti-IL6 therapy on fatigue and the metabolome could be assessed in future studies and changes in metabolites compared with the metabolites found to be associated with CRP in this thesis. Secondly, compared with ESR, the other commonly measure marker of inflammation, CRP levels are less influenced by other variables such as age and sex and are more indicative of inflammation at the particular point in time that the blood is sampled. ESR in contrast changes relatively slowly in response to changing levels of inflammation. Finally, as the conditions in question, inflammatory arthritis and CSA, are associated with systemic inflammation which can be measured by CRP, then studying the correlation between CRP and metabolism could lead to insights into pathoaetiology.

We hypothesized that a metabolomics approach using filtered serum and urine could identify a relationship between metabolic dysfunction and inflammation in patients with inflammatory arthritis and CSA.

## 5.2 Aims & objectives

The aims are as follows:

1) To confirm and extend previous data by investigating the relationship between systemic inflammation and the serum metabolome in adults with newly presenting inflammatory arthritis and clinically suspect arthralgia.

2) To investigate the relationship between systemic inflammation and the urinary metabolome in adults with newly presenting inflammatory arthritis and clinically suspect arthralgia.

The objectives are as follows:

1) Perform NMR spectroscopy metabolomics on filtered serum samples from patients with newly presenting inflammatory arthritis and CSA to investigate the relationship between CRP and metabolism using multivariate analysis.
2) Perform NMR spectroscopy metabolomics on urine samples from patients with newly presenting inflammatory arthritis and CSA to investigate the relationship with CRP and metabolism using multivariate analysis.
3) Identify the metabolic fingerprints in serum and urine which associates with CRP on multivariate analysis – to allow these to be removed in subsequent analyses assessing the relationship between fatigue and the serum and urinary metabolome.

## 5.3 Patients

Sera were available from 270 patients and urine samples from 178 patients from baseline visits for metabolomics analysis with further description of the patients found in **section 2.1**. **Section 2** outlines sample preparation, NMR spectroscopy application and statistical analysis of the resulting metabolomics data.

## 5.4 Results

### 5.4.1 Patient characteristics for study of relationship between serum metabolite profile and CRP level

The diagnoses and characteristics of the patients included in the sera metabolomics analysis are shown in Table 5-1 and Table 5-2 respectively. The median symptom duration was 24 weeks (IQR 12-52 weeks). The largest patient diagnostic group was RA (46.7%). The median Disease Activity Score in 28 joints using the ESR in the RA patients was 5.18 (interquartile range 4.37-6.10). Only 17% of UA and 36.8% CSA patients were seropositive for either RF or ACPA respectively. Table 5-3 shows the diagnostic outcomes at ≥12 months for patients who at baseline were classified as CSA or UA. Table 5-4 shows the various treatment options used to manage patients with persistent CSA, persistent UA and CSA patients who converted to UA.

**Table 5-1 Diagnostic data for patients in whom serum metabolites were assessed by NMR spectroscopy to investigate the relationship between serum metabolome and CRP**

**RA**- rheumatoid arthritis; **UA**- unclassified arthritis; **CSA**- clinically suspect arthralgia; **PsA**- psoriatic arthritis

*Seropositive refers to positive for RF or ACPA. Classification criteria applied as outlined in the **section 2.1**

| Diagnosis | Number (percentage) |
|---|---|
| **RA:** | **126 (46.7)** |
| Seropositive* RA | 79 (29.3) |
| Seronegative RA | 47 (17.4) |
| **Unclassified arthritis:** | **41 (15.2)** |
| Seropositive* | 7 (2.6) |
| Seronegative | 34 (12.6) |
| **CSA:** | **57 (21.1)** |
| Seropositive* | 21 (7.8) |
| Seronegative | 36 (13.3) |
| **PsA** | **29 (10.7)** |
| **Other arthritis:** | **17 (6.3)** |
| Gout | 6 (2.2) |
| Peripheral spondyloarthopathy | 5 (1.9) |
| Post infectious arthritis | 5 (1.9) |
| Sarcoidosis related arthritis | 1 (0.3) |

**Table 5-2 Demographic characteristics by diagnosis for serum metabolomics analysis to investigate the relationship between serum metabolome and CRP**

**RA**- rheumatoid arthritis; **UA**- unclassified arthritis; **CSA**- clinically suspect arthralgia; **PsA**- psoriatic arthritis; **IQR**- interquartile range; **FACIT-F**- functional assessment of chronic illness therapy – fatigue; **CRP**- C-reactive protein; **RF**- rheumatoid factor; **ACPA**- Anti-citrullinated protein antibody; **NSAID**- nonsteroidal anti-inflammatory drug

* p value calculated using Kruskal-Wallis test ** p value calculated using chi-squared test

ł therapies at baseline

| | All Cohort (n=270) | RA (n=126) | PsA (n=29) | UA (n=41) | CSA (n=57) |
|---|---|---|---|---|---|
| Age, median (IQR) years | 52 (41-60) | 55 (47-62) | 48 (35.5-58) | 51 (42-60) | 43 (34.5-53.5) |
| Missing (%) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | | p<0.001* | | | |
| Sex, no. (%) females | 180 (66.7) | 88 (69.8) | 15 (51.7) | 26 (63.4) | 42 (73.7) |
| Missing (%) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | | p=0.182** | | | |
| Symptom duration, median (IQR) weeks | 24 (12-52) | 20.5 (11-47) | 27 (12.5-57) | 21 (12-42) | 32.5 (17.3-103.3) |
| Missing (%) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | | p=0.036* | | | |
| FACIT-F (IQR) | 32 (21-41) | 29 (17-39) | 32 (21.5-40.5) | 41 (28-44) | 33 (23-42) |
| Missing (%) | 18 (6.7) | 10 (7.9) | 0 (0) | 3 (7.3) | 3 (5.4) |
| | | p=0.014* | | | |
| CRP, median (IQR) mg/L | 6 (3-14) | 8 (3-16.3) | 6 (3-21) | 5 (2.5-10) | 6 (3-17) |
| Missing (%) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | | p= 0.064* | | | |
| RF positive, no. (%) | 107 (39.6) | 76 (60.3) | 3 (10.3) | 7 (17.1) | 17 (29.8) |
| Missing (%) | 13 (4.8) | 7 (5.6) | 3 (10.3) | 0 (0) | 3 (5.3) |
| | | p<0.001** | | | |
| ACPA positive, no. (%) | 87 (32.2) | 66 (52.4) | 1 (3.4) | 1 (2.4) | 18 (31.6) |
| Missing (%) | 6 (2.2) | 2 (1.6) | 3 (10.3) | 0 (0) | 1 (1.8) |
| | | p<0.001** | | | |
| NSAIDs, no. (%) | 125 (46.3) | 49 (38.9) | 3 (3.4) | 22 (53.7) | 34 (59.6) |
| Missing (%) ł | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | | p=0.052** | | | |
| Steroids, no. (%) | 42 (15.6) | 7 (5.6) | 1 (3.4) | 13 (31.7) | 8 (14.0) |
| Missing (%) ł | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | | p<0.001** | | | |
| Baseline DMARD | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Missing (%) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| No. (%) who developed RA (at ≥12 months) | -- | -- | -- | 5 (12.2) | 5 (8.8) |

| | | | | | |
|---|---|---|---|---|---|
| Missing (%) | | | | 13 (31.7) | 18 (31.6) |

**Table 5-3 Diagnostic outcomes at ≥12 month follow up in CSA & UA patients in whom serum metabolites were assessed by NMR spectroscopy**

The diagnostic outcomes for clinically suspect arthralgia and unclassified arthritis patients in whom serum metabolites were assessed by NMR spectroscopy to investigate the relationship between serum metabolome and CRP

**UA**- unclassified arthritis; **RA**- rheumatoid arthritis; **CSA**- clinically suspect arthralgia

| Baseline diagnosis | ≥12 month follow up diagnosis | | | | |
|---|---|---|---|---|---|
| | No inflammatory arthritis | UA | RA | Other inflammatory arthropathy | Lost to follow up |
| UA (n=41) | -- | 12 | 5 | 11 | 13 |
| CSA (n=57) | 28 | 3 | 5 | 3 | 18 |

**Table 5-4 Treatment usage in CSA & UA patients in whom serum metabolites were assessed by NMR spectroscopy**

Clinically suspect arthralgia and unclassified arthritis patient treatment in whom serum metabolites were assessed by NMR spectroscopy to investigate the relationship between serum metabolome and CRP

**UA-UA**- baseline and follow up diagnosis of unclassified arthritis; **CSA-NIA-** baseline diagnosis of clinically suspect arthralgia and a follow up diagnosis of no inflammatory arthritis; **CSA-UA-** baseline diagnosis of clinically suspect arthralgia and a follow up diagnosis of unclassified arthritis; **HCQ**- hydroxychloroquine; **pred**- prednisolone; **AZA-** azathioprine; **MTX-** methotrexate; **SFZ-** sulfasalazine

| Patients | HCQ alone | HCQ + pred | HCQ + AZA | MTX + HCQ | MTX alone | MTX + pred | MTX + SFZ | MTX + HCQ + SFZ |
|---|---|---|---|---|---|---|---|---|
| UA-UA | 4 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| CSA-NIA | 8 | 1 | 1 | 1 | 2 | 0 | 1 | 1 |
| CSA-UA | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |

### 5.4.2 Relationship between serum metabolite profile and CRP in the whole cohort

The median CRP value for the entire cohort was 6 (IQR 3-13). Figure 5-2 below shows the distribution of CRP over the whole cohort and also in the four largest diagnostic subgroups. PCA was used to generate an unbiased overview to investigate differences in metabolite profiles between patients in the lowest CRP tertile and the highest CRP tertile (Figure 5-3A). This showed no separation between these groups. However, a supervised analysis using OPLS-DA showed a strong separation with 1+2+0 LV (Figure 5-3B; P <0.000001). Discriminatory bins were identified using the VIP scores. Spectral fitting to identify metabolites was performed using Chenomx (see Figure 5-1) and a published list of metabolites (170).

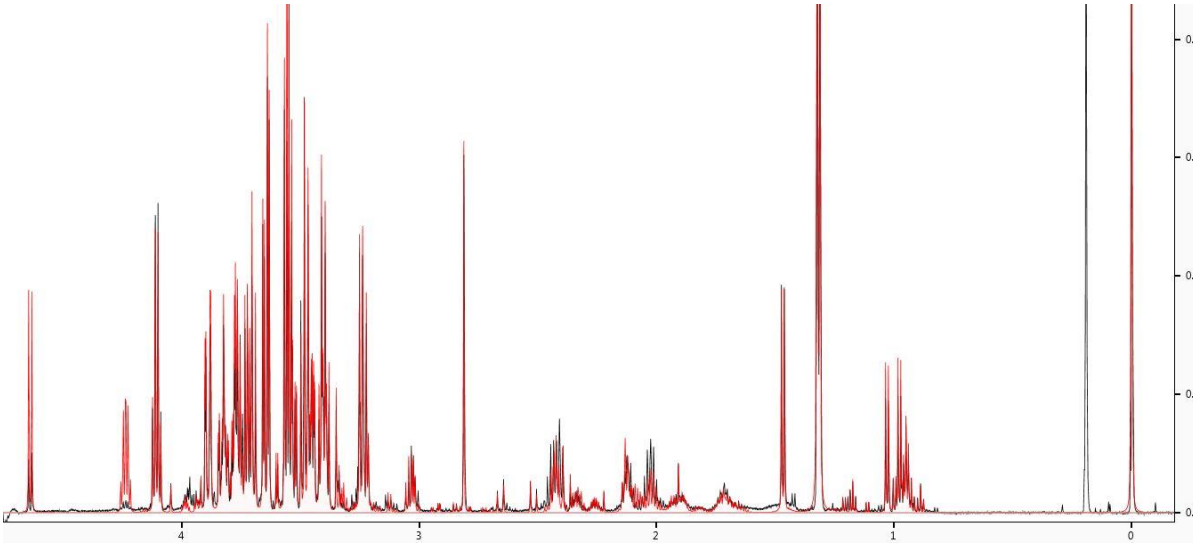



**Figure 5-1 Spectral fitting to identify metabolites**
Metabolites were identified using the reference metabolite library within Chenomx and a published list of metabolites (170).

A VIP score of greater than one was used to identify potential biomarkers (167). The nature of the relationship of the biomarker to the model was elucidated by the post normalised NMR data that were used in the model. This was done by calculating the mean value of NMR data for that bin in the highest and lowest CRP tertile group. The values were then compared between the two groups and if the mean value was greater in the highest CRP tertile group then a ↑ was used to describe the biomarker relationship in the model. A ↓ was used should the mean value be lower in the highest CRP tertile group.

Table 5-5 shows the bins which had VIP scores greater than 1 and their corresponding metabolites. These data are also represented in Table 5-5 by metabolite in order of each NMR bin by VIP score.

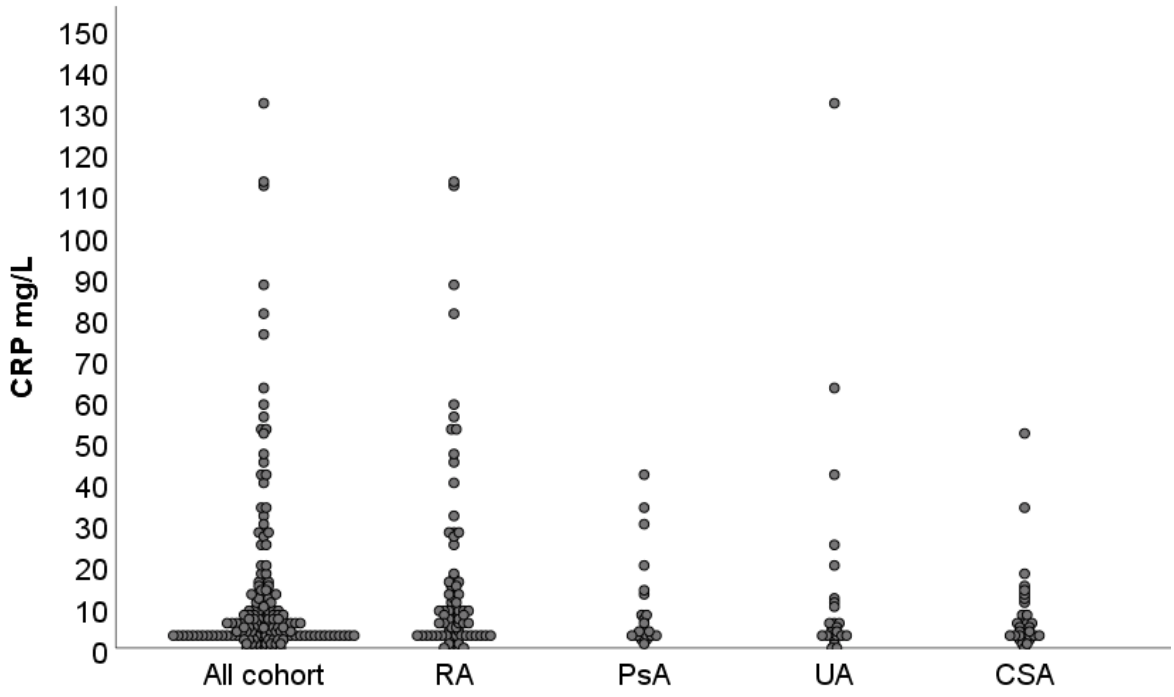



**Figure 5-2 Scatter plot showing the distribution of CRP values across the whole population and diagnostic subgroups used in the serum metabolomics analysis**

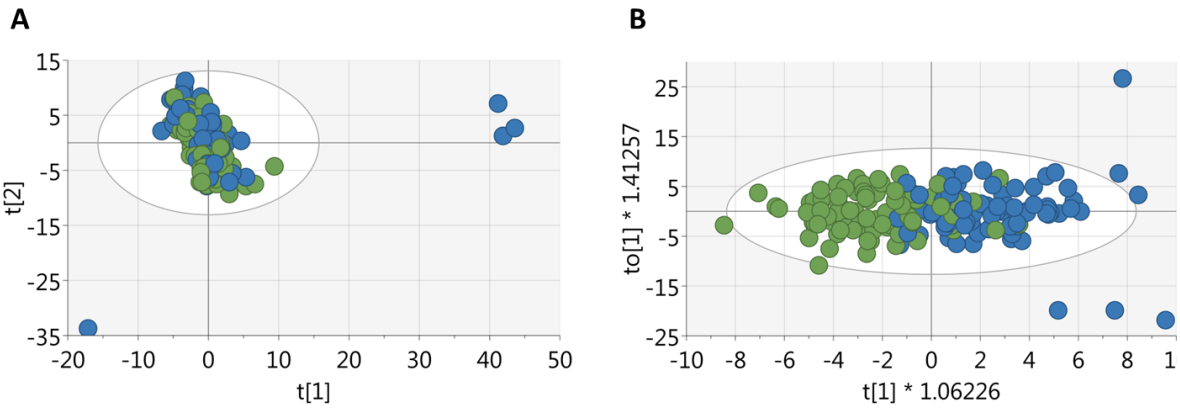



**Figure 5-3 Multivariate analysis of serum metabolite profile by CRP tertiles with data shown for the highest and lowest tertile**

**(A)** PCA plot of metabolic data derived from all patients' (n=270) sera (green=CRP <4 and blue=CRP>10; 20 PC, $r^2$=0.817) showing no separation between the two groups. **(B)** OPLS-DA plot of metabolic data derived from all patients' (n=270) sera (green=CRP <4 and blue=CRP>10; 1+2+0 LV, P value<0.000001) showing a strong separation between the two groups.

**Table 5-5 Serum metabolites with VIP scores greater than 1 from the OPLS-DA model of all patients showing separation between low and high CRP tertiles**

The following metabolites have been ranked by magnitude of the VIP score. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of VIP score. Where the direction of change in metabolite concentration in patients in the higher CRP tertile is indicated by arrows (↑ indicates increased metabolite concentration in patients in the higher CRP tertile and ↓ indicates decreased metabolite concentration in patients in the higher CRP tertile).

| Order | Metabolite | Chemical shift of peak (ppm) | Direction of change in metabolite concentration in patients with higher CRP for each peak |
|---|---|---|---|
| 1 | 3-methylhistidine | 7.053, 3.1948, 7.0472, 3.1896 | ↓↑↓↓ |
| 1 | Histidine | 7.053, 7.0472, 3.9682 | ↓↓↓ |
| 2 | Glucose | 3.1948, 3.1896, 3.9623, 3.9682 | ↑↑↓↓ |
| 3 | Carnitine | 3.1948, 2.4111, 3.1896 | ↑↓↑ |
| 3 | Glycerol | 3.1948, 3.1896, 3.9623, 3.9682 | ↑↑↓↓ |
| 3 | Betaine | 3.1948, 3.1896 | ↑↑ |
| 3 | Arginine | 3.1948, 3.1896, 1.9663, 1.8902 | ↑↑↓↓ |
| 3 | Tyrosine | 3.1948, 3.1896, 3.9623, 7.1463 | ↑↑↓↑ |
| 3 | Cystine | 3.1948, 3.1896 | ↑↑ |
| 3 | Choline | 3.1948, 3.1896 | ↑↑ |
| 4 | Citrate | 2.5106 | ↓ |
| 4 | Glutamine | 2.5106, 2.1706, 2.1067, 2.4111, 1.9663, 2.1536 | ↓↑↓↓↓↑ |
| 4 | Pyruvate | 2.5106, 2.4111 | ↓↓ |
| 5 | Methionine | 2.1706, 2.1067 | ↑↓ |
| 5 | Glutamate | 2.1706, 2.1067, 2.4111 | ↑↓↓ |
| 5 | Valine | 2.1706 | ↑ |
| 5 | Proline | 2.1706, 2.1067, 2.4111, 1.9663, 2.1536 | ↑↓↓↓↑ |
| 6 | Phenylalanine | 7.3691, 3.9623, 3.9682 | ↓↓↓ |
| 7 | 3-hydroxybutyrate | 2.4111 | ↓ |
| 8 | Isoleucine | 1.9663 | ↓ |
| 8 | Ornithine | 1.9663, 1.8902 | ↓↓ |
| 8 | Lysine | 1.9663, 1.8902 | ↓↓ |
| 9 | Acetate | 1.8902 | ↓ |
| 10 | Cysteine | 3.9623, 3.9682 | ↓↓ |
| 10 | Serine | 3.9623, 3.9682 | ↓↓ |
| 10 | Asparagine | 3.9623, 3.9682 | ↓↓ |
| 11 | 2-hydroxybutyrate | 3.9682 | ↓ |
| 11 | Lactate | 3.9682 | ↓ |

To investigate this further, the relationship between serum metabolite profile and CRP was assessed using regression analysis PLS-R. Using the entire 590 bins, a PLS-R analysis of all patient data (Figure 5-4A) showed a statistically significant relationship between the serum metabolite profile and CRP ($r^2$=

0.2897, 7 LV, p<0.001). Forward selection was carried out as described in **section 2.3.6.2** to produce a model containing the top 83 NMR bins with the highest correlation coefficient and so generated the greatest $r^2$ value. A PLS-R using these 83 NMR bins is shown in Figure 5-4B. This enhanced the relationship between metabolite profile and CRP ($r^2$=0.4811, 6 LV, p= <0.001) compared to the original PLS-R. The PLS-R model generated a rank order of NMR bins that identified the regions of the spectra that most strongly predicted the CRP in the PLS-R model. Potential biomarkers were identified using a discriminator of +/- 2 standard deviations from the mean regression coefficient of the entire dataset (168). Potential biomarkers identified by this model are show in Table 5-6. The regression coefficient takes into account the effect of the collinear variation from all the other spectral variables on the continuous variable, in this case CRP, in the PLS-R model (302). Thus, a positive regression coefficient has a positive relationship with the CRP which is represented by a ↑ symbol in the regression coefficient column. By contrast a ↓ symbol represents a negative relationship with CRP.

As inflammation has a significant impact on serum metabolite profiles (152), it would be ideal to control for this relationship when investigating the relationship between fatigue and the serum metabolite profile. Figure 5-4C shows a PLS-R analysis of the remaining 507 NMR bins, with the 83 bins identified by forward selection as strongly correlating with CRP removed. There was no relationship between the remaining 507 bins and CRP ($r^2$= 0.0356, 8 LV, p=0.059), thereby suggesting this serum metabolite profile is devoid of a significant inflammatory signal. This confirms further that the 83 bins previously identified do indeed underpin the relationship between CRP and metabolic profile.

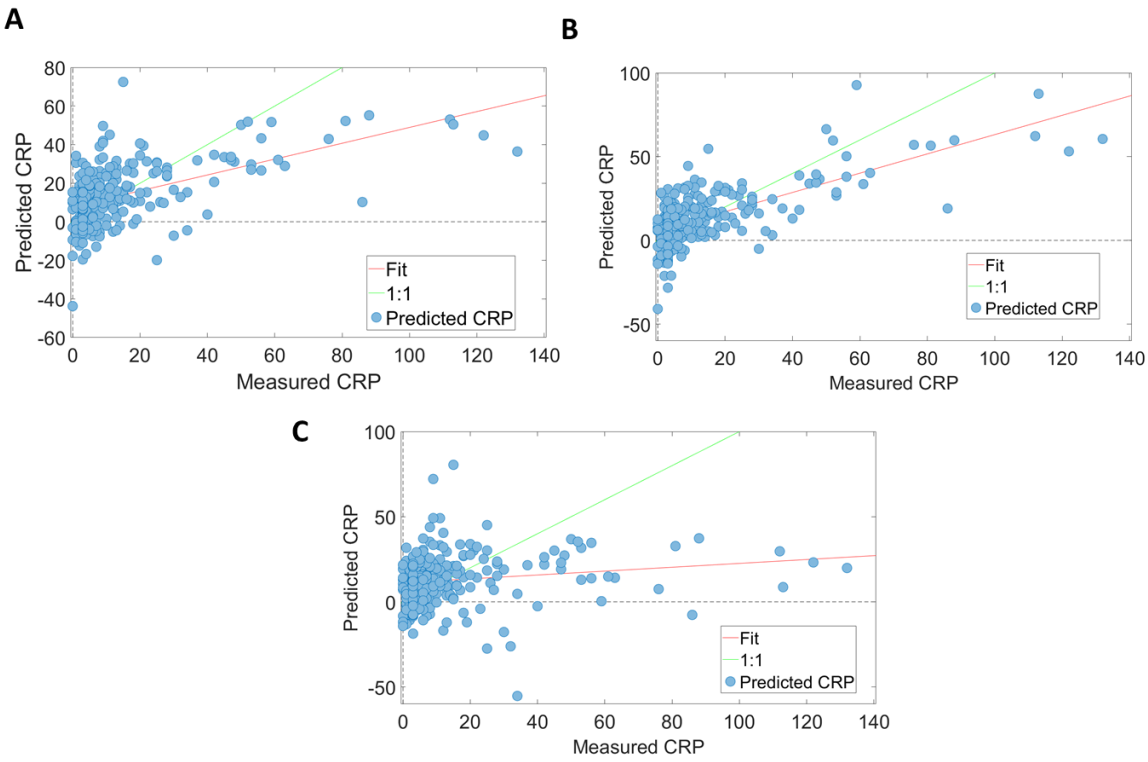



**Figure 5-4 PLS-R analysis showing the relationship between serum metabolite profile and CRP & the removal of the serum metabolite inflammatory signal**
Using the full 590 serum metabolite binned data for all patients (n=270) (**A**) there was a correlation between metabolite data and CRP on PLS-R analysis ($r^2$=0.2897, 7 LV, p<0.001). Using forward selection, 83 bins were identified which correlated with inflammation and a subsequent PLS-R analysis using these bins (**B**) showed a stronger correlation between serum metabolite profile and CRP ($r^2$=0.4811, 6 LV, p<0.001). PLS-

R with the remaining 507 bins (**C**) showed no correlation between serum metabolite profile and CRP ($r^2$=0.0356, 8 LV, p=0.059). The remaining 507 bins can thus be considered a serum metabolite profile without a significant inflammatory signal.

**Table 5-6 Metabolites responsible for the relationship seen in PLS-R between CRP and serum metabolite profile in all patients**

The following metabolites have been ranked by the magnitude of the regression coefficient. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of regression coefficient. The regression coefficient field indicates the nature of correlation (*↑ indicates a regression coefficient greater than 0 and ↓ indicates a regression coefficient less than 0)

| Order | Metabolite | Chemical shift of peak (ppm) | Regression coefficient* |
|---|---|---|---|
| 1 | Glucose | 5.1799, 5.174, 2.8911, 3,1721, 2.903, 2.8741, 3.336, 2.7916, 3.3418, 3.2833 | ↑↑↑↑↑↑↑↑↑↑ |
| 2 | Isoleucine | 1.2585, 1.2349, 1.941, 0.89507, 0.90676 | ↑↓↓↑↑ |
| 2 | Lactate | 1.2585, 1.2349, 4.0805, 1.1702, 1.3633, 1.1643, 1.1586 | ↑↓↑↑↑↑↑ |
| 3 | Citrate | 2.6511, 2.5048, 2.6745, 2.5282, 2.534, | ↓↓↓↓↓ |
| 3 | Aspartate | 2.6511, 2.7916, 2.6745 | ↓↑↓ |
| 4 | Asparagine | 2.8911, 2.903, 2.8741 | ↑↑↑ |
| 4 | Methylguanidine | 2.8911, 2.903, 2.8742, 2.7916 | ↑↑↑↑ |
| 5 | 3-hydroxybutyrate | 1.2349 | ↓ |
| 6 | Proline | 2.3643, 2.3701, 3.336, 3.3418 | ↑↑↑↑ |
| 6 | Glutamine | 2.3643, 2.3701 | ↑↑ |
| 6 | Succinate | 2.3643, 2.3701 | ↑↑ |
| 6 | Glutamate | 2.3643, 2.3701 | ↑↑ |
| 7 | Ornithine | 1.941 | ↓ |
| 8 | Tyrosine | 3.1721 | ↑ |
| 8 | Cystine | 3.1721 | ↑ |
| 8 | 3-methylhistidine | 3.1721, 7.0472, 3.2833 | ↑↓↑ |
| 8 | Choline | 3.1721 | ↑ |
| 9 | Methanol | 3.336, 3.3418 | ↑↑ |
| 10 | Isopropanol | 1.1702, 1.1643, 1.1585 | ↑↑↑ |
| 11 | 2-hydroxybutyrate | 0.89505, 0.90676 | ↑↑ |
| 11 | Valine | 0.89505, 0.90676 | ↑↑ |
| 11 | Leucine | 0.89505, 0.90676 | ↑↑ |
| 12 | Threonine | 1.3633 | ↑ |
| 13 | Phenylalanine | 3.2833, 7.3164 | ↑↑ |

As outlined in **section 2.3.6.3**, MetaboAnalyst 4.0 (http://www.metaboanalyst.ca/MetaboAnalyst/) was used to perform pathway analysis and visualization of all chemical metabolites present at different levels in filtered serum and urine samples (303). Enrichment and pathway analysis using over representation analysis algorithms was performed with a pathway-associated metabolite set library based on normal metabolic pathways to identify biologically meaningful patterns.

As stated in **section 2.3.6.3**, MetaboAnalyst gives each pathway a "total importance" score of 1, the importance measure of each metabolite node is expressed as a percentage with regards to the total pathway importance, and the pathway impact score is the cumulative percentage from the matched metabolite nodes. Figure 5-5 shows all matched metabolite pathways arranged by p values on the

Y-axis and pathway impact score on the x-axis. The node colour is dependent upon its p value and the node radius is determined on its pathway impact value. Only pathways which were statistically significant ($p<0.05$) were labelled. Table 5-7 shows the pathway analysis with all metabolite pathways which were statistically significant in descending order of impact score. Alanine, aspartate and glutamate metabolism and arginine and proline metabolism were the top two-ranking impacted canonical KEGG pathways. Over-representation analysis (Figure 5-6 and Table 5-8) in pathway-associated metabolite sets indicated that some pathways including the Warburg effect, the urea cycle and ammonium recycling are overrepresented in the serum of patients with elevated CRP. These results suggested perturbed energy and amino acid metabolism in the serum are key characteristics of patients with inflammatory arthritis and CSA with elevated CRP.

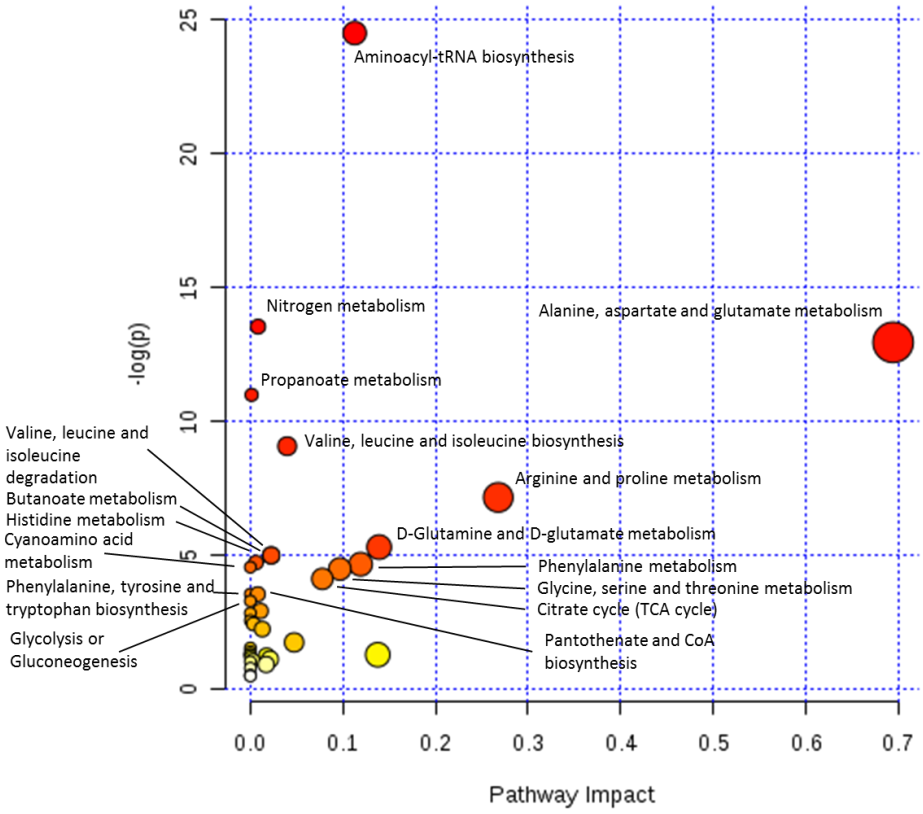



**Figure 5-5 Metaboanalyst pathway analysis of potential biomarkers implicated by PLS-R analysis of CRP and all patients' serum metabolites**

**Table 5-7 Pathway analysis of potential biomarkers implicated by PLS-R analysis of CRP and all patients' serum metabolites.**

**Match status:** The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.
**The impact score:** Each pathway has a total importance score of 1. The importance measure of each metabolite node (a point where pathways intersect within a larger network) is expressed as a fraction with regards to the total pathway importance, and the pathway impact score is the cumulative score of matched metabolite nodes.
* p value calculated using hypergeometric test

| Pathway name | Match status | P value* | Impact score |
|---|---|---|---|
| Alanine, aspartate and glutamate metabolism | 5/24 | 2.38e-06 | 0.69421 |
| Arginine and proline metabolism | 5/77 | 0.000776 | 0.26769 |
| D-glutamine and d-glutamate metabolism | 2/11 | 0.004962 | 0.13904 |
| Phenylalanine metabolism | 3/45 | 0.009394 | 0.11906 |
| Aminoacyl-tRNA biosynthesis | 11/75 | 2.3e-11 | 0.11268 |
| Glycine, serine and threonine metabolism | 3/48 | 0.011228 | 0.09661 |
| Citrate cycle | 2/20 | 0.016229 | 0.07773 |
| Valine, leucine and isoleucine biosynthesis | 4/27 | 0.000115 | 0.03975 |
| Butanoate metabolism | 3/40 | 0.006758 | 0.02254 |
| Valine, leucine and isoleucine degradation | 3/40 | 0.006758 | 0.02232 |
| Nitrogen metabolism | 6/39 | 1.32e-06 | 0.0083 |
| Phenylalanine, tyrosine and tryptophan biosynthesis | 2/27 | 0.028737 | 0.008 |
| Histidine metabolism | 3/44 | 0.008826 | 0.00611 |
| Propanoate metabolism | 5/35 | 1.69e-05 | 0.00134 |

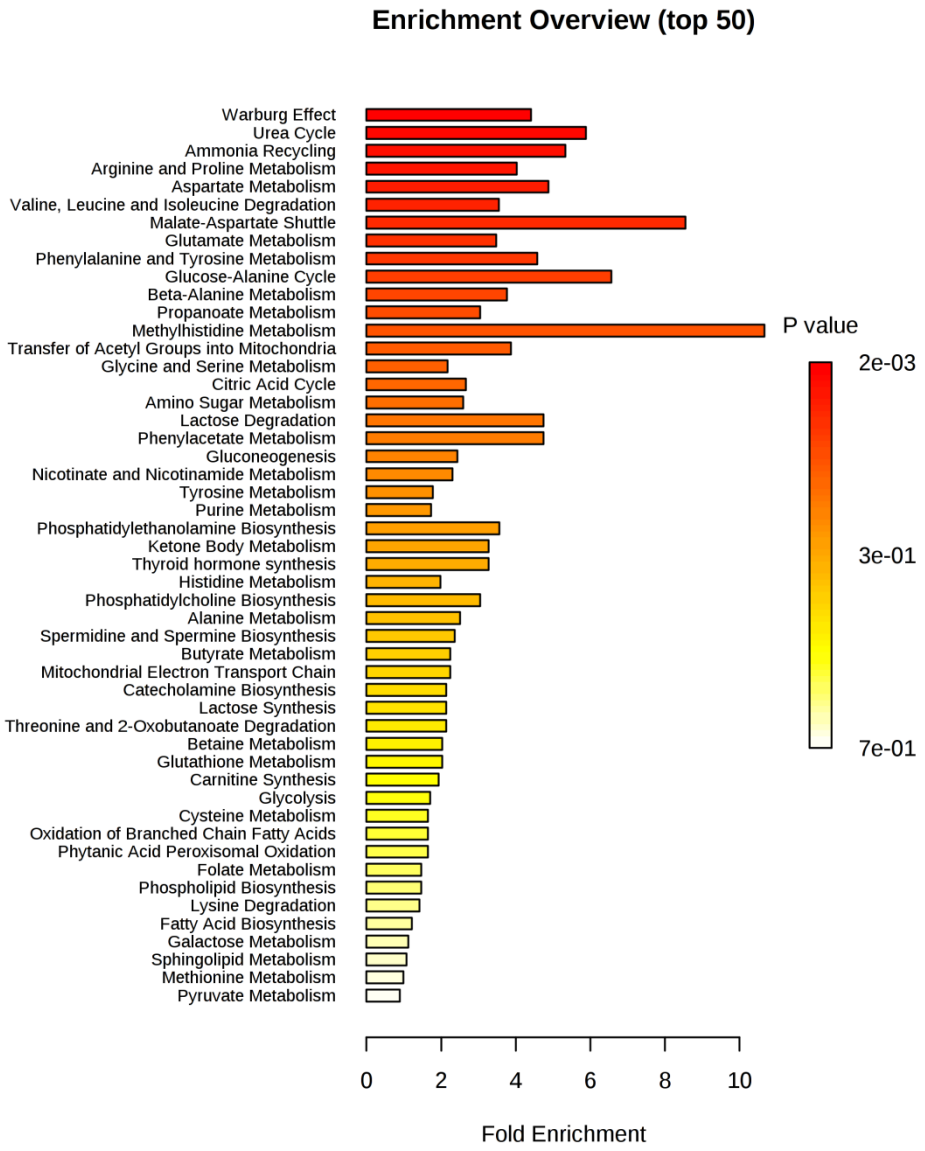



**Figure 5-6 Enrichment analysis of key metabolites in serum implicated as potential biomarkers by the PLS-R analysis of CRP and all patients' serum metabolites**

**Table 5-8 Enrichment analysis of key metabolites in serum implicated as potential biomarkers by the PLS-R analysis of CRP and all patients' serum metabolites.**

**Match status:** The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.
**The fold enrichment value:** the value is generated by the number of matched metabolites divided by the number of metabolites expected to be present in the pathway as per the reference metabolome provided by KEGG.
* p value calculated using hypergeometric test

| Metabolite pathway | Match status | Fold enrichment | Expected | P value* |
|---|---|---|---|---|
| Warburg Effect | 6/58 | 4.41 | 1.36 | 0.00154 |
| Urea Cycle | 4/29 | 5.88 | 0.68 | 0.00373 |
| Ammonia Recycling | 4/32 | 5.33 | 0.75 | 0.00538 |
| Arginine and Proline Metabolism | 5/53 | 4.03 | 1.24 | 0.00615 |
| Aspartate Metabolism | 4/35 | 4.88 | 0.82 | 0.00748 |
| Valine, Leucine and Isoleucine Degradation | 5/60 | 3.55 | 1.41 | 0.0105 |
| Malate-Aspartate Shuttle | 2/10 | 8.55 | 0.234 | 0.0211 |
| Glutamate Metabolism | 4/49 | 3.48 | 1.15 | 0.0242 |
| Phenylalanine and Tyrosine Metabolism | 3/28 | 4.57 | 0.656 | 0.0252 |
| Glucose-Alanine Cycle | 2/13 | 6.56 | 0.305 | 0.0351 |
| Beta-Alanine Metabolism | 3/34 | 3.76 | 0.797 | 0.042 |

### 5.4.3 Relationship between serum metabolite profile and CRP in RA patients

The OPLS-DA was repeated for all the diagnostic subgroups (Figure 5-7). Although a separation was seen between the highest and the lowest CRP tertile amongst all diagnostic groups, this was only significant in RA patients (1+1+0 LV, p=0.03308). It is possible that a statistically significant separation was not seen due to the comparatively small sample sizes of the other diagnostic groups. For the OPLS-DA of serum metabolite data between the highest and the lowest CRP tertiles of RA patients, the bins which had VIP scores greater than 1 and their corresponding metabolites are shown in Table 5-9. Furthermore, Table 5-9 shows each metabolite implicated in this model in descending order of each NMR bin by VIP score.

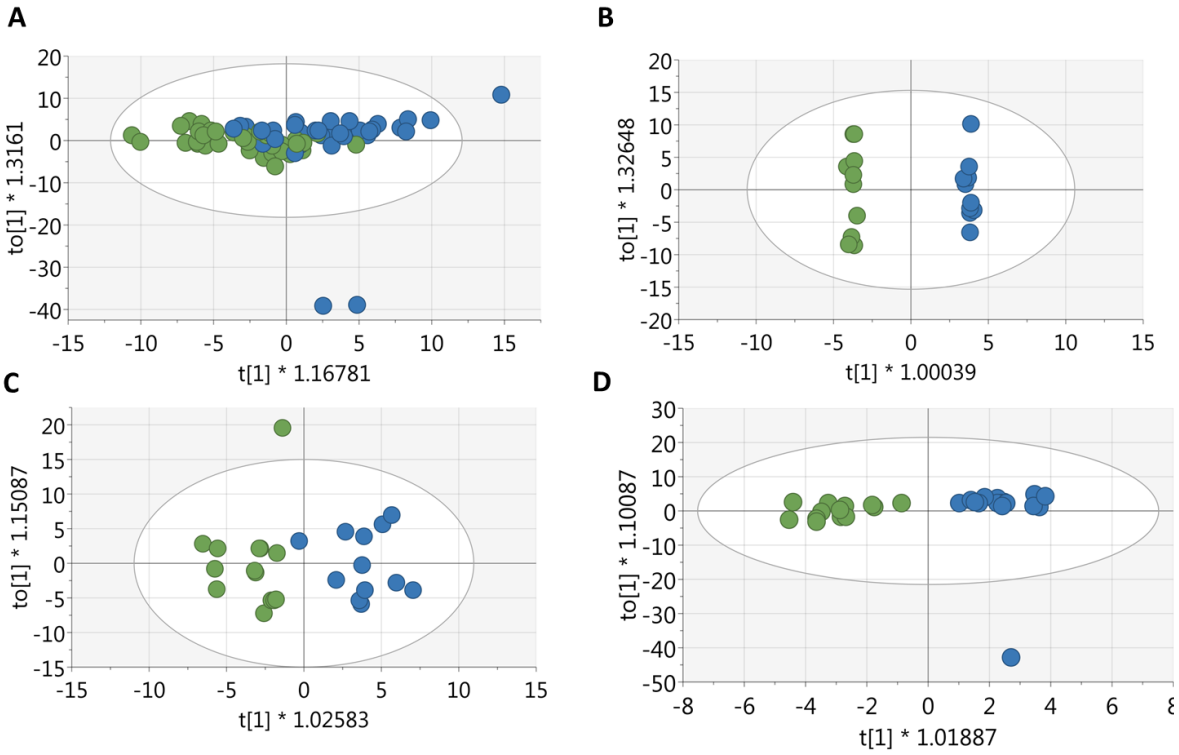



**Figure 5-7 Multivariate analysis of serum metabolite profile by CRP tertiles with data shown for the highest and lowest tertiles by diagnosis**

Across all diagnoses OPLS-DA showed a strong separation between patients in the highest CRP tertile compared to patients in the lowest CRP tertile. However, this relationship was only significant in RA patients. **(A)** OPLS-DA plot of metabolic data derived from RA patients' (n=126) sera (green=CRP <5 and blue=CRP>13; 1+1+0 LV P value= 0.03308). **(B)** OPLS-DA plot of metabolic data derived from PsA patients' (n=29) sera (green=CRP <4 and blue=CRP>11; 1+5+0 LV P value= 0.15838). **(C)** OPLS-DA plot of metabolic data derived from UA patients' (n=41) sera (green=CRP <4 and blue=CRP>10; 1+1+0 LV P value= 0.13007). **(D)** OPLS-DA plot of metabolic data derived from CSA patients' (n=57) sera (green=CRP <3 and blue=CRP>7; 1+3+0 LV P value=0.67464).

**Table 5-9 Serum metabolites with VIP scores greater than 1 from the OPLS-DA model of RA patients showing separation between low and high CRP tertiles.**
The following metabolites have been ranked by magnitude of the VIP score. The following metabolites have been ranked by the magnitude of the regression coefficient. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of VIP score. Where the direction of change in metabolite concentration in patients in the higher CRP tertile is indicated by arrows (↑ indicates increased metabolite concentration in patients in the higher CRP tertile and ↓ indicates decreased metabolite concentration in patients in the higher CRP tertile).

| Order | Metabolite | Chemical shift of peak (ppm) | Regression coefficient |
|---|---|---|---|
| 1 | 3-hydroxybutyrate | 1.1994, 1.1936, 4.1496, 2.3058 | ↑↑↑ |
| 1 | Lactate | 1.1994, 1.1936, 4.2433, 4.1496 | ↑↑↑↑ |
| 1 | Threonine | 1.1994, 4.2433, 4.1496 | ↑↑↑ |
| 1 | Isopropanol | 1.1994, 1.1936 | ↑↑ |
| 2 | 3-methylhistidine | 3.1948, 7.0472, 7.053, 3.1896 | ↑↓↓↑ |
| 2 | Tyrosine | 3.1948, 3.1896 | ↑↑ |
| 2 | Cysteine | 3.1948 | ↑ |
| 2 | Glucose | 3.1948, 4.2433, 4.1496, 3.1896 | ↑↑↑↑ |
| 2 | Choline | 3.1948, 3.1896 | ↑↑ |
| 2 | Carnitine | 3.1948, 3.1896 | ↑↑ |
| 2 | Histidine | 3.1948, 7.0472, 7.053 | ↑↓↓ |
| 2 | Arginine | 3.1948, 1.9663, 3.1896, 1.9523 | ↑↓↑↓ |
| 3 | Valine | 1.1936, 2.1706, 2.3058 | ↑↑↑ |
| 4 | Methionine | 2.1706, 2.095, 2.1594 | ↑↓↑ |
| 4 | Glutamine | 2.1706, 2.3058, 1.9663, 1.9523, 2.095, 2.1594 | ↑↑↓↓↓↑ |
| 4 | Glutamate | 2.1706, 2.3058, 2.095, 2.1594 | ↑↑↓↑ |
| 4 | Proline | 2.1706, 4.1496, 2.3058, 1.9663, 1.9523, 2.095, 2.1594 | ↑↑↑↓↓↓↑ |
| 5 | Glycerol | 3.1896 | ↑ |
| 5 | Betaine | 3.1896 | ↑ |
| 5 | Cystine | 3.1896 | ↑ |
| 6 | Phenylalanine | 7.345 | ↑ |
| 7 | Isoleucine | 1.9663, 1.9523 | ↓↓ |
| 7 | Ornithine | 1.9663, 1.9523 | ↓↓ |
| 7 | Lysine | 1.9663, 1.9523 | ↓↓ |
| 8 | Acetate | 1.9523 | ↓ |

PLS-R analyses with forward selection by diagnostic groups are shown in Figure 5-8. RA ($r^2$=0.5508, 6 LV, p<0.001) and UA ($r^2$=0.7209, 9 LV, p<0.001) patients had a statistically significant correlation between serum metabolite profile and CRP. Table 5-10 & Table 5-13 show the potential biomarkers identified by these models. No statistically significant relationship exists between serum metabolite profile and CRP for both PsA (Figure 5-8 B) and CSA (Figure 5-8 D) patients respectively.

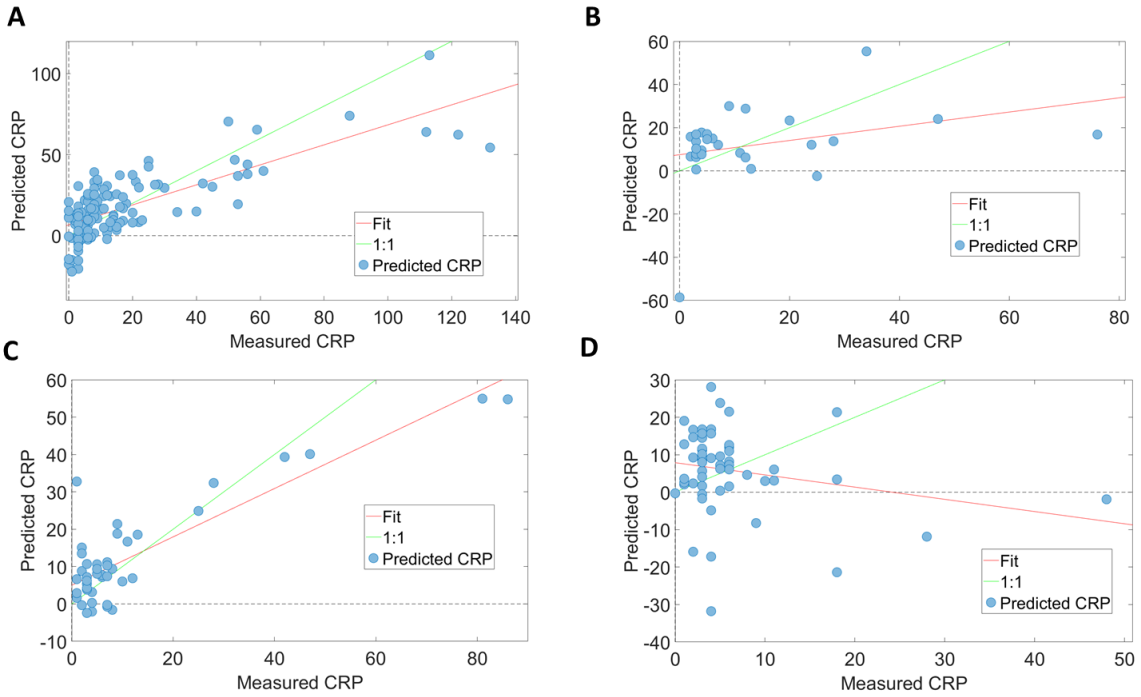



**Figure 5-8 PLS-R analysis of serum metabolites and CRP by diagnosis**
All models shown are post forward selection. Both (**A**) RA (n=126, 36 NMR bins post forward selection, $r^2$=0.5508, 6 LV, p=0.001) and (**C**) UA (n=41, 98 NMR bins post forward selection, $r^2$=0.7209, 9 LV, p<0.001) patients have a statistically significant relationship between serum metabolite profile and CRP. There was no statistically significant relationship between serum metabolite profile and CRP for (**B**) PsA patients (n=29, 3 NMR bins post forward selection, $r^2$=0.0979, 3 LV, p=0.176) and (**D**) CSA (n=57, 85 NMR bins post forward selection, $r^2$=0.0724, 8 LV, p=0.170).

**Table 5-10 Metabolites responsible for the relationship seen in PLS-R analysis between CRP and serum metabolite profile in RA patients.**

The following metabolites have been ranked by the magnitude of the regression coefficient. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of regression coefficient. The regression coefficient field indicates the nature of correlation (↑ indicating a positive relationship with CRP and ↓ indicating a negative relationship with CRP)

| Order | Metabolite | Chemical shift of peak (ppm) | Regression coefficient |
|---|---|---|---|
| 1 | Citrate | 2.534, 2.5048, 2.6511, 2.5106, 2.6745, 2.5282, 2.6394, 2.6453, 2.6886, 2.6687 | ↓↓↓↓↓↓↓↓↓↓ |
| 1 | Glutamine | 2.534, 2.5048, 2.5106, 2.5282, 2.3643, 2.3701, 2.0846, 3.6813 | ↓↓↓↓↑↑↑↓ |
| 2 | Lactate | 1.2585, 1.2349, 1.1116, 1.3633, 1.4921 | ↑↓↑↑↓ |
| 2 | Threonine | 1.2585, 1.2349, 1.3633, 3.6111 | ↑↓↑↓ |
| 2 | Isoleucine | 1.2585, 1.2349, 1.4921, 0.89505, 3.6813 | ↑↓↓↑↓ |
| 3 | Glucose | 5.1799, 2.7916, 3.1948, 5.174, 2.8911, 2.7858, 3.6111, 3.336, 3.3418, 3.6813, 2.8741, 3.1253 | ↑↑↓↑↑↑↓↑↑↓ ↑↓ |
| 4 | Pyruvate | 2.5048, 2.5106, 2.6687 | ↓↓↓ |
| 5 | Aspartate | 2.7916, 2.6511, 2.6745, 2.6394, 2.6453, 2.7858, 2.6886 | ↑↓↓↓↓↑↓ |
| 5 | Methylguanidine | 2.7916, 2.8911, 2.7858, 2.8741 | ↑↑↑↑ |
| 6 | Formate | 8.4462 | ↑ |
| 7 | Carnitine | 3.1948, 3.336, 3.3418, 3.1253 | ↓↑↑↓ |
| 7 | Glycerol | 3.1948, 3.6111, 3.336, 3.3418, 3.6813, 3.1253 | ↓↓↑↑↓↓ |
| 7 | Betaine | 3.1948, 3.336, 3.3418 | ↓↑↑ |
| 7 | 3-methylhistidine | 3.1948, 7.0472, 3.336, 3.6813 | ↓↓↑↓ |
| 7 | Arginine | 3.1948, 3.336, 1.7482 | ↓↑↓ |
| 7 | Tyrosine | 3.1948, 7.1291, 7.1463, 3.1253 | ↓↑↑↓ |
| 7 | Cystine | 3.1948, 3.336, 3.3418 | ↓↑↑ |
| 7 | Choline | 3.1948 | ↓ |
| 8 | Methionine | 2.6511, 2.6394, 2.6453, 2.0846, 2.6687 | ↓↓↓↑↓ |
| 9 | 3-hydroxybutyrate | 1.2349 | ↓ |
| 9 | Isopropanol | 1.2349 | ↓ |
| 10 | Asparagine | 2.8911, 2.8741 | ↑↑ |
| 11 | Phenylalanine | 7.3164, 7.3223, 3.1253, 7.4042, 7.4159, 7.3106 | ↑↑↓↑↑↑ |
| 12 | Histidine | 7.0472, 3.1253 | ↓↓ |
| 13 | Proline | 2.3643, 2.3701, 3.336, 3.3418, 2.0846 | ↑↑↑↑↑ |
| 13 | Succinate | 2.3643, 2.3701 | ↑↑ |
| 13 | Glutamate | 2.3643, 2.3701, 2.0846 | ↑↑↑ |
| 14 | Valine | 1.1116, 3.6111, 0.89505 | ↑↓↑ |

| | | | |
|---|---|---|---|
| 14 | Propylene glycol | 1.1116 | ↑ |
| 15 | Alanine | 1.3633, 1.4921 | ↑↓ |
| 15 | Lysine | 1.3633, 1.4921, 3.6813, 1.7482, 3.1253 | ↑↓↓↓↓ |
| 16 | Glycine | 3.6111 | ↓ |
| 17 | Methanol | 3.336, 3.3418 | ↑↑ |
| 18 | 2-hydroxybutyrate | 0.89505, 1.7482 | ↑↓ |
| 18 | Leucine | 0.89505, 3.6813, 1.7482 | ↑↓↓ |
| 19 | Ornithine | 1.7482 | ↓ |
| 20 | Malonate | 3.1253 | ↓ |
| 20 | Cysteine | 3.1253 | ↓ |
| 21 | Tryptophan | 7.3106 | ↑ |

Figure 5-9 and Table 5-11 shows the Metaboanalyst pathway analysis of the biomarkers identified by PLS-R model of the serum metabolite data of RA patients and CRP. This shows alanine, aspartate and glutamate metabolism, arginine and proline metabolism, pyruvate metabolism and glycine, serine and threonine metabolism are altered in RA patients' serum with elevated CRP. Figure 5-10 and Table 5-12 show the enrichment analysis of the biomarkers implicated by the PLS-R analysis of the RA patients' serum metabolite data and CRP. Amongst the multiple pathways which were implicated, methylhistidine metabolism, the urea cycle and the glucose alanine cycle were the most overrepresented pathways in the enrichment analysis.

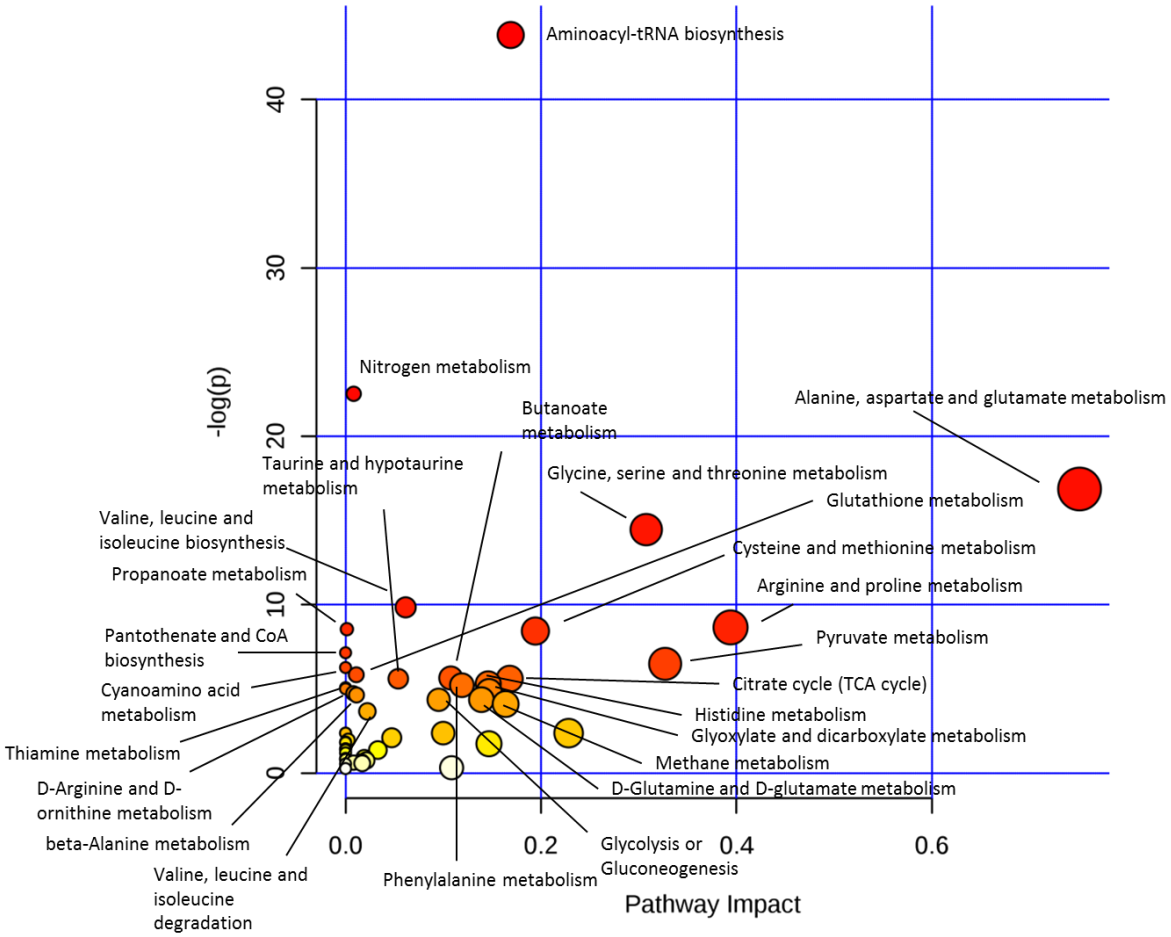



**Figure 5-9 Metaboanalyst pathway analysis of potential biomarkers implicated by PLS-R analysis of CRP and RA patients' serum metabolites**

**Table 5-11 Pathway analysis of potential biomarkers implicated by PLS-R analysis of RA patients' serum metabolites and CRP**

**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.

**The impact score**: Each pathway has a total importance score of 1. The importance measure of each metabolite node (a point where pathways intersect within a larger network) is expressed as a fraction with regards to the total pathway importance, and the pathway impact score is the cumulative score of matched metabolite nodes.

* p value calculated using hypergeometric test

| Pathway Name | Match Status | P value* | Impact score |
|---|---|---|---|
| Alanine, aspartate and glutamate metabolism | 7/24 | 4.73E-08 | 0.75119 |
| Arginine and proline metabolism | 7/77 | 1.76E-04 | 0.39401 |
| Pyruvate metabolism | 4/32 | 0.001528 | 0.32707 |
| Glycine, serine and threonine metabolism | 8/48 | 5.28E-07 | 0.30772 |
| Cysteine and methionine metabolism | 6/56 | 2.18E-04 | 0.19429 |
| Aminoacyl-tRNA biosynthesis | 19/75 | 9.28E-20 | 0.16902 |
| Citrate cycle | 3/20 | 0.003707 | 0.16797 |
| Methane metabolism | 3/34 | 0.016639 | 0.16384 |
| Glyoxylate and dicarboxylate metabolism | 4/50 | 0.007936 | 0.14685 |
| Histidine metabolism | 4/44 | 0.005016 | 0.14599 |
| D-Glutamine and D-glutamate metabolism | 2/11 | 0.012832 | 0.13904 |
| Phenylalanine metabolism | 4/45 | 0.005442 | 0.11906 |
| Butanoate metabolism | 4/40 | 0.003538 | 0.1077 |
| Glycolysis or Gluconeogenesis | 3/31 | 0.012924 | 0.0953 |
| Valine, leucine and isoleucine biosynthesis | 5/27 | 5.34E-05 | 0.06148 |
| Taurine and hypotaurine metabolism | 3/20 | 0.003707 | 0.05395 |
| beta-Alanine metabolism | 3/28 | 0.00974 | 0.01119 |
| Glutathione metabolism | 4/38 | 0.002925 | 0.01095 |
| Nitrogen metabolism | 10/39 | 1.66E-10 | 0.0083 |
| Phenylalanine, tyrosine and tryptophan biosynthesis | 3/27 | 0.008794 | 0.008 |
| Propanoate metabolism | 5/35 | 1.95E-04 | 0.00134 |

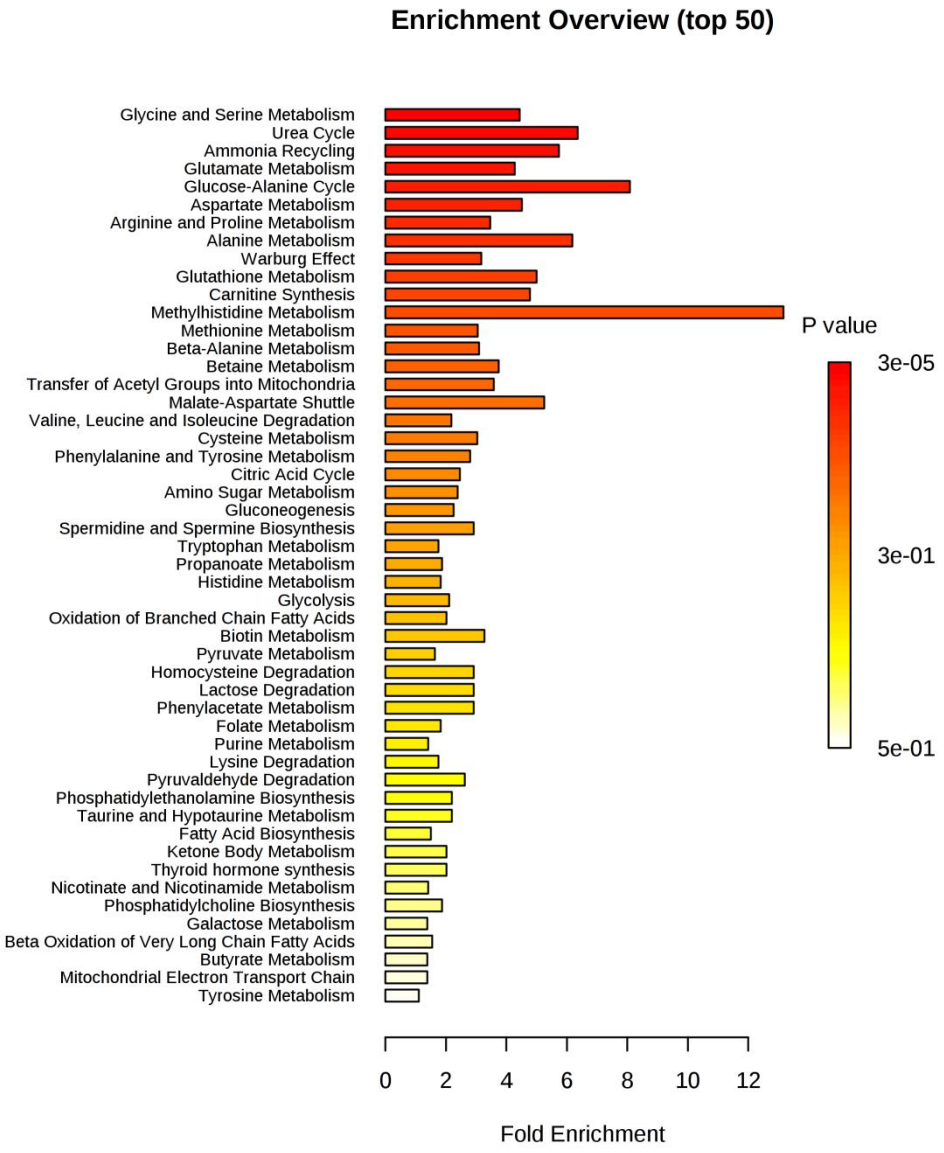



**Figure 5-10 Enrichment analysis of key metabolites in serum implicated as potential biomarkers by the PLS-R analysis of CRP and RA patients' serum metabolites**

**Table 5-12 Enrichment analysis of key metabolites in serum implicated as potential biomarkers by the PLS-R analysis of CRP and RA patients' serum metabolites.**

**Match status:** The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.
**The fold enrichment value:** the value is generated by the number of matched metabolites divided by the number of metabolites expected to be present in the pathway as per the reference metabolome provided by KEGG.
* p value calculated using hypergeometric test

| Metabolite Set | Match status | Fold enrichment | Expected | P value* |
|---|---|---|---|---|
| Glycine and Serine Metabolism | 10/59 | 4.44 | 2.25 | 3.22E-05 |
| Urea Cycle | 7/29 | 6.36 | 1.1 | 5.65E-05 |
| Ammonia Recycling | 7/32 | 5.74 | 1.22 | 0.000112 |
| Glutamate Metabolism | 8/49 | 4.28 | 1.87 | 0.000304 |
| Glucose-Alanine Cycle | 4/13 | 8.08 | 0.495 | 0.00101 |
| Aspartate Metabolism | 6/35 | 4.51 | 1.33 | 0.00148 |
| Arginine and Proline Metabolism | 7/53 | 3.47 | 2.02 | 0.00283 |
| Alanine Metabolism | 4/17 | 6.18 | 0.647 | 0.003 |
| Warburg Effect | 7/58 | 3.17 | 2.21 | 0.00479 |
| Glutathione Metabolism | 4/21 | 5.00 | 0.8 | 0.00674 |
| Carnitine Synthesis | 4/22 | 4.77 | 0.838 | 0.00802 |
| Methylhistidine Metabolism | 2/4 | 13.16 | 0.152 | 0.00808 |
| Methionine Metabolism | 5/43 | 3.05 | 1.64 | 0.0205 |
| Beta-Alanine Metabolism | 4/34 | 3.10 | 1.29 | 0.0365 |
| Betaine Metabolism | 3/21 | 3.75 | 0.8 | 0.0422 |
| Transfer of Acetyl Groups into Mitochondria | 3/22 | 3.58 | 0.838 | 0.0477 |

### 5.4.4 Relationship between serum metabolite profile and CRP in UA patients

Figure 5-11 and Table 5-14 show the pathway analysis of the biomarkers identified by the PLS-R model of UA patients' serum metabolite data and CRP. Alanine, aspartate and glutamate metabolism, glycine, serine and threonine metabolism, pyruvate metabolism and arginine and proline metabolism were identified as the greatest impacted pathways. Figure 5-12 and Table 5-15 show the enrichment analysis of the biomarkers identified by the PLS-R model of UA patients' serum metabolite data and CRP. Amongst the multiple pathways which were implicated, methylhistidine metabolism, glucose alanine cycle, ammonia recycling and the urea cycle were amongst the most overrepresented pathways in the enrichment analysis.

**Table 5-13 Metabolites responsible for the relationship seen in PLS-R between CRP and serum metabolite profile in UA patients.**

The following metabolites have been ranked by the magnitude of the regression coefficient. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of regression coefficient. The regression coefficient field indicates the nature of correlation (↑ indicating a positive relationship with CRP and ↓ indicating a negative relationship with CRP)

| Order | Metabolite | Chemical shift of peak (ppm) | Regression coefficient |
|---|---|---|---|
| 1 | Lactate | 1.3674, 1.3633, 1.0472, 4.0712, 1.1702, 1.0589, 1.4804, 4.009, 1.1994, 4.1613, 1.1585 | ↓↓↑↓↑↑↓↑↑↓↑ |
| 1 | Threonine | 1.3674, 1.3633, 3.6111 | ↓↓↓ |
| 1 | Alanine | 1.3674, 1.3633, 1.4804 | ↓↓↓ |
| 1 | Lysine | 1.3674, 1.3633, 3.1253, 1.4804, 1.941, 1.937 | ↓↓↓↓↓↓ |
| 2 | Formate | 8.4462 | ↓ |
| 3 | Citrate | 2.6511, 2.4989, 2.6745, 2.6628, 2.5048, 2.5282, 2.68 | ↓↓↓↑↓↓↓ |
| 3 | Aspartate | 2.6511, 2.7858, 2.6745, 2.6628, 2.68 | ↓↓↓↑↓ |
| 3 | Methionine | 2.6511, 2.6628, 2.1788 | ↓↑↓ |
| 4 | 3-methylhistidine | 7.053, 7.0687, 7.0647, 3.3184 | ↓↑↑↓ |
| 4 | Histidine | 7.053, 7.0688, 7.0647, 3.1253 | ↓↑↑↓ |
| 5 | Valine | 1.0472, 1.1702, 0.83029, 3.6228, 1.0589, 0.81895, 3.6111, 1.1994, 2.1788, 1.1585, 0.82554 | ↑↑↑↓↑↑↓↑↓↑↑ |
| 5 | Isoleucine | 1.0472, 3.6228, 1.0589, 1.4804, 1.941, 1.1994, 1.937 | ↑↓↑↓↓↑↓ |
| 5 | Leucine | 1.0472, 0.83029, 1.0589, 0.81895, 0.82554 | ↑↑↑↑↑ |
| 6 | Cystine | 4.0712, 3.3184 | ↓↓ |
| 6 | Proline | 4.0712, 3.3184, 1.941, 2.1788, 4.1613, 1.937, 2.3643 | ↓↓↓↓↓↓↑ |
| 6 | Glucose | 4.0712, 3.6228, 3.1253, 3.3184, 2.7858, 4.009, 4.6582, 5.2496, 3.6111, 5.1799, 4.1613 | ↓↓↓↓↓↑↓↑↓↑↓ |
| 6 | Creatinine | 4.0712, 4.009 | ↓↑ |
| 6 | Glycerol | 4.0712, 3.6228, 3.1253, 3.3184, 4.009, 3.6111, 4.1613 | ↓↓↓↓↑↓↓ |
| 6 | Tryptophan | 4.0712, 3.3184 | ↓↓ |
| 6 | Choline | 4.0712 | ↓ |
| 7 | Isopropanol | 1.1702, 4.009, 1.1994, 1.1585 | ↑↑↑↑ |
| 8 | 2-hydroxybutyrate | 0.83029, 4.009 | ↑↑ |
| 9 | Glycine | 3.6228, 3.6111 | ↓↓ |
| 10 | Glutamine | 2.4989, 2.1788, 2.5048, 2.5282, 2.3643 | ↓↓↓↓↑ |
| 10 | Pyruvate | 2.4989, 2.5048 | ↓↓ |
| 11 | Malonate | 3.1253 | ↓ |

| | | | |
|---|---|---|---|
| 11 | Phenylalanine | 3.1253, 3.3184, 4.009 | ↓↓↑ |
| 11 | Cysteine | 3.1253 | ↓ |
| 11 | Carnitine | 3.1253, 3.3184 | ↓↓ |
| 11 | Tyrosine | 3.1253, 6.9061 | ↓↑ |
| 12 | Methanol | 3.3184 | ↓ |
| 12 | Betaine | 3.3184 | ↓ |
| 12 | Arginine | 3.3184, 1.941, 1.937 | ↓↓↓ |
| 13 | Methylguanidine | 2.7858 | ↓ |
| 14 | Serine | 4.009 | ↑ |
| 14 | Asparagine | 4.009 | ↑ |
| 15 | Ornithine | 1.941, 1.937 | ↓↓ |
| 15 | Acetate | 1.941, 1.937 | ↓↓ |
| 16 | 3-hydroxybutyrate | 1.1994, 4.1613 | ↑↓ |
| 17 | Glutamate | 2.1788, 2.3643 | ↓↑ |
| 16 | Succinate | 2.3643 | ↑ |

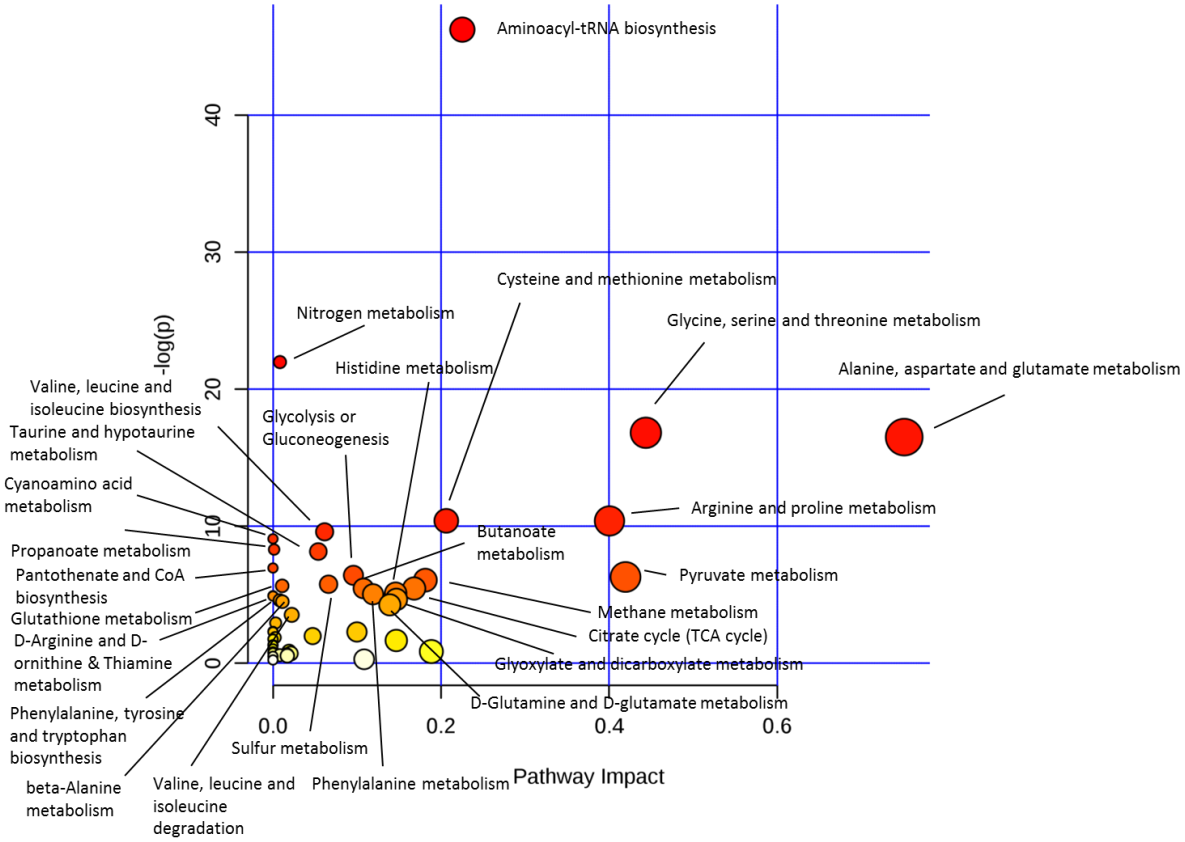



**Figure 5-11 Metaboanalyst pathway analysis of potential biomarkers implicated by PLS-R analysis of CRP and UA patients' serum metabolites**

**Table 5-14 Pathway analysis of potential biomarkers implicated by PLS-R analysis of UA patients' serum metabolites and CRP**

**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.

**The impact score**: Each pathway has a total importance score of 1. The importance measure of each metabolite node (a point where pathways intersect within a larger network) is expressed as a fraction with regards to the total pathway importance, and the pathway impact score is the cumulative score of matched metabolite nodes.

* p value calculated using hypergeometric test

| Pathway Name | Match Status | P value | Impact score |
|---|---|---|---|
| Alanine, aspartate and glutamate metabolism | 7/24 | 6.83E-08 | 0.75119 |
| Glycine, serine and threonine metabolism | 9/48 | 4.97E-08 | 0.44376 |
| Pyruvate metabolism | 4/32 | 0.0018466 | 0.41957 |
| Arginine and proline metabolism | 8/77 | 3.10E-05 | 0.40046 |
| Aminoacyl-tRNA biosynthesis | 20/75 | 8.37E-21 | 0.22536 |
| Cysteine and methionine metabolism | 7/56 | 3.07E-05 | 0.20626 |
| Methane metabolism | 4/34 | 0.0023235 | 0.18135 |
| Citrate cycle | 3/20 | 0.0042778 | 0.16797 |
| Glyoxylate and dicarboxylate metabolism | 4/50 | 0.0094778 | 0.14685 |
| Histidine metabolism | 4/44 | 0.0060138 | 0.14599 |
| D-Glutamine and D-glutamate metabolism | 2/11 | 0.01413 | 0.13904 |
| Phenylalanine metabolism | 4/45 | 0.0065199 | 0.11906 |
| Butanoate metabolism | 4/40 | 0.0042527 | 0.1077 |
| Glycolysis or Gluconeogenesis | 4/31 | 0.0016358 | 0.09576 |
| Sulfur metabolism | 3/18 | 0.0031353 | 0.06614 |
| Valine, leucine and isoleucine biosynthesis | 5/27 | 6.84E-05 | 0.06148 |
| Taurine and hypotaurine metabolism | 4/20 | 2.89E-04 | 0.05395 |
| Valine, leucine and isoleucine degradation | 3/40 | 0.029302 | 0.02232 |
| beta-Alanine metabolism | 3/28 | 0.011185 | 0.01119 |
| Glutathione metabolism | 4/38 | 0.0035204 | 0.01095 |
| Nitrogen metabolism | 10/39 | 2.86E-10 | 0.0083 |
| Phenylalanine, tyrosine and tryptophan biosynthesis | 3/27 | 0.010105 | 0.008 |
| Propanoate metabolism | 5/35 | 2.49E-04 | 0.00134 |

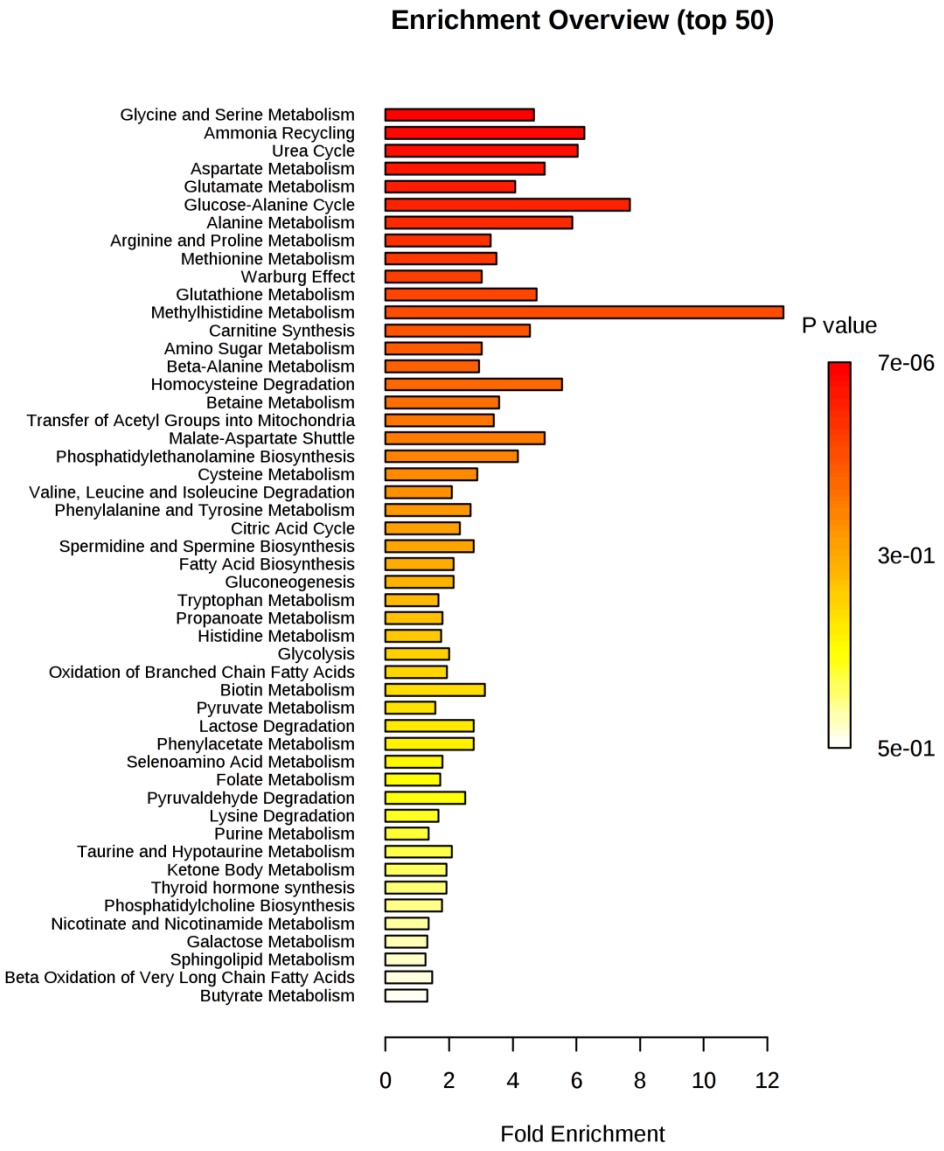



**Figure 5-12 Enrichment analysis of key metabolites in serum implicated as potential biomarkers by the PLS-R analysis of CRP and UA patients' serum metabolites**

**Table 5-15 Enrichment analysis of key metabolites in serum implicated as potential biomarkers by the PLS-R analysis of CRP and UA patients' serum metabolites.**

**Match status:** The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.
**The fold enrichment value:** the value is generated by the number of matched metabolites divided by the number of metabolites expected to be present in the pathway as per the reference metabolome provided by KEGG.
* p value calculated using hypergeometric test

| Metabolite Set | Match status | Fold enrichment | Expected | P value* |
|---|---|---|---|---|
| Glycine and Serine Metabolism | 11/59 | 4.66 | 2.36 | 7.49E-06 |
| Ammonia Recycling | 8/32 | 6.25 | 1.28 | 0.000017 |
| Urea Cycle | 7/29 | 6.03 | 1.16 | 7.94E-05 |
| Aspartate Metabolism | 7/35 | 5.00 | 1.4 | 0.000286 |
| Glutamate Metabolism | 8/49 | 4.08 | 1.96 | 0.000439 |
| Glucose-Alanine Cycle | 4/13 | 7.68 | 0.521 | 0.00122 |
| Alanine Metabolism | 4/17 | 5.87 | 0.681 | 0.00361 |
| Arginine and Proline Metabolism | 7/53 | 3.30 | 2.12 | 0.00381 |
| Methionine Metabolism | 6/43 | 3.49 | 1.72 | 0.00573 |
| Warburg Effect | 7/58 | 3.02 | 2.32 | 0.0064 |
| Glutathione Metabolism | 4/21 | 4.76 | 0.841 | 0.00808 |
| Methylhistidine Metabolism | 2/4 | 12.50 | 0.16 | 0.00892 |
| Carnitine Synthesis | 4/22 | 4.54 | 0.881 | 0.00959 |
| Amino Sugar Metabolism | 4/33 | 3.03 | 1.32 | 0.039 |
| Beta-Alanine Metabolism | 4/34 | 2.94 | 1.36 | 0.0429 |
| Homocysteine Degradation | 2/9 | 5.56 | 0.36 | 0.0471 |
| Betaine Metabolism | 3/21 | 3.57 | 0.841 | 0.048 |

As seen in the analysis using a heterogeneous cohort including a range inflammatory arthritis and CSA patients, the functional interpretation of the serum biomarkers implicated across all multivariate models suggested increased glycolysis, permutations in the citrate cycle, oxidative stress and upregulation of the urea cycle are key characteristics of the patients with elevated CRP. Additionally, protein catabolism, including evidence of articular damage and skeletal muscle degradation related to cachexia may also be present, as methylhistidine metabolism was overrepresented in the sub-analysis of RA and UA patients. In the following section, I will present the functional analysis of the urinary multivariate models to see if similar pathways appear.

### 5.4.5 Patient characteristics for study of relationship between urinary metabolite profile and CRP level

The diagnosis and characteristics of the patients included in the urine metabolomics analysis are shown in Table 5-16 & Table 5-17 respectively. The median symptom duration was 24.5 weeks (IQR 12-52 months). The largest patient diagnostic group was RA (46.6%). The median Disease Activity Score in 28 joints using the erythrocyte sedimentation rate in the RA was 5.11 (interquartile range 4.33-5.92). Only 16% UA and 39.4% CSA patients were seropositive for either RF or ACPA respectively. Table 5-18 shows the diagnostic outcomes at ≥12 months for patients who at baseline were classified as CSA and UA. Table 5-19 shows the various treatment options used to manage patients with persistent CSA, persistent UA and CSA patients who converted to UA.

**Table 5-16 Diagnostic data for patients in whom urinary metabolites were assessed by NMR spectroscopy to investigate the relationship between urinary metabolome and CRP**

**RA**- rheumatoid arthritis; **UA**- unclassified arthritis; **CSA**- clinically suspect arthralgia; **PsA**- psoriatic arthritis

*Seropositive refers to positive for RF or ACPA. Classification criteria applied as outlined in the **section 2.1**

| Diagnosis | Number (percentage) |
|---|---|
| RA<br>Seropositive* RA<br>Seronegative RA | 83 (46.6)<br>51 (28.6)<br>32 (18) |
| Unclassified arthritis<br>Seropositive*<br>Seronegative | 25 (14)<br>4 (2.2)<br>21 (11.8) |
| CSA<br>Seropositive*<br>Seronegative | 33 (18.5)<br>13 (7.3)<br>20 (11.2) |
| PsA | 22 (12.4) |
| Other arthritis<br>Gout<br>Peripheral spondyloarthopathy<br>Post infectious arthritis<br>Sarcoidosis | 15 (8.4)<br>5 (2.8)<br>5 (2.8)<br>4 (2.2)<br>1 (0.6) |

**Table 5-17 Comparison of demographics by diagnosis for urinary metabolomics analysis cohort.**

**RA**- rheumatoid arthritis; **UA**- unclassified arthritis; **CSA**- clinically suspect arthralgia; **PsA**- psoriatic arthritis; **IQR**- interquartile range; **FACIT-F**- functional assessment of chronic illness therapy – fatigue; **CRP**- C-reactive protein; **RF**- rheumatoid factor; **ACPA**- Anti-citrullinated protein antibody; **NSAID**- nonsteroidal anti-inflammatory drug

* p value calculated using Kruskal-Wallis test ** p value calculated using chi-squared test

ł therapies at baseline

| | All Cohort (n=178) | RA (n=83) | PsA (n=22) | UA (n=25) | CSA (n=33) |
|---|---|---|---|---|---|
| Age, median (IQR) years | 52 (40-58.3) | 55 (48-60) | 44 (32.5-58.5) | 51 (38.5-60) | 42 (34.5-53) |
| Missing (%) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | | p=0.002* | | | |
| Sex, no. (%) females | 115 (64.6) | 55 (66.3) | 12 (54.5) | 16 (64) | 24 (72.7) |
| Missing (%) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | | p=0.581** | | | |
| Symptom duration, median (IQR) weeks | 24.5 (12-52) | 24 (12-45) | 26.5 (7.8-72.8) | 28 (14.5-50.5) | 36 (18-91.5) |
| Missing (%) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | | p=0.240* | | | |
| FACIT-F (IQR) | 32 (21-41) | 29 (18-38) | 31.5 (20.3-38) | 36 (29-45.5) | 35 (25-43.5) |
| Missing (%) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | | p=0.014* | | | |
| CRP, median (IQR) mg/L | 6 (3-13) | 8 (3-16) | 4 (3-13.3) | 5 (3-11.5) | 5 (3-9.5) |
| Missing (%) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | | p=0.233* | | | |
| RF positive, no. (%) | 73 (41) | 51 (61.4) | 2 (9.1) | 7 (28) | 10 (30.3) |
| Missing (%) | 10 (5.6) | 6 (7.2) | 2 (9.1) | 1 (4) | 1 (3) |
| | | p<0.001** | | | |
| ACPA positive, no. (%) | 56 (31.5) | 45 (54.2) | 0 (0) | 0 (0) | 10 (30.3) |
| Missing (%) | 3 (1.7) | 1 (1.2) | 1 (4.5) | 0 (0) | 1 (3) |
| | | p<0.001** | | | |
| NSAIDs, no. (%) ł | 78 (43.8) | 41 (49.4) | 5 (22.7) 0 (0) | 11 (44) | 12 (36.4) |
| Missing (%) | 0 (0) | 0 (0) | | 0 (0) | 0 (0) |
| | | p=0.126** | | | |
| Steroids, no. (%) ł | 30 (16.9) | 18 (21.7) | 5 (22.7) | 1 (4) | 2 (6.1) |
| Missing (%) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | | p=0.042** | | | |

| | | | | | |
|---|---|---|---|---|---|
| Baseline DMARD<br>Missing (%) | 0 (0)<br>0 (0) | 0 (0)<br>0 (0) | 0 (0)<br>0 (0) | 0 (0)<br>0 (0) | 0 (0)<br>0 (0) |
| No. (%) who developed RA (at ≥12 months)<br>Missing (%) | -- | -- | -- | 4 (20)<br><br>5 (20) | 3 (11.5)<br><br>7 (21.2) |

**Table 5-18 Diagnostic outcomes at ≥12 month follow up in CSA & UA patients in whom urinary metabolites were assessed by NMR spectroscopy**

The diagnostic outcomes for clinically suspect arthralgia and unclassified arthritis patients in whom urinary metabolites were assessed by NMR spectroscopy to investigate the relationship between urinary metabolome and CRP

**UA**- unclassified arthritis; **RA**- rheumatoid arthritis; **CSA**- clinically suspect arthralgia

| Baseline diagnosis | ≥12 month follow up diagnosis | | | | |
|---|---|---|---|---|---|
| | No inflammatory arthritis | UA | RA | Other inflammatory arthropathy | Lost to follow up |
| UA (n=25) | -- | 9 | 4 | 7 | 5 |
| CSA (n=33) | 20 | 2 | 3 | 1 | 7 |

**Table 5-19 Treatment usage in CSA & UA patients in whom urinary metabolites were assessed by NMR spectroscopy**

Clinically suspect arthralgia and unclassified arthritis patient treatment in whom urinary metabolites were assessed by NMR spectroscopy to investigate the relationship between urinary metabolome and CRP

**UA-UA-** baseline and follow up diagnosis of unclassified arthritis; **CSA-NIA-** baseline diagnosis of clinically suspect arthralgia and a follow up diagnosis of no inflammatory arthritis; **CSA-UA-** baseline diagnosis of clinically suspect arthralgia and a follow up diagnosis of unclassified arthritis; **HCQ-** hydroxychloroquine; **pred**- prednisolone; **AZA-** azathioprine; **MTX-** methotrexate; **SFZ-** sulfasalazine

| Patients | HCQ alone | HCQ + AZA | MTX + HCQ | MTX alone | MTX + pred | MTX + HCQ + SFZ |
|---|---|---|---|---|---|---|
| UA-UA | 3 | 0 | 2 | 1 | 1 | 0 |
| CSA-NIA | 6 | 1 | 1 | 1 | 0 | 1 |
| CSA-UA | 0 | 0 | 1 | 1 | 0 | 0 |

The median value for CRP in the urinary cohort is 6, Figure 5-13 shows the distribution of CRP by diagnostic subgroups.

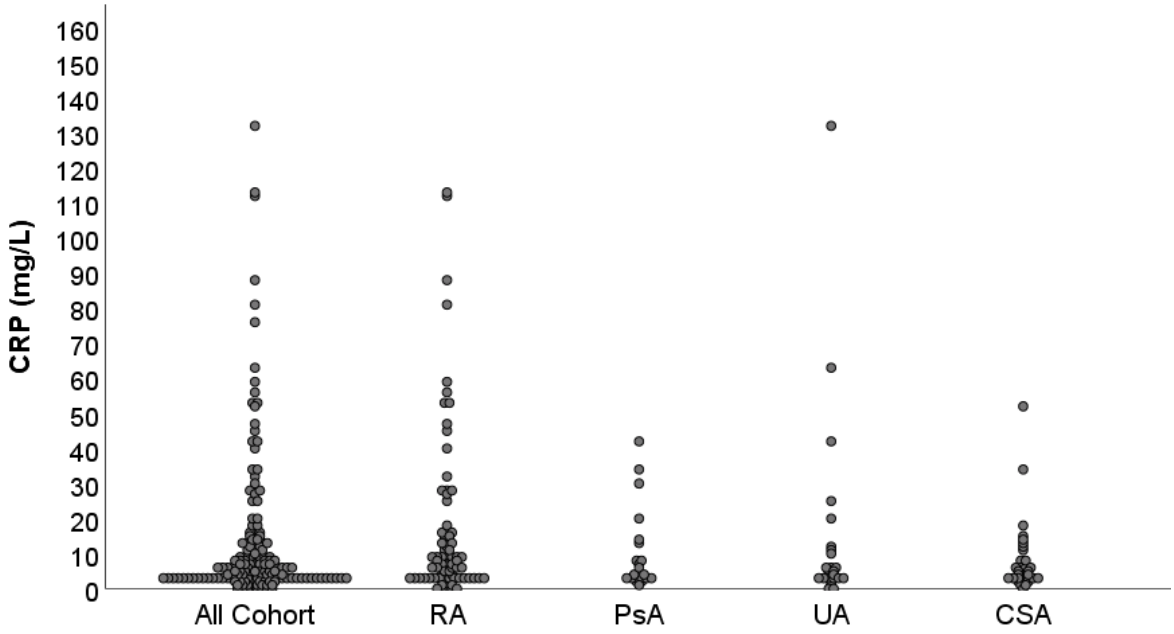



**Figure 5-13 Scatter plot showing the distribution of CRP values across the whole population and diagnostic subgroups used for urinary metabolomics analysis**

### 5.4.6 Relationship between urinary metabolite profile and CRP in the whole cohort

PCA was used to generate an unbiased overview to investigate any differences between patients in the lowest CRP tertile and the highest CRP tertile (Figure 5-14). There was no discernible separation between these groups. However, a supervised analysis using OPLS-DA showed a strong separation with 1+0+0 LVs (p value= 0.00018). Spectral fitting to identify urinary metabolites was performed using Chenomx and a published list of metabolites (171). Table 5-20 shows the bins which had VIP scores greater than 1 and their corresponding urinary metabolites.

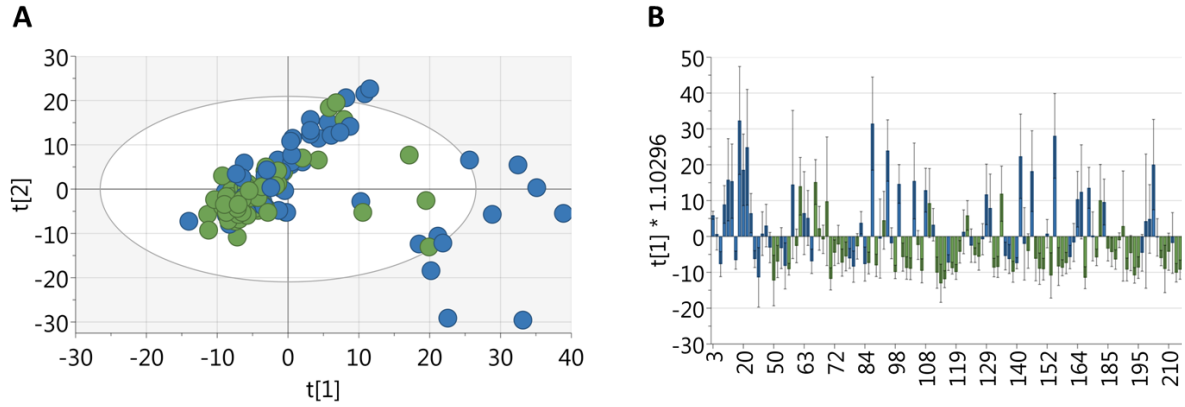



**Figure 5-14 Multivariate analysis of urinary metabolite profile by CRP tertiles with data shown for the highest and lowest tertiles**

**(A)** PCA plot of metabolic data derived from all patients' (n=178) urine (green=CRP <4 and blue=CRP>8; 19 PC, $r^2$=0.673) showing no separation between the two groups. **(B)** OPLS-DA plot of metabolic data derived from all patients' (n=178) urine (green=CRP <4

and blue=CRP>8; 1+0+0 LV, P value= 0.00018) showing a strong separation between the two groups.

**Table 5-20 Urinary metabolites with VIP scores greater than 1 from the OPLS-DA model of all patients showing separation between low and high CRP tertiles.**

The following metabolites have been ranked by magnitude of the VIP score. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of VIP score. Where the direction of change in metabolite concentration in patients in the higher CRP tertile is indicated by arrows (↑ indicates increased metabolite concentration in patients in the higher CRP tertile and ↓ indicates decreased metabolite concentration in patients in the higher CRP tertile).

| Order | Metabolite | Chemical shift of peak (ppm) | Direction of change in metabolite concentration in patients with higher CRP for each peak |
|---|---|---|---|
| 1 | Phenylacetate | 7.3011, 7.225, 7.2425, 7.2367, 7.2484 | ↑↑↑↑↑ |
| 1 | Indoleacetate | 7.3011, 7.3186, 7.225, 7.2308, 7.2425, 7.2367, 7.2484 | ↑↑↑↑↑↑↑ |
| 1 | Indoxyl sulfate | 7.3011, 7.225, 7.2308, 7.2367 | ↑↑↑↑ |
| 1 | Phenol | 7.3011, 7.3186, 7.2367 | ↑↑↑ |
| 1 | Phenylalanine | 7.3011, 7.3186, 7.225, 7.2308, 7.2425, 7.2367, 7.2484 | ↑↑↑↑↑↑↑ |
| 1 | Trans-ferulic acid | 7.3011, 7.225, 7.2308, 7.2367 | ↑↑↑↑ |
| 1 | Tryptophan | 7.3011, 7.3186, 7.225, 7.2308, 7.2367, 7.2484 | ↑↑↑↑↑↑ |
| 1 | Urea | 7.3011, 7.3186, 7.225, 7.2308, 7.2425, 7.2367, 7.2484 | ↑↑↑↑↑↑ |
| 2 | 3-methyladipic acid | 1.4943, 1.4889, 2.3548 | ↑↑↓ |
| 2 | 3-methyl-2-oxovaleric acid | 1.4943, 1.4892, 1.0588 | ↑↑↑ |
| 2 | Adipic acid | 1.4943, 1.4885 | ↑↑ |
| 2 | Azelaic acid | 1.4943, 1.4887 | ↑↑ |
| 2 | Alanine | 1.4943, 1.4891 | ↑↑ |
| 2 | Lysine | 1.4943, 1.4886, 1.3772, 1.3889, 1.3831, 1.8514 | ↑↑↑↑↑ |
| 2 | Sebacic acid | 1.4943, 1.4888 | ↑↑ |
| 2 | Suberic acid | 1.4943, 1.4890 | ↑↑ |
| 3 | 3-hydroxyphenylacetate | 7.225, 7.2308, 7.2425, 7.2367, 7.2484 | ↑↑↑↑↑ |
| 3 | Ortho-hydroxyphenylacetate | 7.225, 7.2308 | ↑↑ |
| 3 | Thymol | 7.225, 7.2308 | ↑↑ |
| 3 | Tyrosine | 7.225, 7.2308, 7.2367 | ↑↑↑ |
| 4 | Alpha-hydroxyisobutyric acid | 1.3772, 1.3889, 1.3831 | ↑↑↑ |
| 4 | Lactate | 1.3772, 1.3889, 1.3831 | ↑↑↑ |
| 5 | 2-ketobutyric acid | 1.0588 | ↑ |

| | | | |
|---|---|---|---|
| 5 | Valine | 1.0588 | ↑ |
| 5 | Methylsuccinate | 1.0588 | ↑ |
| 6 | 3-hydroxyisovaleric acid | 2.3548 | ↓ |
| 6 | Aminoadipic acid | 2.3548, 1.8514 | ↓↑ |
| 6 | Glutaric acid | 2.3548, 1.8514 | ↓↑ |
| 6 | Glutamate | 2.3548 | ↓ |
| 6 | Thymidine | 2.3548, 1.8514 | ↓↑ |
| 7 | Arginine | 1.8514 | ↑ |
| 7 | L-2-hydroxyglutaric acid | 1.8514 | ↑ |
| 7 | Monomethyl glutaric acid | 1.8514 | ↑ |

The urinary metabolite profile was subjected to PLS-R analysis (Figure 5-15). Using the complete 900 bins, a PLS-R analysis (Figure 5-15A) showed a correlation between urinary metabolite profile and serum CRP ($r^2$=0.0947, 1 LV, p=0.008). Potential biomarkers identified from the PLS-R are shown in Table 5-21. Using a forward selection approach, a PLS-R using 6 urinary NMR bins produced the most optimal correlation with CRP (Figure 5-15B- $r^2$=0.1347, 1 LV, p=0.021). In order to isolate a complete inflammation signal, a PLS-R analysis was performed on the remaining 894 urinary NMR bins (Figure 5-15C), which showed a statistically significant correlation between the CRP ($r^2$=0.0845, 1 LV, p=0.014). Forward selection identified 9 urinary NMR bins which were largely responsible for the correlation between CRP and the 894 urinary NMR bins. These 9 bins were removed and a PLS-R was performed on the remaining 885 urinary NMR bins (Figure 5-15D) which nonetheless showed a statistically significant correlation with CRP ($r^2$=0.0744, 1 LV, p=0.017). A final forward selection identified 204 urinary bins, which were then removed and a PLS-R of the remaining 681 bins (Figure 5-15E) showed no correlation between urinary metabolite profile and CRP ($r^2$=0.0187, 1 LV, p=0.241).

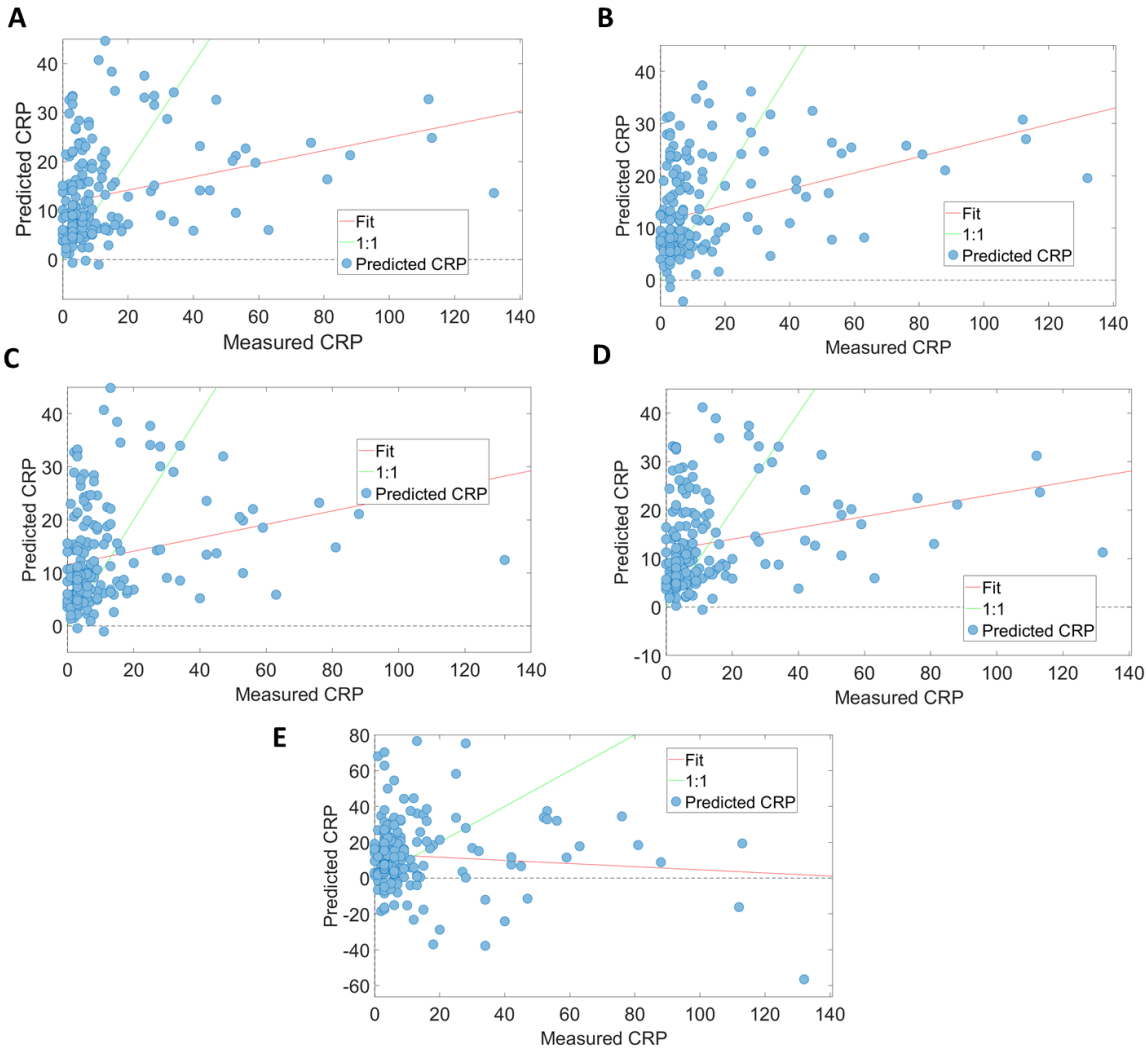



**Figure 5-15 PLS-R analysis showing the relationship between urinary metabolites and CRP & the removal of the urinary metabolite inflammatory signal**

Using the full 900 NMR urinary metabolite bins for all patients (n=178) (**A**) there was a correlation between metabolite profile and CRP ($r^2$=0.0947, 1 LV, p=0.008). Using forward selection, 6 bins were identified which most strongly correlated with CRP and a subsequent PLS-R using these bins (**B**) showed a correlation between urinary metabolite profile and CRP ($r^2$=0.1347, 1 LV, p=0.021). PLS-R with the remaining 894 NMR bins (**C**) showed a correlation between metabolite profile and CRP ($r^2$=0.0845, 1 LV, p=0.014). Another forward selection identified a further 9 NMR bins which correlated with CRP and were removed. A PLS-R with the remaining 885 NMR bins (**D**) showed the correlation between urinary metabolite profile and CRP persisted ($r^2$=0.0744, 1 LV, p=0.017). A final forward selection identified 204 NMR bins which were then removed, a PLS-R of the remaining 681 NMR bins (**E**) showed no correlation between urinary metabolite profile and CRP ($r^2$=0.0187, 1 LV, p=0.241). Thus the remaining 681 NMR bins can be considered a metabolite profile without a significant inflammatory signal.

**Table 5-21 Metabolites responsible for the relationship seen in PLS-R between CRP and urinary metabolite profile in all patients.**

The following metabolites have been ranked by the magnitude of the regression coefficient. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of regression coefficient. The regression coefficient field indicates the nature of correlation (↑ indicating a positive relationship with CRP and ↓ indicating a negative relationship with CRP)

| Order | Metabolite | Chemical shift of peak (ppm) | Regression coefficient |
|---|---|---|---|
| 1 | Indoleacetate | 7.2308, 7.2367, 7.225, 7.2191, 7.2425, 7.2484, 7.3011, 7.2132, 7.4942, 7.3186, 7.143, 7.1372, 7.4825, 7.3128 | ↑↑↑↑↑↑↑↑↑↑↑↑↑ |
| 1 | Urea | 7.2308, 7.2367, 7.225, 7.2191, 7.2425, 7.2484, 7.3011, 7.2132, 7.4942, 7.3186, 7.143, 7.3596, 7.1372, 7.342, 7.7768, 7.4825, 7.3128, 7.3771 | ↑↑↑↑↑↑↑↑↑↑↑↑↑↑↑↑↑ |
| 1 | Phenylalanine | 7.2308, 7.2367, 7.225, 7.2191, 7.2425, 7.2484, 7.4532, 7.3011, 7.2132, 7.4474, 7.4942, 7.3186, 7.4357, 7.143, 7.4415, 3.0923, 7.3596, 3.0982, 7.1372, 7.342, 7.4825, 7.3128, 7.3771 | ↑↑↑↑↑↑↑↑↑↑↑↑↑↑↓↑↓↑↑↑↑↑ |
| 1 | Trans-ferulic acid | 7.2308, 7.2367, 7.225, 7.2191, 7.3011, 7.2132 | ↑↑↑↑↑↑ |
| 1 | Phenylacetate | 7.2308, 7.2367, 7.225, 7.2191, 7.2425, 7.2484, 7.3011, 7.2132, 7.3596, 7.342, 7.3771 | ↑↑↑↑↑↑↑↑↑↑↑ |
| 1 | Tyrosine | 7.2308, 7.2367, 7.225, 7.2191, 7.2132, 7.143 | ↑↑↑↑↑↑ |
| 1 | Tryptophan | 7.2308, 7.2367, 7.225, 7.2191, 7.2484, 7.3011, 7.2132, 7.4942, 7.3186, 7.3128 | ↑↑↑↑↑↑↑↑↑↑ |
| 1 | 3-hydroxyphenyl acetate | 7.2308, 7.2367, 7.225, 7.2425, 7.2484 | ↑↑↑↑↑ |
| 1 | Indoxyl sulfate | 7.2308, 7.2367, 7.225, 7.2191, 7.3011, 7.2132, 7.4942, 7.3596, 7.4825, 7.3771 | ↑↑↑↑↑↑↑↑↑ |
| 1 | Ortho-hydroxyphenyl acetate | 7.2308, 7.2367, 7.225, 7.2191, 7.2132 | ↑↑↑↑↑ |
| 1 | Thymol | 7.2308, 7.2367, 7.225, 7.2191, 7.2132, 1.1431 | ↑↑↑↑↑↓ |
| 2 | Phenol | 7.2367, 7.3011, 7.3186, 7.342, 7.3128 | ↑↑↑↑↑ |
| 3 | Adipic acid | 1.4943, 1.5002, 1.4885, 1.506, 2.1558, 1.5111 | ↑↑↑↑↑↑ |
| 3 | Lysine | 1.4943, 1.8514, 1.5002, 1.4885, 1.506, 1.8568, 1.8455, 1.5111, 1.8338 | ↑↑↑↑↑↑↑↑↑ |
| 3 | Azelaic acid | 1.4943, 1.5002, 1.4885, 1.506, 2.1558, 1.5111 | ↑↑↑↑↑↑ |
| 3 | Sebacic acid | 1.4943, 1.5002, 1.4885, 1.506, 2.1558, 1.5111 | ↑↑↑↑↑↑ |
| 3 | 3-methyladipic acid | 1.4943, 1.5002, 1.4885, 1.506, 2.1558, 1.5111, 1.6055 | ↑↑↑↑↑↑↑ |
| 3 | Suberic acid | 1.4943, 1.5002, 1.4885, 1.506, 1.5938, 2.1558, 1.5111 | ↑↑↑↑↑↑↑ |
| 3 | Alanine | 1.4943, 1.5002, 1.4885, 1.506, 1.5111 | ↑↑↑↑↑ |
| 3 | 3-methyl-2-oxovaleric acid | 1.4943, 1.5002, 1.4885, 1.127 | ↑↑↑↓ |

| | | | |
|---|---|---|---|
| 4 | Arginine | 1.8514, 1.8568, 1.5938, 1.5997, 1.8455, 1.6055, 1.8338 | ↑↑↑↑↑↑↑ |
| 4 | L-2-hydroxyglutaric acid | 1.8514, 1.8568, 1.8455, 2.1558, 1.8338 | ↑↑↑↑↑ |
| 4 | Thymidine | 1.8514, 1.8568, 1.8455, 3.8357 | ↑↑↑↓ |
| 4 | Aminoadipic acid | 1.8514, 1.8568, 1.5938, 1.5997, 1.8455, 1.6055, 1.8338 | ↑↑↑↑↑↑↑ |
| 4 | Glutaric acid | 1.8514, 1.8568, 1.8455, 1.8338 | ↑↑↑↑ |
| 4 | Monomethyl glutaric acid | 1.8514, 1.8568, 1.8455, 2.1558, 1.8338 | ↑↑↑↑↑ |
| 5 | Vanillic acid | 7.4532, 7.4474, 7.4942, 7.4357, 7.4415, 7.4825 | ↑↑↑↑↑↑ |
| 5 | Cinnamic acid | 7.4532, 7.4474, 7.4942, 7.4357, 7.4415, 7.4825, 7.3771 | ↑↑↑↑↑↑↑ |
| 5 | Benzoic acid | 7.4532, 7.4474, 7.4942, 7.4825 | ↑↑↑↑ |
| 6 | D-alpha-aminobutyric acid | 1.8568 | ↑ |
| 7 | Histidine | 7.2132, 7.143, 7.1372 | ↑↑↑ |
| 8 | Quinolinic acid | 7.4474, 7.4357 , 7.4415 | ↑↑↑ |
| 9 | Cytosine | 7.4942, 7.4825 | ↑↑ |
| 9 | Hippuric acid | 7.4942, 7.7768, 7.4825, 7.8045 | ↑↑↑↑ |
| 9 | Uracil | 7.4942 | ↑ |
| 9 | Kynurenic acid | 7.4942, 7.4357, 7.3596, 7.342 | ↑↑↑↑ |
| 10 | N-acetylputrescine | 1.5938, 1.5997, 1.6055, 1.8338 | ↑↑↑↑ |
| 11 | P-hydroxyphenyl acetate | 7.143, 7.1372 | ↑↑ |
| 11 | P-cresol sulfate | 7.143, 7.1372 | ↑↑ |
| 11 | Carnosine | 7.143, 7.1372, 2.6826, 3.8357, 2.6767, 2.6533 | ↑↑↓↓↓↓ |
| 11 | Hydroxyphenyl lactate | 7.143, 7.1372 | ↑↑ |
| 11 | Anserine | 7.143, 7.1372, 2.6826, 2.6592, 2.6767, 2.6533 | ↑↑↓↓↓↓ |
| 12 | 4-hydroxyproline | 2.1558, 2.5187, 2.5128, 2.507 | ↑↓↓↓ |
| 12 | Isovalerylglycine | 2.1558 | ↑ |
| 12 | L-cystathionine | 2.1558, 3.0923, 3.0982, 2.6826, 3.8357, 2.6592, 2.6767, 2.6533 | ↑↓↓↓↓↓↓↓ |
| 12 | Methionine | 2.1558 | ↑ |
| 12 | Pimelic acid | 2.1558, 1.5111 | ↑↑ |
| 12 | Glutamate | 2.1558 | ↑ |
| 12 | Glutamine | 2.1558 | ↑ |
| 13 | Creatinine | 3.0923, 3.0982, 2.6826, 2.6592, 2.6767, 2.6533 | ↓↓↓↓↓↓ |
| 13 | 1,3-diaminopropane | 3.0923, 3.0982 | ↓↓ |

| | | | |
|---|---|---|---|
| 13 | 3-aminoisobutanoic acid | 3.0923, 3.0982, 1.1431, 1.127, 2.6592, 2.6533 | ↓↓↓↓↓↓ |
| 13 | Malonate | 3.0923, 3.0982 | ↓↓ |
| 13 | Cysteine | 3.0923, 3.0982 | ↓↓ |
| 13 | 3-methylhistidine | 3.0923 | ↓ |
| 14 | Mandelic acid | 7.3596, 7.3771 | ↑↑ |
| 15 | 5-aminopentanoic acid | 1.6055 | ↑ |
| 16 | Phenylglyoxylic acid | 7.7768 | ↑ |
| 16 | 4-hydroxybenzoic acid | 7.7768, 7.8045 | ↑↑ |
| 16 | 7-methylxanthine | 7.7768, 7.8045 | ↑↑ |
| 17 | Citrate | 2.6826, 2.5187, 2.5245, 2.5128, 2.6592, 2.507, 2.6767, 2.6533 | ↓↓↓↓↓↓↓↓ |
| 17 | Dihydrouracil | 2.6826, 2.6592, 2.6767, 2.6533 | ↓↓↓↓ |
| 17 | Aspartate | 2.6826, 2.6592, 2.6767, 2.6533 | ↓↓↓↓ |
| 18 | Beta-alanine | 2.5187, 2.5245, 2.5128, 2.507 | ↓↓↓↓ |
| 18 | Isocitrate | 2.5187, 2.5245, 2.5128, 2.507 | ↓↓↓↓ |
| 18 | Pyroglutamic acid | 2.5187, 2.5245, 2.5128, 2.507 | ↓↓↓↓ |
| 18 | Methylsuccinate | 2.5187, 2.5245, 2.5128, 2.507 | ↓↓↓↓ |
| 18 | 5-aminolevulinic acid | 2.5187, 2.5128, 2.507 | ↓↓↓ |
| 18 | Dimethylamine | 2.5187, 2.5128, 2.507 | ↓↓↓ |
| 19 | Ethanol | 1.1431, 1.127 | ↓↓ |
| 19 | Propylene glycol | 1.1431, 1.127 | ↓↓ |
| 20 | D-threitol | 3.584 | ↓ |
| 20 | Myo-inositol | 3.584 | ↓ |
| 20 | D-maltose | 3.584, 3.8357 | ↓↓ |
| 20 | Alpha-lactose | 3.584 | ↓ |
| 20 | Phosphorylcholine | 3.584 | ↓ |
| 20 | Erythritol | 3.584 | ↓ |
| 20 | L-arabitol | 3.584, 3.8357 | ↓↓ |
| 21 | Serine | 3.8357 | ↓ |
| 21 | Sorbitol | 3.8357 | ↓ |
| 21 | Glyceric acid | 3.8357 | ↓ |
| 21 | Hydroxypropionic acid | 3.8357, 2.6592, 2.6533 | ↓↓ |

| | | | |
|---|---|---|---|
| 21 | D-galactose | 3.8357 | ↓ |
| 21 | Ethanolamine | 3.8357 | ↓ |
| 21 | D-xylitol | 3.8357 | ↓ |
| 21 | Gluconic acid | 3.8357 | ↓ |
| 21 | L-arabinose | 3.8357 | ↓ |
| 21 | Pseudouridine | 3.8357 | ↓ |
| 21 | Sucrose | 3.8357 | ↓ |
| 21 | N-acetylneuraminic acid | 3.8357 | ↓ |
| 21 | Inosine | 3.8357 | ↓ |
| 21 | Dehydroascorbic acid | 3.8357 | ↓ |
| 11 | Anserine | 7.143, 7.1372, 2.6826, 2.6592, 2.6767, 2.6533 | ↑↑↓↓↓↓ |
| 12 | 4-hydroxyproline | 2.1558, 2.5187, 2.5128, 2.507 | ↑↓↓↓ |
| 12 | Isovalerylglycine | 2.1558 | ↑ |
| 12 | L-cystathionine | 2.1558, 3.0923, 3.0982, 2.6826, 3.8357, 2.6592, 2.6767, 2.6533 | ↑↓↓↓↓↓↓↓ |
| 12 | Methionine | 2.1558 | ↑ |
| 12 | Pimelic acid | 2.1558, 1.5111 | ↑↑ |
| 12 | Glutamate | 2.1558 | ↑ |
| 12 | Glutamine | 2.1558 | ↑ |
| 13 | Creatinine | 3.0923, 3.0982, 2.6826, 2.6592, 2.6767, 2.6533 | ↓↓↓↓↓↓ |
| 13 | 1,3-diaminopropane | 3.0923, 3.0982 | ↓↓ |
| 13 | 3-aminoisobutanoic acid | 3.0923, 3.0982, 1.1431, 1.127, 2.6592, 2.6533 | ↓↓↓↓↓↓ |
| 13 | Malonate | 3.0923, 3.0982 | ↓↓ |
| 13 | Cysteine | 3.0923, 3.0982 | ↓↓ |
| 13 | 3-methylhistidine | 3.0923 | ↓ |
| 14 | Mandelic acid | 7.3596, 7.3771 | ↑↑ |
| 15 | 5-aminopentanoic acid | 1.6055 | ↑ |
| 16 | Phenylglyoxylic acid | 7.7768 | ↑ |
| 16 | 4-hydroxybenzoic acid | 7.7768, 7.8045 | ↑↑ |
| 16 | 7-methylxanthine | 7.7768, 7.8045 | ↑↑ |
| 17 | Citrate | 2.6826, 2.5187, 2.5245, 2.5128, 2.6592, 2.507, 2.6767, 2.6533 | ↓↓↓↓↓↓↓↓ |

| | | | |
|---|---|---|---|
| 17 | Dihydrouracil | 2.6826, 2.6592, 2.6767, 2.6533 | ↓↓↓↓ |
| 17 | Aspartate | 2.6826, 2.6592, 2.6767, 2.6533 | ↓↓↓↓ |
| 18 | Beta-alanine | 2.5187, 2.5245, 2.5128, 2.507 | ↓↓↓↓ |
| 18 | Isocitrate | 2.5187, 2.5245, 2.5128, 2.507 | ↓↓↓↓ |
| 18 | Pyroglutamic acid | 2.5187, 2.5245, 2.5128, 2.507 | ↓↓↓↓ |
| 18 | Methylsuccinate | 2.5187, 2.5245, 2.5128, 2.507 | ↓↓↓↓ |
| 18 | 5-aminolevulinic acid | 2.5187, 2.5128, 2.507 | ↓↓↓ |
| 18 | Dimethylamine | 2.5187, 2.5128, 2.507 | ↓↓↓ |
| 19 | Ethanol | 1.1431, 1.127 | ↓↓ |
| 19 | Propylene glycol | 1.1431, 1.127 | ↓↓ |
| 20 | D-threitol | 3.584 | ↓ |
| 20 | Myo-inositol | 3.584 | ↓ |
| 20 | D-maltose | 3.584, 3.8357 | ↓↓ |
| 20 | Alpha-lactose | 3.584 | ↓ |
| 20 | Phosphorylcholine | 3.584 | ↓ |
| 20 | Erythritol | 3.584 | ↓ |
| 20 | L-arabitol | 3.584, 3.8357 | ↓↓ |
| 21 | Serine | 3.8357 | ↓ |
| 21 | Sorbitol | 3.8357 | ↓ |
| 21 | Glyceric acid | 3.8357 | ↓ |
| 21 | Hydroxypropionic acid | 3.8357, 2.6592, 2.6533 | ↓↓ |
| 21 | D-galactose | 3.8357 | ↓ |
| 21 | Ethanolamine | 3.8357 | ↓ |
| 21 | D-xylitol | 3.8357 | ↓ |
| 21 | Gluconic acid | 3.8357 | ↓ |
| 21 | L-arabinose | 3.8357 | ↓ |
| 21 | Pseudouridine | 3.8357 | ↓ |
| 21 | Sucrose | 3.8357 | ↓ |
| 21 | N-acetylneuraminic acid | 3.8357 | ↓ |
| 21 | Inosine | 3.8357 | ↓ |
| 21 | Dehydroascorbic acid | 3.8357 | ↓ |

Figure 5-16 and Table 5-22 show the pathway analysis of the biomarkers identified by the PLS-R analysis of all patients' urinary metabolite data and CRP. This indicated alanine, aspartate and glutamate metabolism and beta-alanine metabolism were the most impacted metabolic pathways. Figure 5-17 and Table 5-23 show the enrichment analysis using the biomarkers isolated by the PLS-R analysis of all patients' urinary metabolite data and CRP. Beta-alanine metabolism, the urea cycle and homocysteine degradation were the only overrepresented metabolic pathways that reached statistical significance. Similar to the functional interpretation of all patients' serum biomarkers, the

urinary biomarkers also suggest altered amino acid metabolism is the main attribute of inflammatory arthritis and CSA patients with elevated CRP.

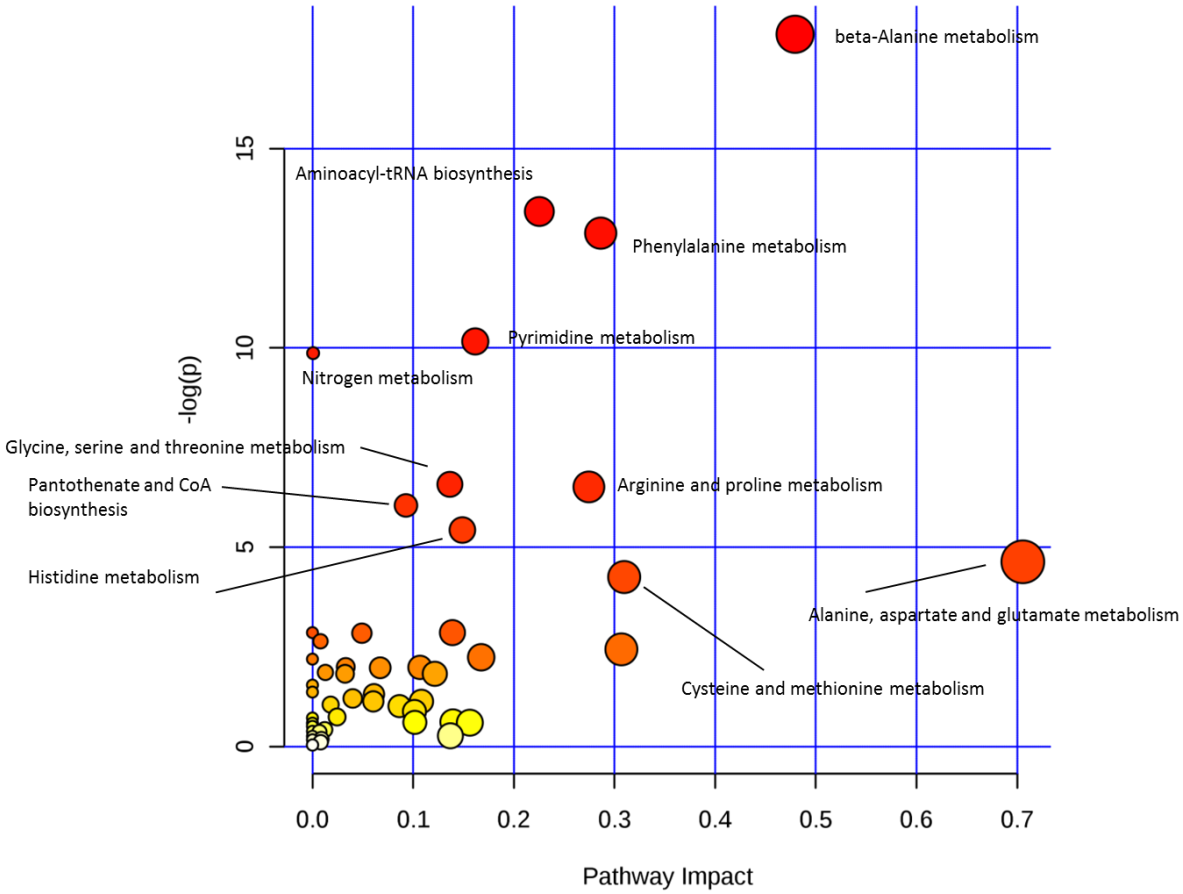



**Figure 5-16 Metaboanalyst pathway analysis of potential biomarkers implicated by PLS-R analysis of CRP and all patients' urinary metabolites**

**Table 5-22 Pathway analysis of potential biomarkers implicated by PLS-R analysis of all patients' urinary metabolites and CRP**

**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.

**The impact score**: Each pathway has a total importance score of 1. The importance measure of each metabolite node (a point where pathways intersect within a larger network) is expressed as a fraction with regards to the total pathway importance, and the pathway impact score is the cumulative score of matched metabolite nodes.

* p value calculated using hypergeometric test

| Pathway Name | Match Status | P value* | Impact score |
|---|---|---|---|
| Alanine, aspartate and glutamate metabolism | 4/24 | 0.009727 | 0.70561 |
| beta-Alanine metabolism | 10/28 | 1.74E-08 | 0.47941 |
| Cysteine and methionine metabolism | 6/56 | 0.014257 | 0.30965 |
| Phenylalanine metabolism | 10/45 | 2.56E-06 | 0.28632 |
| Arginine and proline metabolism | 9/77 | 0.001487 | 0.27458 |
| Aminoacyl-tRNA biosynthesis | 13/75 | 1.49E-06 | 0.22536 |
| Pyrimidine metabolism | 10/60 | 3.87E-05 | 0.16173 |
| Histidine metabolism | 6/44 | 0.004383 | 0.14893 |
| Glycine, serine and threonine metabolism | 7/48 | 0.001394 | 0.13651 |
| Pantothenate and CoA biosynthesis | 5/27 | 0.002366 | 0.09288 |
| Nitrogen metabolism | 8/39 | 5.18E-05 | 6.70E-04 |

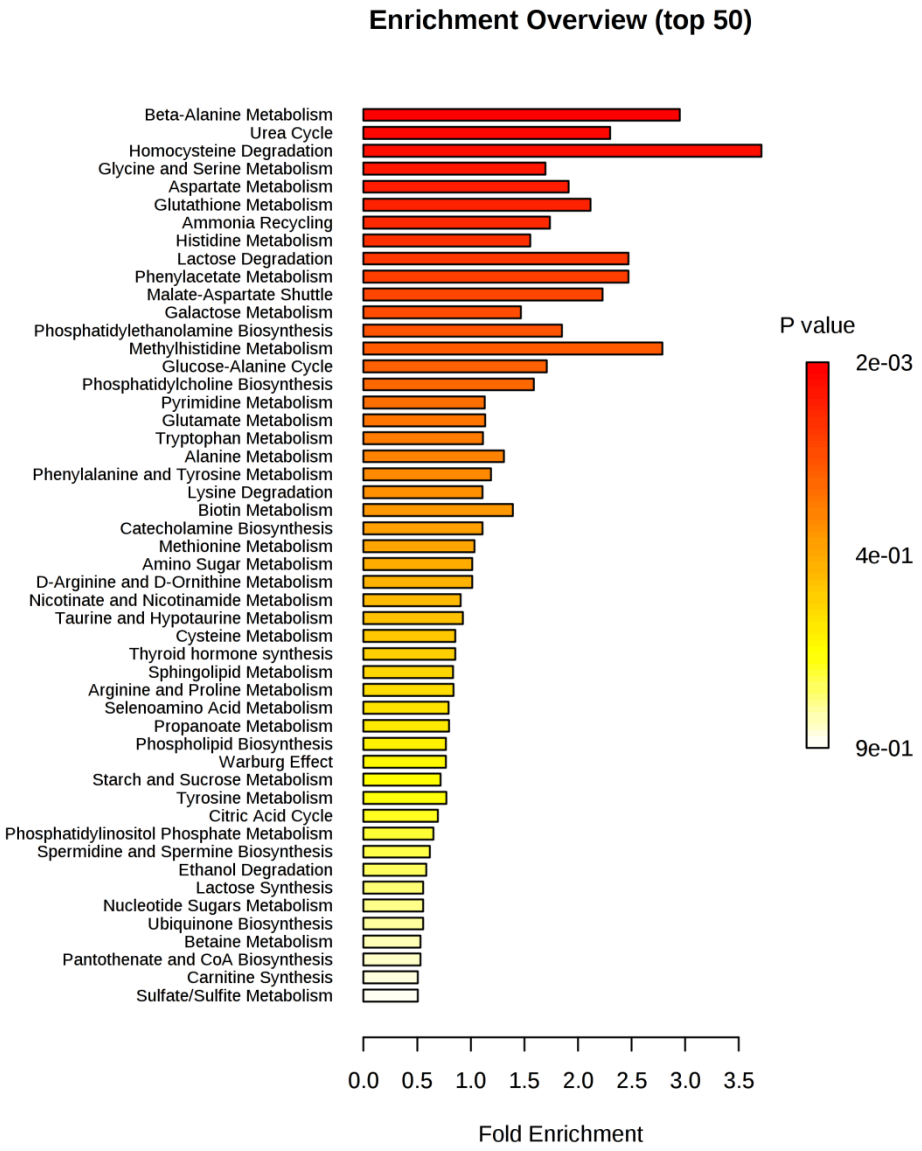



**Figure 5-17 Enrichment analysis of key metabolites in urine implicated as potential biomarkers by the PLS-R analysis of CRP and all patients' urinary metabolites**

**Table 5-23 Enrichment analysis of key metabolites in urine implicated as potential biomarkers by the PLS-R analysis of CRP and all patients' urinary metabolites.**
**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.
**The fold enrichment value**: the value is generated by the number of matched metabolites divided by the number of metabolites expected to be present in the pathway as per the reference metabolome provided by KEGG.
* p value calculated using hypergeometric test

| Metabolite Set | Match status | Fold enrichment | Expected | P value* |
|---|---|---|---|---|
| Beta-Alanine Metabolism | 9/34 | 2.95 | 3.05 | 0.00205 |
| Urea Cycle | 6/29 | 2.30 | 2.61 | 0.0389 |
| Homocysteine Degradation | 3/9 | 3.71 | 0.809 | 0.0396 |

## 5.4.7 Relationship between urinary metabolite profile and CRP in RA patients

The OPLS-DA of those in the highest CRP tertile versus those in the lowest CRP tertile was repeated for RA, UA, PsA and CSA (Figure 5-18). Although a separation was seen between the highest and the lowest CRP tertile amongst all diagnostic groups, this was only significant in RA patients (1+0+0 LV, p= 0.00001).

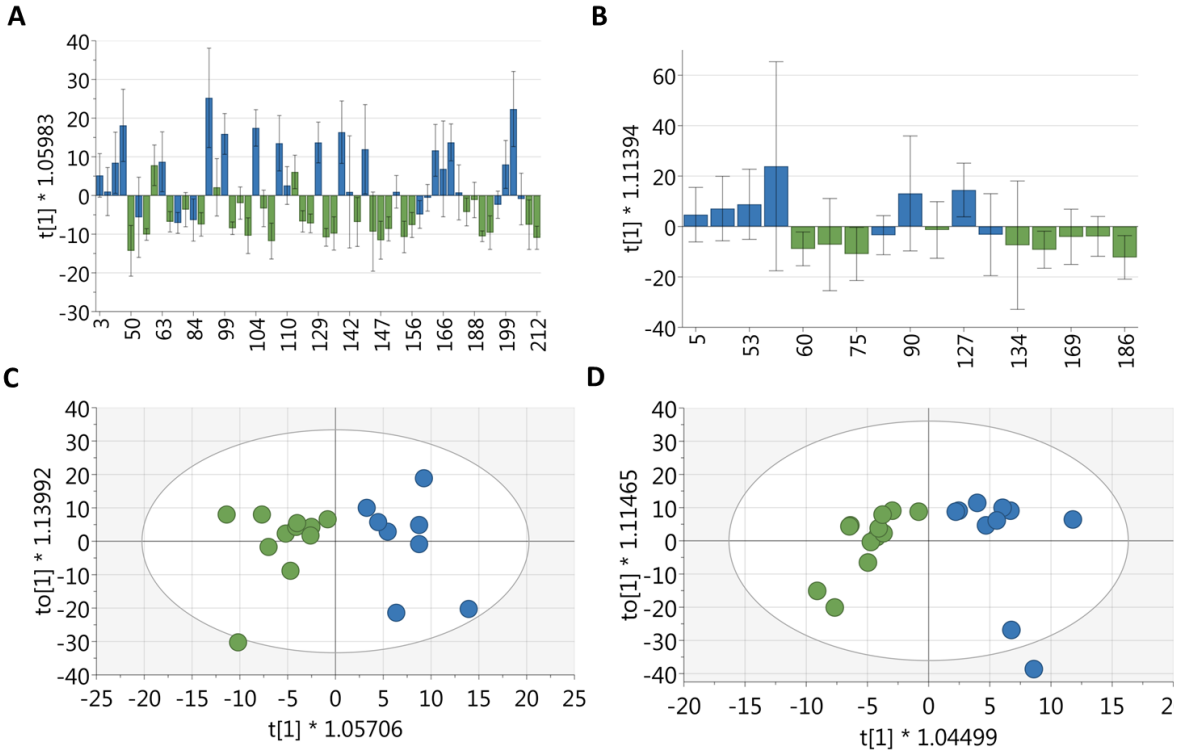



**Figure 5-18 Multivariate analysis of urinary metabolite profile by CRP tertiles with data shown for the highest and lowest tertiles by diagnosis**
Across all diagnoses OPLS-DA showed separation between patients in the highest CRP tertile compared to patients in the lowest CRP tertile. However, similar to the results seen in serum, this relationship was only significant in RA patients: **(A)** OPLS-DA plot of RA (n=83) patients' urinary metabolite profile (green=CRP <5 and blue=CRP>11; 1+0+0 LV P value= 0.00001). **(B)** OPLS-DA plot of PsA (n=22) patients' urinary metabolite profile (green=CRP <4 and blue=CRP>7; 1+0+0 LV P value= 0.26997).

**(C)** OPLS-DA plot of UA (n=25) patients' urinary metabolite profile (green=CRP <4 and blue=CRP>6; 1+1+0 LV P value= 0.92805). **(D)** OPLS-DA plot of CSA (n=33) patients' urinary metabolite profile (green=CRP <4 and blue=CRP>7; 1+1+0 LV P value=1).

**Table 5-24 Urinary metabolites with VIP scores greater than 1 from the OPLS-DA model of RA patients showing separation between low and high CRP tertiles.**

The following metabolites have been ranked by magnitude of the VIP score. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of VIP score. Where the direction of change in metabolite concentration in patients in the higher CRP tertile is indicated by arrows (↑ indicates increased metabolite concentration in patients in the higher CRP tertile and ↓ indicates decreased metabolite concentration in patients in the higher CRP tertile).

| Order | Metabolite | Chemical shift of peak (ppm) | Regression coefficient |
|---|---|---|---|
| 1 | Phenol | 7.3011, 7.3186, 7.2367 | ↑↑↑ |
| 1 | Tryptophan | 7.3011, 7.3186, 7.225, 7.2308, 7.2367, 7.2484 | ↑↑↑↑↑↑ |
| 1 | Phenylalanine | 7.3011, 7.3186, 7.225, 7.2308, 7.2425, 7.2367, 7.2484 | ↑↑↑↑↑↑↑ |
| 1 | Indoleacetate | 7.3011, 7.3186, 7.225, 7.2308, 7.2425, 7.2367, 7.2484 | ↑↑↑↑↑↑↑ |
| 1 | Urea | 7.3011, 7.3186, 7.225, 7.2308, 7.2425, 7.2367, 7.2484 | ↑↑↑↑↑↑↑ |
| 1 | Trans-ferulic acid | 7.3011, 7.225, 7.2308, 7.2367 | ↑↑↑↑ |
| 1 | Indoxyl sulfate | 7.3011, 7.225, 7.2308, 7.2367 | ↑↑↑↑ |
| 1 | phenylacetate | 7.3011, 7.225, 7.2425, 7.2367, 7.2484 | ↑↑↑↑↑ |
| 2 | Adipic acid | 1.4943, 1.4885 | ↑↑ |
| 2 | Lysine | 1.4943, 1.4886, 1.3772, 1.3889, 1.3831, 1.8514 | ↑↑↑↑↑ |
| 2 | Azelaic acid | 1.4943, 1.4887 | ↑↑ |
| 2 | Sebacic acid | 1.4943, 1.4888 | ↑↑ |
| 2 | 3-methyladipic acid | 1.4943, 1.4889, 2.3548 | ↑↑↓ |
| 2 | Suberic acid | 1.4943, 1.4890 | ↑↑ |
| 2 | Alanine | 1.4943, 1.4891 | ↑↑ |
| 2 | 3-methyl-2-oxovaleric acid | 1.4943, 1.4892, 1.0588 | ↑↑↑ |
| 3 | Tyrosine | 7.225, 7.2308, 7.2367 | ↑↑↑ |
| 3 | Ortho-hydroxyphenylacetate | 7.225, 7.2308 | ↑↑ |
| 3 | 3-hydroxyphenylacetate | 7.225, 7.2308 | ↑↑ |
| 3 | Thymol | 7.225, 7.2308 | ↑↑ |
| 4 | Lactate | 1.3772, 1.3889, 1.3831 | ↑↑↑ |
| 4 | Alpha-hydroxyisobutyric acid | 1.3772, 1.3889, 1.3831 | ↑↑↑ |
| 5 | 2-ketobutyric acid | 1.0588 | ↑ |
| 5 | Methylsuccinate | 1.0588 | ↑ |
| 5 | Valine | 1.0588 | ↑ |

| | | | |
|---|---|---|---|
| 6 | 3-hydroxyphenylacetate | 7.2425, 7.2367, 7.2484 | ↑↑↑ |
| 7 | Glutamate | 2.3548 | ↓ |
| 7 | Glutaric acid | 2.3548, 1.8514 | ↓↑ |
| 7 | Thymidine | 2.3548, 1.8514 | ↓↑ |
| 7 | 3-hydroxyisovaleric acid | 2.3548 | ↓ |
| 7 | Aminoadipic acid | 2.3548, 1.8514 | ↓↑ |
| 8 | Arginine | 1.8514 | ↑ |
| 8 | L-2-hydroxyglutaric acid | 1.8514 | ↑ |
| 8 | Monomethyl glutaric acid | 1.8514 | ↑ |

Separate PLS-R of urinary metabolites and CRP by diagnostic groups with forward selection are shown in Figure 5-19. RA ($r^2$=0.4292, 3 LV, p<0.001), PsA ($r^2$=0.6625, 6 LV, p=0.028) and UA ($r^2$=0.6117, 8 LV, p=0.025) patients had a statistically significant correlation between urinary metabolite profile and CRP. Table 5-25, Table 5-31 and Table 5-28 show the potential biomarkers identified by these analyses.

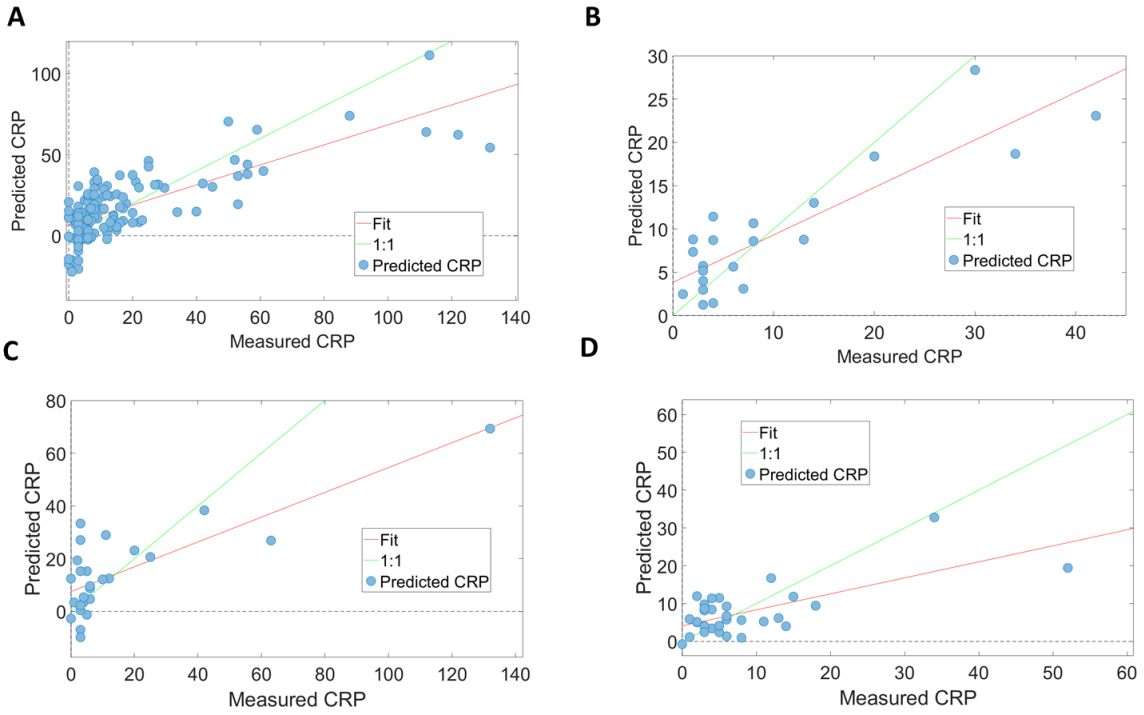



**Figure 5-19 PLS-R analysis of urinary metabolites and CRP by diagnosis**
All models shown are post forward selection. (**A**) RA (n=83, 144 NMR bins post forward selection, $r^2$=0.4292, 3 LV, p<0.001), (**B**) PsA patients (n=22, 102 NMR bins post forward selection, $r^2$=0.6625, 6 LV, p=0.028) and (**C**) UA (n=25, 90 NMR bins post forward selection, $r^2$=0.6117, 8 LV, p=0.025) patients have a statistically significant relationship between urinary metabolite profile and CRP respectively. There was no statistically

significant relationship between urinary metabolite profile and CRP for (D) CSA (n=33, 2 NMR bins post forward selection, $r^2$=0.3446, 1 LV, p=0.074) patients.

**Table 5-25 Metabolites responsible for the relationship seen in PLS-R between CRP and urinary metabolite profile in RA patients.**

The following metabolites have been ranked by the magnitude of the regression coefficient. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of regression coefficient. The regression coefficient field indicates the nature of correlation (↑ indicating a positive relationship with CRP and ↓ indicating a negative relationship with CRP)

| order | Metabolite | Chemical shift of peak (ppm) | Regression coefficient |
|---|---|---|---|
| 1 | Ethanol | 1.1431, 1.1376, 1.1303, 1.127, 1.1607, 1.1468, 1.1665, 1.155 | ↓↓↓↓↓↓↓↓ |
| 1 | 3-Aminoisobutanoic acid | 1.1431, 1.1376, 1.1303, 1.127, 3.0865, 1.0904, 1.1019, 1.1607, 3.0923, 1.1468, 1.1665, 3.0982, 1.155, 3.104 | ↓↓↓↓↓↑↑↓↓↓↓↓↓ |
| 1 | Propylene glycol | 1.1431, 1.1376, 1.1303, 1.127, 1.1468 | ↓↓↓↓↓ |
| 1 | Thymol | 1.1431, 1.1607, 1.1468, 1.1665, 7.2191, 1.155, 7.225 | ↓↓↓↓↑↓↑ |
| 2 | Adipic acid | 1.4943, 1.4885, 1.506, 1.5002, 2.1733 | ↑↑↑↑↓ |
| 2 | Lysine | 1.4943, 1.4885, 1.506, 1.5002, 1.4416 | ↑↑↑↑↑ |
| 2 | Azelaic acid | 1.4943, 1.4885, 1.506, 1.5002, 2.1733 | ↑↑↑↑↓ |
| 2 | Sebacic acid | 1.4943, 1.4885, 1.506, 1.5002, 2.1733 | ↑↑↑↑↓ |
| 2 | 3-Methyladipic acid | 1.4943, 1.4885, 1.506, 1.5002, 2.1733 | ↑↑↑↑↓ |
| 2 | Suberic acid | 1.4943, 1.4885, 1.506, 1.5002, 2.1733 | ↑↑↑↑↓ |
| 2 | Alanine | 1.4943, 1.4885, 1.506, 1.5002, 1.4416 | ↑↑↑↑↑ |
| 2 | 3-Methyl-2-oxovaleric acid | 1.4943, 1.1303, 1.127, 1.4885, 1.0904, 1.1019, 1.5002, 1.4416, 1.0558 | ↑↓↓↑↑↑↑↑↓ |
| 3 | L-Cystathionine | 3.0865, 3.0923, 3.0982, 2.7353, 2.7294, 2.1733, 2.706, 2.7587, 3.104, 3.8474, 3.8416 | ↓↓↓↑↑↓↑↑↓↓↓ |
| 3 | Creatinine | 3.0865, 2.7879, 3.0923, 2.8172, 3.0982, 2.7353, 2.7294, 3.1567, 2.8406, 2.706, 2.7587, 2.7938, 3.104 | ↓↑↓↑↓↑↑↓↑↑↑↑↓ |
| 3 | Phenylalanine | 3.0865, 3.0923, 7.2191, 3.0982, 7.3819, 7.2484, 7.5001, 7.2425, 7.225, 7.3771, 3.104, 7.4123 | ↓↓↑↓↑↑↑↑↑↑↓↓ |
| 3 | 1,3-Diaminopropane | 3.0865, 3.0923, 3.0982, 3.104 | ↓↓↓↓ |
| 3 | Cysteine | 3.0865, 3.0923, 3.0982, 3.104 | ↓↓↓↓ |
| 3 | 3-methylhistidine | 3.0865, 7.7518, 3.0923, 3.1567 | ↓↑↓↓ |
| 4 | 2-Ketobutyric acid | 1.0904, 1.1019, 2.7879, 2.7587, 1.0558 | ↑↑↑↑↓ |

| | | | |
|---|---|---|---|
| 4 | Methylsuccinate | 1.0904, 1.1019, 1.0558 | ↑↑↓ |
| 5 | Hippuric acid | 7.7518, 7.9918, 7.7768, 7.9332, 7.8513, 7.5001, 7.9976, 7.781 | ↑↓↑↑↑↑↓↑ |
| 5 | Tryptophan | 7.7518, 7.2484, 7.5001, 7.225 | ↑↑↑↑ |
| 5 | Phenylglyoxylic acid | 7.7518, 7.9918, 7.7768, 7.9497, 7.9332, 7.9976, 7.781, 7.947 | ↑↓↑↑↑↓↑↑ |
| 5 | Urea | 7.7518, 7.9918, 7.7768, 7.2191, 7.3819, 7.9497, 7.9332, 7.2484, 7.5001, 7.9976, 7.781, 7.2425, 7.225, 7.3771, 7.947 | ↑↓↑↑↑↑↑↑↑↓↑↑↑↑↑ |
| 5 | 7-Methylxanthine | 7.7518, 7.9918, 7.7768, 7.9332, 7.8513, 7.9976, 7.781, 3.8474, 3.8416 | ↑↓↑↑↑↓↑↓↓ |
| 6 | Dihydrothymine | 1.1607, 2.7879, 1.1665, 3.1567, 1.155, 2.7587, 2.7938 | ↓↑↓↓↓↑↑ |
| 7 | Quinolinic acid | 7.9918, 7.9976 | ↓↓ |
| 7 | Carnosine | 7.9918, 2.7294, 3.1567, 7.9976, 2.706 | ↓↑↓↓↑ |
| 7 | Picolinic acid | 7.9918, 7.8981, 7.9497, 7.9332, 7.9976, 7.947 | ↓↑↑↑↓↑ |
| 7 | Histidine | 7.9918, 7.8981, 7.9332, 3.1567, 7.9976 | ↓↑↑↓↓ |
| 8 | Succinylacetone | 2.7879, 2.8172, 2.8406, 2.7938, 3.8474, 3.8416 | ↑↑↑↑↓↓ |
| 8 | Aspartate | 2.7879, 2.8172, 2.8406, 2.7938 | ↑↑↑↑ |
| 8 | Methylguanidine | 2.7879, 2.8172, 2.7938 | ↑↑↑ |
| 8 | Citrate | 2.7879, 2.8172, 2.7353, 2.7294, 2.8406, 2.706, 2.7587, 2.7938 | ↑↑↑↑↑↑↑ |
| 8 | 5-Aminolevulinic acid | 2.7879, 2.7587, 2.7938 | ↑↑↑ |
| 8 | Levulinic acid | 2.7879, 2.7587 | ↑↑ |
| 9 | Malonate | 3.0923, 3.0982, 3.104 | ↓↓↓ |
| 10 | Symmetric dimethylarginine | 2.8172 | ↑ |
| 11 | 4-Hydroxybenzoic acid | 7.7768, 7.781 | ↑↑ |
| 12 | Indoleacetate | 7.2191, 7.2484, 7.5001, 7.2425, 7.225 | ↑↑↑↑↑ |
| 13 | trans-Ferulic acid | 7.2191, 7.225 | ↑↑ |
| 13 | Tyrosine | 7.2191, 7.225 | ↑↑ |
| 13 | Ortho-Hydroxyphenylacetate | 7.2191, 7.225 | ↑↑ |
| 13 | Indoxyl sulfate | 7.2191, 7.3819, 7.5001, 7.225, 7.3771 | ↑↑↑↑↑ |
| 13 | Tryptophan | 7.2191 | ↑ |
| 13 | Phenylacetate | 7.2191, 7.3819, 7.2484, 7.2425, 7.225, 7.3771 | ↑↑↑↑↑↑ |
| 14 | Mandelic acid | 7.3819, 7.3771, 7.4123 | ↑↑↓ |

| | | | |
|---|---|---|---|
| 15 | Cinnamic acid | 7.3819, 7.5001, 7.3771, 7.4123 | ↑↑↑↓ |
| 16 | Cystine | 3.3792, 3.1567, 3.385 | ↓↓↓ |
| 16 | 4-Hydroxyproline | 3.3792, 2.1733, 3.385 | ↓↓↓ |
| 16 | Pantothenic acid | 3.3792 | ↓ |
| 17 | Anserine | 2.7353, 2.7294, 2.706 | ↑↑↑ |
| 17 | Sarcosine | 2.7353, 2.7294, 2.7587 | ↑↑↑ |
| 17 | Citramalic acid | 2.7353, 2.7587 | ↑↑ |
| 18 | Kynurenic acid | 7.8981, 7.9332, 7.5001 | ↑↑↑ |
| 18 | 3-Methylhistidine | 7.8981, 7.9332 | ↑↑ |
| 19 | Benzoic acid | 7.9332, 7.8513, 7.5001 | ↑↑↑ |
| 20 | 3-Hydroxyphenylacetate | 7.2484, 7.2425, 7.225 | ↑↑↑ |
| 21 | L-Kynurenine | 7.8513, 7.4123 | ↑↓ |
| 22 | Ethanolamine | 3.1567, 3.8474, 3.8416 | ↓↓↓ |
| 22 | Beta-Alanine | 3.1567 | ↓ |
| 23 | Asparagine | 2.8406 | ↑ |
| 24 | Vanillic acid | 7.5001 | ↑ |
| 24 | Uracil | 7.5001 | ↑ |
| 24 | 4-Pyridoxic acid | 7.5001 | ↑ |
| 24 | Cytosine | 7.5001 | ↑ |
| 25 | Monomethyl glutaric acid | 2.1733 | ↓ |
| 25 | Pimelic acid | 2.1733 | ↓ |
| 25 | Methionine | 2.1733, 3.8474, 3.8416 | ↓↓↓ |
| 25 | Isovalerylglycine | 2.1733 | ↓ |
| 25 | Glutamate | 2.1733 | ↓ |
| 25 | Methylglutaric acid | 2.1733 | ↓ |
| 25 | L-2-Hydroxyglutaric acid | 2.1733 | ↓ |
| 25 | Glutamine | 2.1733 | ↓ |
| 26 | Isoleucine | 1.4416 | ↑ |
| 27 | Dihydrouracil | 2.706 | ↑ |
| 28 | Valine | 1.0558 | ↓ |
| 29 | L-Arabitol | 3.8474, 3.8416 | ↓↓ |
| 29 | Serine | 3.8474, 3.8416 | ↓↓ |
| 29 | N-Acetylneuraminic acid | 3.8474, 3.8416 | ↓↓ |
| 29 | D-Maltose | 3.8474, 3.8416 | ↓↓ |
| 29 | Pseudouridine | 3.8474, 3.8416 | ↓↓ |
| 29 | Thymidine | 3.8474, 3.8416 | ↓↓ |
| 29 | Hydroxypropionic acid | 3.8474, 3.8416 | ↓↓ |

| | | | |
|---|---|---|---|
| 29 | Alpha-Lactose | 3.8474, 3.8416 | ↓↓ |
| 29 | Adenosine | 3.8474, 3.8416 | ↓↓ |
| 29 | Sorbitol | 3.8474, 3.8416 | ↓↓ |
| 29 | D-Galactose | 3.8474, 3.8416 | ↓↓ |
| 29 | Homovanillic acid | 3.8474, 3.8416 | ↓↓ |
| 29 | D-Xylitol | 3.8474, 3.8416 | ↓↓ |
| 29 | Gluconic acid | 3.8474, 3.8416 | ↓↓ |
| 29 | L-Arabinose | 3.8474, 3.8416 | ↓↓ |
| 29 | Sucrose | 3.8474, 3.8416 | ↓↓ |
| 29 | Dehydroascorbic acid | 3.8474, 3.8416 | ↓↓ |
| 29 | 1-Methyladenosine | 3.8474, 3.8416 | ↓↓ |
| 29 | Glyceric acid | 3.8474, 3.8416 | ↓↓ |

Figure 5-20 and Table 5-26 show pathway analysis of metabolites identified as biomarkers by PLS-R analysis of RA patients' urinary metabolite data and CRP. This indicated alanine, aspartate and glutamate metabolism and beta-alanine metabolism were the most impacted metabolic pathways. Figure 5-21 and Table 5-27 show the enrichment analysis using metabolites identified as biomarkers by PLS-R analysis of RA patients' urinary metabolite data and CRP. Beta-alanine metabolism, glycine and serine metabolism, homocysteine degradation and methylhistidine metabolism were the only overrepresented metabolic pathways that reached statistical significance.

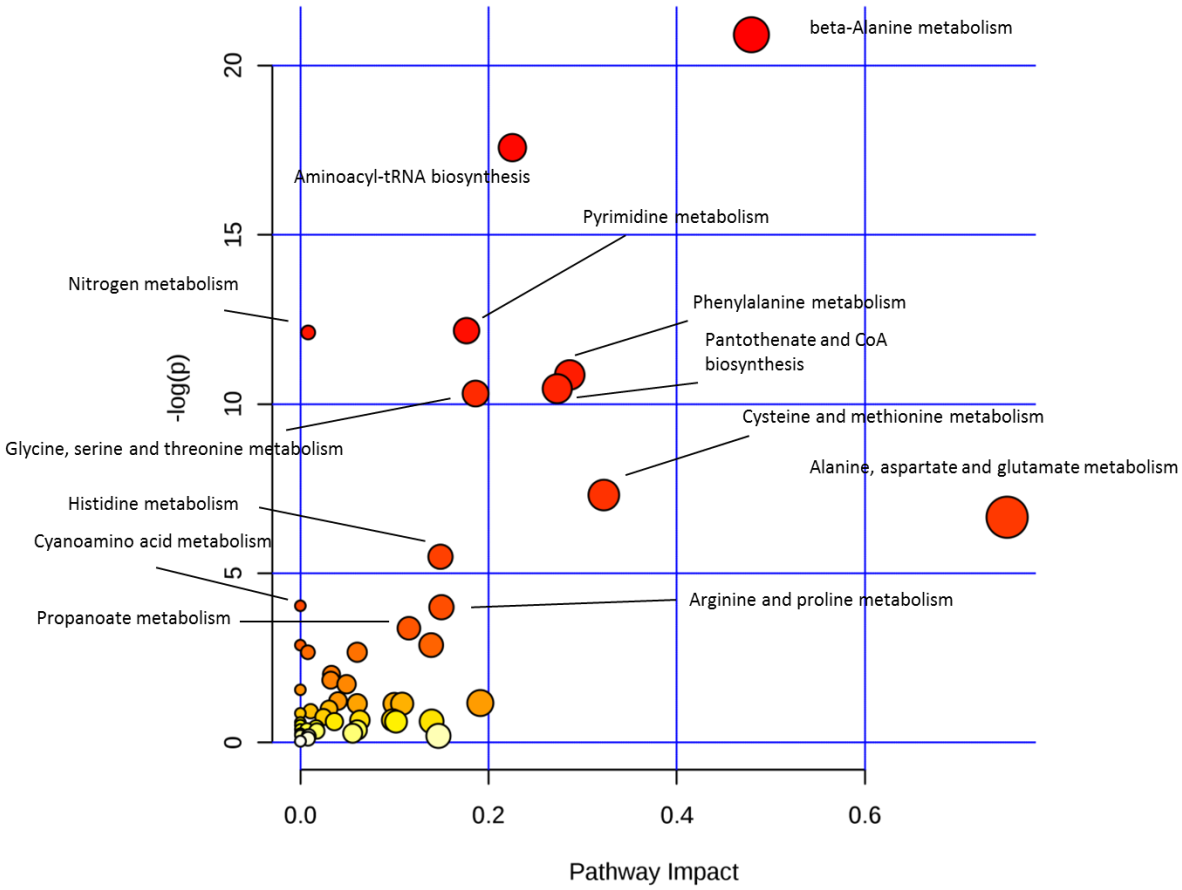



**Figure 5-20 Metaboanalyst pathway analysis of potential biomarkers implicated by PLS-R analysis of CRP and RA patients' urinary metabolites**

**Table 5-26 Pathway analysis of potential biomarkers implicated by PLS-R analysis of RA patients' urinary metabolites and CRP**

**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.

**The impact score**: Each pathway has a total importance score of 1. The importance measure of each metabolite node (a point where pathways intersect within a larger network) is expressed as a fraction with regards to the total pathway importance, and the pathway impact score is the cumulative score of matched metabolite nodes.

* p value calculated using hypergeometric test

| Pathway Name | Match Status | P value* | Impact score |
|---|---|---|---|
| Alanine, aspartate and glutamate metabolism | 5/24 | 0.001288 | 0.75119 |
| beta-Alanine metabolism | 11/28 | 8.32E-10 | 0.47941 |
| Cysteine and methionine metabolism | 8/56 | 6.69E-04 | 0.32254 |
| Phenylalanine metabolism | 9/45 | 1.92E-05 | 0.28632 |
| Pantothenate and CoA biosynthesis | 7/27 | 2.89E-05 | 0.27302 |
| Aminoacyl-tRNA biosynthesis | 15/75 | 2.33E-08 | 0.22536 |
| Glycine, serine and threonine metabolism | 9/48 | 3.33E-05 | 0.18624 |
| Pyrimidine metabolism | 11/60 | 5.20E-06 | 0.17665 |
| Arginine and proline metabolism | 7/77 | 0.018381 | 0.14997 |
| Histidine metabolism | 6/44 | 0.004137 | 0.14893 |
| Propanoate metabolism | 4/35 | 0.034197 | 0.11538 |
| Nitrogen metabolism | 9/39 | 5.49E-06 | 0.0083 |

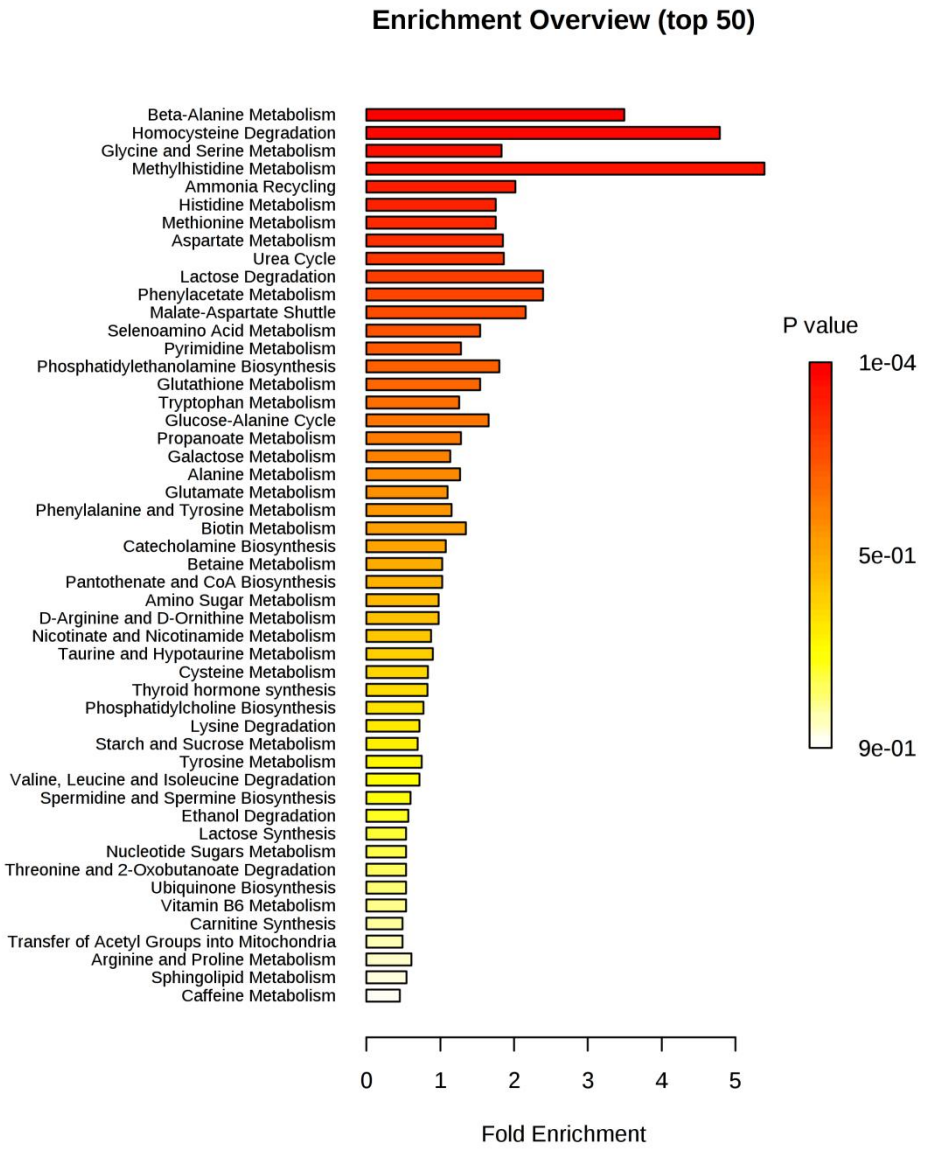



**Figure 5-21 Enrichment analysis of key metabolites in urine implicated as potential biomarkers by the PLS-R analysis of CRP and RA patients' urinary metabolites**

**Table 5-27 Enrichment analysis of key metabolites in urine implicated as potential biomarkers by the PLS-R analysis of CRP and RA patients' urinary metabolites.**
**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.
**The fold enrichment value**: the value is generated by the number of matched metabolites divided by the number of metabolites expected to be present in the pathway as per the reference metabolome provided by KEGG.
* p value calculated using hypergeometric test

| Metabolite Set | Match status | Fold enrichment | Expected | P value* |
|---|---|---|---|---|
| Beta-Alanine Metabolism | 11/34 | 3.49 | 3.15 | 0.000117 |
| Homocysteine Degradation | 4/9 | 4.79 | 0.835 | 0.00609 |
| Glycine and Serine Metabolism | 10/59 | 1.83 | 5.47 | 0.039 |
| Methylhistidine Metabolism | 2/4 | 5.39 | 0.371 | 0.0452 |

### 5.4.8 Relationship between urinary metabolite profile and CRP in PsA patients

Figure 5-22 and Table 5-29 show pathway analysis of metabolites identified as biomarkers by PLS-R analysis of PsA patients' urinary metabolite data and CRP. This showed alanine, aspartate and glutamate metabolism and beta-alanine metabolism were the most impacted metabolic pathways. Figure 5-23 and Table 5-23 show the enrichment analysis using metabolites identified as biomarkers by PLS-R analysis of PsA patients' urinary metabolite data and CRP. Beta-alanine metabolism, homocysteine degradation and methylhistidine metabolism were the only overrepresented metabolic pathways that reached statistical significance.

**Table 5-28 Metabolites responsible for the relationship seen in PLS-R between CRP and urinary metabolite profile in PsA patients.**

The following metabolites have been ranked by the magnitude of the regression coefficient. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of regression coefficient. The regression coefficient field indicates the nature of correlation (↑ indicating a positive relationship with CRP and ↓ indicating a negative relationship with CRP)

| Order | Metabolite | Chemical shift of peak (ppm) | Regression coefficient |
|---|---|---|---|
| 1 | Trans-aconitic acid | 6.9506, 6.9557, 6.9615, 6.9733, 6.9674 | ↑↑↑↑↑ |
| 1 | Phenol | 6.9506, 6.9557, 7.0084, 6.9615, 6.9733, 6.9967, 7.0435, 6.9674, 6.9138, 7.0318 | ↑↑↑↑↑↓↓↑↑↑ |
| 1 | Pyrocatechol | 6.9506, 6.9557, 6.9615, 6.9733, 6.9674, 6.9138 | ↑↑↑↑↑↑ |
| 1 | Vanillic acid | 6.9506, 6.9557, 7.0084, 7.5052, 6.9615, 6.9733, 6.9967, 7.4942, 6.9674, 6.9138, 7.4884, 7.4298 | ↑↑↑↓↑↑↓↓↑↑↓↓ |
| 1 | Sumiki's acid | 6.9506, 6.9557, 6.9615 | ↑↑↑ |
| 1 | Urea | 6.9506, 6.9557, 7.0084, 7.5052, 6.9615, 6.9733, 6.9967, 7.4942, 7.9918, 7.0903, 7.0435, 6.9674, 6.9138, 7.4884, 7.9859, 7.0318, 7.947 | ↑↑↑↓↑↑↓↓↓↑↓↑↑↓↓↑↓ |
| 1 | Ortho-hydroxyphenylacetate | 6.9506, 6.9557, 6.9615, 6.9674, 6.9138 | ↑↑↑↑↑ |
| 1 | Homovanillic acid | 6.9506, 6.9557, 6.9615, 6.9138 | ↑↑↑↑ |
| 1 | 4-hydroxybenzoic acid | 6.9506, 6.9557, 6.9138 | ↑↑↑ |
| 1 | 3-methylhistidine | 6.9506, 6.9557, 7.0084, 6.9615, 6.9733, 6.9967, 3.0572, 7.0903, 7.0435, 6.9674, 3.0514, 7.0318, 3.0631 | ↑↑↑↑↑↓↓↑↓↑↓↑↓ |
| 1 | L-kynurenine | 6.9506, 6.9138, 7.4298 | ↑↑↓ |
| 1 | Tyrosine | 6.9506, 3.0572, 6.9138, 3.0514, 3.0631 | ↑↓↑↓↓ |
| 2 | Lysine | 1.372, 1.465, 1.3772, 1.8338, 1.4767, 1.8568, 1.4709, 1.6582, 1.4826 | ↑↓↑↑↓↑↓↓↓ |
| 2 | Lactate | 1.372, 1.365, 1.3772, 1.3421 | ↑↑↑↑ |
| 2 | Alpha-hydroxyisobutyric acid | 1.372, 1.365, 1.3772, 1.3421 | ↑↑↑↑ |
| 3 | 3-methylhistidine | 7.0084, 6.9967, 7.0903, 7.0435, 7.0318 | ↑↓↑↓↑ |
| 3 | Histidine | 7.0084, 6.9967, 7.9918, 7.0903, 7.0435, 7.9859, 7.0318 | ↑↓↓↑↓↓↑ |
| 3 | Phenylalanine | 7.0084, 7.5052, 7.4942, 7.0903, 7.0435, 7.4884, 7.4298, 7.0318 | ↑↓↓↑↓↓↓↑ |
| 4 | Glutamate | 1.9684, 1.9919, 1.9626, 1.9977 | ↑↑↑↑ |

| | | | |
|---|---|---|---|
| 4 | Citraconic acid | 1.9684, 1.9626 | ↑↑ |
| 4 | L-2-hydroxyglutaric acid | 1.9684, 1.8338, 1.9919, 1.8568, 1.9626, 1.9977 | ↑↑↑↑↑↑ |
| 4 | Methylglutaric acid | 1.9684, 1.9919, 1.9626, 1.9977 | ↑↑↑↑ |
| 4 | Isovalerylglycine | 1.9684, 1.9919, 1.9977 | ↑↑↑ |
| 4 | N-acetylputrescine | 1.9684, 1.9919, 1.9626, 1.6406, 1.6582, 1.6348, 1.9977, 1.6465 | ↑↑↑↓↓↓↑↓ |
| 4 | Arginine | 1.9684, 1.8338, 1.9919, 1.8568, 1.9626, 1.6406, 1.6582, 1.6348, 1.9977, 1.6465 | ↑↑↑↑↑↓↓↓↑↓ |
| 4 | Isoleucine | 1.9684, 1.465, 1.4767, 1.9919, 1.4709, 1.9626, 1.4826 | ↑↓↓↑↓↑↓ |
| 5 | Hippuric acid | 7.5052, 7.4942, 7.9918, 7.4884, 7.9859 | ↓↓↓↓↓ |
| 5 | Indoleacetate | 7.5052, 7.4942, 7.0903, 7.0435, 7.4884 | ↓↓↑↓↓ |
| 5 | Uracil | 7.5052, 7.4942, 7.4884 | ↓↓↓ |
| 5 | Tryptophan | 7.5052, 7.4942 | ↓↓ |
| 5 | Benzoic acid | 7.5052, 7.4942, 7.4884, 7.9859 | ↓↓↓↓ |
| 5 | 4-pyridoxic acid | 7.5052 | ↓ |
| 5 | Kynurenic acid | 7.5052, 7.4942, 7.9859 | ↓↓↓ |
| 5 | Cytosine | 7.5052, 7.4942, 7.4884 | ↓↓↓ |
| 5 | Indoxyl sulfate | 7.5052, 7.4942, 7.4884 | ↓↓↓ |
| 5 | Picolinic acid | 7.5052, 7.9918, 7.9859, 7.947 | ↓↓↓↓ |
| 5 | Cinnamic acid | 7.5052, 7.4942, 7.4884. 7.4298 | ↓↓↓↓ |
| 6 | Asparagine | 2.8406, 2.8348, 2.8465, 2.8523, 6.9138, 2.864, 2.8231 | ↑↑↑↑↑↑↓ |
| 6 | Creatinine | 2.8406, 2.8348, 2.7587, 3.0572, 2.6592, 2.8055, 2.6826, 2.8465, 2.8523, 2.7821, 2.864, 3.0514, 2.7879, 2.7353, 3.0631, 2.8231 | ↑↑↑↓↓↑↓↑↑↑↑↓ ↑↑↓↓ |
| 6 | Succinylacetone | 2.8406, 2.8348, 2.8055, 2.7821, 2.7879, 2.8231 | ↑↑↑↑↑↓ |
| 6 | Citrate | 2.8406, 2.8348, 2.7587, 2.6592, 2.8055, 2.6826, 2.7821, 2.5538, 2.7879, 2.7353, 2.8231 | ↑↑↑↓↑↓↑↓↑↑↓ |
| 6 | Aspartate | 2.8406, 2.8348, 2.6592, 2.8055, 2.6826, 2.7821, 2.7879, 2.8231 | ↑↑↓↑↓↑↑↓ |
| 7 | Methylguanidine | 2.8348, 2.8055, 2.7879, 2.8231 | ↑↑↑↓ |
| 7 | Symmetric dimethylarginine | 2.8348, 2.8055, 1.6406, 1.6582, 1.6348, 2.8231, 1.6465 | ↑↑↓↓↓↓↓ |
| 8 | Alanine | 1.465, 1.4767, 1.4709, 1.4826 | ↓↓↓↓ |
| 8 | 3-methyl-2-oxovaleric acid | 1.465, 1.4767, 1.4709, 1.4826 | ↓↓↓↓ |
| 8 | Adipic acid | 1.465, 1.4767, 1.4709, 1.4826 | ↓↓↓↓ |
| 9 | Citramalic acid | 1.365, 2.7587, 1.3421, 2.7353 | ↑↑↑↑ |
| 9 | Threonine | 1.365, 1.3421 | ↑↑ |
| 9 | Azelaic acid | 1.365, 1.4767, 1.4709, 1.3421, 1.4826 | ↑↓↓↑↓ |

| | | | |
|---|---|---|---|
| 10 | Dihydrothymine | 2.7587, 2.8055, 2.7821, 2.7879 | ↑↑↑↑ |
| 10 | 5-aminolevulinic acid | 2.7587, 2.8055, 2.7821, 2.7879 | ↑↑↑↑ |
| 10 | 2-ketobutyric acid | 2.7587, 2.7821, 2.7879 | ↑↑↑ |
| 10 | Levulinic acid | 2.7587, 2.7821, 2.7879 | ↑↑↑ |
| 10 | L-cystathionine | 2.7587, 3.0572, 2.6592, 2.6826, 2.7821, 3.0514, 2.7353, 3.0631 | ↑↓↓↓↑↓↑↓ |
| 10 | Sarcosine | 2.7587, 2.7353 | ↑↑ |
| 11 | Cysteine | 3.0572, 3.0514, 3.0631 | ↓↓↓ |
| 11 | Glutaconic acid | 3.0572, 3.0631 | ↓↓ |
| 12 | Aminoadipic acid | 1.8338, 1.8568, 1.6406, 1.6582, 1.6348, 1.6465 | ↑↑↓↓↓↓ |
| 12 | Glutaric acid | 1.8338, 1.8568 | ↑↑ |
| 12 | Monomethyl glutaric acid | 1.8338, 1.8568 | ↑↑ |
| 12 | N-acetylneuraminic acid | 1.8338 | ↑ |
| 13 | Carnosine | 2.6592, 2.6826, 7.9918, 7.0903, 7.0435, 3.0514, 7.9859 | ↓↓↓↑↓↓↓ |
| 13 | Dihydrouracil | 2.6592, 2.6826 | ↓↓ |
| 13 | Anserine | 2.6592, 2.6826, 7.0903, 2.7353, 3.0631 | ↓↓↑↑↓ |
| 13 | 3-aminoisobutanoic acid | 2.6592, 2.5538, 1.1376 | ↓↓↓ |
| 13 | Hydroxypropionic acid | 2.6592, 2.5538 | ↓↓ |
| 14 | Sebacic acid | 1.4767, 1.3421, 1.4826 | ↓↑↓ |
| 14 | 3-methyladipic acid | 1.4767, 1.6406, 1.6348, 1.4826, 1.6465 | ↓↓↓↓↓ |
| 14 | Suberic acid | 1.4767, 1.4709, 1.3421, 1.4826 | ↓↓↑↓ |
| 15 | 1,3-diaminopropane | 1.9919, 1.9977 | ↑↑ |
| 15 | Pyroglutamic acid | 1.9919, 1.9977 | ↑↑ |
| 16 | Quinolinic acid | 7.9918, 8.4484, 7.4298, 7.9859 | ↓↓↓↓ |
| 16 | Phenylglyoxylic acid | 7.9918, 7.9859, 7.947 | ↓↓↓ |
| 16 | 7-methylxanthine | 7.9918, 7.9859 | ↓↓ |
| 17 | Thymidine | 1.8568 | ↑ |
| 17 | D-alpha-aminobutyric acid | 1.8568 | ↑ |
| 18 | Vanillylmandelic acid | 7.0435, 6.9138, 7.0318 | ↓↑↑ |
| 19 | Trans-ferulic acid | 6.9138 | ↑ |

| | | | |
|---|---|---|---|
| 19 | 4-aminohippuric acid | 6.9138 | ↑ |
| 20 | Hydroxyphenyllactate | 2.864 | ↑ |
| 20 | Trimethylamine | 2.864 | ↑ |
| 21 | Beta-alanine | 2.5538 | ↓ |
| 22 | Formate | 8.4484 | ↓ |
| 23 | 3-hydroxymethylglutaric acid | 1.3421 | ↑ |
| 24 | 5-aminopentanoic acid | 1.6406, 1.6582, 1.6348, 1.6465 | ↓↓↓↓ |
| 25 | Mandelic acid | 7.4884, 7.4298 | ↓↓ |
| 26 | Leucine | 1.6582 | ↓ |
| 27 | Ethanol | 1.1376 | ↓ |
| 27 | Propylene glycol | 1.1376 | ↓ |

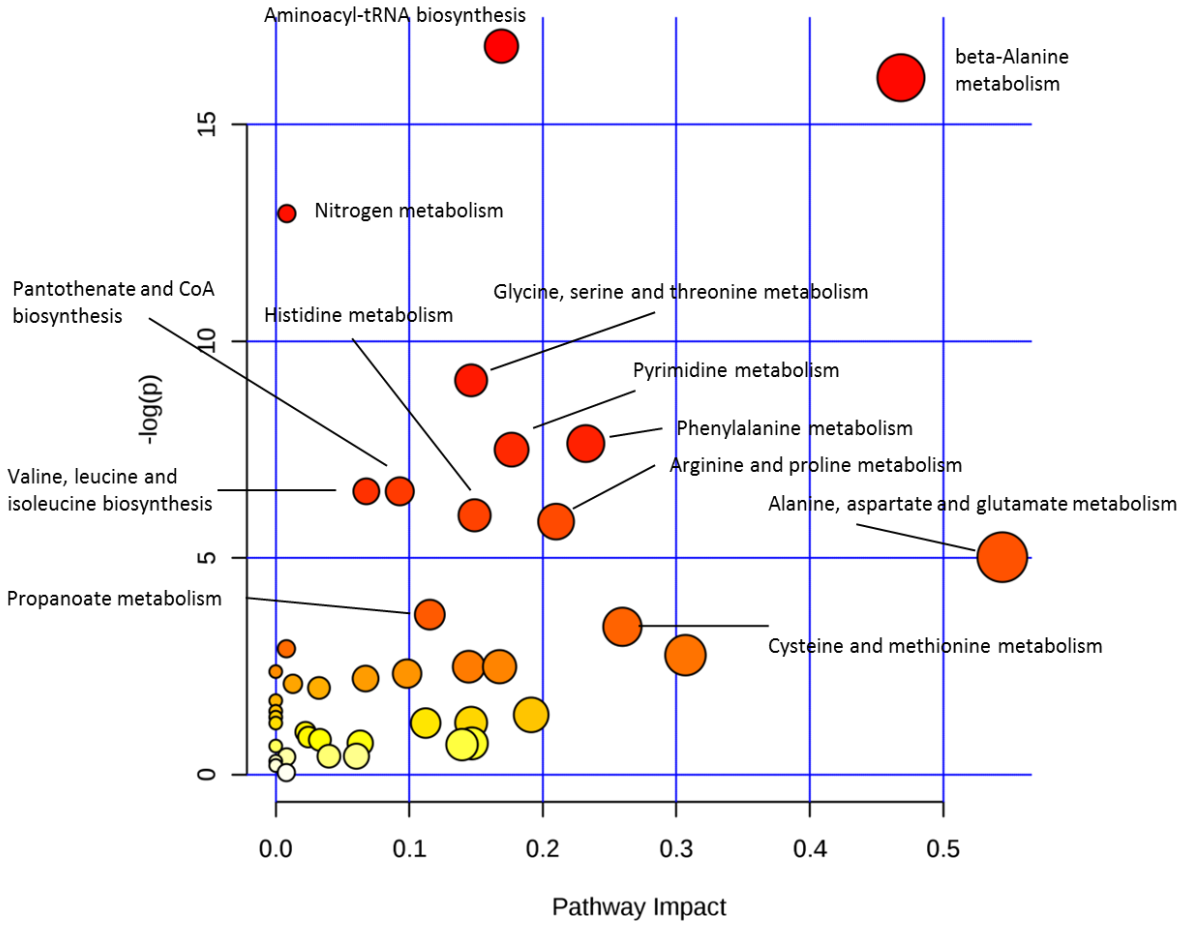



**Figure 5-22 Metaboanalyst pathway analysis of potential biomarkers implicated by PLS-R analysis of CRP and PsA patients' urinary metabolites**

**Table 5-29 Pathway analysis of potential biomarkers implicated by PLS-R analysis of PsA patients' urinary metabolites and CRP**

**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.

**The impact score**: Each pathway has a total importance score of 1. The importance measure of each metabolite node (a point where pathways intersect within a larger network) is expressed as a fraction with regards to the total pathway importance, and the pathway impact score is the cumulative score of matched metabolite nodes.

* p value calculated using hypergeometric test

| Pathway name | Match status | P value* | Impact score |
|---|---|---|---|
| Alanine, aspartate and glutamate metabolism | 4/24 | 0.006621 | 0.54416 |
| Beta-alanine metabolism | 9/28 | 1.04e-07 | 0.46822 |
| Cysteine and methionine metabolism | 5/56 | 0.033023 | 0.25962 |
| Phenylalanine metabolism | 7/45 | 4.81e-04 | 0.2322 |
| Arginine and proline metabolism | 8/77 | 0.002915 | 0.20996 |
| Pyrimidine metabolism | 8/60 | 5.54e-04 | 0.17665 |
| Aminoacyl-trna biosynthesis | 14/75 | 5.01e-08 | 0.16902 |
| Histidine metabolism | 6/44 | 0.002522 | 0.14893 |
| Glycine, serine and threonine metabolism | 8/48 | 1.12e-04 | 0.14634 |
| Propanoate metabolism | 4/35 | 0.024934 | 0.11538 |
| Pantothenate and coa biosynthesis | 5/27 | 0.001449 | 0.09288 |
| Valine, leucine and isoleucine biosynthesis | 5/27 | 0.001449 | 0.06784 |
| Nitrogen metabolism | 9/39 | 2.39e-06 | 0.0083 |

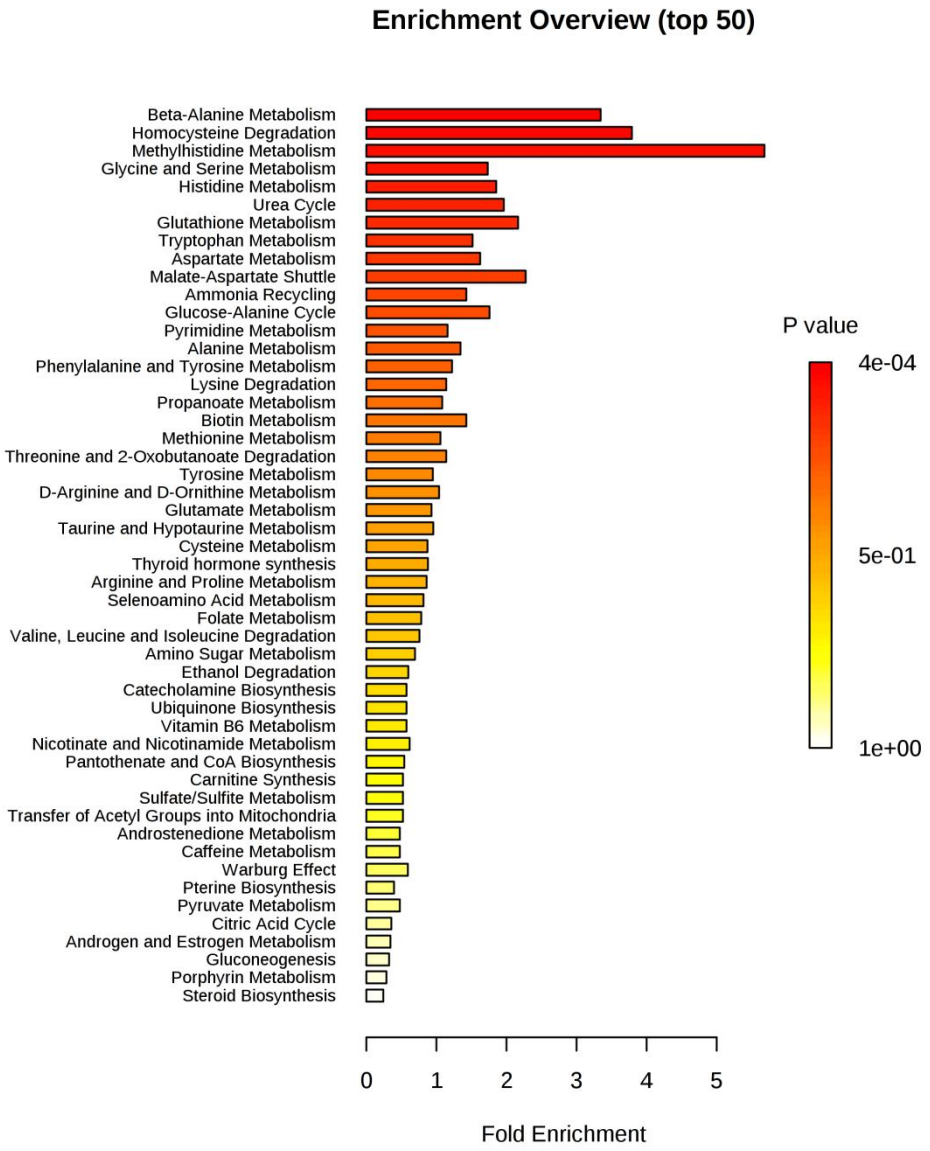



**Figure 5-23 Enrichment analysis of key metabolites in urine implicated as potential biomarkers by the PLS-R analysis of CRP and PsA patients' urinary metabolites**

**Table 5-30 Enrichment analysis of key metabolites in urine implicated as potential biomarkers by the PLS-R analysis of CRP and PsA patients' urinary metabolites.**
**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.
**The fold enrichment value**: the value is generated by the number of matched metabolites divided by the number of metabolites expected to be present in the pathway as per the reference metabolome provided by KEGG.
* p value calculated using hypergeometric test

| Metabolite set | Match status | Fold enrichment | Expected | P value* |
|---|---|---|---|---|
| Beta-alanine metabolism | 10/34 | 3.34 | 2.99 | 0.000375 |
| Homocysteine degradation | 3/9 | 3.79 | 0.791 | 0.0374 |
| Methylhistidine metabolism | 2/4 | 5.68 | 0.352 | 0.0408 |

### 5.4.9 Relationship between urinary metabolite profile and CRP in UA patients

Figure 5-24 and Table 5-32 show pathway analysis of metabolites identified as biomarkers by PLS-R analysis of UA patients' urinary metabolite data and CRP. This showed alanine, aspartate and glutamate metabolism and beta-alanine metabolism were the most impacted metabolic pathways. Figure 5-25 and Table 5-33 show the enrichment analysis using metabolites identified as biomarkers by PLS-R analysis of UA patients' urinary metabolite data and CRP. Beta-alanine metabolism, homocysteine degradation and methylhistidine metabolism were the only overrepresented metabolic pathways that reached statistical significance.

**Table 5-31 Metabolites responsible for the relationship seen in PLS-R between CRP and urinary metabolite profile in UA patients.**

The following metabolites have been ranked by the magnitude of the regression coefficient. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of regression coefficient. The regression coefficient field indicates the nature of correlation (↑ indicating a positive relationship with CRP and ↓ indicating a negative relationship with CRP)

| Order | Metabolite | Chemical shift of peak (ppm) | Regression coefficient |
|---|---|---|---|
| 1 | Phenol | 6.9733, 6.9615, 6.9557, 6.9674, 7.0435, 6.9138, 6.9791, 7.2367 | ↓↓↓↓↑↓↓↑ |
| 1 | Urea | 6.9733, 6.9615, 7.9332, 6.9557, 0.74505, 6.9674, 7.9742, 7.0435, 0.73334, 7.9684, 7.2308, 6.9138, 4.3333, 7.0847, 7.1313, 7.9859, 7.0805, 4.3274, 6.9791, 7.9801, 7.2367, 7.7635 | ↓↓↓↓↑↓↓↑↑↓↑↓↓↑ ↑↑↑↓↓↓↑↓ |
| 1 | 3-methylhistidine | 6.9733, 6.9615, 6.9557, 6.9674, 3.081, 7.0435, 3.0865, 3.0572, 7.0805, 6.9791, 3.0631 | ↓↓↓↓↑↑↑↓↑↓↓ |
| 1 | Vanillic acid | 6.9733, 6.9615, 6.9557, 6.9674, 6.9138, 6.9791 | ↓↓↓↓↓↓ |
| 1 | Trans-aconitic acid | 6.9733, 6.9615, 6.9557, 6.9674, 6.9791 | ↓↓↓↓↓ |
| 1 | Pyrocatechol | 6.9733, 6.9615, 6.9557, 6.9674, 6.9138, 6.9791 | ↓↓↓↓↓↓ |
| 2 | Citrate | 2.5187, 2.5128, 2.507, 2.5245, 2.6826, 2.6884 | ↓↓↓↓↓↓ |
| 2 | Beta-alanine | 2.5187, 2.5128, 2.507, 2.5245 | ↓↓↓↓ |
| 2 | Isocitrate | 2.5187, 2.5128, 2.507, 2.5245 | ↓↓↓↓ |
| 2 | Pyroglutamic acid | 2.5187, 2.5128, 2.507, 2.5245 | ↓↓↓↓ |
| 2 | Methylsuccinate | 2.5187, 2.5128, 1.0904, 2.507, 2.5245, 1.0703, 1.0588, 1.0675, 1.1019 | ↓↓↑↓↓↓↓↓↑ |
| 2 | 5-aminolevulinic acid | 2.5187, 2.5128, 2.507 | ↓↓↓ |
| 2 | Dimethylamine | 2.5187, 2.5128, 2.507 | ↓↓↓ |
| 2 | 4-hydroxyproline | 2.5187, 2.5128, 2.507, 4.3333, 4.3274 | ↓↓↓↓↓ |
| 3 | Trimethylamine n-oxide | 3.2621, 3.2562, 3.2679 | ↑↑↑ |
| 3 | Taurine | 3.2621, 3.4201, 3.2562, 3.2679, 3.4084 | ↑↑↑↑↑ |
| 3 | D-maltose | 3.2621, 3.5957, 3.4201, 3.2562, 3.2679, 3.5899, 3.6016 | ↑↑↑↑↑↑↑ |
| 3 | Phenylalanine | 3.2621, 3.2562, 3.081, 7.0435, 3.0865, 3.2679, 7.2308, 7.0847, 7.1313, 7.0805, 7.2367 | ↑↑↑↑↑↑↑↑↑↑↑ |

| | | | |
|---|---|---|---|
| 3 | Myo-inositol | 3.2621, 3.5957, 3.2562, 3.2679, 3.5899, 3.6016 | ↑↑↑↑↑↑ |
| 3 | Betaine | 3.2621, 3.2562, 3.2679 | ↑↑↑ |
| 3 | Histidine | 3.2621, 7.9332, 3.2562, 7.9742, 7.0435, 7.1079, 3.2679, 7.0847, 7.1137, 7.1313, 7.9859, 7.0805, 7.9801 | ↑↓↑↓↑↓↑↑↑↓↑↑↓ |
| 3 | Arginine | 3.2621, 3.2562, 1.9567, 1.7636, 1.7577 | ↑↑↓↓↓ |
| 4 | Ortho-hydroxyphenylacetate | 6.9615, 6.9557, 6.9674, 7.2308, 6.9138 | ↓↓↓↑↓ |
| 4 | Homovanillic acid | 6.9615, 6.9557, 6.9138 | ↓↓↓ |
| 4 | Sumiki's acid | 6.9615, 6.9557 | ↓↓ |
| 5 | 3-aminoisobutanoic acid | 1.0904, 3.081, 1.155, 3.0865, 1.7636, 1.7577, 1.1019 | ↑↑↑↑↓↓↑ |
| 5 | 3-methyl-2-oxovaleric acid | 1.0904. 1.0703, 1.0588, 1.0675, 1.1019 | ↑↓↓↓↑ |
| 5 | 2-ketobutyric acid | 1.0904. 1.0703, 1.0588, 1.0675, 1.1019 | ↑↓↓↓↑ |
| 6 | Picolinic acid | 7.9332, 7.9742, 7.9684, 7.9859, 7.9801 | ↓↓↓↑↓ |
| 6 | Kynurenic acid | 7.9332, 7.9742 | ↓↓ |
| 6 | 3-methylhistidine | 7.9332, 7.9742, 7.0435, 3.2679, 7.0847, 7.0805 | ↓↓↑↑↑↑ |
| 6 | Hippuric acid | 7.9332, 7.9742, 7.9859, 7.9801, 7.8571, 7.7635 | ↓↓↑↓↓↓ |
| 6 | 7-methylxanthine | 7.9332, 7.9742, 7.9859, 7.9801, 7.8571, 7.7635 | ↓↓↑↓↓↓ |
| 6 | Phenylglyoxylic acid | 7.9332, 7.9742, 7.9684, 7.9859, 7.9801, 7.7635 | ↓↓↓↑↓↓ |
| 6 | Benzoic acid | 7.9332, 7.9742, 7.9859, 7.9801, 7.8571 | ↓↓↑↓↓ |
| 7 | D-threitol | 3.5957, 3.5899, 3.6016 | ↑↑↑ |
| 7 | Alpha-lactose | 3.5957, 3.2679, 3.5899, 7.1313 | ↑↑↑↑ |
| 7 | Phosphorylcholine | 3.5957, 3.5899 | ↑↑ |
| 7 | D-xylose | 3.5957, 3.4201 | ↑↑ |
| 7 | Erythritol | 3.5957, 3.5899 | ↑↑ |
| 7 | N-acetylneuraminic acid | 3.5957 | ↑ |
| 8 | 4-hydroxybenzoic acid | 6.9557, 6.9138, 7.7635 | ↓↓↓ |
| 9 | Cis-aconitic acid | 3.4201 | ↑ |
| 10 | Ethylmalonate | 0.74505, 1.7636, 1.7577 | ↑↓↓ |

| | | | |
|---|---|---|---|
| 11 | Carnosine | 8.0637, 7.9742, 7.0435, 7.1079, 2.6826, 7.0847, 7.1137, 7.1313, 7.9859, 2.6884, 7.0805, 7.9801 | ↑↓↑↓↓↑↑↓↑↓↑↓ |
| 11 | Trigonelline | 8.0637 | ↑ |
| 12 | L-cystathionine | 3.081, 3.0865, 3.0572, 2.6826, 2.6884, 3.0631 | ↑↑↓↓↓↓ |
| 12 | Creatinine | 3.081, 3.0865, 3.0572, 2.6826, 4.3333, 2.6884, 4.3274, 3.0631 | ↑↑↓↓↓↓↓↓ |
| 12 | 1,3-diaminopropane | 3.081, 3.0865 | ↑↑ |
| 12 | Cysteine | 3.081, 3.0865, 3.0572, 3.0631 | ↑↑↓↓ |
| 12 | Glutaconic acid | 3.081, 3.0572, 3.0631 | ↑↓↓ |
| 13 | Quinolinic acid | 7.9742, 7.9684, 7.9859, 7.9801 | ↓↓↑↓ |
| 14 | Valine | 1.0703, 1.0588, 1.0675 | ↓↓↓ |
| 15 | Ethanol | 1.155 | ↑ |
| 15 | Dihydrothymine | 1.155 | ↑ |
| 15 | Thymol | 1.155, 7.2308 | ↑↑ |
| 16 | Vanillylmandelic acid | 7.0435, 6.9138 | ↑↓ |
| 16 | Indoleacetate | 7.0435, 7.2308, 7.1313, 7.0805, 7.2367 | ↑↑↑↑↑ |
| 17 | Glutamate | 1.9567 | ↓ |
| 17 | Citraconic acid | 1.9567 | ↓ |
| 17 | L-2-hydroxyglutaric acid | 1.9567, 1.7636 | ↓↓ |
| 17 | Methylglutaric acid | 1.9567 | ↓ |
| 17 | N-acetylputrescine | 1.9567 | ↓ |
| 17 | Isoleucine | 1.9567 | ↓ |
| 18 | Trans-ferulic acid | 7.2308, 6.9138, 7.1137, 7.1313, 7.2367 | ↑↓↑↓↑ |
| 18 | Phenylacetate | 7.2308, 7.2367 | ↑↑ |
| 18 | Tyrosine | 7.2308, 6.9138, 3.0572, 7.2367, 3.0631 | ↑↓↓↑↓ |
| 18 | Tryptophan | 7.2308, 7.2367, 7.7635 | ↑↑↓ |
| 18 | 3-hydroxyphenylacetate | 7.2308, 7.2367 | ↑↑ |
| 18 | Indoxyl sulfate | 7.2308, 7.2367 | ↑↑ |
| 19 | Acetoacetate | 3.4084 | ↑ |
| 19 | Carnitine | 3.4084 | ↑ |
| 20 | Lysine | 1.7636, 1.7577 | ↓↓ |
| 20 | Aminoadipic acid | 1.7636, 1.7577 | ↓↓ |

| | | | |
|---|---|---|---|
| 21 | L-arabitol | 3.5899 | ↑ |
| 22 | L-kynurenine | 6.9138, 7.8571 | ↓↓ |
| 22 | Asparagine | 6.9138 | ↓ |
| 22 | 4-aminohippuric acid | 6.9138 | ↓ |
| 23 | Anserine | 2.6826, 7.1137, 7.1313, 2.6884, 3.0631 | ↓↑↓↓↓ |
| 23 | Dihydrouracil | 2.6826, 2.6884 | ↓↓ |
| 23 | Aspartate | 2.6826, 2.6884 | ↓↓ |
| 24 | D-tartaric acid | 4.3333, 4.3274 | ↓↓ |
| 24 | Gluconic acid | 4.3333, 4.3274 | ↓↓ |
| 24 | Adenosine | 4.3333, 4.3274 | ↓↓ |
| 24 | Pseudouridine | 4.3333, 4.3274 | ↓↓ |
| 24 | Hydroxyphenyllactate | 4.3333, 4.3274 | ↓↓ |
| 25 | P-cresol sulfate | 7.1313 | ↑ |
| 25 | P-hydroxyphenylacetate | 7.1313 | ↑ |
| 26 | Threonic acid | 3.6016 | ↑ |
| 26 | Sarcosine | 3.6016 | ↑ |
| 27 | Kynurenic acid | 7.9859, 7.9801 | ↑↓ |

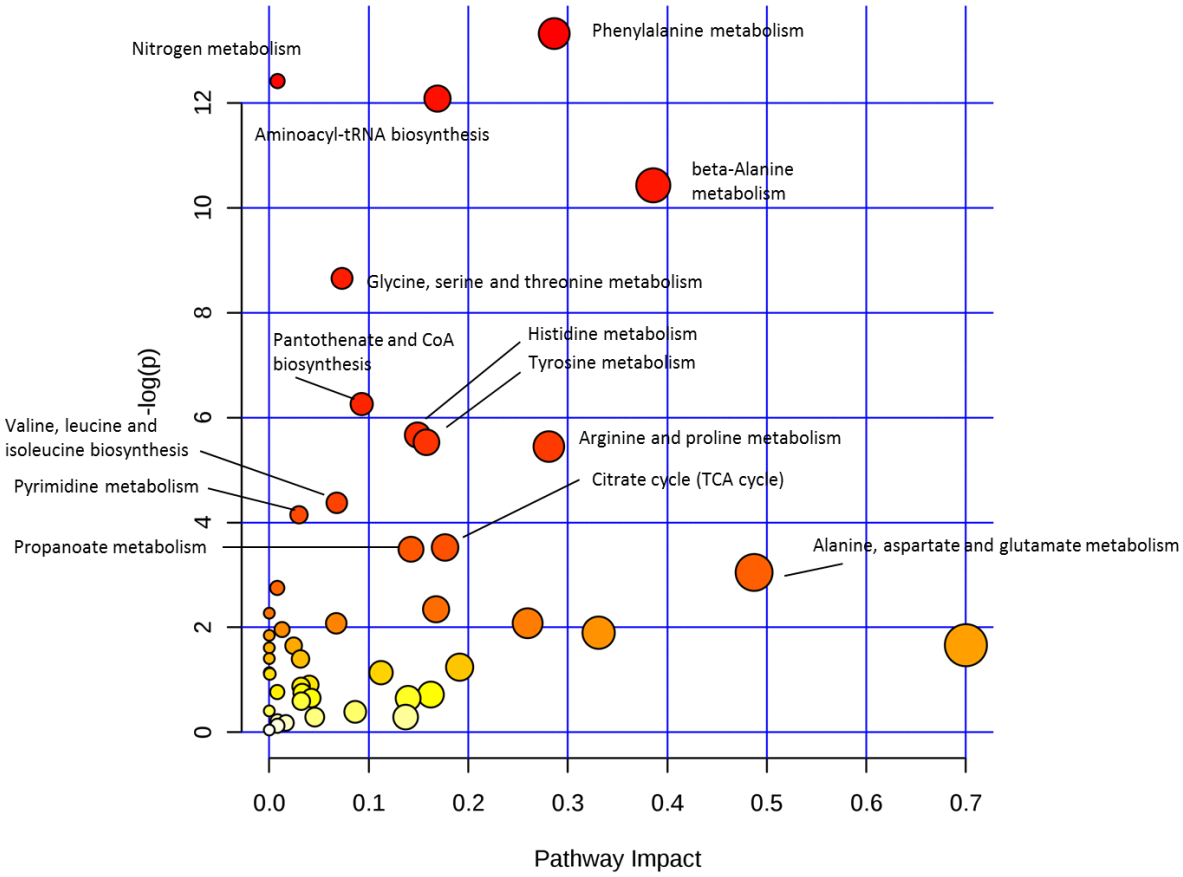



**Figure 5-24 Metaboanalyst pathway analysis of potential biomarkers implicated by PLS-R analysis of CRP and UA patients' urinary metabolites**

**Table 5-32 Pathway analysis of potential biomarkers implicated by PLS-R analysis of UA patients' urinary metabolites and CRP**

**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.

**The impact score**: Each pathway has a total importance score of 1. The importance measure of each metabolite node (a point where pathways intersect within a larger network) is expressed as a fraction with regards to the total pathway importance, and the pathway impact score is the cumulative score of matched metabolite nodes.

* p value calculated using hypergeometric test

| Pathway Name | Match Status | P value* | Impact score |
|---|---|---|---|
| Alanine, aspartate and glutamate metabolism | 3/24 | 0.047488 | 0.48718 |
| beta-Alanine metabolism | 7/28 | 2.96E-05 | 0.38586 |
| Phenylalanine metabolism | 10/45 | 1.64E-06 | 0.28632 |
| Arginine and proline metabolism | 8/77 | 0.004309 | 0.28094 |
| Citrate cycle | 3/20 | 0.02951 | 0.17664 |
| Aminoacyl-tRNA biosynthesis | 12/75 | 5.67E-06 | 0.16902 |
| Tyrosine metabolism | 8/76 | 0.003971 | 0.15793 |
| Histidine metabolism | 6/44 | 0.003461 | 0.14893 |
| Propanoate metabolism | 4/35 | 0.030516 | 0.14241 |
| Pantothenate and CoA biosynthesis | 5/27 | 0.001918 | 0.09288 |
| Glycine, serine and threonine metabolism | 8/48 | 1.75E-04 | 0.0731 |
| Valine, leucine and isoleucine biosynthesis | 4/27 | 0.012595 | 0.06784 |
| Pyrimidine metabolism | 6/60 | 0.015819 | 0.02984 |
| Nitrogen metabolism | 9/39 | 4.06E-06 | 0.0083 |

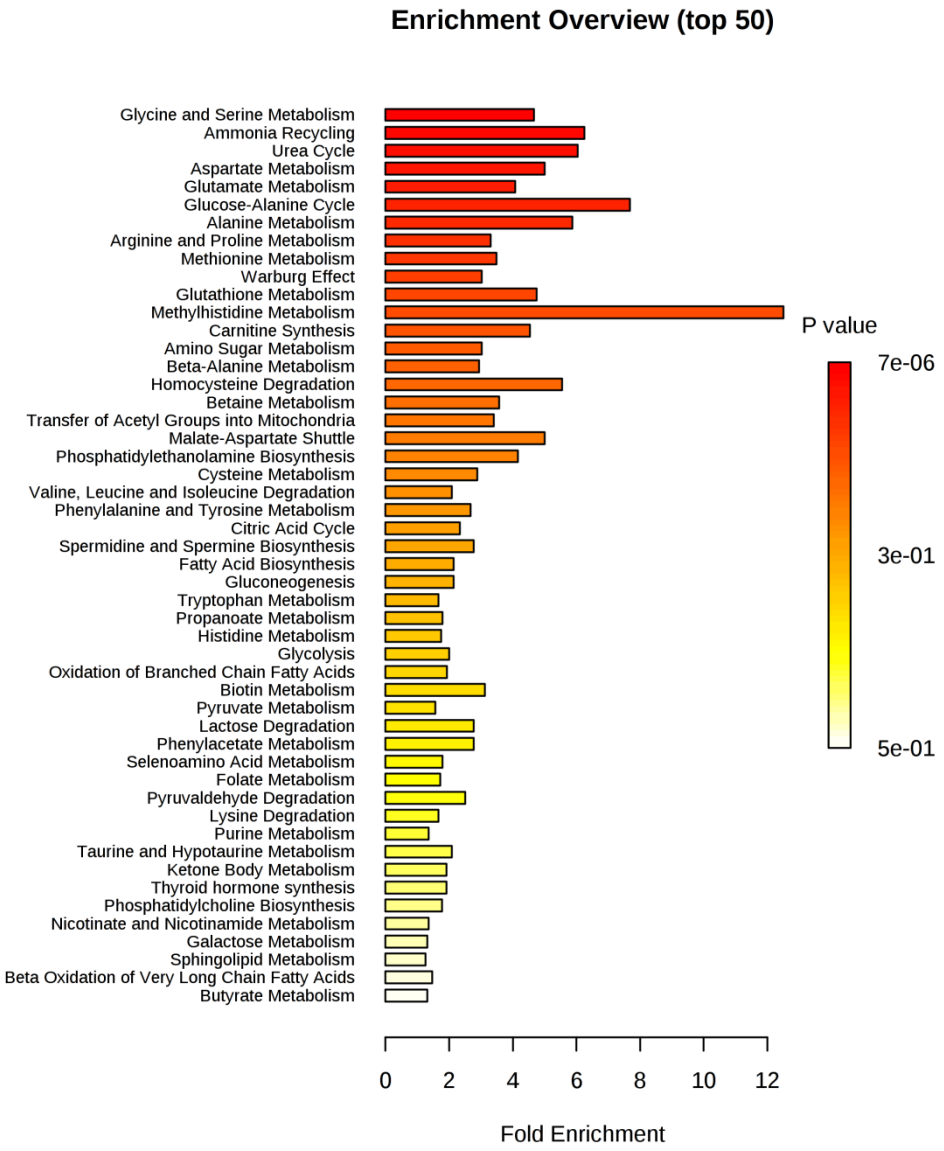



**Figure 5-25 Enrichment analysis of key metabolites in urine implicated as potential biomarkers by the PLS-R analysis of CRP and UA patients' urinary metabolites**

**Table 5-33 Enrichment analysis of key metabolites in urine implicated as potential biomarkers by the PLS-R analysis of CRP and UA patients' urinary metabolites.**
**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.
**The fold enrichment value**: the value is generated by the number of matched metabolites divided by the number of metabolites expected to be present in the pathway as per the reference metabolome provided by KEGG.
* p value calculated using hypergeometric test

| Metabolite set | Match status | Fold enrichment | Expected | P value* |
|---|---|---|---|---|
| Beta-alanine metabolism | 9/34 | 3.01 | 2.99 | 0.00174 |
| Homocysteine degradation | 3/9 | 3.79 | 0.791 | 0.0374 |
| Methylhistidine metabolism | 2/4 | 5.68 | 0.352 | 0.0408 |

Findings from the urinary metabolites functional analysis of biomarkers implicated by multivariate models largely validated the findings of the functional analysis of serum metabolites. As seen across all urinary sub analyses, there is evidence to suggest oxidative stress, upregulation of the urea cycle and protein catabolism are key characteristics of the patients with elevated CRP. I will now discuss these findings in more depth in the next section.

## 5.5 Discussion

In this study, we applied $^{1}$H-NMR metabolomics to study the relationship between systemic inflammation, as assessed by the serum CRP, and the serum and urinary metabolome in a cohort of DMARD naïve patients with inflammatory arthritis and CSA at presentation. Figure 5-26 and Figure 5-27 summarises how the metabolic changes we observed to be correlated with CRP at presentation can be related to increased urea cycle activity, oxidative stress, increased glycolysis, collagen degradation, and skeletal muscle degradation related to cachexia.

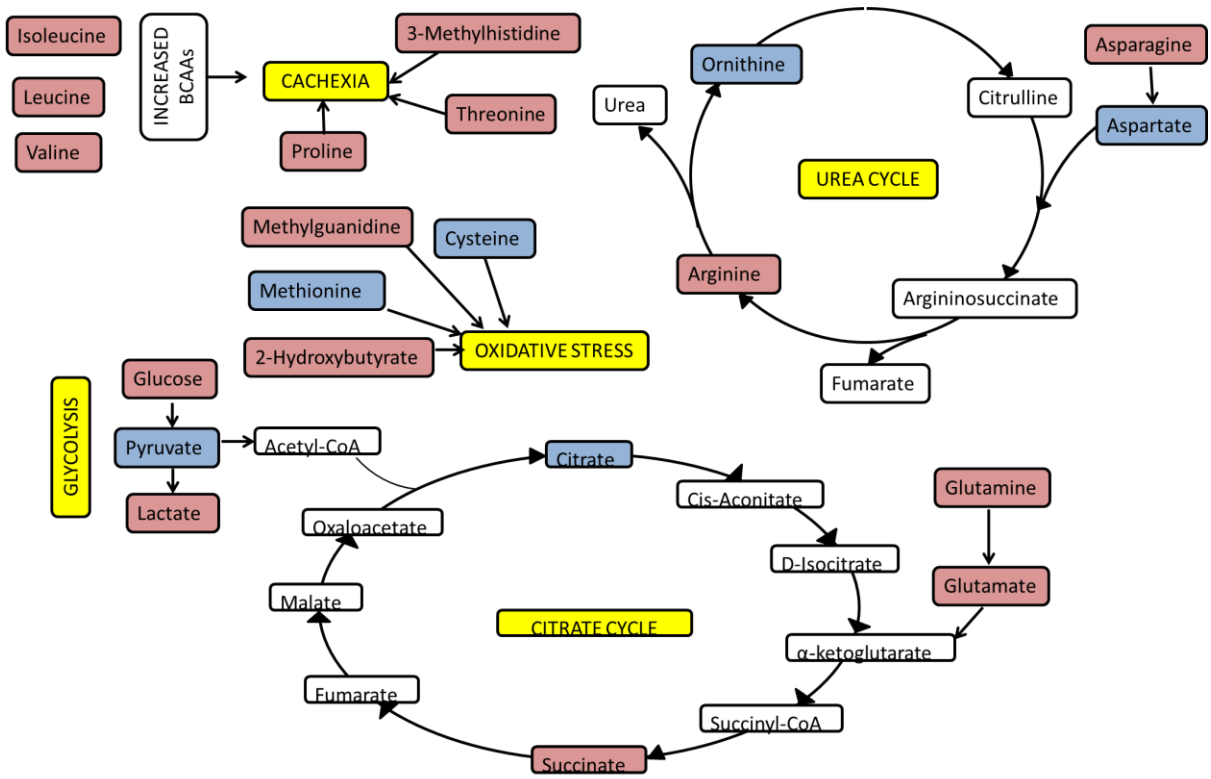



**Figure 5-26 Overview of key pathways and serum metabolites correlating with CRP.**
A summary of the metabolic permutations shown by the PLS-R analysis between CRP and the serum metabolome of inflammatory arthritis and CSA patients as assessed by NMR spectroscopy. Red metabolites had a positive correlation with CRP and blue metabolites had a negative correlation with CRP.

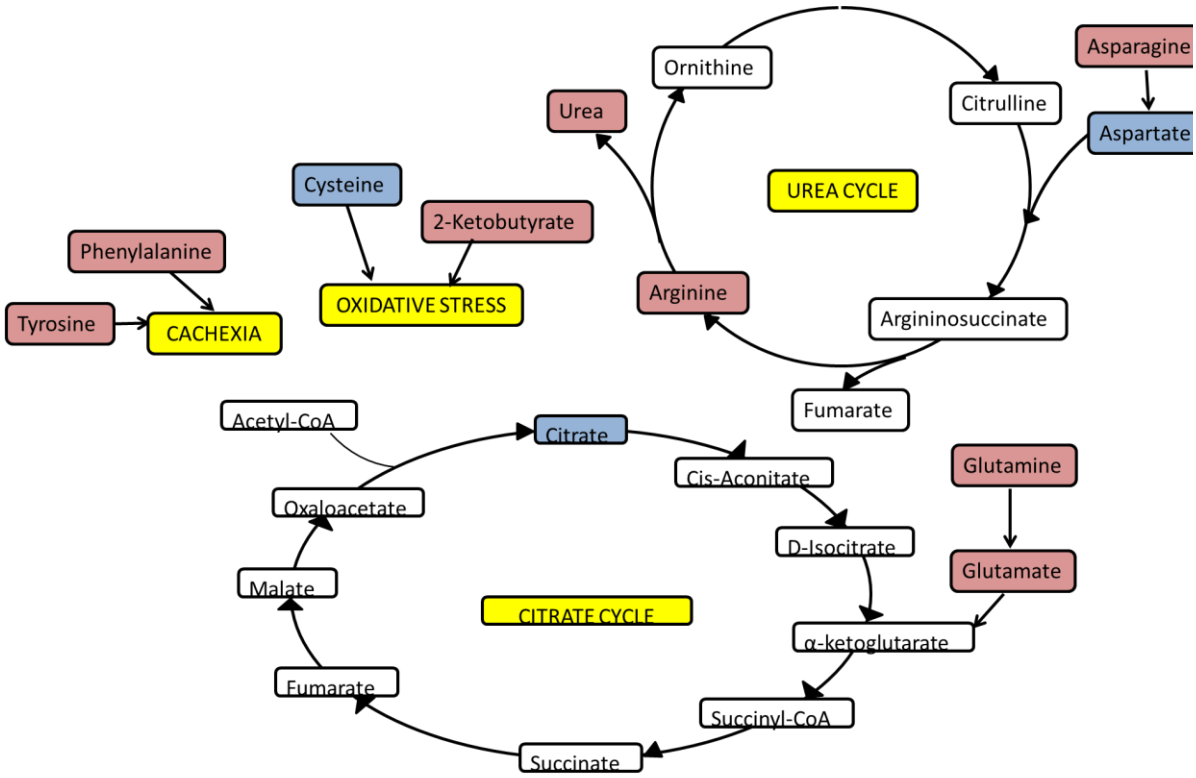



**Figure 5-27 Overview of key pathways and urinary metabolites correlating with CRP**
A summary of the metabolic permutations shown by the PLS-R analysis between CRP and the urinary metabolome of inflammatory arthritis and CSA patients as assessed by NMR spectroscopy. Red metabolites had a positive correlation with CRP and blue metabolites had a negative correlation with CRP.

Young et al (152) undertook PLS-R analyses of unfiltered serum metabolites of two separate groups of patients with early arthritis and showed a significant relationship between serum metabolites and CRP (group 1 $r^2$= 0.671, p <0.001 and group 2 $r^2$= 0.4157, p <0.001). The metabolites which contributed to this relationship included low-density lipoprotein lipids, lactate, glucose, methylguanidine and amino acids and their derivatives (taurine, acetylglycine, choline, threonine and methylhistidine). Many of the metabolites which correlated with CRP are associated with lipid metabolism (152). In the present study filtered serum was used which is devoid of large proteins and lipoproteins. This was done to avoid the significant overlap of the broad NMR signals of proteins and lipoproteins with the metabolites in the spectra (304), which can lead to difficulty in identifying metabolites. Therefore, despite losing information provided by proteins and lipoproteins, filtered serum results in spectra with fewer overlapping metabolites which can make metabolite identification less problematic. Loss of lipoproteins notwithstanding, the filtered serum PLS-R resulted in a significant relationship between serum metabolites ($r^2$=0.4811, p<0.001) and CRP. The most highly weighted metabolites in the model included glucose, amino acids, lactate, and citrate. This validates the previously seen relationship between metabolites and CRP (152). Furthermore, it shows a definitive relationship between CRP and metabolism which persists in filtered serum.

My data also showed a relationship between urinary metabolites and CRP. Blood concentrations of metabolites are strictly regulated, while urine metabolite concentrations can vary widely and can provide complementary information about systemic metabolism. In addition to filtration, the kidney

has imperative role in the generation, breakdown, and active reabsorption and secretion of metabolites, processes which determine urinary metabolite concentrations. (305, 306).

Urinary metabolomics have been used in the past to predict the response to anti TNF treatment (153) and to diagnose inflammatory rheumatic conditions (299, 307). Pietzner et al demonstrated a serum and urinary metabolic signature of chronic low grade inflammation in apparently healthy individuals (195). Our findings validate this and show there is a relationship between non-low grade inflammatory states and urinary metabolome in inflammatory arthritis and CSA patients. The functional interpretation of biomarkers generated by PLS-R analyses of all patients' urinary metabolome and CRP largely confirmed the findings seen in the serum analyses namely increased urea cycle activity, oxidative stress and protein catabolism.

I will discuss the major metabolites identified in both serum and urinary functional analyses of the multivariate models in the context of inflammation and musculoskeletal disease. For ease, I will group the discussion according to the metabolic pathways used by both KEGG (308) and MetaboAnalyst (172).

### 5.5.1 Alanine, aspartate and glutamate metabolism (including nitrogen metabolism)

Alanine, aspartate and glutamate metabolism pathway play a role in nitrogen handling, energy production and the management of oxidative stress (309). PLS-R models of the serum metabolome and CRP revealed a positive correlation between CRP and glutamine and glutamate. Both metabolites positively associate with CRP in the PLS-R model of all patients' urinary metabolite data and CRP. This could be as a result of increased protein mobilisation from skeletal muscle, which is the primary source of glutamine synthesis in the body (310). Amino acid demand increases during the acute phase response (287) as a result of changes in liver protein synthesis. Additionally, lymphocytes, macrophages, neutrophils and fibroblasts have high glutamine requirements and depend upon an adequate supply for their function (310-313). Increased demand for glutamate may reflect the need for amino acids for synthesis of proteins for the acute phase response, nucleic acids for immune cell proliferation and transcription of proteins, or the need for other high-energy, versatile metabolites such as pyruvate or oxaloacetate formed during transamination.

PLS-R models of the serum metabolome and CRP revealed a positive correlation between CRP and asparagine. Furthermore, PLS-R models of the serum metabolome and CRP showed a negative correlation between CRP and aspartate and ornithine. The negative correlation between aspartate and CRP was also seen in the PLS-R model of all patients' urinary metabolite data and CRP. Aspartate, synthesised from asparagine, is used in the urea cycle (Figure 5-28) to donate an amino group for the production of urea. It is possible that there is increased urea cycle activity during inflammation (314) may account for aspartate utilisation.

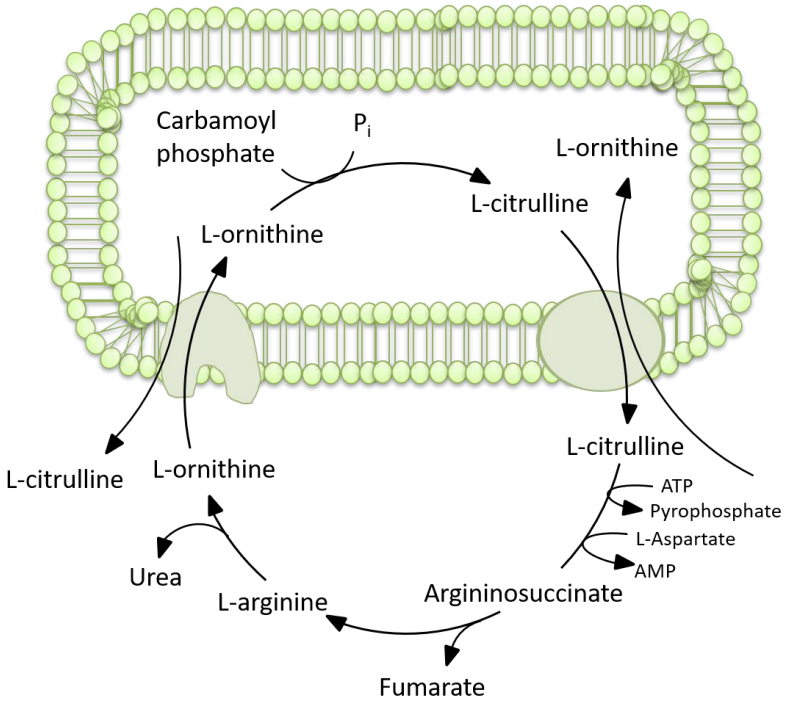



**Figure 5-28 Urea cycle**
The urea cycle was overrepresented in the enrichment analysis when examining the correlation between serum metabolome and CRP. PLS-R models of the serum metabolome and CRP showed a negative correlation between CRP and aspartate and may be explained by aspartate utilisation by the urea cycle and thus suggest increased urea cycle activity during inflammatory states.

The urea cycle and the malate-aspartame shuttle were highlighted as pathways of interest (Figure 5-6) when examining the correlation between serum metabolome and CRP. Both aspartate and glutamate also play a role as intermediaries in the malate-aspartate shuttle. The malate-aspartate shuttle is a series of reactions (Figure 5-29) which take place in the mitochondria which translocate electron produced by glycolysis across the inner membrane.

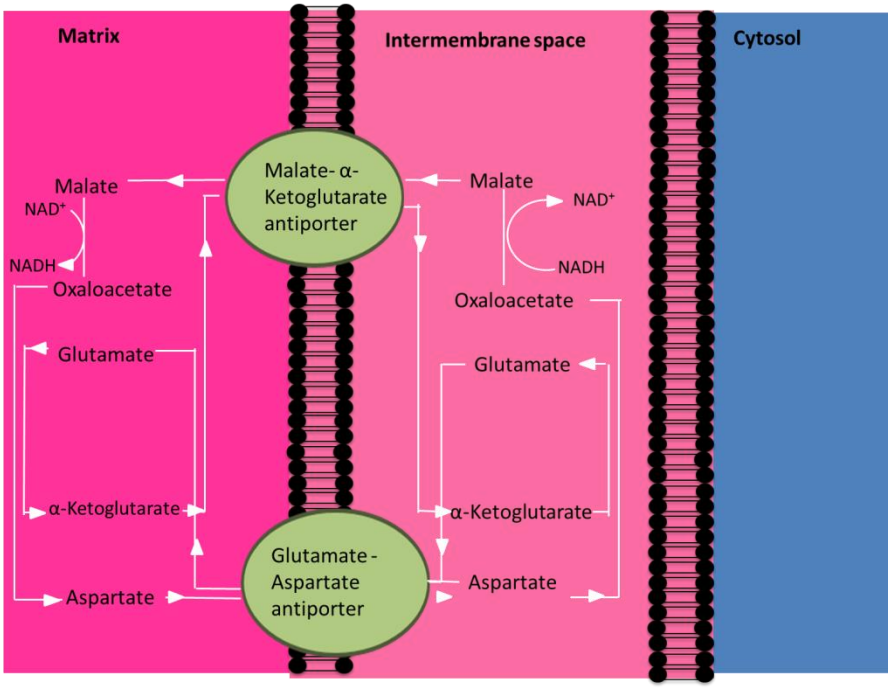



**Figure 5-29 The malate-aspartate shuttle.**
Malate acts as a reducing agent to allow electrons to be translocated across the inner membrane of the mitochondria during oxidative phosphorylation.

Systemic inflammation has been shown to increase glycolysis and fatty acid oxidation (315). The acetyl-CoA produced by fatty acid oxidation upregulates malate-aspartame shuttle activity and glycolysis via nonenzymatic acetylation during the inflammatory stress response (316). This may account for the findings seen in the overrepresentation analysis.

### 5.5.2 Arginine and proline metabolism

PLS-R models of the serum metabolome and CRP showed a negative correlation between CRP and arginine. Two important pathways of arginine metabolism in inflammation include the conversion of arginine to NO, and the breakdown of arginine to urea and ornithine (317-319), which could account for the utilisation of arginine as depicted in Figure 5-30. However, the PLS-R and OPLS-DA models of the urinary metabolome and CRP revealed a positive correlation between CRP and arginine. One potential explanation for this finding is altered renal arginine metabolism during systemic inflammation. The kidneys play a role in arginine synthesis and reabsorption, furthermore it also contributes to creatine synthesis, which utilises arginine (320). One can postulate the positive association between urinary arginine and CRP may result from impaired tubular reabsorption in patients with active inflammation in patients with active inflammation.

PLS-R models of the serum metabolome and CRP showed a negative correlation between CRP and ornithine. Ornithine is converted to the pro-proliferative polyamines via ornithine decarboxylase (ODC), and to proline, a constituent of extracellular matrix, via ornithine aminotransferase (OAT) as seen in Figure 5-30. Upregulation of ODC leads to impaired M1 macrophage function and could lead to persistence of inflammation (321). Thus, decreased ornithine in inflammatory states may be explained by the need for polyamines, which are vital for cellular growth and as ODC upregulates to meet this need, may lead to the perpetuation of the inflammatory response.

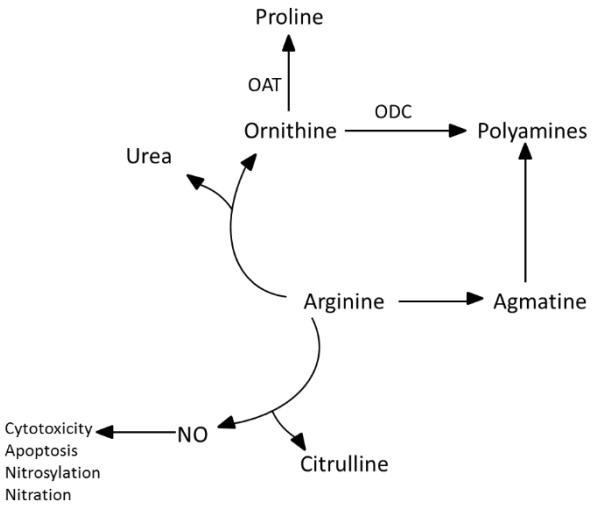



**Figure 5-30 A summary of arginine and ornithine metabolism.**
PLS-R models of the serum metabolome and CRP showed a negative correlation between CRP and both arginine and ornithine. It is possible that during systemic inflammation arginine and ornithine are utilised as depicted.

PLS-R analysis of serum metabolite data in relation to CRP showed a positive correlation between CRP and proline. Proline is an important substrate for varying cellular processes. In addition to being synthesised from ornithine and glutamate, proline, which makes up over 25% of collagen amino acids, can be readily liberated from collagen by matrix metalloproteinases which can be upregulated during pro-inflammatory states (322). Evidence suggests that free proline availability determines the rate of collagen biosynthesis (323). However, proinflammatory cytokines promote chondrocyte catabolic pathways and matrix destruction, also inhibit new cartilage formation. To investigate the relationship between serum proline and disease activity further, serum proline concentration was estimated using Chenomx NMR suite (Chenomx, professional version 8.5) from 1H NMR metabolomics analysis from sera collected from newly presenting RA patients as outlined in **section 2.3.2**. Post-identification analysis of metabolites was performed using Chenomx profiler, by manual matching of representative spectra against the Chenomx database of known spectral signatures. Manual peak fitting was used for quantification of metabolites and peak clusters were adjusted to account for variation in pH. To provide further clarity, handling and identification of metabolites with the data, water peaks were removed using Chenomx automated deletion tool. Proline was identified with peaks at 4.1, 3.4, 3.3, 2.3, 2.1 and 2.0 ppm (see Figure 5-31)

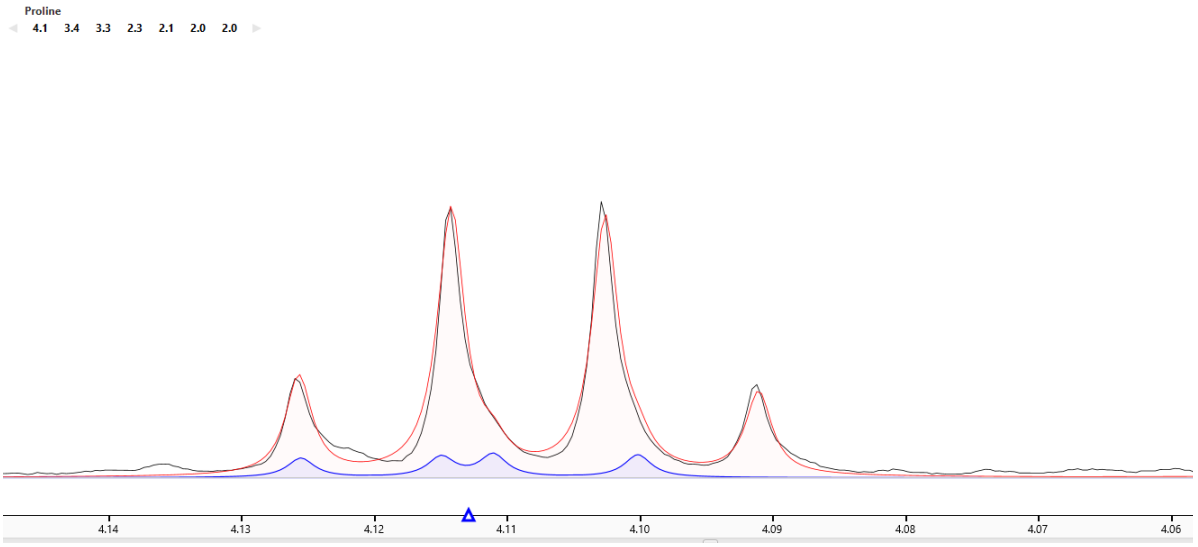



**Figure 5-31 Spectral fitting of proline at 4.1ppm**
The blue shaded area indicates proline, whilst the red area shows the overall peak shape. 4.1ppm is shared with lactate which is more abundant.

The relationship between proline concentration and swollen joint count is shown in Figure 5-32. There were no correlations between serum proline concentration and SJC66 score (p=0.358), DAS28ESR (p=0.634), and CRP (p=0.649).

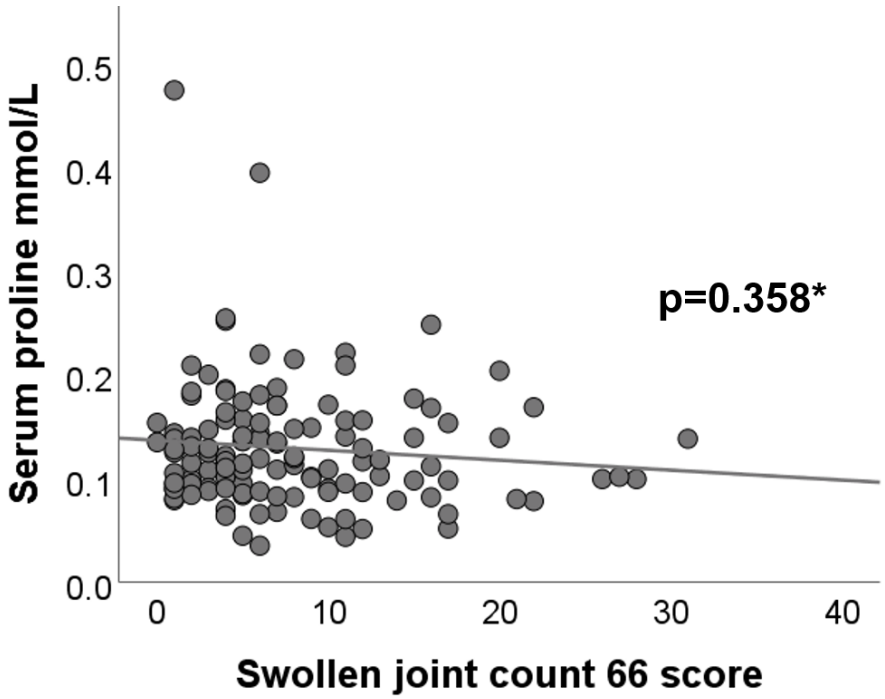



**Figure 5-32 Scatter plot between serum proline & SJC66 score in RA patients.**
There was no correlation between the serum proline concentration and the swollen joint count in all RA patients (n=126, p=0.358*).
*p value calculated using Spearman's rank correlation

Like 4-hydroxyproline, it is possible that a relationship between serum proline concentration may have a relationship with articular damage, which is only evident longitudinally. Future work investigating this relationship should account for patient body composition which may act a confounding factor as circulating proline appears to correlate well with changes in body composition (324).

### 5.5.3 Phenylalanine, tyrosine & tryptophan biosynthesis

PLS-R and OPLS-DA models of all patients' serum metabolome and CRP revealed a positive correlation between CRP and phenylalanine and tyrosine. Furthermore, a positive correlation between CRP and phenylalanine and tyrosine was also seen across all multivariate analyses of urinary metabolome and CRP. Positive acute phase protein production during inflammatory states is associated with anorexia and catabolism. Therefore, the source of amino acids is likely to be from protein stores such as skeletal muscle rather than dietary protein. The amino acid profile for acute phase proteins contrasts with that of skeletal muscle. Due to the high demand for phenylalanine, tryptophan and tyrosine three times the amount of protein from skeletal muscle must be catabolised to synthesise acute phase proteins (325). PLS-R models of UA and all patients' serum metabolome and CRP revealed a positive correlation between CRP and the BCAAs valine, leucine and isoleucine. These three amino acids are thought to be the most abundant amino acids in skeletal muscle (326), which lends further support to the observation of protein catabolism in skeletal muscle.

In addition to acute phase proteins, phenylalanine has been proposed to be involved in the release of prostaglandins important in initiating inflammatory processes. Phenylalanine has been associated with the development of type 2 diabetes mellitus (327, 328), cardiovascular disease (329) and the reduction in telomere length (330), suggesting phenylalanine metabolism could be integral in the chronic low grade inflammation associated with ageing (331), known as "inflammaging". Data in this thesis also suggests there is an association between inflammation, as measured by CRP, and phenylalanine.

### 5.5.4 Glycine, serine & threonine metabolism

PLS-R models of all patients' serum metabolome and CRP revealed a positive correlation between CRP and threonine and choline. Threonine is an essential amino acid which is important for modulating immune function (332-335). However during intestinal inflammation which is commonly present during a systemic inflammatory response, increased threonine bioavailability is secondary to liberation from skeletal muscle rather than intestinal absorption (336). The positive correlation between CRP and threonine may be a consequence of protein catabolism present in inflammation associated cachexia. Threonine itself is also important for preventing apoptosis, cell growth and antibody production in lymphocytes (337).

Choline is an essential amine nutrient which can be used to synthesis acetylcholine in cholinergic neurones and phosphatidylcholine (PC) in non-neurogenic cells. Choline availability and CTL1 upregulation is critical for cytokine production in LPS-stimulated macrophages (338). Choline like-transport protein 1 (CTL1) is responsible for transporting choline intracellularly in non-neuronal cells. Once in the cytoplasm, choline is shuttled along by the Kennedy pathway, combined with diacylglycerol to form PC. PC is the major outer leaflet of phospholipid. Lipopolysaccharide (LPS) stimulated macrophages results in significant increase in PC synthesis (339, 340). Furthermore, PC synthesis inhibition results in a reduction of pro-inflammatory cytokine secretion by LPS activated

macrophages (341). Choline availability and CTL1 upregulation is critical for cytokine production in LPS-stimulated macrophages (338).

### 5.5.5 Citrate cycle

PLS-R and OPLS-DA models of the serum metabolome and CRP revealed a negative correlation between CRP and citrate. PLS-R models of the serum metabolome and CRP revealed a positive correlation between CRP and succinate. There are several potential explanations for these findings. For instance, it is possible that high energy demands from infiltrating immune cells could explain the increased citrate utilisation as postulated by Ani et al, who observed a relationship between hypocituria and active or relapsing renal vasculitis (342). An alternative explanation however could be immune metabolic reprogramming. Cells of the innate immune system, such as M1 macrophages and dendritic cells, have been shown to upregulate glycolysis and the pentose phosphate pathway, in addition to downregulating the citrate cycle (Figure 5-33), oxidative phosphorylation and fatty acid oxidation (343). This increased glycolytic flux may represent a need to generate more ATP and also to generate other intermediates from the citrate cycle, as the citrate cycle switches from primarily a catabolic to an anabolic pathway, one consequence of which is the accumulation of both citrate and succinate in the mitochondria (344). Citrate is transported to the cytosol and broken down to acetyl-CoA for both fatty-acid synthesis and protein acetylation, both of which have been linked to macrophage and DC activation (344). Furthermore, citrate utilisation has also been associated with the production of a number of pro-inflammatory mediators such as nitric oxide, ROS and prostaglandin E2 (345-347). These mechanisms may explain the negative correlation seen between CRP and citrate in the present study.

The increase in succinate, by glutamine-dependent anaplerosis, leads to HIF 1α activation and ultimately enhanced IL-1β production during inflammation (348). Finally, succinate has been shown to post-translationally modify proteins, a process known as succinylation, which also perpetuates the inflammatory response. Although succinate appears to be utilised by these processes, it is possible that overproduction may outweigh utilisation of succinate as the present study identifies a positive correlation between CRP and succinate, which has been reported previously (349).

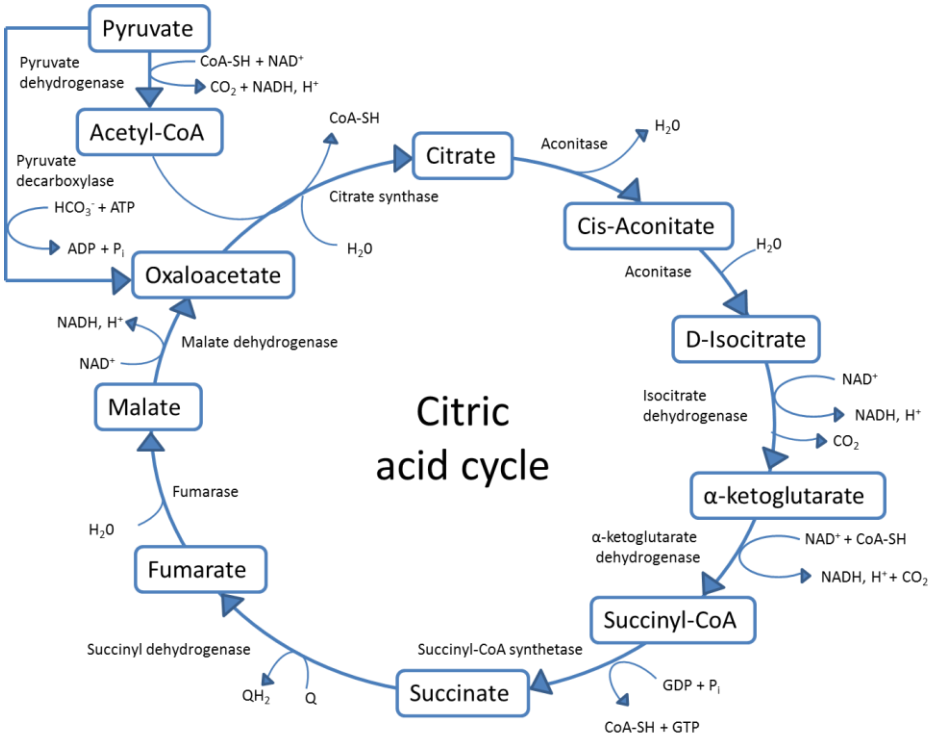



**Figure 5-33 Citrate cycle.**
PLS-R and OPLS-DA models of the serum metabolome and CRP revealed a negative correlation between CRP and citrate. PLS-R models of the serum metabolome and CRP revealed a positive correlation between CRP and succinate. It is possible these findings can be explained by metabolic reprogramming which switches the citrate cycle into an anabolic pathway.

### 5.5.6 Pyruvate metabolism

PLS-R and OPLS-DA models of the serum metabolome and CRP revealed a negative correlation between CRP and pyruvate. This could reflect immune metabolism reprogramming. For instance, the macrophage environment is likely to be in a hypoxic environment as these cells infiltrate sites of inflammation (350), which would lead to significantly upregulated transcription of HIF1-α (351). Macrophages undergo significant metabolic changes upon activation mediated by HIF1-α stabilisation (348). This metabolic reprogramming includes increased glycolysis, decreased oxygen consumption and lactate production. HIF1-α is ordinarily responsible for upregulating transcription of pyruvate dehydrogenase kinase 1 (PDK1), which inhibits pyruvate dehydrogenase (PDH) via phosphorylation in cells in a hypoxic environment (352). However pyruvate continues to be oxidised by PDH in pro-inflammatory macrophages as a result of downregulating PDK1 expression (353). Sustained PDH flux plays an important role in maintaining macrophages in an activated state (353) and could be responsible for the negative association between pyruvate and CRP shown in the PLS-R models. In addition to scavenging ROS, pyruvate also inhibits TNF-α production and NF-κB signalling pathways (354). Pyruvate also inhibits IL-6 mRNA expression in liver and intestinal mucosa (355). Thus, the reduction of pyruvate availability may be a pre-requisite to propagate the inflammatory response.

### 5.5.7 Cysteine, methionine and glutathione metabolism

PLS-R models of RA and UA patients' serum metabolome and CRP revealed a negative correlation between CRP and cysteine and methionine. Furthermore PLS-R models of urinary metabolome and CRP revealed a negative correlation between CRP and cysteine and cystathionine. It is possible the cystathionine and methionine are being utilised to produce more cysteine and in turn cysteine is being utilised to synthesise GSH. GSH is a ubiquitous intracellular tri-peptide that has multiple functions including detoxification and antioxidant defence. The synthesis of GSH from glutamate, cysteine and glycine involves two ATP-requiring enzymatic steps:

L-glutamate + Cysteine + ATP → γ-glutamyl-Cysteine + ADP + Pi

γ-glutamyl-Cysteine + L-glycine + ATP → GSH + ADP + Pi

The first step is catalysed by glutamate cysteine ligase (GCL) which, in addition to the availability of its substrate Cysteine, is the rate limiting step for the synthesis of GSH. In addition to being available from dietary sources, cysteine can be synthesised from the essential amino acid methionine in hepatocytes as seen in Figure 5-34:

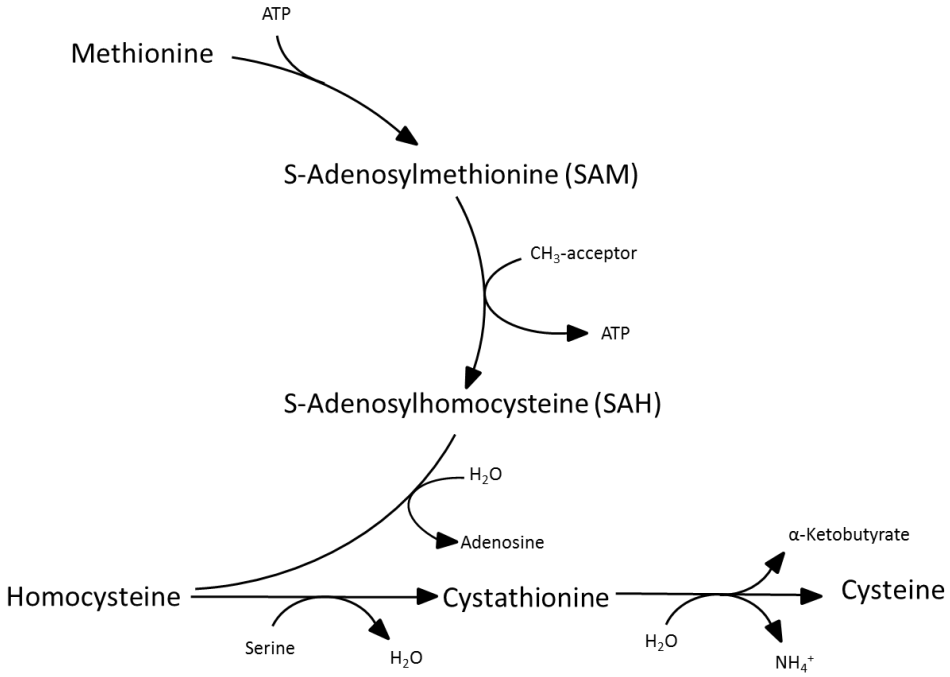



**Figure 5-34 Hepatic methionine and cysteine synthesis**

GSH reacts with exogenously and endogenously formed electrophilic compounds to form GSH conjugates. The process of GSH conjugation irreversibly expends intracellular GSH (356). GSH is used to reduce oxidative stress, severe oxidative stress can deplete glutathione stores (357, 358). Whilst oxidative stress can induce inflammation and vice versa, the two processes commonly co-exist and thus can account for the negative correlation between CRP cysteine and its precursors, methionine and cystathionine.

### 5.5.8 Valine, leucine and isoleucine biosynthesis & degradation

PLS-R models of all patients' serum metabolome and CRP revealed a positive correlation between CRP and the BCAAs valine, leucine and isoleucine. Furthermore, a positive association between CRP and

valine and isoleucine was also identified by the OPLS-DA models of serum metabolite data showing separation between low and high CRP tertiles for both all the cohort and RA patients, respectively. There is a close relationship between the essential amino acids BCAA's metabolism and immune function. In vitro studies have shown a reduction in BCAA metabolism in response to pro-inflammatory cytokines including TNFα (359). Proinflammatory states are responsible for decreased metabolism of BCAA directly through reduced the expression of the genes linked to BCAA transport and oxidation and indirectly through oxidative stress leading to carbonylation of enzymes responsible for BCAA metabolism (359, 360). Therefore, in addition to cachexia driven protein catabolism, there may be an inflammation mediated reduction in BCAA metabolism. This may explain the positive correlation between CRP and BCAA.

### 5.5.9 Histidine metabolism

PLS-R model of all patients' serum metabolome and CRP showed a positive correlation between CRP and methylguanidine and 3-methylhistidine. These findings may broadly indicate inflammation induced cachexia. 3-methylhistidine, the methylated analogue of histidine, is an amino acid which is present in actin and myosin (361-363). Catabolism of this complex result in 3-methylhistidine excretion and thus urinary and plasma 3-methylhistidine has been suggested as marker of skeletal muscle turnover (364-368). Although meat is the main exogenous form of 3-methylhistidine and thus to accurately measure endogenous 3-methylhistidine production one should abstain from meat and soya products for 3 days before sampling (364). Methylguanidine is a product of protein catabolism (369). It is a product of creatinine oxidation by the hydroxyl radical and accumulates in renal failure (370). Creatinine is a breakdown product of creatine phosphate in muscle and is renally eliminated. Creatinine can be used as a surrogate marker of renal function. Thus, methylguanidine production relies upon renal function and muscle catabolism. Increased methylguanidine production may indicate oxidative stress (371, 372), which can co-exist with pro-inflammatory states. These findings suggest proinflammatory states are associated with protein catabolism, a finding which has been reported previously (373-375).

### 5.5.10 Propanoate metabolism

Propanoate is a short chain fatty acid produced predominately by the gut microbiome digesting fibre. Propanoate, like other short chain fatty acids, can be metabolised to propionyl-CoA by beta-oxidation. Propionyl CoA is a substrate which can be metabolised into succinate as seen in Figure 5-35 below:

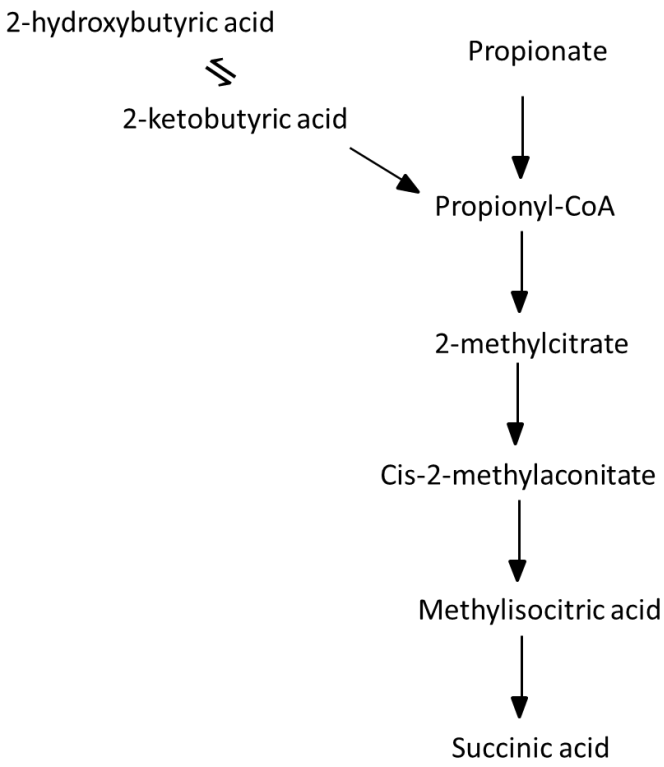



**Figure 5-35 Propanoate metabolism**

The PLS-R model of all patients' serum metabolome and CRP showed a positive correlation between CRP and 2-hydroxybutyrate and succinate. Furthermore, PLS-R models of RA and PsA patients' urinary metabolome and CRP showed a positive correlation between CRP and 2-ketobutyrate. 2-Hydroxybutyrate is formed as a result of the formation of 2-ketobutyrate (376). 2-Ketobutyrate is a product of either amino acid catabolism or GSH formation (377). GSH is integral for uptake of amino acids, synthesis of leukotrienes and converting dangerous substances such as free radicals to inactive products (378). The observed increase in 2-hydroxybutyrate may be because of the cleaving of cystathionine to cysteine that is required for GSH synthesis.

### 5.5.11 Glycolysis & gluconeogenesis

PLS-R models of RA and all patients' serum metabolome and CRP showed a positive correlation between CRP and glucose. Studies have reported an association between impaired glucose metabolism and inflammatory arthritis (379-382). Several mechanisms are likely to be responsible for insulin resistance including TNF-α inhibiting insulin signalling pathways (383), diminished expression of both adipocyte glucose transport protein-4 (384) and insulin receptor substrate-1 (385). Furthermore, islet β-cell dysfunction (113, 114) and apoptosis may also play a role in impaired glucose metabolism (386-388) in the context of raised inflammatory states. An animal model of arthritis also showed IL-6 induces islet β-cell dysfunction (389). RA patients have a tendency to have impaired islet β-cell function and insulin sensitivity (390), which was also seen in RA patients without diagnosed diabetes mellitus (382). In addition to direct effect on the islet β-cell function and insulin sensitivity, liver organ dysfunction may also play a role in impaired glucose metabolism. ROS associated with the development of RA (391, 392), may be associated with elevated ROS levels in the liver (393, 394). This may be associated with a number of key hepatocellular abnormalities (395-397), which may lead to

reduced hepatic gluconeogenesis (398) and increased hepatic uptake of glucose and glycolysis (399). Dysfunction of the hypothalamic-pituitary-axis in chronic inflammatory states leading to inappropriately low cortisol in response to inflammation, in the absence of synthetic glucocorticoids, which may also contribute to impaired glucose metabolism (400). Systemic inflammation has a well-documented effect on glucose metabolism with numerous complex mechanisms and metabolic connotations. The relationship between elevated serum glucose and elevated CRP seen in the PLS-R models is most likely pro-inflammatory cytokine mediated and the resulting glucose may serve to meet increased metabolic demands of immune cells.

In further support of altered glucose metabolism, the serum PLS-R model of all cohorts', RA and UA patients' serum metabolite profile and CRP shows a positive association between lactate and CRP respectively. A positive association between lactate and CRP is also seen in the OPLS-DA model of all patients' serum metabolite data and CRP tertiles. Highly active immune and cancerous cells have a large uptake of glucose and rely upon glycolysis as their main form of energy production. In rapidly proliferating immune cells and malignant cells, pyruvate is reduced to lactate even in an aerobic environment, this is known as the Warburg effect or aerobic glycolysis (401). In healthy individuals, serum and tissue lactate is kept between 1.5-3 nM, however the lactate in synovial fluid of RA patients can be as elevated as 10nM (402) with accompanying increased activity of lactate dehydrogenase (LDH) in the synovial fluid of RA patients compared to controls (403), suggesting impaired glycolytic metabolism. Lactate has immunomodulatory properties as it has been shown to regulate T-cell effector function by stopping migration and increasing production of the pro-inflammatory cytokine IL-17 in CD4+ T cells and diminishing CD8+ T-cell mediated killing (404, 405). Increased LDH activity may also play a role in promoting T-cell effector function (406). PDH inhibition in CD4+ T cells can lead to reduced pyruvate oxidation and contribute to reduced lactate clearance (407). Increased glycolysis may therefore not only directly serve highly active immune cells need for ATP, but may have downstream effects which propagate the inflammatory response through lactate and LDH activity.

## 5.6 Limitations

Despite many advantages of using NMR spectroscopy to assess the metabolome such as minimal, non-destructive sample preparation with high reproducibility (187), there are several important limitations. Firstly, there is low sensitivity for identifying metabolites in NMR spectroscopy, leading to failure to identify metabolites present at a lower concentration. Secondly, overlapping $^{1}H$ signals pose a challenge to metabolite identification and can provide a barrier to completely understand the biological processes behind the metabolomic multivariate analysis correlations observed. Due to the overlapping $^{1}H$ signals, individual peaks in each spectrum may be as a result of a combination of metabolites rather than a single metabolite. Thus, when a chemical shift of peak (ppm) of interest is identified, there is no method of elucidating which of the possible metabolites present at that ppm drives association. This could explain why a proportion of the associations between each metabolite and CRP varied in each model. As a result, it may not be possible to comment on the exact change in the metabolites with respect to the CRP, rather a change in each metabolic pathway in relation with CRP. In addition to this, filtered serum was used instead of unfiltered serum which limited the assessment of lipid metabolism, as large proteins and lipoproteins are removed from filtered serum. Other confounders which are known to influence studies on metabolism were not controlled for include comorbidities, medications, diet and time of sample collection.

Furthermore, the cohort of patients used to investigate the relationship between serum metabolome and CRP (n=270) did not entirely overlap with the cohort of patients used to investigate the relationship between urinary metabolome and CRP (n=178). This is a confounding factor when comparing findings between biofluids.

Lastly, the distribution of CRP in the clinical population was skewed with most of the values falling in the low to normal range. Our data thus provide insight into metabolic permutations associated in particular with low grade inflammation. As patients with moderate to severe inflammation as measured by CRP are underrepresented, it is possible that metabolic permutations associated with high levels of CRP may not have been captured in this study.

## 5.7 Conclusion

The PLS-R models between metabolite profiles and CRP has given significant insights into metabolic derangements during inflammatory states such as oxidative stress, cachexia and impaired glycolytic metabolism. Oxidative stress is an important process during inflammation. Elevated 2-hydroxybutyrate may reflect the need for glutathione synthesis to neutralise products of oxidative stress. Furthermore methylguanidine, in addition to reflecting protein catabolism may also indicate the presence of oxidative stress. The increase in amino acids, likely from skeletal muscle and hepatocytes, could reflect cachexia driven protein catabolism. This is further confirmed by increased 3-methylhistidine which is typically used as a surrogate marker of skeletal muscle turnover.

Amino acids play important roles in the onset and persistence of inflammation. Increased amino acid metabolism is accompanied by an increase in urea cycle activity as evidenced by the decrease in aspartate and increase in urea. Increased glucose could reflect cytokine mediated insulin resistance, impaired β-cell function and altered glucose transport and uptake. Elevated lactate could represent the Warburg effect as rapidly proliferating immune cells switch to glycolysis as their main source of ATP production even in absence of hypoxia. Decreased citrate and increased succinate may reflect citrate cycle changes as it switches from primarily a catabolic function to anabolic in inflammatory states and citrate consumption may be as a result of increased lipolysis. Finally, alteration in 3-hydroxybutyrate metabolism in response to inflammation is likely to represent changes in lipolysis in inflammatory states although further information pertaining to lipid metabolism is limited in filtered serum. These metabolites have significant downstream effects which propagate inflammation.

The relationship between CRP and metabolites, were seen across RA, PsA and UA patients in addition to the entire cohort. The metabolites implicated as potential biomarkers reveal broadly similar findings across each diagnostic group. However, there are some notable differences. For instance, skeletal muscle breakdown products 3-methylhistidine and methylguanidine, had positive correlation with CRP in the PLS-R analysis of RA patients' serum metabolite data, whilst a negative correlation with CRP was observed in PLS-R analysis of UA patients' serum metabolite data. It is possible that protein catabolism and cachexia is more advanced in RA patients rather than UA patients. Furthermore, although a relationship between CRP and urinary metabolome was seen in PsA patients and many of the metabolites implicated as potential biomarkers were similar to other sub-analyses by diagnosis, the relationship between multiple metabolites and CRP varied in comparison with other diagnoses and the whole cohort. For example, PLS-R analysis of PsA patients' urinary metabolite data in relation to CRP showed a negative correlation between CRP and phenylalanine, tyrosine, isoleucine and leucine, whilst also showing a positive correlation between CRP and aspartate. Whilst the converse findings were seen amongst the whole cohort and other diagnoses. There is a possibility that

these findings may be accounted by the small sample size of PsA patients, however, there is also a chance these findings could indeed reflect metabolic permutation related to inflammation. The pathoaeitology of PsA, like RA, is incompletely understood, however studies suggest there are significant differences between the two conditions (408). For instance, IL-6, the essential stimulator of CRP, does not play a significant role in PsA development as a result the CRP level is often low or absent in PsA patients (409). Thus, the difference between the metabolic permutations in relation to CRP seen in PsA patients and in RA and pre-RA patients may reflect differences in underlying disease mechanisms.

These findings validate a known relationship between serum metabolite profile and inflammation as measured by CRP, provide novel evidence that this relationship persists despite using filtered serum and finally, shows there is relationship between urinary metabolite profile and inflammation.

# 6 Relationship between FACIT-F and metabolism

## 6.1 Introduction

Fatigue is a highly prevalent and poorly managed symptom of inflammatory rheumatic conditions. Up to 70% of patients with RA report fatigue and more than 50% of patients feel that physicians inadequately address this symptom (197, 410, 411). RA patients consider fatigue as only secondary to pain in order of symptom importance and a feature of active disease (412). Fatigue experienced by RA patients has been described as "being stuck in mud" (413) with life changing social, emotional, cognitive and physical consequences (414). The cause of fatigue in rheumatic conditions is poorly understood but is likely to be multifactorial. As outlined in **chapter 4**, many clinical parameters associate with fatigue, however epidemiological studies are unable to identify whether these associations are causative or consequential.

Inflammation associated with rheumatic conditions has been proposed as a cause of fatigue; proinflammatory cytokines and chemokines have themselves been associated with sickness behaviour (415). However an inconsistent relationship between fatigue and serum CRP or ESR (39, 416, 417) has been reported in the literature. Levels of the cytokines IL-1β and IFN-γ have been shown to predict changes in fatigue in 80 RA patients over a 6 month period (418). It has been proposed that the effects of low grade inflammation both directly and indirectly contribute to the symptom of fatigue (419). Pro-inflammatory cytokines IL-6 and TNF-α have both been associated with disturbed sleep wake cycles (420, 421). Disruption in circadian rhythm, both through inflammation and sleep deprivation, mediate altered cellular energy availability and decreased behavioural-energy expenditure. Inflammation has been shown to switch from the energy efficient oxidative phosphorylation to the rapid but less efficient oxidative glycolysis. A pro-inflammatory state is also associated with increased ROS (422, 423) and reduced insulin sensitivity (424, 425). These changes lead to decreased glucose, and thus cellular energy, availability which is then insufficient to meet behavioural-energy demand and may therefore lead to the symptom of fatigue.

The association between metabolism and fatigue has been extensively investigated in CFS, using both blood (426-431) and urine (426-428, 432). MS metabolomics analysis of CFS patients' plasma has shown abnormalities in cholesterol, branch chain amino, proline/glutamate and mitochondrial metabolites (433). Furthermore, another study using MS metabolomics on CFS patients' plasma found alterations in metabolites related to the citrate cycle and urea cycles (434). The finding of citrate cycle impairment has been seen in subsequent studies on CFS patients and may be secondary to PDH dysfunction (435). These findings have been replicated using CFS patients' plasma using NMR metabolomics approaches (429, 436). Animal models of fatigue, which are based upon exhaustion following intense exercise such as immersion into water, have also revealed impaired energy metabolism (437, 438).

One study has applied MS metabolomics to plasma of 24 bDMARD naïve RA patients with increased fatigue as measured by FVAS. In that study, fatigue was associated with a reduction in metabolites from the urea cycle, fatty acids, tocopherols, aromatic amino acids, and hypoxanthine (439). These findings broadly indicate that fatigued states are associated with increased oxidative stress and dysregulated niacin metabolism. Besides its small overall sample size, 19 patients were taking methotrexate and 10 patients were taking prednisolone, which could have influenced the results. More recently, a study applied gas chromatography mass spectrometry plasma metabolomics to evaluate the impact of moderate-to-high intensity, aerobic and resistance exercise on fatigue, anxiety and depression on RA patients ≥65 years of age (440). Physical fatigue, as assessed by a subdomain of the MFI-20, was associated with changes in the metabolism of lipids, bile acids, the urea cycle and several sugars. Plasma sampling was conducted on 48 patients of 74 patients for the metabolomics

sub analysis, yet clinical and demographic data were reported for the 74 subjects only. However, the overall cohort had low disease activity (mean DAS 28 ESR 2.33 ± 1.10) and only 4 subjects were DMARD free, which could represent limitations in applying these findings to newly presenting RA and pre-RA patients. A metabolomics study investigating the association between fatigue and plasma metabolome in SLE, also showed potential niacin dysregulation (441).

As already shown in this thesis (**chapter 5**) and also in previous studies (152, 195), there is a significant association between CRP and the serum and urinary metabolome. The association between fatigue and serum and urinary metabolome in patients with inflammatory arthritis and CSA is poorly characterised and requires further investigation which should take account for the effect of inflammation on the metabolome.

We hypothesized that a metabolomics approach using filtered serum and urine could identify a relationship between metabolic dysfunction and fatigue in patients with inflammatory arthritis and CSA, which persists despite controlling for the effect of inflammation on metabolism.

## 6.2 Aims & objectives

1. To identify serum and urinary metabolites that may correlate with the FACIT-F score of adults with newly presenting inflammatory arthritis and CSA, using the **entire serum and urinary metabolome** respectively.
2. To identify serum and urinary metabolites that may correlate with the FACIT-F score of adults with newly presenting inflammatory arthritis and CSA, using the **serum and urinary metabolome devoid of those NMR bins that strongly correlate with CRP.**

## 6.3 Patients

Sera were available from 252 patients and urine samples from 178 patients from baseline visits for metabolomics analysis. These patients were identified from the overall cohort which has previously been described (**section 2.1)** and included those for whom CRP and FACIT-F data were available. **Section 2** outlines sample preparation, NMR spectroscopy application and statistical analysis of the resulting metabolomics data.

## 6.4 Results

### 6.4.1 Patient characteristics for study of relationship between serum metabolite profile and FACIT-F

The same cohort that was used for serum metabolomics analysis to investigate the relationship between the serum metabolome profile and CRP was used to investigate the relationship between serum metabolome and FACIT-F. FACIT-F, as opposed to FVAS, was used as it was normally distributed as assessed by Q-Q plots. However, 18 of the 270 patients had missing FACIT-F data. Table 6-1 and Table 6-2 show the diagnostics and clinical characteristics of these 252 patients, respectively. Table 6-3 shows the diagnostic outcomes at ≥12 months for patients who at baseline were classified as CSA and UA. Table 6-4 shows the various treatment options used to manage patients with persistent CSA, persistent UA and CSA patients who converted to UA.

**Table 6-1 Diagnostic data at baseline for patients in whom serum metabolites were assessed by NMR spectroscopy to investigate the relationship between serum metabolome and FACIT-F**

**RA**- rheumatoid arthritis; **UA**- unclassified arthritis; **CSA**- clinically suspect arthralgia; **PsA**- psoriatic arthritis

*Seropositive refers to positive for RF or ACPA. Classification criteria applied as outlined in the methods section (see **section 2.1**)

| Diagnosis | Number (percentage) |
|---|---|
| RA<br>Seropositive* RA<br>Seronegative RA | 113 (44.8)<br>69 (27.4)<br>44 (17.4) |
| Unclassified arthritis<br>Seropositive*<br>Seronegative | 39 (15.5)<br>6 (2.4)<br>33 (13.1) |
| CSA<br>Seropositive*<br>Seronegative | 55 (21.8)<br>20 (7.9)<br>35 (13.9) |
| PsA | 29 (11.5) |
| Other arthritis<br>Gout<br>Peripheral spondyloarthopathy<br>Post infectious arthritis<br>Sarcoidosis | 16 (6.3)<br>6 (2.4)<br>5 (2)<br>4 (1.6)<br>1 (0.3) |

**Table 6-2 Demographic characteristics by diagnosis for patients whose serum was used for metabolomics analysis to investigate the relationship between serum metabolome and FACIT-F**

**RA**- rheumatoid arthritis; **UA**- unclassified arthritis; **CSA**- clinically suspect arthralgia; **PsA**- psoriatic arthritis; **IQR**- interquartile range; **FACIT-F**- functional assessment of chronic illness therapy – fatigue; **CRP**- C-reactive protein; **RF**- rheumatoid factor; **ACPA**- Anti-citrullinated protein antibody; **NSAID**- nonsteroidal anti-inflammatory drug

* p value calculated using Kruskal-Wallis test ** p value calculated using chi-squared test

ł therapies at baseline

| | All Cohort (n=252) | RA (n=113) | PsA (n=29) | UA (n=39) | CSA (n=55) |
|---|---|---|---|---|---|
| Age, median (IQR) years | 52 (41-60) | 55 (47-62) | 48 (35.5-58) | 51 (42-60) | 43 (34.5-53.5) |
| Missing (%) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | | p=0.002* | | | |
| Sex, no. (%) females | 168 (66.7) | 79 (69.9) | 15 (51.7) | 25 (64.1) | 40 (72.7) |
| Missing (%) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |

| | | | | | |
|---|---|---|---|---|---|
| | | p=0.223** | | | |
| Symptom duration, median (IQR) weeks | 24 (12-52) | 20.5 (11-47) | 27 (12.5-57) | 21 (12-42) | 32.5 (17.3-103.3) |
| Missing (%) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | | p= 0.072* | | | |
| FACIT-F (IQR) | 32 (21-41) | 29 (17-39) | 32 (21.5-40.5) | 41 (28-44) | 33 (23-42) |
| Missing (%) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | | p=0.014* | | | |
| CRP, median (IQR) mg/L | 6 (3-14) | 8 (3-16.3) | 4 (3-13.3) | 6 (3-21) | 5 (2.5-10) |
| Missing (%) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | | p=0.126* | | | |
| RF positive, no. (%) | 96 (38.1) | 68 (60.2) | 2 (9.1) | 6 (15.4) | 16 (29.1) |
| Missing (%) | 11 (4.4) | 6 (5.3) | 3 (10.3) | 0 (0) | 2 (3.6) |
| | | p<0.001** | | | |
| ACPA positive, no. (%) | 77 (30.6) | 57 (50.4) | 0 (0) | 1 (2.6) | 17 (30.9) |
| Missing (%) | 5 (2.2) | 1 (0.9) | 3 (10.3) | 0 (0) | 1 (1.8) |
| | | p<0.001** | | | |
| NSAIDs, no. (%) | 117 (46.4) | 46 (40.7) | 3 (10.3) | 20 (51.3) | 32 (58.2) |
| Missing (%) ł | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | | p=0.184** | | | |
| Steroids, no. (%) | 41 (16.3) | 7 (6.2) | 1 (3.4) | 12 (30.8) | 8 (14.5) |
| Missing (%) ł | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | | p=0.001** | | | |
| Baseline DMARD | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Missing (%) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| No. (%) who developed RA (at ≥12 months) | -- | -- | -- | 5 (12.8) | 5 (9.1) |
| Missing (%) | | | | 12 (30.8) | 17 (30.9) |

**Table 6-3 Diagnostic outcomes at ≥12 month follow up in CSA & UA patients in whom serum metabolites were assessed by NMR spectroscopy**

The diagnostic outcomes for clinically suspect arthralgia and unclassified arthritis patients in whom serum metabolites were assessed by NMR spectroscopy to investigate the relationship between serum metabolome and FACIT-F

**UA**- unclassified arthritis; **RA**- rheumatoid arthritis; **CSA**- clinically suspect arthralgia

| Baseline diagnosis | ≥12 month follow up diagnosis | | | | |
|---|---|---|---|---|---|
| | No inflammatory arthritis | UA | RA | Other inflammatory arthropathy | Lost to follow up |
| UA (n=41) | -- | 11 | 5 | 13 | 12 |
| CSA (n=57) | 28 | 3 | 5 | 4 | 17 |

**Table 6-4 Treatment usage in CSA & UA patients in whom serum metabolites were assessed by NMR spectroscopy**

Clinically suspect arthralgia and unclassified arthritis patient treatment in whom serum metabolites were assessed by NMR spectroscopy to investigate the relationship between serum metabolome and FACIT-F

**UA-UA-** baseline and follow up diagnosis of unclassified arthritis; **CSA-NIA-** baseline diagnosis of clinically suspect arthralgia and a follow up diagnosis of no inflammatory arthritis; **CSA-UA-** baseline diagnosis of clinically suspect arthralgia and a follow up diagnosis of unclassified arthritis; **HCQ-** hydroxychloroquine; **pred-** prednisolone; **AZA-** azathioprine; **MTX-** methotrexate; **SFZ**- sulfasalazine

| Patients | HCQ alone | HCQ + pred | HCQ + AZA | MTX + HCQ | MTX alone | MTX + pred | MTX + SFZ | MTX + HCQ + SFZ | None |
|---|---|---|---|---|---|---|---|---|---|
| UA-UA | 4 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 5 |
| CSA-NIA | 8 | 1 | 1 | 1 | 2 | 0 | 1 | 1 | 13 |
| CSA-UA | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |

The median FACIT-F value for the entire population was 32 (IQR 21-41). Figure 6-1 shows the distribution of FACIT-F scores across the whole cohort and in the four largest diagnostic subgroups used in serum metabolomics analysis.

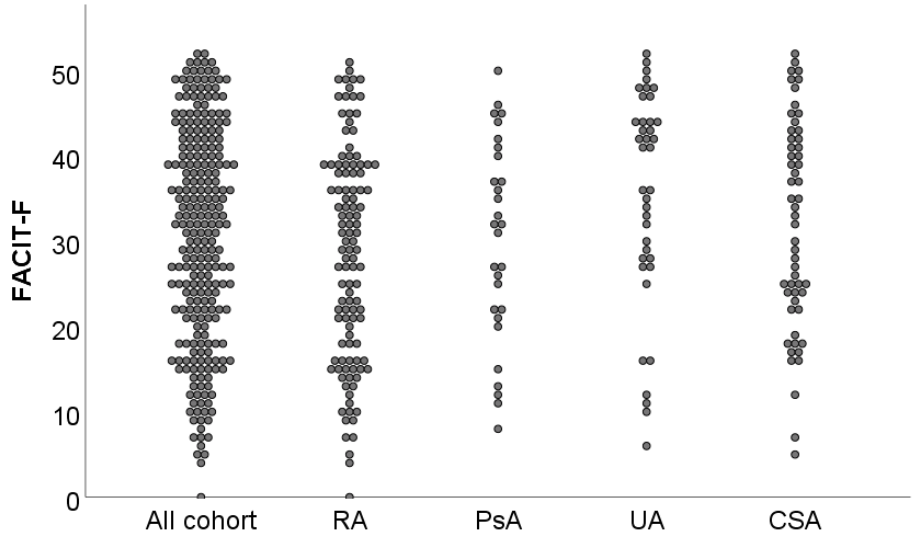



**Figure 6-1 Scatter plot showing the distribution of FACIT-F values across the whole population and diagnostic subgroups used in the serum metabolomics analysis**

### 6.4.2 Relationship between serum metabolite profile and FACIT-F in the whole cohort

A similar approach to investigate the relationship between CRP and metabolism was utilised for investigating the relationship between FACIT-F and serum metabolite profile. A PCA and OPLS-DA and showed no separation between those patients who scored in the highest and lowest FACIT-F tertile (Figure 6-2).

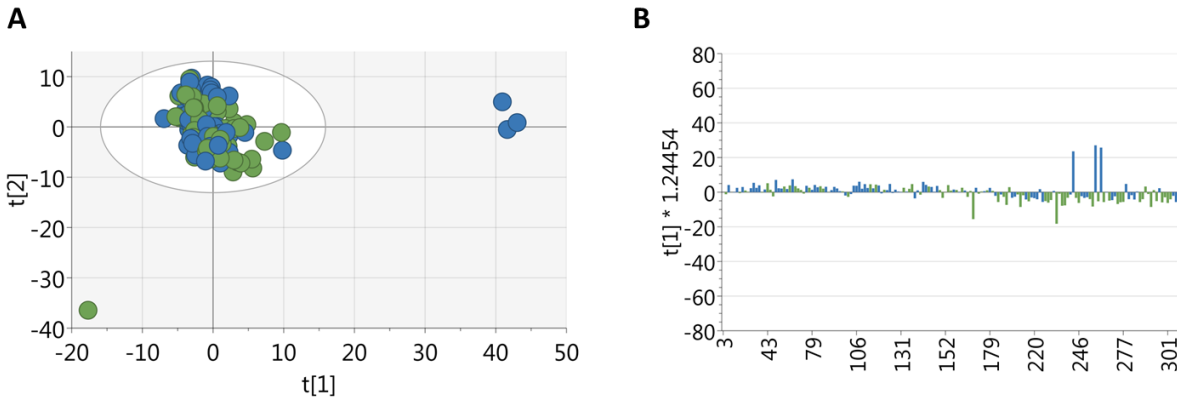



**Figure 6-2 Multivariate analysis of serum metabolite profile by FACIT-F tertiles with data shown for the highest and lowest tertiles**
**(A)** PCA plot of metabolic data derived from all patients' (n=252) sera (green= FACIT-F<25 and blue=FACIT-F>38; 21 PC, $r^2$=0.818) showing no separation between the two groups. **(B)** OPLS-DA plot of metabolic data derived from all patients' (n=252) sera (green= FACIT-F<25 and blue=FACIT-F>38; 1+0+0 LV, P value=1) showing separation between highest FACIT-F tertile and the lowest FACIT-F tertile which is not statistically significant.

To investigate this further, the relationship between serum metabolite profile and FACIT-F was assessed using PLS-R. PLS-R analysis revealed a correlation between serum metabolite profile and

fatigue as measured by FACIT-F with application of forward selection as described in **section 2.3.6.2**. Figure 6-3A shows a post forward selection PLS-R analysis with an $r^2$ value of 0.1809 (7 LV and $p<0.001$) using the serum metabolome. Similarly, Figure 6-3B shows a post forward selection PLS-R analysis with an $r^2$ value of 0.1227 (4 LV and $p<0.001$) using serum metabolome with the inflammation signal removed. Therefore, a correlation between FACIT-F and i) the entire serum metabolite dataset and ii) the serum metabolite dataset devoid of those bins which highly correlate with CRP exists. This indicates that the correlation between fatigue and serum metabolite profile may be independent from the relationship between inflammation (measured by CRP) and metabolism. As outlined in **section 2.3.6.3**, NMR bins were identified as potential biomarkers if their regression coefficient was +/- 2 standard deviations of the mean regression coefficient of the entire dataset (168). The regression coefficient takes into account the effect of the collinear variation from all the other spectral variables on the continuous variable, in this case FACIT-F, in the PLS-R model (302). Thus, a positive regression coefficient has a positive relationship with the FACIT-F which is represented by a ↑ symbol in the regression coefficient column. In contrast, a ↓ symbol represents a negative relationship with FACIT-F. Each NMR bin was translated into a list of metabolites using labelled spectra using Chenomx suite, which is shown in Table 6.4.

As outlined in **section 2.3.6.3**, MetabolAnalyst 4.0 (http://www.metaboanalyst.ca/MetaboAnalyst/) was used to perform pathway analysis and visualization of all chemical metabolites present at different levels in filtered serum and urine samples (303). Enrichment and pathway analysis using over representation analysis algorithms was performed with a pathway-associated metabolite set library based on normal metabolic pathways to identify biologically meaningful patterns. Pathway analyses for these potential biomarkers are shown in Figure 6-4 and Table 6-6 and respectively, whilst enrichment analyses are shown in Figure 6-5 and Table 6-7 respectively.

Table 6-6 shows the pathway analysis with all metabolite pathways which were statistically significant in descending order of impact score. Figure 6-4 shows all matched metabolite pathways arranged by p values on the Y-axis and pathway impact score on the x-axis. The node colour is dependent upon its p value and the node radius is determined on its pathway impact value. Only pathways which were statistically significant ($p<0.05$) were labelled. Over-representation analysis is shown in Figure 6-5 and Table 6-7. The pathway analyses reveal that glycine, serine and threonine, alanine, aspartate and glutamine and arginine and proline metabolism are implicated as the top three most impacted pathways. There is an overlap of the large proportion of the pathways implicated between both pathway analyses. Synthesis and degradation of ketone bodies pathway was the most impacted pathway implicated using the serum metabolome devoid of CRP biomarkers whereas it was not significantly impacted (1/6 metabolites present, impact score 0, $p=0.08847$) using all the serum metabolome biomarkers. Enrichment analyses were similar, with the glucose-alanine cycle the most overrepresented pathway in both enrichment analyses.

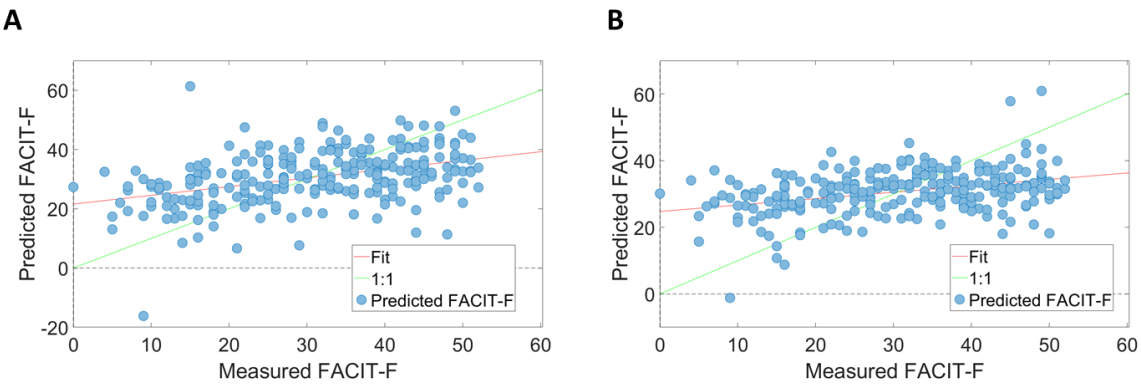



**Figure 6-3 PLS-R analysis of serum metabolites and FACIT-F**

All models shown are post forward selection. PLS-R analyses of metabolic data derived from all patients' (n=252) sera and FACIT-F. There is a correlation between serum metabolite profile and FACIT-F (**A**) using the full 590 NMR bins from the serum metabolite data (149 NMR bins post forward selection, $r^2$=0.1809, 7 LVs, p<0.001). (**B**) A PLS-R analysis of the remaining 507 NMR bins after the 83 NMR bins that correlated with CRP were removed, showed a correlation between FACIT-F and serum metabolite profile (126 NMR bins post forward selection, $r^2$=0.1227, 4 LV, p<0.001).

**Table 6-5 Metabolites responsible for the relationship between FACIT-F and serum metabolite profile in all patients**
Results from the PLS-R models using the complete metabolite dataset and the serum metabolome devoid of the inflammatory signal in PLS-R analysis. The following metabolites have been ranked by the magnitude of the regression coefficient. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of regression coefficient. The regression coefficient field indicates the nature of correlation (↑ indicating a positive relationship with FACIT-F and ↓ indicating a negative relationship with FACIT-F)

| | | | PLS-R model using all serum metabolome | | PLS-R model using serum metabolome devoid of the inflammatory signal | |
|---|---|---|---|---|---|---|
| Order in PLS-R using all serum metabolome | Order in PLS-R using serum metabolome devoid of the inflammatory signal | Metabolite | Chemical shift of peak (ppm) implicated in PLS-R Model | Regression coefficient | Chemical shift of peak (ppm) implicated in PLS-R Model | Regression coefficient |
| 1 | N/A | Formate | 8.4462 | ↓ | N/A | N/A |
| 2 | 1 | Glucose | 2.9094, 3.9213, 3.5818, 3.0257, 3.6287, 3.087, 2.9145, 3.1948, 2.8092, 4.6296, 3.857, 4.2168, 4.0805, 3.6813, 4.2316 | ↑↓↑↓↓↑ ↑↓↓↓↓↓ ↑↓↑ | 3.5818, 3.1948, 2.8628, 2.8092, 4.6296, 3.857, 2.9145, 2.8209, 2.815, 3.087, 4.2316, 2.8315 3.576, 3.3126, 3.0901, 2.9094 | ↑↓↓↓↓ ↓↑↓↓↑ ↑↑↑↑↑ ↑ |
| 2 | 4 | Asparagine | 2.9094, 2.9145 | ↑↑ | 2.8628, 2.8092, 2.9145, 2.8209, 2.815, 2.8315, 2.9094 | ↓↓↑↓↓ ↑↑ |
| 2 | 4 | Methylguanidine | 2.8092, 2.9145, 2.8092 | ↓↑↓ | 2.8628, 2.8092, 2.9145, 2.8209, 2.815, 2.8315, 2.9094 | ↓↓↑↓↓ ↑↑ |
| 3 | N/A | Creatine | 3.9213, 3.0257 | ↓↓ | N/A | N/A |
| 3 | 2 | Tyrosine | 3.9213, 3.0257, 3.087, 3.1948, 7.1463, 7.1291 | ↓↓↑↓↓↓ | 7.1463, 3.1948, 3.087, 3.0901 | ↓↓↑↑ |
| 3 | 3 | 3-Methylhistidine | 3.9213, 3.1948, 7.7781, 3.6813 | ↓↓↓↓ | 3.1948, 7.7781, 3.3126 | ↓↓↑ |
| 3 | 8 | Serine | 3.9213, 3.857 | ↓↓ | 3.857 | ↓ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 3 | 5 | Aspartate | 3.9213, 2.8092, 2.6687 | ↓↓↓ | 2.8092, 2.8209, 2.815, 2.8315 | ↓↓↓↑ |
| 3 | 3 | Betaine | 3.9213, 3.1948, 3.857 | ↓↓↓ | 3.1948, 3.857, 3.3126 | ↓↓↑ |
| 3 | 1 | Glycerol | 3.9213, 3.5818, 3.6287, 3.087, 3.1948, 3.857, 4.0805, 3.6813 | ↓↑↓↑↓↓↑↓ | 3.5818, 3.1948, 3.857, 3.087, 3.576, 3.3126, 3.0901 | ↑↓↓↑↑↑↑ |
| 3 | 12 | Lactate | 3.9213, 4.2168, 4.0805, 1.3633, 1.2585, 1.2633, 1.0697, 4.2316, 1.1994 | ↓↓↑↓↓↓↑↑↑ | 1.2633, 4.2316, 1.0697, 1.2404 | ↓↑↑↑ |
| 4 | 1 | Threonine | 3.5818, 4.2168, 1.3633, 1.2585, 1.2633, 4.2316 | ↑↓↓↓↓↑ | 3.5818, 1.2633, 4.2316, 3.576, 1.2404 | ↑↓↑↑↑ |
| 4 | 1 | Valine | 3.5818, 3.6287, 2.1788, 1.0697, 1.1994 | ↑↓↓↑↑ | 3.5818, 2.2238, 3.576, 1.0697, 0.83029 | ↑↓↑↑ |
| 4 | 1 | Glycine | 3.5818, 3.6287 | ↑↓ | 3.5818, 3.576 | ↑↑ |
| 5 | 10 | Lysine | 3.0257, 3.087, 2.9145, 3.6813, 1.3633, 1.8744, 1.937 | ↓↑↑↓↓↑↑ | 2.9145, 3.087, 1.9613, 3.0901 | ↑↑↑↑ |
| 5 | 11 | Cysteine | 3.0257, 3.087 | ↓↑ | 3.087, 3.0901 | ↑↑ |
| 5 | N/A | Creatinine | 3.0257, 4.0805 | ↓↑ | N/A | N/A |
| 5 | 11 | Ornithine | 3.0257, 3.087, 1.8744, 1.937 | ↓↑↑↑ | 3.087, 1.9613, 3.0902 | ↑↑↑ |
| 6 | 12 | Isoleucine | 3.6287, 3.6813, 1.2585, 1.2633, 1.0697, 1.1994, 1.937 | ↓↓↓↓↑↑↑ | 1.2633, 1.9613, 1.0697, 1.2404 | ↓↑↑↑ |
| 7 | 11 | Phenylalanine | 3.087, 7.375 | ↑↑ | 3.087, 3.3126, 7.375, 3.0901 | ↑↑↑↑ |
| 8 | 3 | Carnitine | 3.1948, 2.417 | ↓↓ | 3.1948, 2.417, 2.4111, 3.3126 | ↓↓↓↑ |
| 8 | 3 | Arginine | 3.1948, 1.8744, 1.937 | ↓↑↑ | 3.1948, 1.9613, 3.3126 | ↓↑↑ |
| 8 | 3 | Cystine | 3.1948, 4.0805 | ↓↑ | 3.1948 | ↓ |
| 8 | 3 | Choline | 3.1948 | ↓ | 3.1948 | ↓ |
| 9 | N/A | Citrate | 2.5106, 2.6687, 2.5165, 2.543 | ↓↓↓↑ | N/A | N/A |
| 9 | 6 | Glutamine | 2.5106, 2.1788, 3.6813, 2.5165, 2.417, 2.543 | ↓↓↓↓↓↑ | 2.417, 2.4111, 2.0716 | ↓↓↓ |
| 9 | 6 | Pyruvate | 2.5106, 2.417 | ↓↓ | 2.417, 2.4111 | ↓↓ |
| 10 | 9 | Propylene glycol | 3.857 | ↓ | 3.857 | ↓ |
| 10 | 7 | Methionine | 3.857, 2.1788, 2.6687 | ↓↓↓ | 2.2238, 3.857, 2,0716, | ↓↓↓ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 11 | 6 | Glutamate | 2.1788, 2.417 | ↓↓ | 2.417, 2.2238, 2.4111, 2.0716, 1.9613 | ↓↓↓↓↑ |
| 11 | 6 | Proline | 2.1788, 4.0805, 2.417, 1.8744, 1.937 | ↓↑↓↑↑ | 2.417, 2.2238, 2.4111, 2.0716, 1.9613, 3.3126 | ↓↓↓↓↑↑ |
| 12 | 14 | Leucine | 3.6813, 1.0697 | ↓↑ | 0.83029 | ↑ |
| 13 | N/A | Alanine | 1.3633 | ↓ | N/A | N/A |
| 14 | 6 | 3-hydroxybutyrate | 2.417, 1.1994 | ↓↑ | 2.417, 2.4111, 1.2404 | ↓↓↑ |
| 15 | N/A | Acetate | 1.8744 | ↑ | N/A | N/A |
| 16 | N/A | Isopropanol | 1.1994 | ↑ | N/A | N/A |
| N/A | 6 | Glutamine | N/A | N/A | 2.417, 2.2238, 2.4111, 2.0716, 1.9613 | ↓↓↓↓↑ |
| N/A | 6 | Proline | N/A | N/A | 2.417, 2.2238, 2.4111, 2.0716, 1.9613, 3.3126 | ↓↓↓↓↑↑ |
| N/A | 7 | Acetone | N/A | N/A | 2.2238 | ↓ |
| N/A | 7 | Acetoacetate | N/A | N/A | 2.2238 | ↓ |
| N/A | 11 | Phenylalanine | N/A | N/A | 3.087, 3.3126, 7.375, 3.0901 | ↑↑↑↑ |
| N/A | 13 | Methanol | N/A | N/A | 3.3126 | ↑ |
| N/A | 14 | 2-hydroxybutyrate | N/A | N/A | 0.83029 | ↑ |

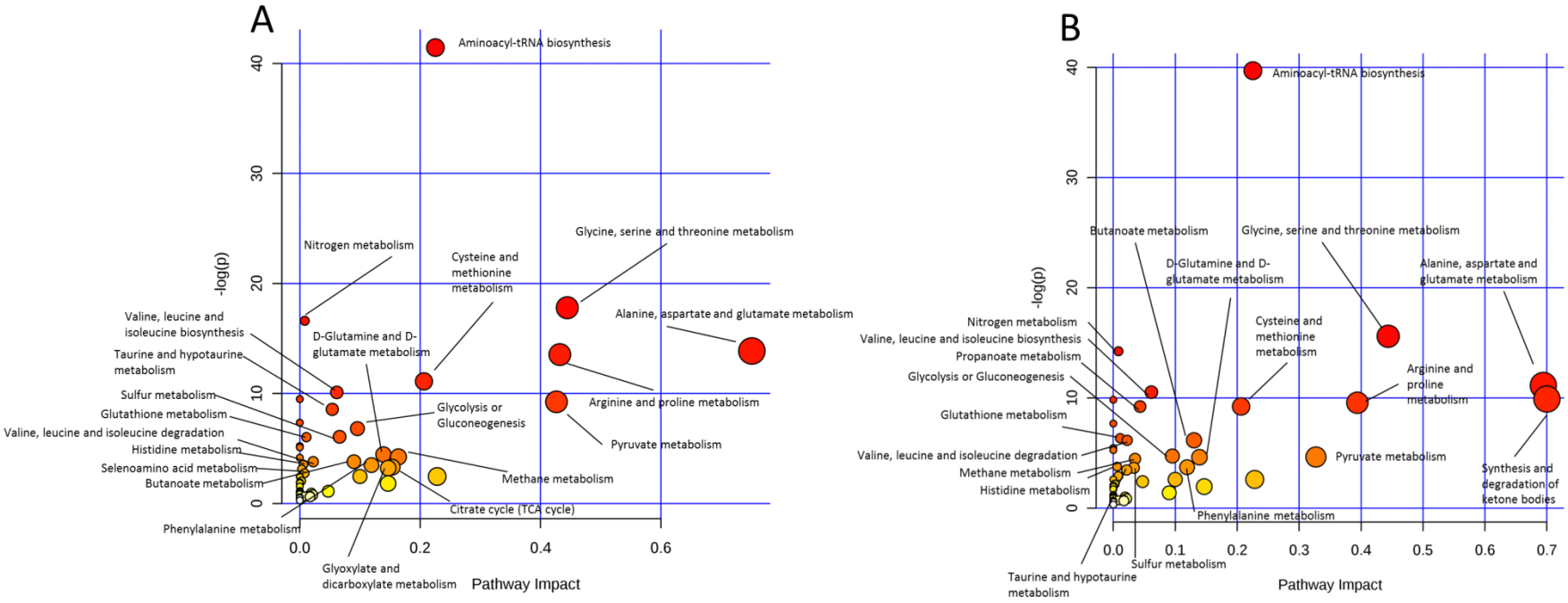



**Figure 6-4 Pathway analysis of potential biomarkers implicated by PLS-R analysis of FACIT-F and all patients' serum metabolites**
Pathway analysis of potential biomarkers implicated by the PLS-R of (A) all the serum metabolome and (B) the serum metabolome devoid of the inflammatory signal

**Table 6-6 Pathway analysis of potential biomarkers implicated by PLS-R analysis of FACIT-F and all patients' serum metabolites**
Results from PLS-R models using all the serum metabolome and the serum metabolome devoid of the inflammatory signal.
**Match status:** The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.
**The impact score:** Each pathway has a total importance score of 1. The importance measure of each metabolite node (a point where pathways intersect within a larger network) is expressed as a fraction with regards to the total pathway importance, and the pathway impact score is the cumulative score of matched metabolite nodes.
* p value calculated using hypergeometric test

| | PLS-R model using all serum metabolome | | | PLS-R model using serum metabolome devoid of the inflammatory signal | | |
|---|---|---|---|---|---|---|
| Pathway name | Match status | P value* | Impact score | Match status | P value* | Impact score |
| Alanine, aspartate and glutamate metabolism | 6/24 | 9.54E-07 | 0.75119 | 5/24 | 1.46E-05 | 0.69421 |
| Glycine, serine and threonine metabolism | 9/48 | 1.87E-08 | 0.44447 | 8/48 | 1.68E-07 | 0.44376 |
| Arginine and proline metabolism | 9/77 | 1.32E-06 | 0.43209 | 7/77 | 7.00E-05 | 0.39401 |
| Pyruvate metabolism | 5/32 | 9.68E-05 | 0.42654 | 3/32 | 0.009659 | 0.32707 |
| Aminoacyl-tRNA biosynthesis | 18/75 | 1.01E-18 | 0.22536 | 17/75 | 5.75E-18 | 0.22536 |
| Cysteine and methionine metabolism | 7/56 | 1.51E-05 | 0.20626 | 6/56 | 9.83E-05 | 0.20626 |
| Methane metabolism | 3/34 | 0.014421 | 0.16384 | 3/34 | 0.011431 | 0.03502 |
| Citrate cycle | 2/20 | 0.036718 | 0.15351 | 1/20 | 2.48E-01 | 0.09024 |
| Glyoxylate and dicarboxylate metabolism | 3/50 | 0.03994 | 0.14685 | 1/50 | 0.51262 | 0 |
| D-Glutamine and D-glutamate metabolism | 11/19 | 0.011591 | 0.13904 | 2/11 | 0.009837 | 0.13904 |
| Phenylalanine metabolism | 3/45 | 0.030467 | 0.11906 | 3/45 | 0.024392 | 0.11906 |
| Glycolysis or Gluconeogenesis | 4/31 | 0.001106 | 0.09576 | 3/31 | 0.008838 | 0.0953 |
| Butanoate metabolism | 3/40 | 0.022355 | 0.08996 | 4/40 | 0.002118 | 0.13026 |
| Sulfur metabolism | 3/18 | 0.002329 | 0.06614 | 2/18 | 0.025727 | 0.03307 |
| Valine, leucine and isoleucine biosynthesis | 5/27 | 4.11E-05 | 0.06148 | 5/27 | 2.68E-05 | 0.06148 |
| Taurine and hypotaurine metabolism | 4/20 | 1.92E-04 | 0.05395 | 2/20 | 0.031392 | 0.02158 |
| Valine, leucine and isoleucine degradation | 3/40 | 0.022355 | 0.02232 | 4/40 | 0.002118 | 0.02232 |
| Glutathione metabolism | 4/38 | 0.002402 | 0.01095 | 4/38 | 0.001745 | 0.01095 |
| Nitrogen metabolism | 8/39 | 6.15E-08 | 0.0083 | 7/39 | 6.54E-07 | 0.0083 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Histidine metabolism | 3/44 | 0.028735 | 0.00611 | 3/44 | 0.022985 | 0.00611 |
| Selenoamino acid metabolism | 2/22 | 0.043795 | 0.00321 | -- | -- | -- |
| Synthesis and degradation of ketone bodies | 1/6 | 0.08847 | 0 | 3/6 | 5.01E-05 | 0.7 |
| Propanoate metabolism | 3/35 | 0.015608 | 0 | 5/35 | 9.94E-05 | 0.04317 |

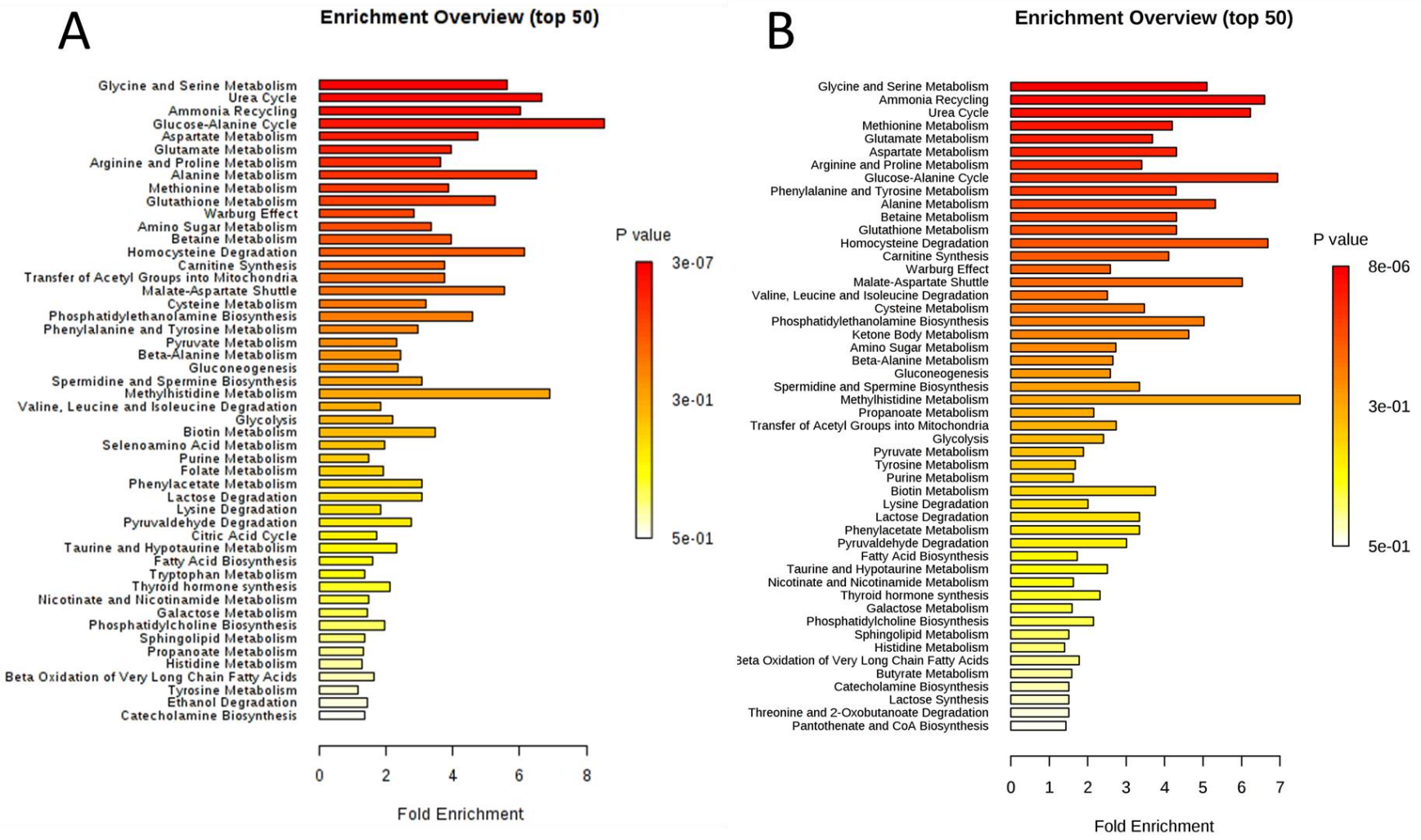



**Figure 6-5 Enrichment analysis of key metabolites in serum implicated as potential biomarkers by the PLS-R analysis of FACIT-F.** Enrichment analysis of potential biomarkers implicated by the PLS-R of (A) all the serum metabolome and (B) the serum metabolome devoid of the inflammatory signal

**Table 6-7 Enrichment analysis of key metabolites in serum implicated as potential biomarkers by the PLS-R analysis of FACIT-F and all patients' serum metabolites**

Results from PLS-R models using all the serum metabolome and the serum metabolome devoid of the inflammatory signal.

**Match status:** The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.

**The fold enrichment value:** the value is generated by the number of matched metabolites divided by the number of metabolites expected to be present in the pathway as per the reference metabolome provided by KEGG.

* p value calculated using hypergeometric test

| | PLS-R model using all serum metabolome | | | | PLS-R model using serum metabolome devoid of the inflammatory signal | | | |
|---|---|---|---|---|---|---|---|---|
| Metabolite pathway | Match status | Fold enrichment | Expected | P value* | Match status | Fold enrichment | Expected | P value* |
| Glycine and Serine Metabolism | 12/59 | 5.63 | 2.13 | 2.64E-07 | 10/59 | 5.10 | 1.96 | 8.33E-06 |
| Urea Cycle | 7/29 | 6.67 | 1.05 | 3.93E-05 | 6/29 | 6.23 | 0.963 | 2.33E-04 |
| Ammonia Recycling | 7/32 | 6.03 | 1.16 | 7.83E-05 | 7/32 | 6.60 | 1.06 | 4.37E-05 |
| Glucose-Alanine Cycle | 4/13 | 8.51 | 0.47 | 8.19E-04 | 3/13 | 6.94 | 0.432 | 0.00762 |
| Aspartate Metabolism | 6/35 | 4.76 | 1.26 | 0.00111 | 5/35 | 4.31 | 1.16 | 0.00473 |
| Glutamate Metabolism | 7/49 | 3.95 | 1.77 | 0.00127 | 6/49 | 3.68 | 1.63 | 0.00424 |
| Arginine and Proline Metabolism | 7/53 | 3.65 | 1.92 | 0.00205 | 6/53 | 3.41 | 1.76 | 0.00633 |
| Alanine Metabolism | 4/17 | 6.51 | 0.614 | 0.00246 | 3/17 | 5.32 | 0.564 | 0.0165 |
| Methionine Metabolism | 6/43 | 3.87 | 1.55 | 0.00336 | 6/43 | 4.20 | 1.43 | 0.00214 |
| Glutathione Metabolism | 4/21 | 5.27 | 0.759 | 0.00556 | 3/21 | 4.30 | 0.697 | 0.0296 |
| Warburg Effect | 6/58 | 2.86 | 2.1 | 0.015 | 5/58 | 2.59 | 1.93 | 0.0384 |
| Amino Sugar Metabolism | 4/33 | 3.36 | 1.19 | 0.0279 | 3/33 | 2.52 | 1.19 | 0.0992 |
| Betaine Metabolism | 3/21 | 3.95 | 0.759 | 0.0369 | 3/21 | 4.30 | 0.697 | 0.0296 |
| Homocysteine Degradation | 2/9 | 6.15 | 0.325 | 0.039 | 2/9 | 6.69 | 0.299 | 0.0333 |
| Carnitine Synthesis | 3/22 | 3.77 | 0.795 | 0.0417 | 3/22 | 4.11 | 0.73 | 0.0335 |
| Transfer of Acetyl Groups into Mitochondria | 3/22 | 3.77 | 0.795 | 0.0417 | 2/22 | 2.52 | 0.795 | 0.164 |
| Malate-Aspartate Shuttle | 2/10 | 5.54 | 0.361 | 0.0476 | 2/10 | 6.02 | 0.332 | 0.0408 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Phenylalanine and Tyrosine Metabolism | 3/28 | 2.97 | 1.01 | 0.0764 | 4/28 | 4.30 | 0.93 | 0.0118 |
| Valine, Leucine and Isoleucine Degradation | 4/60 | 1.84 | 2.17 | 0.166 | 5/60 | 2.51 | 1.99 | 0.0436 |

### 6.4.3 Relationship between serum metabolite profile and FACIT-F in RA patients

PCA and OPLS-DA were undertaken for RA (Figure 6-6A), PsA (Figure 6-6B), UA (Figure 6-6C) and CSA (Figure 6-6D) which showed no statistically significant separation between those patients whose FACIT-F score was in the lowest tertile and those who score was in the highest tertile.

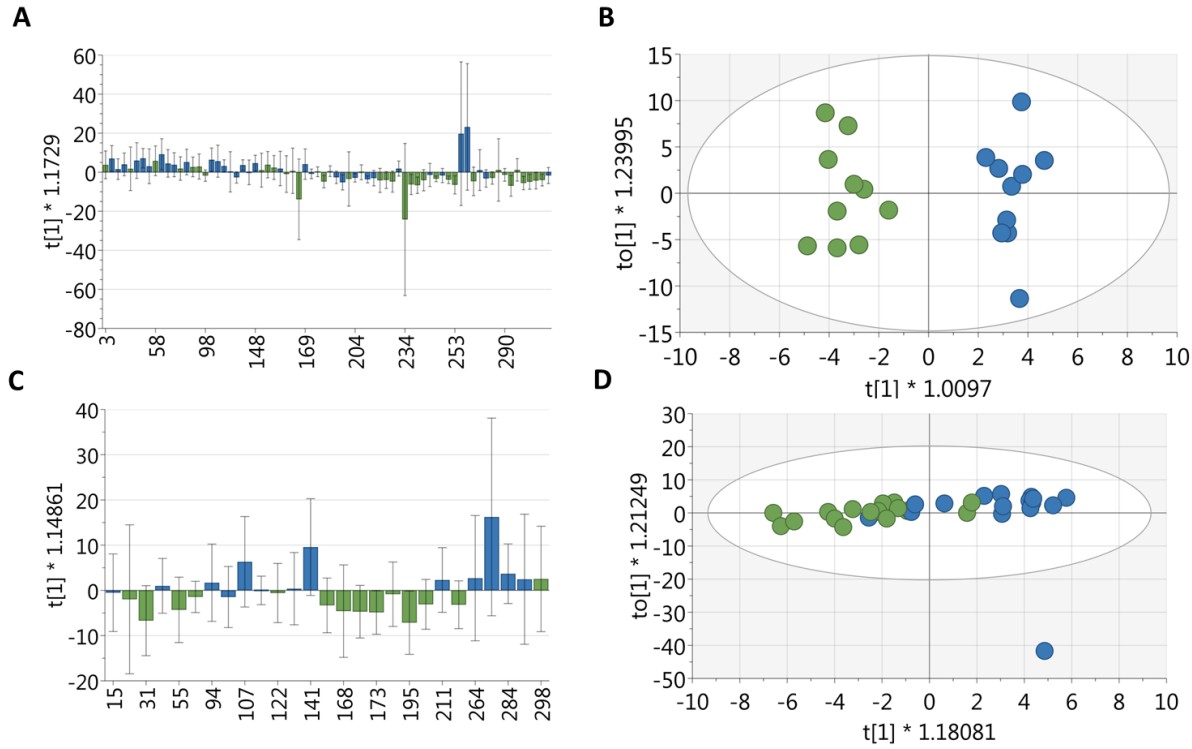



**Figure 6-6 Multivariate analysis of serum metabolite profile by FACIT-F tertiles with data shown for the highest and lowest tertiles by diagnosis**
Across all diagnoses OPLS-DA showed no statistically significant relationship between patients in the highest FACIT-F tertile compared to patients in the lowest FACIT-F tertile. **(A)** OPLS-DA plot of metabolic data derived from RA patients' (n=127) sera (green= FACIT-F<22 and blue=FACIT-F>36; 1+0+0 LV P value= 0.26267). **(B)** OPLS-DA plot of metabolic data derived from PsA patients' (n=29) sera (green= FACIT-F<26 and blue= FACIT-F>37; 1+3+0 LV P value= 0.62129). **(C)** OPLS-DA plot of metabolic data derived from UA patients' (n=42) sera (green= FACIT-F<33 and blue= FACIT-F>43; 1+0+0 LV P value= 1). **(D)** OPLS-DA plot of metabolic data derived from CSA patients' (n=53) sera CSA (green= FACIT-F<25 and blue= FACIT-F>39; 1+1+0 LV P value=1).

Figure 6-7 shows PLS-R analyses with forward selection by diagnosis. For each diagnosis there are two PLS-R analyses, one using the entire 590 bins (sub labelled 1) and the other using 507 bins (sub labelled 2) with the 83 bins which strongly correlate to CRP removed. Each PLS-R analysis has utilised forward selection.

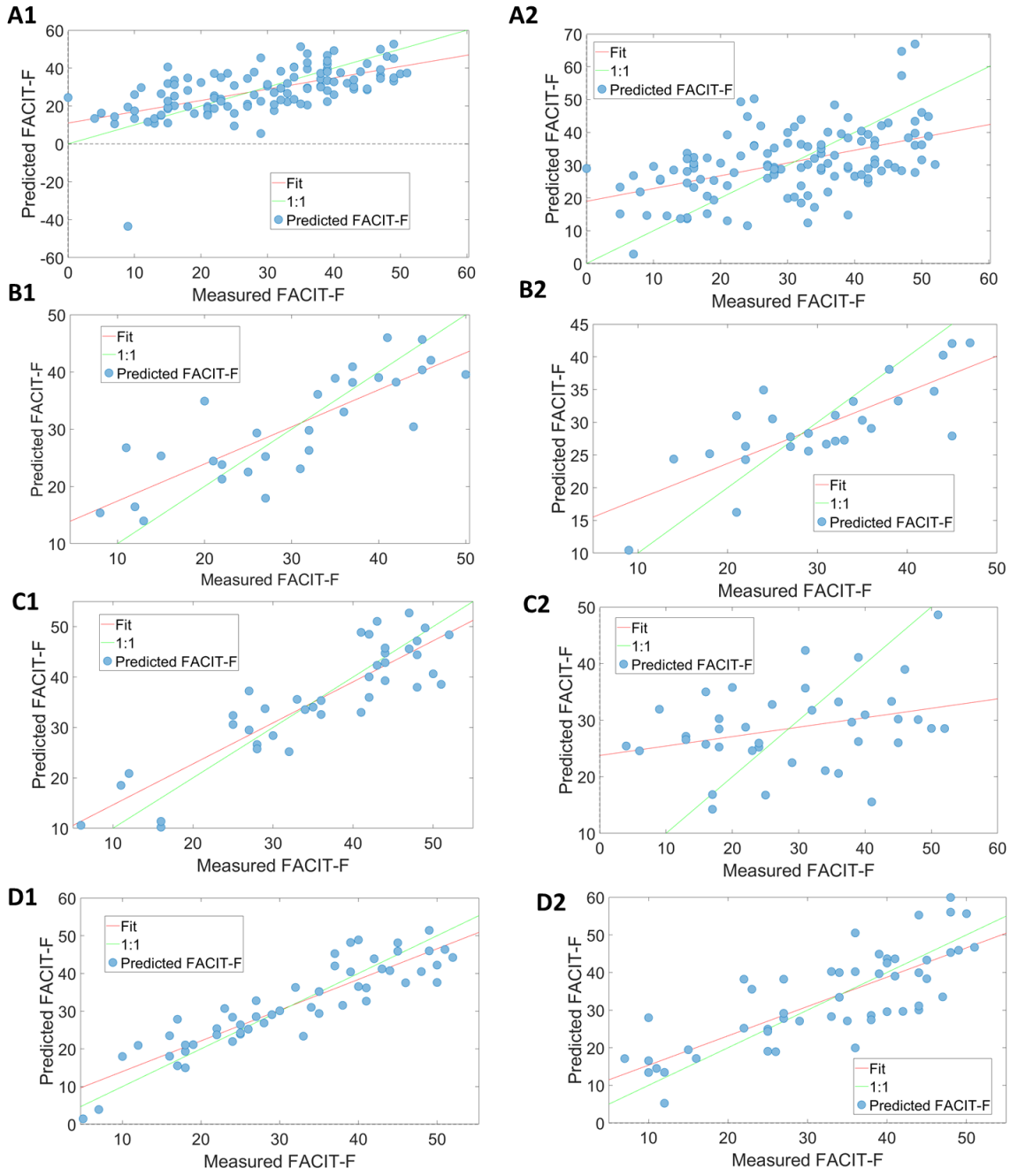



**Figure 6-7 PLS-R analysis of serum metabolites and FACIT-F by diagnosis**

All models shown are post forward selection. All figures sub labelled "1" show PLS-R analyses using the full 590 NMR bins of serum metabolite data before forward selection. All figures sub labelled "2" show PLS-R analyses using 507 NMR bins of serum metabolite data before forward selection (83 bins that correlated with CRP removed). PLS-R showed a correlation between serum metabolite profile and FACIT-F across all diagnosis using the full serum metabolite profile. A correlation between FACIT-F and serum metabolite profile with the CRP signal removed existed for RA and PsA patients. (**A1)** PLS-R of RA patients (n=115) showed a correlation between FACIT-F and the serum metabolite profile (147 NMR bins post forward selection, $r^2$=0.3835, 10 LV, p<0.001) and (**A2)** serum metabolite profile with CRP signal removed (47 NMR bins post forward selection, $r^2$=0.2173, 8 LV, p=0.003). (**B1)** PLS-R of PsA patients (n=29) showed a correlation between FACIT-F and the serum metabolite profile (98 NMR bins post forward selection, $r^2$=0.6219, 10 LV,

p=0.004) and (**B2)** the serum metabolite profile with CRP signal removed (170 NMR bins post forward selection, $r^2$=0.5282, 10 LV, p=0.018). (**C1)** PLS-R of UA patients (n=42) showed a correlation between FACIT-F and the serum metabolite profile (19 NMR bins post forward selection, $r^2$=0.7307, 6 LV, p<0.001). **(C2)** This relationship did not persist with the serum metabolite profile with CRP signal removed (11 NMR bins post forward selection, $r^2$=0.1012, 2 LV, p=0.231). (**D1)** PLS-R of CSA patients (n=53) showed a correlation between FACIT-F and the serum metabolite profile (48 NMR bins post forward selection, $r^2$=0.2831, 8 LV, p=0.017) and (**D2)** serum metabolite profile with CRP signal removed (67 NMR bins post forward selection, $r^2$=0.5967, 10 LV, p<0.001).

The relationship between serum metabolome and FACIT-F was investigated further with PLS-R. For RA patients, there is a statistically significant correlation between FACIT-F and the complete serum metabolite dataset (Figure 6-7A1- 147 NMR bins, $r^2$=0.3835, 10 LV, p<0.001). A relationship was also seen between the serum metabolite dataset devoid of the NMR bins that correlate strongly with CRP and FACIT-F in RA patients (Figure 6-7A2- 47 NMR bins, $r^2$=0.2173, 8 LV, p=0.003). Table 6-8 shows potential biomarkers for these analyses. Pathway analyses for these potential biomarkers are shown in Figure 6-8 and Table 6-9 respectively, whilst enrichment analyses are shown in Figure 6-9 and Table 6-10 respectively.

Pathway and enrichment analyses for the biomarkers implicated in the PLS-R analysis between FACIT-F and RA patients' serum metabolome and the serum metabolome without the bins that strongly correlate with CRP are shown in Figure 6-8, Table 6-9 and Figure 6-9, Table 6-10 respectively. The large proportion of the same metabolite pathways was present in both pathway and enrichment analyses. Ketone body metabolism and alanine, aspartate and glutamate metabolism were the two most impacted metabolic pathways in both pathway analyses. Pyruvate metabolism had greater impact using the biomarkers of serum metabolome devoid of the CRP signal. Methylhistidine metabolism and the glucose alanine cycle were the most overrepresented pathways in the enrichment analyses.

**Table 6-8 Metabolites responsible for the relationship between FACIT-F and serum metabolite profile in RA patients**

Results from PLS-R models using the complete metabolite dataset and the serum metabolome devoid of the inflammatory signal in PLS-R analysis. The following metabolites have been ranked by the magnitude of the regression coefficient. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of regression coefficient. The regression coefficient field indicates the nature of correlation (↑ indicating a positive relationship with FACIT-F and ↓ indicating a negative relationship with FACIT-F)

| | | | PLS-R model using all serum metabolome | | PLS-R model using serum metabolome devoid of the inflammatory signal | |
|---|---|---|---|---|---|---|
| Order in PLS-R using all serum metabolome | Order in PLS-R using serum metabolome devoid of the inflammatory signal | Metabolite | Chemical shift of peak (ppm) implicated in PLS-R Model | Regression coefficient | Chemical shift of peak (ppm) implicated in PLS-R Model | Regression coefficient |
| 1 | 9 | Methionine | 2.1788, 2.6687, 2.1803, 2.2141, 2.6453, 2.2238 | ↓↓↓↑↓↓ | 2.1594 | ↑ |
| 1 | 6 | Glutamine | 2.1788, 2.1803, 2.2141, 2.2238, 2.376 | ↓↓↑↓↓ | 1.9523, 1.9663, 1.9613, 2.4111, 2.5242, 2.1594, 2.543 | ↓↓↓↓↑↑↑ |
| 1 | 7 | Glutamate | 2.1788, 2.1803, 2.376 | ↓↓↓ | 2.4111, 2.1594 | ↓↑ |
| 1 | 5 | Valine | 2.1788, 1.1702, 2.1803, 2.2141, 1.1292, 0.90676, 2.2238, 1.1643, 1.1585, 1.176 | ↓↑↓↑↓↑↓↑↑↑ | 3.617, 3.6228, 3.576 | ↓↓↑ |
| 1 | 6 | Proline | 2.1788, 3.3126, 2.1803, 2.2141, 2.2238, 4.0805, 3.2946, 1.9136, 2.376, 4.0852, 3.3025 | ↓↑↓↑↓↑↑↓↓↑↑ | 1.9523, 1.9663, 1.9613, 2.4111, 2.1594, 1.937, 3.4355 | ↓↓↓↓↑↑↑ |
| 2 | 10 | Lactate | 1.1702, 1.3516, 1.1292, 4.2168, 1.1643, 4.0805, 1.1585, 4.0401, 4.0852, 1.176, 4.0033 | ↑↓↓↓↑↑↑↓↑↑↑ | 1.2931 | ↑ |
| 2 | N/A | Isopropanol | 1.1702, 1.1292, 1.1643, 1.1585, 4.0401, 1.176, 4.0033 | ↑↓↑↑↓↑↑ | N/A | N/A |

| | | | | | | |
|---|---|---|---|---|---|---|
| 3 | 8 | Citrate | 2.6687, 2.6453, 2.6745, 2.7016 | ↓↓↓↓ | 2.5242, 2.543 | ↑↑ |
| 3 | 4 | Aspartate | 2.6687, 2.6453, 2.6745, 2.8315 | ↓↓↓↑ | 2.8315 | ↑ |
| 4 | 2 | Glucose | 3.3126, 3.2023, 4.2168, 4.0805, 3.087, 3.2946, 2.7016, 4.0401, 2.9295, 4.0852, 2.8315, 5.2106, 3.3025, 2.9094, 2.903, 4.0033, 5.2443 | ↑↓↓↑↑↑ ↓↓↑↑↑↑ ↑↑↑↑↑ | 2.8628, 3.1779, 2.8315, 3.617, 2.9197, 3.1611, 2.9262, 3.6228, 2.9145, 3.4355, 3.576 | ↓↑↑↓↓ ↓↓↓↑↑ ↑ |
| 4 | N/A | Methanol | 3.3126, 3.2946, 3.3025 | ↑↑↑ | N/A | N/A |
| 4 | 3 | Carnitine | 3.3126, 3.2023, 3.2946, 2.376, 3.3025 | ↑↓↑↓↑ | 3.1779, 3.1611, 2.4111, 3.4355 | ↑↓↓↑ |
| 4 | 3 | Glycerol | 3.3126, 3.2023, 4.0805, 3.087, 3.2946, 4.0401, 4.0852, 3.3025, 4.0033 | ↑↓↑↑↑↓ ↑↑↑ | 3.1779, 3.617, 3.1611, 3.6228, 3.4355, 3.576 | ↑↓↓↓↑ ↑ |
| 4 | 3 | Betaine | 3.3126, 3.2023, 3.2946, 3.3025, 3.3025 | ↑↓↑↑↑ | 3.1779 | ↑ |
| 4 | 1 | 3-Methylhistidine | 3.3126, 3.2023, 7.7869, 3.2946, 3.3025 | ↑↓↓↑↑ | 7.0647, 3.1779, 3.1611, 7.0687, 7.053 | ↓↑↓↓↑ |
| 4 | 3 | Arginine | 3.3126, 3.2023, 3.2946, 1.9136, 3.3025 | ↑↓↑↓↑ | 3.1779, 1.9523, 1.9663, 3.1611, 1.9613, 1.937 | ↑↓↓↓↓ ↑ |
| 4 | N/A | Tryptophan | 3.3126, 3.2946, 4.0401, 3.3025 | ↑↑↓↑ | N/A | N/A |
| 4 | 3 | Phenylalanine | 3.3126, 3.087, 3.2946, 7.375, 3.3025, 4.0033 | ↑↑↑↑↑↑ | 3.1779, 3.1611, 7.375 | ↑↓↑ |
| 5 | 5 | Threonine | 1.3516, 4.2168 | ↓↓ | 3.617, 1.2931, 3.576 | ↓↑↑ |
| 5 | N/A | Alanine | 1.3516 | ↓ | N/A | N/A |
| 5 | 6 | Lysine | 1.3516, 3.087, 1.9136, 2.9295 | ↓↑↓↑ | 1.9523, 2.9197, 1.9663, 1.9613, 2.9262, 2.9145, 1.937 | ↓↓↓↓↓ ↑ |
| 6 | 3 | Tyrosine | 3.2023, 3.087, 6.9061 | ↓↑↑ | 3.1779, 3.1611 | ↑↓ |
| 6 | 3 | Cystine | 3.2023, 4.0805, 4.0852 | ↓↑↑ | 3.1779, 3.1611 | ↑↓ |
| 6 | 1 | Histidine | 3.2023 | ↓ | 7.0647, 3.1611, 7.0687, 7.053 | ↓↓↓↑ |
| 6 | 3 | Choline | 3.2023, 4.0401 | ↓↓ | 3.1779, 3.1611 | ↑↓ |
| 7 | N/A | Acetone | 2.2141, 2.2238 | ↑↓ | N/A | N/A |
| 8 | 11 | Propylene glycol | 1.1292 | ↓ | 3.4355 | ↑ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 9 | N/A | 2-hydroxybutyrate | 0.90676, 4.0033 | ↑↑ | N/A | N/A |
| 9 | 6 | Isoleucine | 0.90676, 1.9136, 1.176 | ↑↓↑ | 1.9523, 1.9663, 1.9613, 3.6228, 1.2931, 1.937 | ↓↓↓↓↑↑ |
| 9 | N/A | Leucine | 0.90676 | ↑ | N/A | N/A |
| 10 | 11 | Acetoacetate | 2.2238 | ↓ | 3.4355 | ↑ |
| 11 | N/A | Creatinine | 4.0805, 4.0401, 4.0852, 4.0033 | ↑↓↑↑ | N/A | N/A |
| 12 | N/A | Cysteine | 3.087 | ↑ | N/A | N/A |
| 12 | 6 | Ornithine | 3.087, 1.9136 | ↑↓ | 1.9523, 1.9663, 1.9613, 1.937 | ↓↓↓↑ |
| 13 | N/A | Adenine | 8.1795 | ↑ | N/A | N/A |
| 13 | N/A | Hypoxanthine | 8.1795 | ↑ | N/A | N/A |
| 14 | 2 | Methylguanidine | 2.7016, 2.9295, 2.8315, 2.9094, 2.903 | ↓↑↑↑↑ | 2.8628, 2.8315, 2.9197, 2.9262, 2.9145 | ↓↑↓↓↑ |
| 15 | 6 | Acetate | 1.9136 | ↓ | 1.9523, 1.937 | ↓↑ |
| 16 | N/A | Succinate | 2.376 | ↓ | N/A | N/A |
| 17 | N/A | Serine | 4.0401, 4.0033 | ↓↑ | N/A | N/A |
| 18 | 2 | Asparagine | 2.9295, 2.8315, 2.9094, 2.903, 4.0033 | ↑↑↑↑↑ | 2.8628, 2.8315, 2.9197, 2.9262, 2.9145 | ↓↑↓↓↑ |
| 19 | 7 | 3-hydroxybutyrate | 1.176 | ↑ | 2.4111 | ↓ |
| N/A | 5 | Glycine | N/A | N/A | 3.617, 3.6228, 3.576 | ↓↓↑ |
| N/A | 7 | Pyruvate | N/A | N/A | 2.4111 | ↓ |

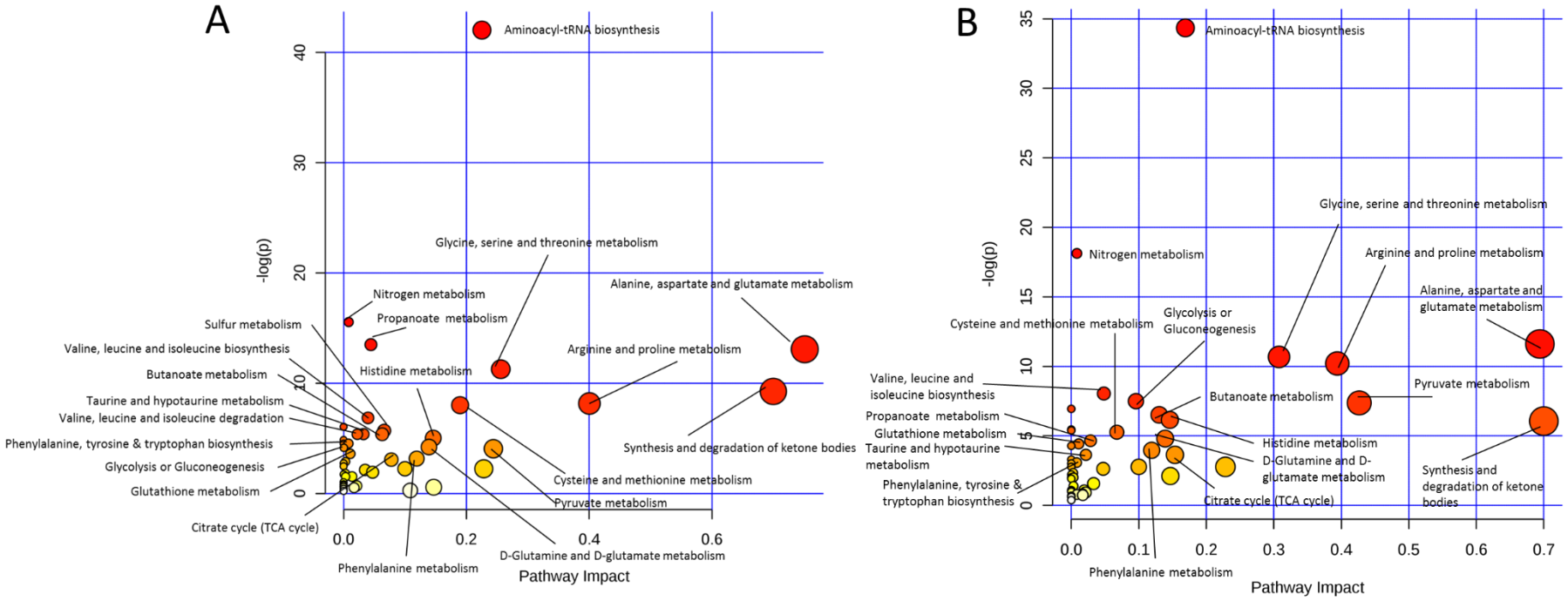



**Figure 6-8 Pathway analysis of potential biomarkers implicated by PLS-R analysis of FACIT-F and RA patients' serum metabolites**
Pathway analysis of potential biomarkers implicated by the PLS-R of (A) all the serum metabolome and (B) the serum metabolome devoid of the inflammatory signal.

**Table 6-9 Pathway analysis of potential biomarkers implicated by PLS-R analysis of FACIT-F and RA patients' serum metabolites**
Results from PLS-R models using all the serum metabolome and the serum metabolome devoid of the inflammatory signal
**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.
**The impact score**: Each pathway has a total importance score of 1. The importance measure of each metabolite node (a point where pathways intersect within a larger network) is expressed as a fraction with regards to the total pathway importance, and the pathway impact score is the cumulative score of matched metabolite nodes.
* p value calculated using hypergeometric test

| | PLS-R model using all serum metabolome | | | PLS-R model using serum metabolome devoid of the inflammatory signal | | |
|---|---|---|---|---|---|---|
| Pathway Name | Match status | P value* | Impact score | Match status | P value* | Impact score |
| Alanine, aspartate and glutamate metabolism | 6/24 | 2.08E-06 | 0.75119 | 5/24 | 9.07E-06 | 0.69421 |
| Synthesis and degradation of ketone bodies | 3/6 | 9.53E-05 | 0.7 | 2/6 | 0.002332 | 0.7 |
| Arginine and proline metabolism | 7/77 | 2.85E-04 | 0.40046 | 7/77 | 3.69E-05 | 0.39401 |
| Glycine, serine and threonine metabolism | 7/48 | 1.27E-05 | 0.25602 | 6/48 | 2.31E-05 | 0.30772 |
| Pyruvate metabolism | 3/32 | 0.017244 | 0.244 | 4/32 | 6.30E-04 | 0.42654 |
| Aminoacyl-tRNA biosynthesis | 19/75 | 5.62E-19 | 0.22536 | 15/75 | 1.21E-15 | 0.16902 |
| Cysteine and methionine metabolism | 6/56 | 3.32E-04 | 0.18977 | 4/56 | 0.005183 | 0.06744 |
| Histidine metabolism | 4/44 | 0.006559 | 0.14599 | 4/44 | 0.002134 | 0.14599 |
| D-Glutamine and D-glutamate metabolism | 2/11 | 0.014799 | 0.13904 | 2/11 | 0.008215 | 0.13904 |
| Phenylalanine metabolism | 3/45 | 0.042205 | 0.11906 | 3/45 | 0.019049 | 0.11906 |
| Citrate cycle | 2/20 | 0.046311 | 0.07773 | 2/20 | 0.026409 | 0.15351 |
| Sulfur metabolism | 3/18 | 0.003361 | 0.06614 | 1/18 | 0.20877 | 0.03307 |
| Butanoate metabolism | 4/40 | 0.004644 | 0.06284 | 4/40 | 0.00149 | 0.13026 |
| Propanoate metabolism | 7/35 | 1.37E-06 | 0.04451 | 3/35 | 0.009583 | 0.02848 |
| Valine, leucine and isoleucine biosynthesis | 4/27 | 0.001051 | 0.03975 | 4/27 | 3.22E-04 | 0.04823 |
| Taurine and hypotaurine metabolism | 3/20 | 0.004583 | 0.03237 | 2/20 | 0.026409 | 0.02158 |
| Valine, leucine and isoleucine degradation | 4/40 | 0.004644 | 0.02232 | 3/40 | 0.013851 | 0 |
| Glutathione metabolism | 3/38 | 0.027286 | 0.01095 | 3/38 | 0.012034 | 0.01095 |
| Nitrogen metabolism | 8/39 | 1.77E-07 | 0.0083 | 8/39 | 1.35E-08 | 0.0083 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Phenylalanine, tyrosine and tryptophan biosynthesis | 3/27 | 0.010802 | 0.008 | 2/27 | 0.046148 | 0.008 |
| Glycolysis or Gluconeogenesis | 3/31 | 0.015817 | 4.60E-04 | 4/31 | 5.56E-04 | 0.09576 |

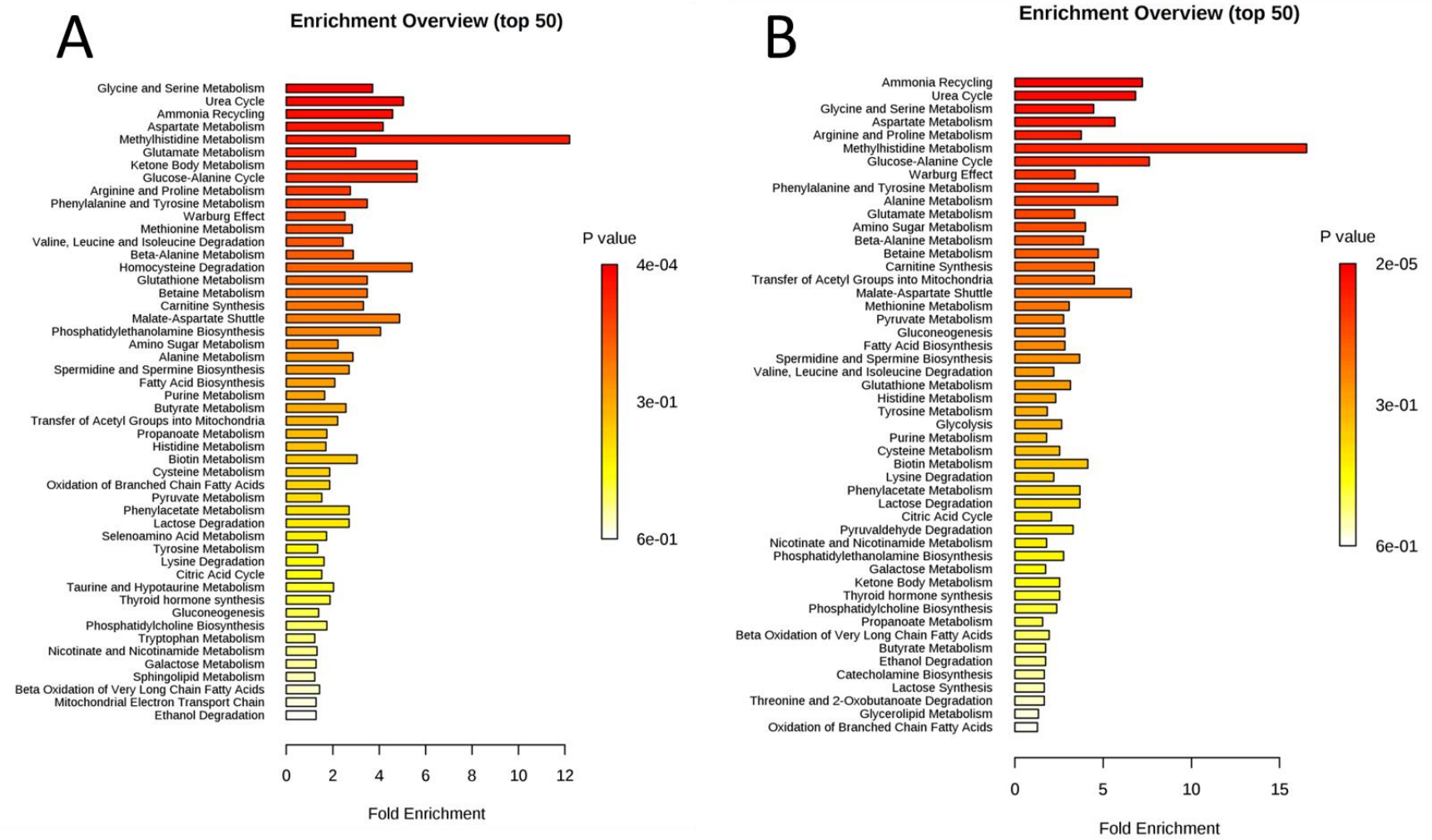



**Figure 6-9 Enrichment analysis of key metabolites in serum implicated as potential biomarkers by the PLS-R analysis of FACIT-F and RA patients'**

Enrichment analysis of potential biomarkers implicated by the PLS-R of (A) all the serum metabolome and (B) the serum metabolome devoid of the inflammatory signal

**Table 6-10 Enrichment analysis of key metabolites in serum implicated as potential biomarkers by the PLS-R analysis of FACIT-F and RA patients' serum metabolites**

Results from PLS-R models using all the serum metabolome and the serum metabolome devoid of the inflammatory signal

**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.

**The fold enrichment value**: the value is generated by the number of matched metabolites divided by the number of metabolites expected to be present in the pathway as per the reference metabolome provided by KEGG.

* p value calculated using hypergeometric test

| | PLS-R model using all serum metabolome | | | | PLS-R model using serum metabolome devoid of the inflammatory signal | | | |
|---|---|---|---|---|---|---|---|---|
| Metabolite pathway | Match status | Fold enrichment | Expected | P value* | Match status | Fold enrichment | Expected | P value* |
| Glycine and Serine Metabolism | 9/59 | 3.72 | 2.42 | 3.77E-04 | 8/59 | 4.47 | 1.79 | 2.09E-04 |
| Urea Cycle | 6/29 | 5.04 | 1.19 | 7.77E-04 | 6/29 | 6.83 | 0.878 | 1.36E-04 |
| Ammonia Recycling | 6/32 | 4.58 | 1.31 | 0.00135 | 7/32 | 7.22 | 0.969 | 2.28E-05 |
| Aspartate Metabolism | 6/35 | 4.17 | 1.44 | 0.00221 | 6/35 | 5.66 | 1.06 | 4.07E-04 |
| Methylhistidine Metabolism | 2/4 | 12.20 | 0.164 | 0.00936 | 2/4 | 16.53 | 0.121 | 0.00513 |
| Glutamate Metabolism | 6/49 | 2.99 | 2.01 | 0.0123 | 5/49 | 3.38 | 1.48 | 0.0135 |
| Glucose-Alanine Cycle | 3/13 | 5.63 | 0.533 | 0.0138 | 3/13 | 7.61 | 0.394 | 0.00586 |
| Ketone Body Metabolism | 3/13 | 5.63 | 0.533 | 0.0138 | 1/13 | 2.54 | 0.394 | 0.331 |
| Arginine and Proline Metabolism | 6/53 | 2.76 | 2.17 | 0.0179 | 6/53 | 3.75 | 1.6 | 0.00391 |
| Phenylalanine and Tyrosine Metabolism | 4/28 | 3.48 | 1.15 | 0.0245 | 4/28 | 4.72 | 0.848 | 0.00847 |
| Warburg Effect | 6/58 | 2.52 | 2.38 | 0.027 | 6/58 | 3.41 | 1.76 | 0.00618 |
| Methionine Metabolism | 5/43 | 2.84 | 1.76 | 0.0275 | 4/43 | 3.08 | 1.3 | 0.0371 |
| Valine, Leucine and Isoleucine Degradation | 6/60 | 2.44 | 2.46 | 0.0314 | 4/60 | 2.20 | 1.82 | 0.102 |
| Beta-Alanine Metabolism | 4/34 | 2.88 | 1.39 | 0.0463 | 4/34 | 3.88 | 1.03 | 0.0169 |
| Homocysteine Degradation | 2/9 | 5.42 | 0.369 | 0.0493 | -- | -- | -- | -- |
| Betaine Metabolism | 3/21 | 3.48 | 0.861 | 0.051 | 3/21 | 4.72 | 0.636 | 0.0231 |
| Carnitine Synthesis | 3/22 | 3.33 | 0.902 | 0.0574 | 3/22 | 4.50 | 0.666 | 0.0262 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Malate-Aspartate Shuttle | 2/10 | 4.88 | 0.41 | 0.06 | 2/10 | 6.60 | 0.303 | 0.0343 |
| Amino Sugar Metabolism | 3/33 | 2.22 | 1.35 | 0.149 | 4/33 | 4.00 | 0.999 | 0.0152 |
| Alanine Metabolism | 2/17 | 2.89 | 0.697 | 0.152 | 3/17 | 5.83 | 0.515 | 0.0128 |
| Transfer of Acetyl Groups into Mitochondria | 2/22 | 2.22 | 0.902 | 0.227 | 3/22 | 4.50 | 0.666 | 0.0262 |

### 6.4.4 Relationship between serum metabolite profile and FACIT-F in PsA patients

Similarly, patients with PsA had a statistically significant correlation between the all serum metabolite bins and FACIT-F (Figure 6-7B1- 98 NMR bins, $r^2$=0.6219, 10 LV, p=0.004). A statistically significant correlation between FACIT-F and serum metabolite data devoid of NMR bins that strongly correlate with CRP persisted for PsA patients (Figure 6-7B2- 170 NMR bins, $r^2$= 0.5282, 10 LV, p=0.018). Table 6-11 shows potential biomarkers for these analyses.

Pathway and enrichment analyses for the biomarkers implicated in the PLS-R analysis between FACIT-F and PsA patients' serum metabolome and the serum metabolome without the bins that strongly correlate with CRP are shown in Figure 6-10, Table 6-12 and Figure 6-11, Table 6-13 respectively. The greater proportion of the pathways implicated between both pathway analyses are the same, however, synthesis and degradation of ketone bodies pathway was the most impacted pathway implicated using the serum metabolome devoid of CRP biomarkers whereas it was not impacted using all the serum metabolome biomarkers. Enrichment analysis using the entire serum metabolome biomarkers revealed carnitine synthesis, glucose-alanine cycle and arginine and proline metabolism as the most overrepresented pathways. Whereas enrichment analysis using the biomarkers of the serum metabolome devoid of CRP indicated methylhistidine metabolism, malate-aspartate shuttle and ammonia recycling as the most overrepresented pathways.

**Table 6-11 Metabolites responsible for the relationship between FACIT-F and serum metabolite profile in PsA patients**

Results from PLS-R models using the complete metabolite dataset and the serum metabolome devoid of the inflammatory signal in PLS-R analysis. The following metabolites have been ranked by the magnitude of the regression coefficient. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of regression coefficient. The regression coefficient field indicates the nature of correlation (↑ indicating a positive relationship with FACIT-F and ↓ indicating a negative relationship with FACIT-F)

| | | | PLS-R model using all serum metabolome | | PLS-R model using serum metabolome devoid of the inflammatory signal | |
|---|---|---|---|---|---|---|
| Order in PLS-R using all serum metabolome | Order in PLS-R using serum metabolome devoid of the inflammatory signal | Metabolite | Chemical shift of peak (ppm) implicated in PLS-R Model | Regression coefficient | Chemical shift of peak (ppm) implicated in PLS-R Model | Regression coefficient |
| 1 | 8 | Asparagine | 2.9197, 2.8651, 2.8315, 2.8209, 2.8092, 2.8033 | ↓↑↑↓↓↓ | 2.8315, 2.8628 | ↑↓ |
| 1 | 6 | Methylguanidine | 2.9197, 2.8651, 2.8315, 2.8209, 2.8092, 2.8033 | ↓↑↑↓↓↓ | 2.6998, 2.8315, 2.7016, 2.8628 | ↓↑↓↓ |
| 1 | 1 | Glucose | 2.9197, 2.8651, 3.1948, 3.3126, 3.1428, 3.1896, 2.8315, 4.2316, 2.8209, 3.1487, 2.8092, 3.1611, 3.1779, 4.6413, 2.8033 | ↓↑↓↑↓↓ ↑↑↓↓↓↓ ↑↓↓ | 3.3711, 3.6228, 2.6998, 2.8315, 4.0401, 2.7016, 3.6287, 3.3535, 4.6296, 2.8628, 3.576, 3.1779 | ↑↑↓↑↑ ↓↓↓↓↓ ↑↓ |
| 1 | 13 | Lysine | 2.9197, 1.978, 1.9077, 1.4746, 1.457, 1.4628, 1.5303, 2.0072 | ↓↑↑↑↑↑ ↓↓ | 1.6641 | ↑ |
| 2 | 6 | Citrate | 2.6687, 2.6394, 2.5399, 2.6453 | ↓↓↓↓ | 2.6998, 2.7016 | ↓↓ |
| 2 | 6 | Aspartate | 2.6687, 2.8315, 2.6394, 2.8209, 2.6453, 2.8092, 2.8033 | ↓↑↓↓↓↓ ↓ | 2.6998, 2.8315 | ↓↑ |
| 2 | 2 | Methionine | 2.6687, 2.6394, 2.6453, 2.1641, 2.1594 | ↓↓↓↓↓ | 2.218, 2.095, 2.1706, 2.0716, 2.2238 | ↓↓↑↓↓ |
| 3 | 1 | Carnitine | 3.1948, 3.3126, 3.1428, 3.1896, 3.1487, 3.1611, 3.1779 | ↓↑↓↓↓↓ ↑ | 3.3711, 3.3535, 3.1779 | ↑↓↓ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 3 | 1 | Glycerol | 3.1948, 3.3126, 3.1428, 3.1896, 3.1487, 3.1611, 3.1779 | ↓↑↓↓↓↓↑ | 3.3711, 3.6228, 4.0401, 3.6287, 3.3535, 3.576, 3.1779 | ↑↑↑↓↓↑↓ |
| 3 | 15 | Betaine | 3.1948, 3.3126, 3.1896, 3.1779 | ↓↑↓↑ | 3.1779 | ↓ |
| 3 | 15 | 3-Methylhistidine | 3.1948, 3.3126, 7.0472, 3.1896, 3.1487, 3.1611, 3.1779 | ↓↑↑↓↓↓↑ | 3.1779 | ↓ |
| 3 | 13 | Arginine | 3.1948, 1.978, 3.3126, 3.1428, 3.1896, 1.9077, 2.0072, 3.1487, 3.1611, 3.1779 | ↓↑↑↓↓↑↓↓↓↑ | 1.6641, 3.1779 | ↑↓ |
| 3 | 10 | Tyrosine | 3.1948, 3.1428, 3.1896, 3.1487, 3.1611, 3.1779 | ↓↓↓↓↓↑ | 7.1417, 7.1818, 3.1779 | ↓↓↓ |
| 3 | 1 | Cystine | 3.1948, 3.1428, 3.1896, 3.1487, 3.1611, 3.1779 | ↓↓↓↓↓↑ | 3.3711, 3.3535, 3.1779 | ↑↓↓ |
| 3 | 9 | Choline | 3.1948, 3.1896, 3.1487, 3.1611, 3.1779 | ↓↓↓↓↑ | 4.0401, 3.1779 | ↑↓ |
| 4 | 1 | Proline | 1.978, 2.3643, 3.3126, 1.9077, 2.0072, 2.1641, 2.1594 | ↑↑↑↑↓↓↓ | 3.3711, 2.218, 2.095, 2.2297, 3.3535, 2.1706, 2.0716, 2.2238 | ↑↓↓↓↓↑↓↓ |
| 4 | 4 | Isoleucine | 1.978, 1.4746, 1.457, 1.4628, 2.0072, 0.88919 | ↑↑↑↑↓↑ | 1.217, 3.6228, 1.2053, 3.6287, 1.1994, 1.2111, 1.1936 | ↑↑↑↓↑↑↑ |
| 4 | 13 | Ornithine | 1.978, 1.9077, 2.0072 | ↑↑↓ | 1.6641 | ↑ |
| 4 | 3 | Glutamate | 1.978, 2.3643, 2.0072, 2.1641, 2.1594 | ↑↑↓↓↓ | 2.095, 2.1706, 2.0716 | ↓↑↓ |
| 4 | 2 | Glutamine | 1.978, 2.3643, 2.5399, 2.0072, 2.1641, 2.1594 | ↑↑↓↓↓↓ | 2.218, 2.095, 2.2297, 2.1706, 2.0716, 2.2238 | ↓↓↓↑↓↓ |
| 5 | N/A | Succinate | 2.3643 | ↑ | N/A | N/A |
| 6 | 1 | Methanol | 3.3126 | ↑ | 3.3711, 3.3535 | ↑↓ |
| 6 | 9 | Tryptophan | 3.3126 | ↑ | 4.0401, 7.1818 | ↑↓ |
| 6 | 15 | Phenylalanine | 3.3126, 3.1428, 3.1487, 3.1611, 3.1779 | ↑↓↓↓↑ | 3.1779 | ↓ |
| 7 | N/A | Histidine | 7.0472, 3.1428, 3.1487, 3.1611 | ↑↓↓↓ | N/A | N/A |
| 8 | N/A | Malonate | 3.1428, 3.1487 | ↓↓ | N/A | N/A |
| 8 | N/A | Cysteine | 3.1428 | ↓ | N/A | N/A |
| 9 | 4 | Threonine | 4.2316 | ↑ | 1.217, 3.576 | ↑↑ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 9 | 4 | Lactate | 4.2316, 1.4746, 1.457, 1.4628, 1.5303, 1.1409, 1.1292 | ↑↑↑↑↓↓↓ | 1.217, 4.0401, 1.2053, 1.1292, 1.135, 1.1994, 1.2111, 1.6641, 1.1936, 1.1233, 1.1409 | ↑↑↑↑↑↑↑↑↑↑↑ |
| 10 | N/A | Acetate | 1.9077 | ↑ | N/A | N/A |
| 11 | N/A | Alanine | 1.4746, 1.457, 1.4628, 1.5303 | ↑↑↑↓ | N/A | N/A |
| 12 | 4 | Isopropanol | 1.1409, 1.1292 | ↓↓ | 1.217, 4.0401, 1.2053, 1.2053, 1.1292, 1.135, 1.1994, 1.2111, 1.1936, 1.1409 | ↑↑↑↑↑↑↑↑↑↑ |
| 12 | 2 | Valine | 1.1409, 0.88919, 1.1292 | ↓↑↓ | 2.218, 3.6228, 0.81895, 0.83029, 1.2053, 3.6287, 1.1292, 1.135, 1.1994, 1.1936, 2.2297, 2.1706, 1.1233, 3.576, 1.1409, 2.2238 | ↓↑↓↑↑↓↑↑↑↓↑↑↑↓ |
| 12 | 12 | Propylene glycol | 1.1409, 1.1292 | ↓↓ | 1.1292, 1.135, 1.1233, 1.1409 | ↑↑↑↑ |
| 13 | N/A | Formate | 8.4462 | ↓ | N/A | N/A |
| 14 | 11 | 2-hydroxybutyrate | 0.88919 | ↑ | 0.83029, 1.6641 | ↑↑ |
| 14 | 7 | Leucine | 0.88919 | ↑ | 0.81895, 0.83029, 1.6641 | ↑↑↑ |
| N/A | 1 | Glycerol | N/A | N/A | 3.3711, 3.6228, 4.0401, 3.6287, 3.3535, 3.576, 3.1779 | ↑↑↑↓↓↑↓ |
| N/A | 2 | Acetone | N/A | N/A | 2.218, 2.2297, 2.2238 | ↓↓↓ |
| N/A | 4 | 3-hydroxybutyrate | N/A | N/A | 1.217, 1.2053, 1.1994, 1.2111, 1.1936 | ↑↑↑↑↑ |
| N/A | 5 | Glycine | N/A | N/A | 3.6228, 3.6287, 3.576 | ↑↓↑ |
| N/A | 9 | Creatinine | N/A | N/A | 4.0401 | ↑ |
| N/A | 9 | Serine | N/A | N/A | 4.0401 | ↑ |
| N/A | 13 | Lysine | N/A | N/A | 1.6641 | ↑ |
| N/A | 14 | Acetoacetate | N/A | N/A | 2.2297, 2.2238 | ↓↓ |

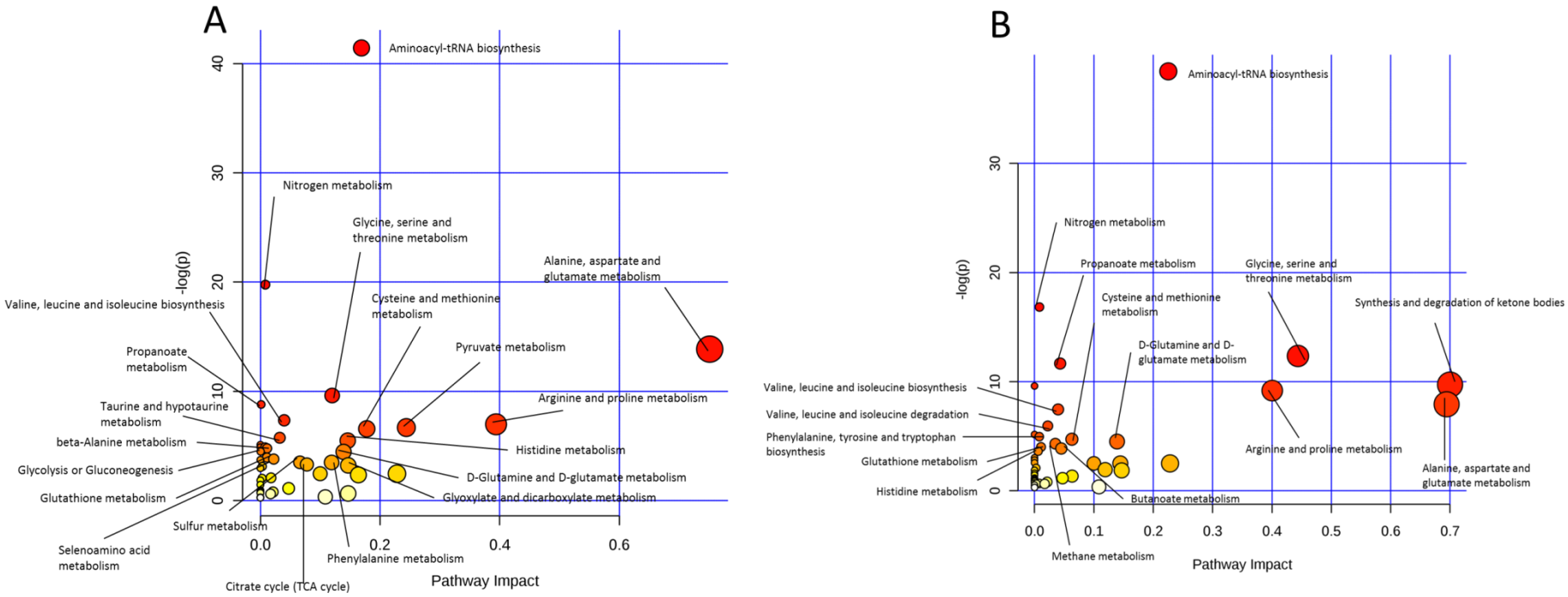



**Figure 6-10 Metaboanalyst pathway analysis of potential biomarkers implicated by PLS-R analysis of FACIT-F and PsA patients' serum metabolites**

Pathway analysis of potential biomarkers implicated by the PLS-R of (A) all the serum metabolome and (B) the serum metabolome devoid of the inflammatory signal

**Table 6-12 Pathway analysis of potential biomarkers implicated by PLS-R analysis of FACIT-F and PsA patients' serum metabolites**
Results from PLS-R models using all the serum metabolome and the serum metabolome devoid of the inflammatory signal
**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.
**The impact score**: Each pathway has a total importance score of 1. The importance measure of each metabolite node (a point where pathways intersect within a larger network) is expressed as a fraction with regards to the total pathway importance, and the pathway impact score is the cumulative score of matched metabolite nodes.
* p value calculated using hypergeometric test

| | PLS-R model using all serum metabolome | | | PLS-R model using serum metabolome devoid of the inflammatory signal | | |
|---|---|---|---|---|---|---|
| Pathway name | Match status | P value* | Impact score | Match status | P value* | Impact score |
| Alanine, aspartate and glutamate metabolism | 6/24 | 9.54E-07 | 0.75119 | 4/24 | 3.62E-04 | 0.69421 |
| Arginine and proline metabolism | 6/77 | 9.29E-04 | 0.39401 | 7/77 | 1.03E-04 | 0.40046 |
| Pyruvate metabolism | 4/32 | 0.00125 | 0.244 | 2/32 | 0.081536 | 0.14453 |
| Cysteine and methionine metabolism | 5/56 | 0.001405 | 0.1778 | 4/56 | 0.008902 | 0.06292 |
| Aminoacyl-tRNA biosynthesis | 18/75 | 1.01E-18 | 0.16902 | 17/75 | 2.02E-17 | 0.22536 |
| Methane metabolism | 2/34 | 0.094889 | 0.16384 | 3/34 | 0.01338 | 0.03502 |
| Glyoxylate and dicarboxylate metabolism | 3/50 | 0.03994 | 0.14685 | 1/50 | 0.53294 | 0.00326 |
| Histidine metabolism | 4/44 | 0.004136 | 0.14599 | 3/44 | 0.026741 | 0.00611 |
| D-Glutamine and D-glutamate metabolism | 2/11 | 0.011591 | 0.13904 | 2/11 | 0.010992 | 0.13904 |
| Glycine, serine and threonine metabolism | 6/48 | 6.65E-05 | 0.11998 | 7/48 | 4.31E-06 | 0.44376 |
| Phenylalanine metabolism | 3/45 | 0.030467 | 0.11906 | 2/45 | 0.1445 | 0.11906 |
| Citrate cycle | 2/20 | 0.036718 | 0.07773 | 1/20 | 2.61E-01 | 0.06327 |
| Sulfur metabolism | 2/18 | 0.030141 | 0.06614 | 1/18 | 0.23831 | 0 |
| Valine, leucine and isoleucine biosynthesis | 4/27 | 6.45E-04 | 0.03975 | 4/27 | 5.79E-04 | 0.03975 |
| Taurine and hypotaurine metabolism | 3/20 | 0.003185 | 0.03237 | -- | -- | -- |
| Valine, leucine and isoleucine degradation | 3/40 | 0.022355 | 0.02232 | 4/40 | 0.002626 | 0.02232 |
| Butanoate metabolism | 2/40 | 0.12468 | 0.01774 | 3/40 | 0.020778 | 0.0451 |
| beta-Alanine metabolism | 3/28 | 0.00841 | 0.01119 | 1/28 | 3.46E-01 | 0 |
| Glutathione metabolism | 3/38 | 0.019493 | 0.01095 | 3/38 | 0.018107 | 0.01095 |
| Nitrogen metabolism | 9/39 | 2.60E-09 | 0.0083 | 8/39 | 4.87E-08 | 0.0083 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Phenylalanine, tyrosine and tryptophan biosynthesis | 3/27 | 0.007589 | 0.008 | 3/27 | 0.007026 | 0.008 |
| Selenoamino acid metabolism | 2/22 | 0.043795 | 0.00321 | -- | -- | -- |
| Propanoate metabolism | 5/35 | 1.51E-04 | 0.00134 | 6/35 | 8.61E-06 | 0.04317 |
| Synthesis and degradation of ketone bodies | -- | -- | -- | 3/6 | 5.96E-05 | 0.7 |
| Glycolysis or Gluconeogenesis | 3/31 | 0.01118 | 4.60E-04 | 2/31 | 0.077147 | 0 |

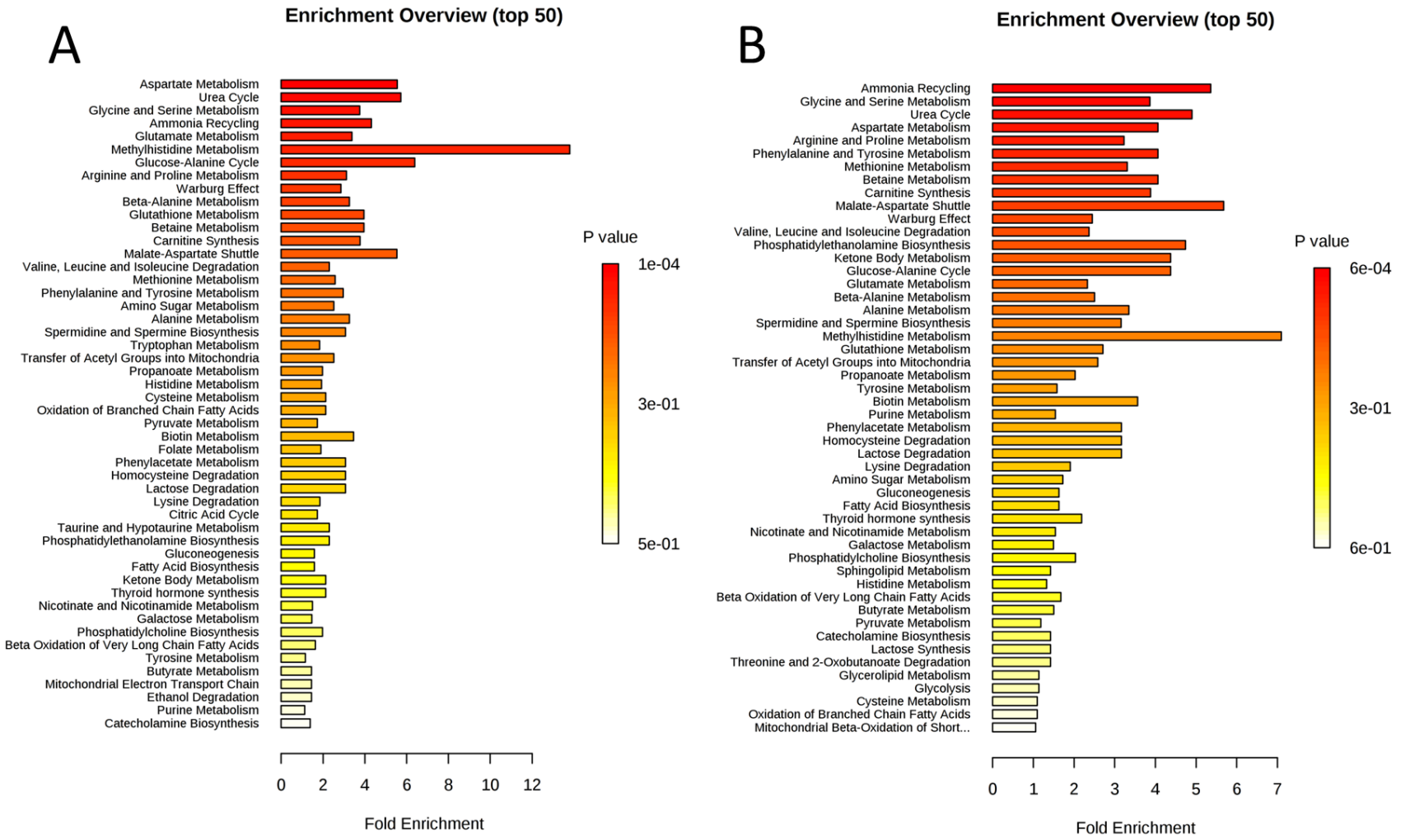



**Figure 6-11 Enrichment analysis of key metabolites in serum implicated as potential biomarkers by the PLS-R analysis of FACIT-F and PsA patients' serum metabolites**

Enrichment analysis of potential biomarkers implicated by the PLS-R of (A) all the serum metabolome and (B) the serum metabolome devoid of the inflammatory signal

**Table 6-13 Enrichment analysis of key metabolites in serum implicated as potential biomarkers by the PLS-R analysis of FACIT-F and PsA patients' serum metabolites**

Results from PLS-R model using all the serum metabolome and the serum metabolome devoid of the inflammatory signal

**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.

**The fold enrichment value**: the value is generated by the number of matched metabolites divided by the number of metabolites expected to be present in the pathway as per the reference metabolome provided by KEGG.

* p value calculated using hypergeometric test

| | PLS-R model using all serum metabolome | | | | PLS-R model using serum metabolome devoid of the inflammatory signal | | | |
|---|---|---|---|---|---|---|---|---|
| Metabolite pathway | Match status | Fold enrichment | Expected | P value* | Match status | Fold enrichment | Expected | P value* |
| Aspartate Metabolism | 7/35 | 3.76 | 1.26 | 1.44E-04 | 5/35 | 4.07 | 1.23 | 0.00609 |
| Urea Cycle | 6/29 | 3.77 | 1.05 | 3.80E-04 | 5/29 | 4.90 | 1.02 | 0.0026 |
| Glycine and Serine Metabolism | 8/59 | 3.25 | 2.13 | 7.77E-04 | 8/59 | 3.86 | 2.07 | 6.38E-04 |
| Ammonia Recycling | 5/32 | 5.56 | 1.16 | 0.00462 | 6/32 | 5.36 | 1.12 | 5.74E-04 |
| Glutamate Metabolism | 6/49 | 3.13 | 1.77 | 0.00657 | 4/49 | 2.33 | 1.72 | 0.0882 |
| Methylhistidine Metabolism | 2/4 | 3.95 | 0.145 | 0.00729 | 1/4 | 7.09 | 0.141 | 0.134 |
| Glucose-Alanine Cycle | 3/13 | 6.38 | 0.47 | 0.00968 | 2/13 | 4.38 | 0.457 | 0.0745 |
| Arginine and Proline Metabolism | 6/53 | 5.71 | 1.92 | 0.00969 | 6/53 | 3.23 | 1.86 | 0.00845 |
| Warburg Effect | 6/58 | 5.54 | 2.1 | 0.015 | 5/58 | 2.45 | 2.04 | 0.0477 |
| Beta-Alanine Metabolism | 4/34 | 4.31 | 1.23 | 0.0308 | 3/34 | 2.50 | 1.2 | 0.113 |
| Betaine Metabolism | 3/21 | 3.39 | 0.759 | 0.0369 | 3/21 | 4.07 | 0.738 | 0.0343 |
| Glutathione Metabolism | 3/21 | 2.86 | 0.759 | 0.0369 | 2/21 | 2.71 | 0.738 | 0.166 |
| Carnitine Synthesis | 3/22 | 13.79 | 0.795 | 0.0417 | 3/22 | 3.88 | 0.773 | 0.0388 |
| Malate-Aspartate Shuttle | 2/10 | 3.95 | 0.361 | 0.0476 | 2/10 | 5.68 | 0.352 | 0.0453 |
| Phenylalanine and Tyrosine Metabolism | 3/28 | 2.97 | 1.01 | 0.0764 | 4/28 | 4.07 | 0.984 | 0.0144 |
| Methionine Metabolism | 4/43 | 2.58 | 1.55 | 0.0649 | 5/43 | 3.31 | 1.51 | 0.0147 |

### 6.4.5 Relationship between serum metabolite profile and FACIT-F in UA patients

For UA patients there was a correlation between all serum metabolite bins and FACIT-F (Figure 6-7C1- 19 NMR bins, $r^2$=0.2173, 6 LV, p<0.001). Table 6-14 shows the potential biomarkers for this relationship. The relationship between FACIT-F and serum metabolite profile without bins which strongly correlate with CRP removed was not present (11 NMR bins, $r^2$=0.1012, 2 LV, p=0.231).

Pathway and enrichment analysis for the biomarkers implicated in the PLS-R analysis between FACIT-F and UA patients' serum metabolome are shown in Figure 6-12, Table 6-15 and Figure 6-13, Table 6-16 respectively. Ketone body, pyruvate and alanine, aspartate and glutamine metabolism were the most impacted pathways in the pathway analysis. Methylhistidine metabolism, urea cycle and ammonia recycling are the most overrepresented pathways.

**Table 6-14 Potential biomarkers for PLS-R analysis between FACIT-F and serum metabolite profile in UA patients using the complete metabolite dataset**

| Order | Metabolite | Chemical shift of peak (ppm) | Regression coefficient |
|---|---|---|---|
| 1 | Glucose | 3.2072, 2.6998, 2.8911, 2.9094, 3.1948, 2.9145, 3.1611, 4.2168, 2.7989, 3.3711, 2.8787, 5.2496, 3.2892, 2.7916, 2.903, 4.0712, 3.3594 | ↑↑↓↑↑↑↑↓↑↑↓↓↓↓↓↑↓ |
| 1 | Carnitine | 3.2072, 3.1948, 3.1611, 3.3711, 3.2892, 3.3594 | ↑↑↑↑↓↓ |
| 1 | Glycerol | 3.2072, 3.1948, 3.1611, 3.3711, 3.2892, 4.0712, 3.3594 | ↑↑↑↑↓↑↓ |
| 1 | Betaine | 3.2072, 3.1948, 3.2892 | ↑↑↓ |
| 1 | 3-Methylhistidine | 3.2072, 3.1948, 3.1611, 7.0647, 3.2892, 7.053 | ↑↑↑↓↓↑ |
| 1 | Arginine | 3.2072, 3.1948, 1.8744, 3.1611, 1.9077, 3.2892, 1.6524, 1.937 | ↑↑↑↑↓↓↑↑ |
| 1 | Phenylalanine | 3.2072, 3.1611, 3.2892 | ↑↑↓ |
| 1 | Tyrosine | 3.2072, 3.1948, 3.1611, 7.1333 | ↑↑↑↑ |
| 1 | Cystine | 3.2072, 3.1948, 3.1611, 3.3711, 4.0712, 3.3594 | ↑↑↑↑↑↓ |
| 1 | Histidine | 3.2072, 3.1611, 7.0647, 7.053 | ↑↑↓↑ |
| 1 | Choline | 3.2072, 3.1948, 3.1611, 4.0712 | ↑↑↑↑ |
| 2 | Citrate | 2.6998, 2.691, 2.5106 | ↑↓↓ |
| 2 | Aspartate | 2.6998, 2.691, 2.7989, 2.7916 | ↑↓↑↓ |
| 2 | Methylguanidine | 2.6998, 2.8911, 2.9094, 2.9145, 2.7989, 2.8787, 2.7916, 2.903 | ↑↓↑↑↑↓↓↓ |
| 3 | Isoleucine | 1.2274, 1.176, 1.2931, 1.937 | ↓↑↑↑ |
| 3 | Lactate | 1.2274, 4.2168, 1.1702, 1.1643, 1.2931, 1.1233, 1.6524, 1.1585, 4.0712 | ↓↓↑↑↑↑↑↑↑ |

| | | | |
|---|---|---|---|
| 3 | 3-hydroxybutyrate | 1.2274 | ↓ |
| 3 | Isopropanol | 1.2274, 1.176, 1.1702, 1.1643, 1.1585 | ↓↑↑↑↑ |
| 3 | Threonine | 1.2274, 4.2168, 1.2931 | ↓↓↑ |
| 4 | Asparagine | 2.8911, 2.9094, 2.9145, 2.7989, 2.8787, 2.903 | ↓↑↑↑↓↓ |
| 5 | Acetate | 1.8744, 1.9077, 1.937 | ↑↓↑ |
| 5 | Lysine | 1.8744, 2.9145, 1.9077, 1.6524, 1.937 | ↑↑↓↑↑ |
| 5 | Ornithine | 1.8744, 1.9077, 1.937 | ↑↓↑ |
| 5 | Proline | 1.8744, 2.2238, 1.9077, 3.3711, 3.2892, 1.937, 4.0712, 3.3594 | ↑↑↓↑↓↑↑↓ |
| 6 | Valine | 2.2238, 1.1702, 1.1643, 1.1233, 1.1585 | ↑↑↑↑↑ |
| 6 | Acetone | 2.2238 | ↑ |
| 6 | Methionine | 2.2238 | ↑ |
| 6 | Glutamine | 2.2238, 2.5106 | ↑↓ |
| 6 | Acetoacetate | 2.2238 | ↑ |
| 7 | Pyruvate | 2.5106 | ↓ |
| 8 | Methanol | 3.3711, 3.3594 | ↑↓ |
| 9 | Tryptophan | 3.2892, 4.0712 | ↓↑ |
| 10 | Propylene glycol | 1.1233 | ↑ |
| 11 | 2-hydroxybutyrate | 1.6524 | ↑ |
| 11 | Leucine | 1.6524 | ↑ |
| 12 | Creatinine | 4.0712 | ↑ |

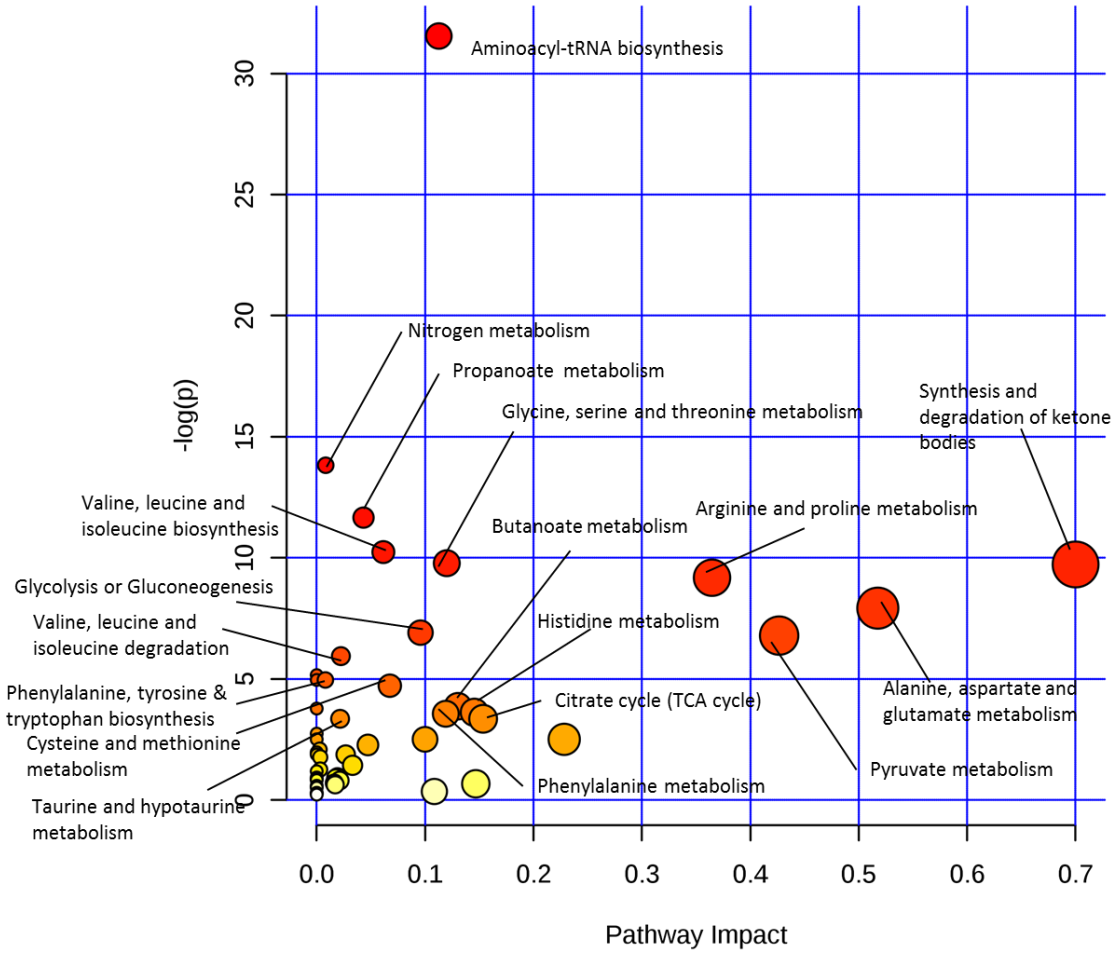



**Figure 6-12 Metaboanalyst pathway analysis of potential biomarkers implicated by PLS-R analysis of FACIT-F and UA patients' serum metabolites**

**Table 6-15 Pathway analysis of potential biomarkers implicated by PLS-R analysis of FACIT-F and UA patients' serum metabolites using all the serum metabolome**

**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.

**The impact score**: Each pathway has a total importance score of 1. The importance measure of each metabolite node (a point where pathways intersect within a larger network) is expressed as a fraction with regards to the total pathway importance, and the pathway impact score is the cumulative score of matched metabolite nodes.

* p value calculated using hypergeometric test

| Pathway Name | Match Status | P value* | Impact score |
|---|---|---|---|
| Synthesis and degradation of ketone bodies | 3/6 | 5.96E-05 | 0.7 |
| Alanine, aspartate and glutamate metabolism | 4/24 | 3.62E-04 | 0.51757 |
| Pyruvate metabolism | 4/32 | 0.001125 | 0.42654 |
| Arginine and proline metabolism | 7/77 | 1.03E-04 | 0.36464 |
| Citrate cycle | 2/20 | 0.034906 | 0.15351 |
| Histidine metabolism | 3/44 | 0.026741 | 0.14548 |
| Butanoate metabolism | 3/40 | 0.020778 | 0.13026 |
| Glycine, serine and threonine metabolism | 6/48 | 5.66E-05 | 0.11998 |
| Phenylalanine metabolism | 3/45 | 0.028361 | 0.11906 |
| Aminoacyl-tRNA biosynthesis | 15/75 | 1.99E-14 | 0.11268 |
| Glycolysis or Gluconeogenesis | 4/31 | 9.95E-04 | 0.09576 |
| Cysteine and methionine metabolism | 4/56 | 0.008902 | 0.06744 |
| Valine, leucine and isoleucine biosynthesis | 5/27 | 3.58E-05 | 0.06148 |
| Propanoate metabolism | 6/35 | 8.61E-06 | 0.04317 |
| Valine, leucine and isoleucine degradation | 4/40 | 0.002626 | 0.02232 |
| Taurine and hypotaurine metabolism | 2/20 | 0.034906 | 0.02158 |
| Nitrogen metabolism | 7/39 | 9.91E-07 | 0.0083 |
| Phenylalanine, tyrosine and tryptophan biosynthesis | 3/27 | 0.007026 | 0.008 |

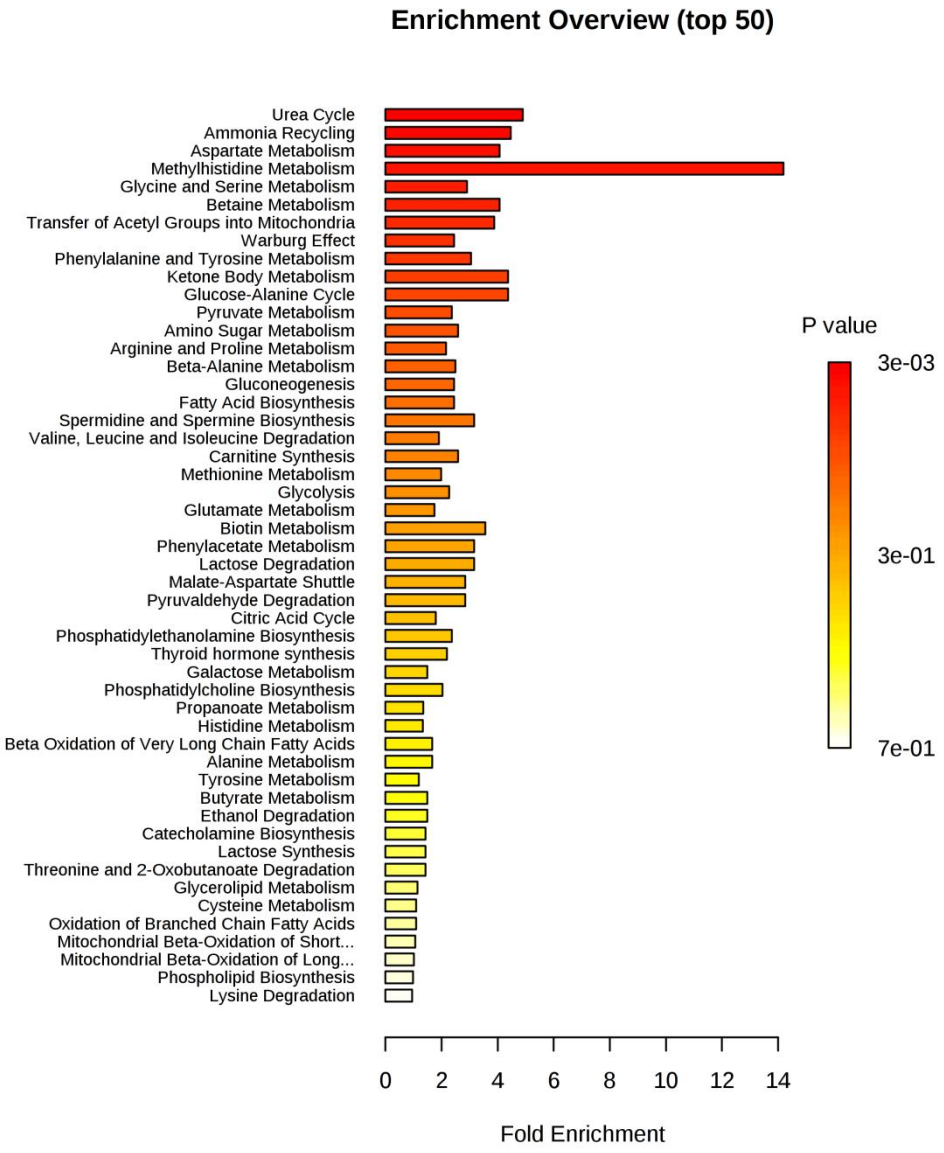



**Figure 6-13 Enrichment analysis of key metabolites in serum implicated as potential biomarkers by the PLS-R analysis of FACIT-F and UA patients' serum metabolites**

**Table 6-16 Enrichment analysis of key metabolites in serum implicated as potential biomarkers by the PLS-R analysis of FACIT-F and UA patients' serum metabolites**
**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.
**The fold enrichment value**: the value is generated by the number of matched metabolites divided by the number of metabolites expected to be present in the pathway as per the reference metabolome provided by KEGG.
* p value calculated using hypergeometric test

| Metabolite pathway | Match status | Enrichment | Expected | P value* |
|---|---|---|---|---|
| Urea Cycle | 5/29 | 4.90 | 1.02 | 0.0026 |
| Ammonia Recycling | 5/32 | 4.46 | 1.12 | 0.00408 |
| Aspartate Metabolism | 5/35 | 4.07 | 1.23 | 0.00609 |
| Methylhistidine Metabolism | 2/4 | 14.18 | 0.141 | 0.0069 |
| Glycine and Serine Metabolism | 6/59 | 2.90 | 2.07 | 0.0142 |
| Betaine Metabolism | 3/21 | 4.07 | 0.738 | 0.0343 |
| Transfer of Acetyl Groups into Mitochondria | 3/22 | 3.88 | 0.773 | 0.0388 |
| Warburg Effect | 5/58 | 2.45 | 2.04 | 0.0477 |

### 6.4.6 Relationship between serum metabolite profile and FACIT-F in CSA patients

There was a correlation between all serum metabolite data bins and FACIT-F in CSA patients (Figure 6-7D1- 48 NMR bins, $r^2$=0.2831, 8 LV, p=0.017) and a relationship between FACIT-F and the serum metabolite profile devoid of the bins that strongly correlate with CRP in CSA (Figure 6-7D2- 67 NMR bins, $r^2$=0.5967, 10 LV, p<0.001). Table 6-17 shows potential biomarkers for these analyses.

Pathway and enrichment analyses for the biomarkers implicated in the PLS-R analysis between FACIT-F and CSA patients' serum metabolome and the serum metabolome without the bins that strongly correlate with CRP are shown in Figure 6-14, Table 6-18 and Figure 6-15, Table 6-19 respectively. Enrichment analyses showed methylhistidine metabolism, glucose-alanine cycle and homocysteine degradation were the most overrepresented pathways. Pathway analyses showed alanine, aspartate and glutamate metabolism was the most impacted pathway across both pathway analyses. Synthesis and degradation of ketone bodies was the second most impacted pathway implicated using the serum metabolome biomarkers (3/6 metabolites present, impact score 0.7, p=8.22E-05) whereas it was not impacted using all the serum metabolome devoid of CRP biomarkers (1/6 metabolites present, impact score 0, p=0. 093456).

**Table 6-17 Metabolites responsible for the relationship between FACIT-F and serum metabolite profile in CSA patients**

Results from PLS-R models using the complete metabolite dataset and the serum metabolome devoid of the inflammatory signal in PLS-R analysis. The following metabolites have been ranked by the magnitude of the regression coefficient. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of regression coefficient. The regression coefficient field indicates the nature of correlation (↑ indicating a positive relationship with FACIT-F and ↓ indicating a negative relationship with FACIT-F)

| | | | PLS-R model using all serum metabolome | | PLS-R model using serum metabolome devoid of the inflammatory signal | |
|---|---|---|---|---|---|---|
| Order in PLS-R using all serum metabolome | Order in PLS-R using serum metabolome devoid of the inflammatory signal | Metabolite | Chemical shift of peak (ppm) implicated in PLS-R Model | Regression coefficient | Chemical shift of peak (ppm) implicated in PLS-R Model | Regression coefficient |
| 1 | 1 | Glycerol | 3.6813, 3.6755, 3.857, 3.3711, 3.7926, 3.1136, 3.4355, 3.6696, 3.1611, 4.0443, 3.7867, 3.8687 | ↓↓↓↑↓↓ ↑↓↓↓↓↓ | 4.009, 3.3711, 4.0618, 4.0033, 3.617, 3.2072, 3.1662, 3.1428, 3.9974, 3.3126 | ↑↓↑↑↑ ↑↓↓↑↑ |
| 1 | 1 | Glucose | 3.6813, 2.8315, 3.6755, 3.857, 2.9197, 4.6238, 3.3711, 2.7016, 2.8267, 3.7926, 3.1136, 4.6413, 3.4355, 2.8628, 3.6696, 3.1611, 4.0443, 3.7867, 5.215, 2.9145, 2.6998, 3.8687 | ↓↑↓↓↓↑ ↑↑↑↓↓↑ ↑↓↓↓↓↓ ↑↑↑↓ | 4.009, 2.6998, 3.3711, 4.0618, 4.0033, 4.653, 3.617, 2.8092, 2.8209, 4.6413, 3.2072, 3.1662, 3.1428, 3.9974, 3.3126 | ↑↑↓↑↑ ↓↑↓↓↑ ↑↓↓↑↑ |
| 1 | 6 | Glutamine | 3.6813, 2.2238, 2.2297, 3.6755, 2.218, 3.7926, 2.2706, 3.7867, 1.978 | ↓↓↓↓↓↓ ↑↓↑ | 1.9613, 1.9663, 2.1706, 1.9523 | ↑↑↓↑ |
| 1 | 6 | Lysine | 3.6813, 1.9077, 3.6755, 2.9197, 1.9136, 3.7926, 3.1136, 3.6696, 3.7867, 2.9145, 1.978 | ↓↑↓↓↑↓ ↓↓↓↑↑ | 1.9613, 1.9663, 1.9077, 1.6319, 1.9523 | ↑↑↑↑↑ |
| 1 | 11 | 3-Methylhistidine | 3.6813, 3.6755, 3.6696, 3.1611 | ↓↓↓↓ | 7.053, 3.2072, 3.1662, 3.3126 | ↓↑↓↑ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 6 | Isoleucine | 3.6813, 3.6755, 1.9136, 3.6696, 0.86395, 0.88919, 1.2111, 1.978 | ↓↓↑↓↓↑↑↑ | 1.9613, 1.9663, 1.0414, 1.2404, 1.9523, 1.1877 | ↑↑↓↑↑↑ |
| 1 | 9 | Leucine | 3.6813, 3.6755, 0.86395, 0.88919 | ↓↓↓↑ | 1.0414, 1.6319 | ↓↑ |
| 2 | 4 | Valine | 2.2238, 2.2297, 2.218, 2.2706, 3.6696, 0.86395, 0.88919, 1.135, 1.1409 | ↓↓↓↑↓↓↑↑↑ | 1.135, 3.617, 1.0414, 1.1292, 2.1706, 1.1877 | ↓↑↓↓↓↑ |
| 2 | N/A | Acetone | 2.2238, 2.2297, 2.218 | ↓↓↓ | N/A | N/A |
| 2 | 14 | Methionine | 2.2238, 3.857, 2.218, 3.8687 | ↓↓↓↓ | 2.1706, 2.6167 | ↓↓ |
| 2 | 3 | Proline | 2.2238, 1.9077, 2.2297, 3.3711, 1.9136, 2.218, 2.2706, 3.4355, 4.0443, 1.978 | ↓↑↓↑↑↓↑↑↓↑ | 3.3711, 4.0618, 1.9613, 1.9663, 1.9077, 2.1706, 1.9523, 3.3126 | ↓↑↑↑↑↓↑↑ |
| 2 | N/A | Acetoacetate | 2.2238, 2.2297, 2.2706, 3.4355 | ↓↓↑↑ | N/A | N/A |
| 3 | 12 | Acetate | 1.9077, 1.9136 | ↑↑ | 1.9077, 1.9523 | ↑↑ |
| 3 | 6 | Arginine | 1.9077, 1.9136, 3.7926, 3.1611, 3.7867, 1.978 | ↑↑↓↓↓↑ | 1.9613, 1.9663, 1.9077, 3.2072, 1.6319, 3.1662, 1.9523, 3.1428, 3.3126 | ↑↑↑↑↑↓↑↓↑ |
| 3 | 6 | Ornithine | 1.9077, 1.9136, 3.7926, 3.1136, 3.7867, 1.978 | ↑↑↓↓↓↑ | 1.9613, 1.9663, 1.9077, 1.9523 | ↑↑↑↑ |
| 4 | 1 | Asparagine | 2.8315, 2.9197, 2.8267, 2.8628, 2.9145 | ↑↓↑↓↑ | 4.009, 4.0033, 2.8092, 2.8209, 3.9974 | ↑↑↓↓↑ |
| 4 | 2 | Methylguanidine | 2.8315, 2.9197, 2.7016, 2.8267, 2.8628, 2.9145, 2.6998 | ↑↓↑↑↓↑↑ | 2.6998, 2.8092, 2.8209 | ↑↓↓ |
| 4 | 2 | Aspartate | 2.8315, 2.8267, 2.6998, 3.8687 | ↑↑↑↓ | 2.6998, 2.8092, 2.8209, 2.6167 | ↑↓↓↓ |
| 5 | 4 | Propylene glycol | 3.857, 3.4355, 1.135, 1.1409, 3.8687 | ↓↑↑↑↓ | 1.135, 1.1292 | ↓↓ |
| 5 | 1 | Serine | 3.857, 3.7926, 3.7867, 3.8687 | ↓↓↓↓ | 4.009, 4.0033, 3.9974 | ↑↑↑ |
| 5 | 13 | Betaine | 3.857, 3.8687 | ↓↓ | 3.2072, 3.1662, 3.3126 | ↑↓↑ |
| 6 | 3 | Cystine | 3.3711, 3.1611 | ↑↓ | 3.3711, 4.0618, 3.2072, 3.1662, 3.1428 | ↓↑↑↓↓ |
| 7 | 3 | Methanol | 3.3711 | ↑ | 3.3711, 3.3126 | ↓↑ |
| 7 | 3 | Carnitine | 3.3711, 3.1136, 3.4355, 3.1611 | ↑↓↑↓ | 3.3711, 3.2072, 3.1662, 3.1428, 3.3126 | ↓↑↓↓↑ |
| 8 | 2 | Citrate | 2.7016, 2.6998 | ↑↑ | 2.6998, 2.6167 | ↑↓ |
| 9 | 7 | Alanine | 3.7926, 3.7867 | ↓↓ | 1.3341 | ↓ |
| 9 | 14 | Glutamate | 3.7926, 2.2706, 3.7867, 1.978 | ↓↑↓↑ | 2.1706 | ↓ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 10 | 1 | Phenylalanine | 3.1136, 3.1611 | ↓↓ | 4.009, 4.0033, 3.2072, 3.1662, 3.1428, 3.9974, 3.3126 | ↑↑↑↓↓↑↑ |
| 10 | 15 | Cysteine | 3.1136 | ↓ | 3.1428 | ↓ |
| 10 | 11 | Histidine | 3.1136, 3.1611 | ↓↓ | 7.053, 3.2072, 3.1428 | ↓↑↓ |
| 10 | 15 | Malonate | 3.1136 | ↓ | 3.1428 | ↓ |
| 10 | 13 | Tyrosine | 3.1136, 3.1611 | ↓↓ | 3.2072, 3.1662, 3.1428 | ↑↓↓ |
| 11 | 1 | 2-hydroxybutyrate | 0.86395, 0.88919 | ↓↑ | 4.009, 4.0033, 1.6319, 3.9974 | ↑↑↑↑ |
| 12 | 5 | Choline | 3.1611, 4.0443 | ↓↓ | 4.0618, 3.2072, 3.1662 | ↑↑↓ |
| 13 | 1 | Lactate | 4.0443, 1.2111, 1.135, 1.1409 | ↓↓↑↑ | 4.009, 1.135, 4.0618, 4.0033, 1.3341, 1.0414, 1.2404, 1.1292, 1.6319, 3.9974, 1.1877 | ↑↓↑↑↓↓↑↓↑↑↑ |
| 13 | 1 | Creatinine | 4.0443 | ↓ | 4.009, 4.0618, 4.0033 | ↑↑↑ |
| 13 | 5 | Tryptophan | 4.0443 | ↓ | 4.0618, 3.3126 | ↑↑ |
| 13 | 1 | Isopropanol | 4.0443, 1.2111, 1.135, 1.1409 | ↓↓↑↑ | 4.009, 1.135, 4.0033, 1.1292, 3.9974, 1.1877 | ↑↓↑↓↑↑ |
| 14 | 10 | 3-hydroxybutyrate | 1.2111 | ↓ | 1.2404, 1.1877 | ↑↑ |
| 15 | N/A | Formate | 8.4462 | ↓ | N/A | N/A |
| N/A | 7 | Threonine | N/A | N/A | 1.3341, 3.617, 1.2404 | ↓↑↑ |
| N/A | 8 | Glycine | N/A | N/A | 3.617 | ↑ |

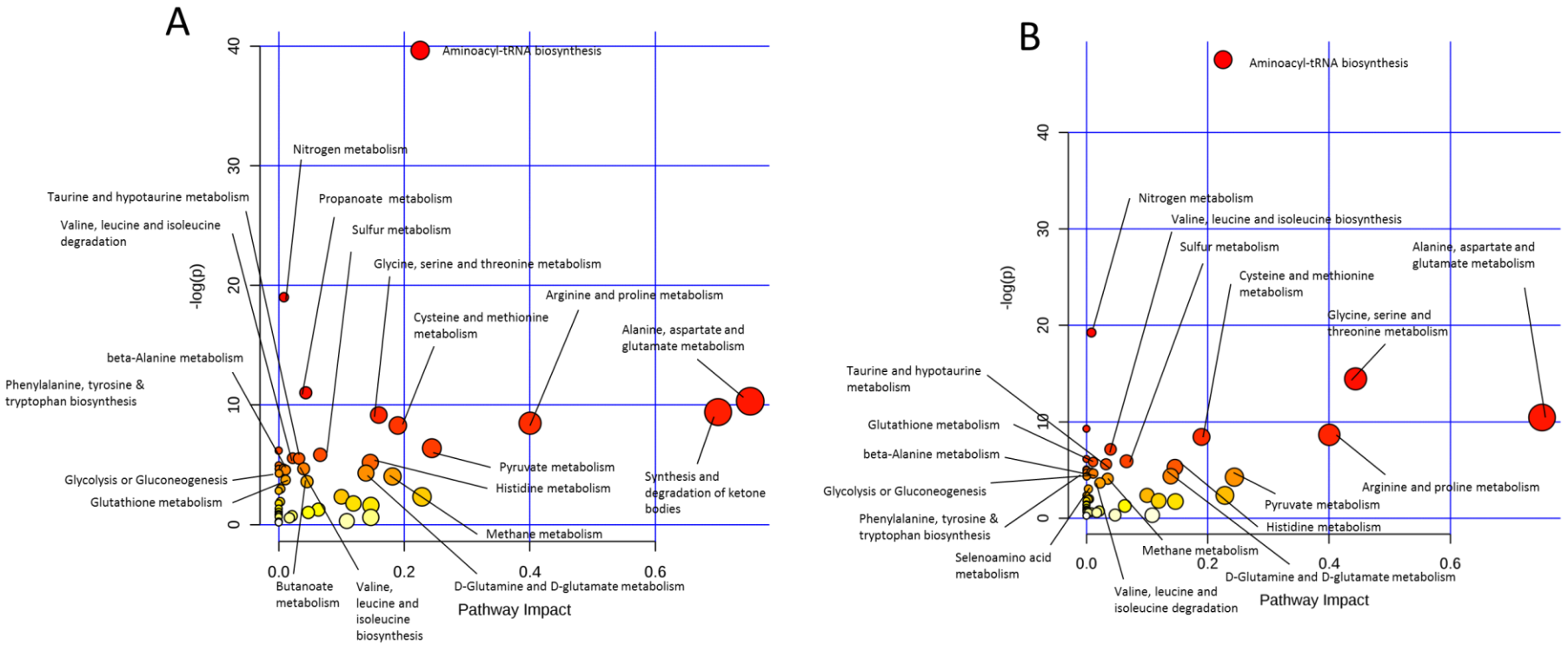



**Figure 6-14 Pathway analysis of potential biomarkers implicated by PLS-R analysis of FACIT-F and CSA patients' serum metabolites**
Pathway analysis of potential biomarkers implicated by the PLS-R of (A) all the serum metabolome and (B) the serum metabolome devoid of the inflammatory signal.

**Table 6-18 Pathway analysis of potential biomarkers implicated by PLS-R analysis of FACIT-F and CSA patients' serum metabolites**
Results from PLS-R models using all the serum metabolome and the serum metabolome devoid of the inflammatory signal.
**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.
**The impact score**: Each pathway has a total importance score of 1. The importance measure of each metabolite node (a point where pathways intersect within a larger network) is expressed as a fraction with regards to the total pathway importance, and the pathway impact score is the cumulative score of matched metabolite nodes.
* p value calculated using hypergeometric test

| | PLS-R model using all serum metabolome | | | PLS-R model using serum metabolome devoid of the inflammatory signal | | |
|---|---|---|---|---|---|---|
| Pathway name | Match status | P value* | Impact score | Match status | P value* | Impact score |
| Alanine, aspartate and glutamate metabolism | 5/24 | 3.31E-05 | 0.75119 | 5/24 | 2.91E-05 | 0.75119 |
| Synthesis and degradation of ketone bodies | 3/6 | 8.22E-05 | 0.7 | 1/6 | 0.093456 | 0 |
| Arginine and proline metabolism | 7/77 | 2.08E-04 | 0.40046 | 7/77 | 1.76E-04 | 0.40046 |
| Pyruvate metabolism | 4/32 | 0.001682 | 0.244 | 3/32 | 0.014103 | 0.244 |
| Aminoacyl-tRNA biosynthesis | 18/75 | 6.07E-18 | 0.22536 | 20/75 | 2.24E-21 | 0.22536 |
| Cysteine and methionine metabolism | 6/56 | 2.52E-04 | 0.18977 | 6/56 | 2.18E-04 | 0.18977 |
| Methane metabolism | 3/34 | 0.017817 | 0.18135 | 3/34 | 0.016639 | 0.03502 |
| Glycine, serine and threonine metabolism | 6/48 | 1.05E-04 | 0.15941 | 8/48 | 5.28E-07 | 0.44376 |
| Histidine metabolism | 4/44 | 0.0055 | 0.14599 | 4/44 | 0.005016 | 0.14599 |
| D-Glutamine and D-glutamate metabolism | 2/11 | 0.013474 | 0.13904 | 2/11 | 0.012832 | 0.13904 |
| Sulfur metabolism | 3/18 | 0.00292 | 0.06614 | 3/18 | 0.002713 | 0.06614 |
| Butanoate metabolism | 3/40 | 0.027469 | 0.0451 | 2/40 | 0.13593 | 0.0048 |
| Propanoate metabolism | 6/35 | 1.63E-05 | 0.04317 | 4/35 | 0.0021485 | 0 |
| Valine, leucine and isoleucine biosynthesis | 3/27 | 0.009436 | 0.03975 | 4/27 | 7.91E-04 | 0.03975 |
| Taurine and hypotaurine metabolism | 3/20 | 0.003986 | 0.03237 | 3/20 | 0.003707 | 0.03237 |
| Valine, leucine and isoleucine degradation | 4/40 | 0.003884 | 0.02232 | 3/40 | 0.0257 | 0.02232 |
| beta-Alanine metabolism | 3/28 | 0.010448 | 0.01119 | 3/28 | 0.00974 | 0.01119 |
| Glutathione metabolism | 3/38 | 0.023995 | 0.01095 | 4/38 | 0.002925 | 0.01095 |
| Nitrogen metabolism | 9/39 | 5.53E-09 | 0.0083 | 9/39 | 4.34E-09 | 0.0083 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Phenylalanine, tyrosine and tryptophan biosynthesis | 3/27 | 0.009436 | 0.008 | 3/27 | 0.008794 | 0.008 |
| Glycolysis or Gluconeogenesis | 3/31 | 0.013851 | 4.60E-04 | 3/31 | 0.012924 | 4.60E-04 |
| Selenoamino acid metabolism | 2/22 | 0.050455 | 0.00321 | 2/22 | 0.048196 | 0.00321 |

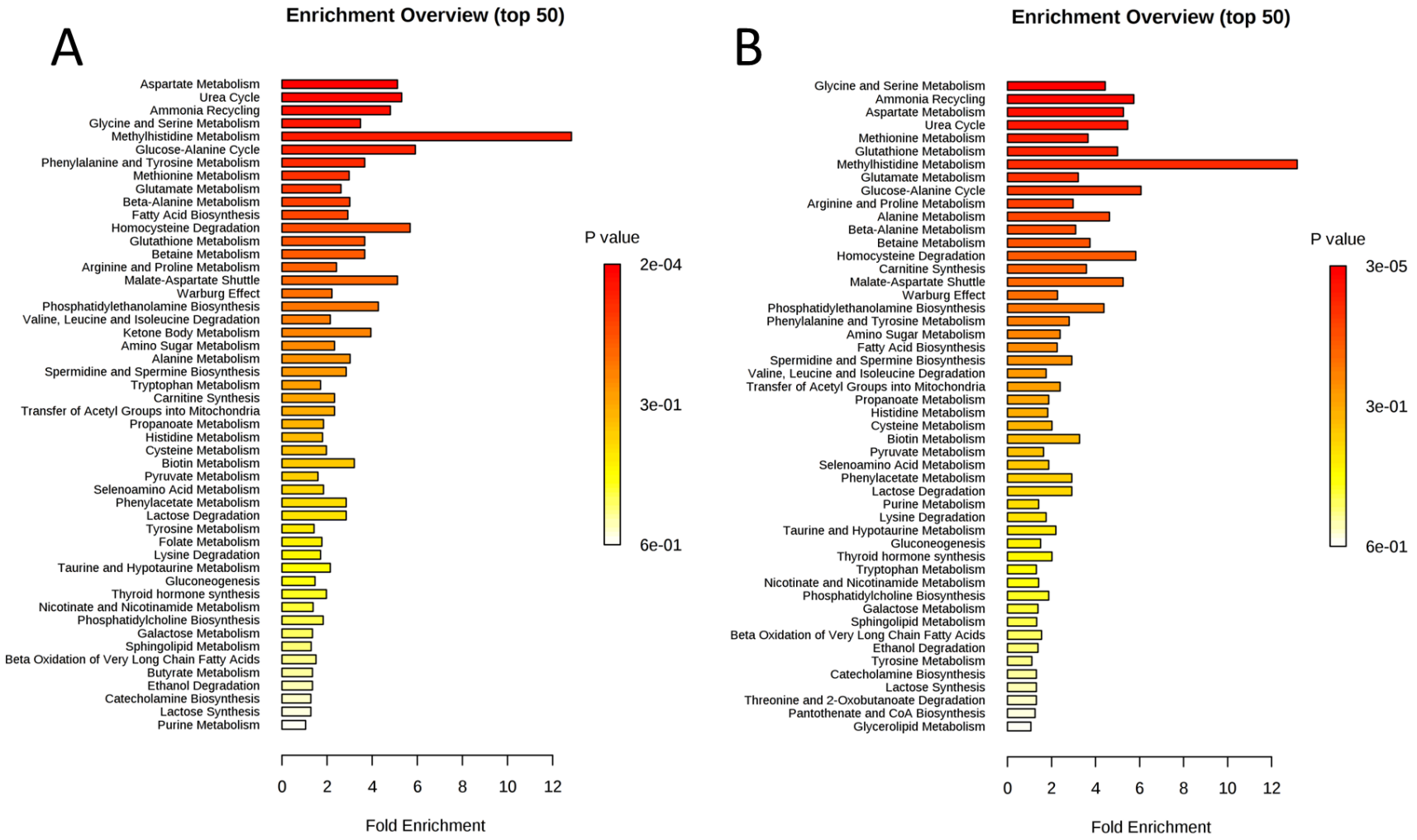



**Figure 6-15 Enrichment analysis of key metabolites in serum implicated as potential biomarkers by the PLS-R analysis of FACIT-F and CSA patients' serum metabolites**

Enrichment analysis of potential biomarkers implicated by the PLS-R of (A) all the serum metabolome and (B) the serum metabolome devoid of the inflammatory signal

**Table 6-19 Enrichment analysis of key metabolites in serum implicated as potential biomarkers by the PLS-R analysis of FACIT-F and CSA patients' serum metabolites**

Results from PLS-R models using all the serum metabolome and the serum metabolome devoid of the inflammatory signal

**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.

**The fold enrichment value**: the value is generated by the number of matched metabolites divided by the number of metabolites expected to be present in the pathway as per the reference metabolome provided by KEGG.

* p value calculated using hypergeometric test

| | PLS-R model using all serum metabolome | | | | PLS-R model using serum metabolome devoid of the inflammatory signal | | | |
|---|---|---|---|---|---|---|---|---|
| Metabolite pathway | Match status | Fold enrichment | Expected | P value* | Match status | Fold enrichment | Expected | P value* |
| Aspartate Metabolism | 7/35 | 5.11 | 1.37 | 2.43E-04 | 7/35 | 5.26 | 1.33 | 2.05E-04 |
| Urea Cycle | 6/29 | 5.31 | 1.13 | 5.92E-04 | 6/29 | 5.45 | 1.1 | 5.13E-04 |
| Ammonia Recycling | 6/32 | 4.80 | 1.25 | 0.00103 | 7/32 | 5.74 | 1.22 | 1.12E-04 |
| Glycine and Serine Metabolism | 8/59 | 3.48 | 2.3 | 0.00135 | 10/59 | 4.44 | 2.25 | 3.22E-05 |
| Methylhistidine Metabolism | 2/4 | 12.82 | 0.156 | 0.0085 | 2/4 | 13.16 | 0.152 | 0.00808 |
| Glucose-Alanine Cycle | 3/13 | 5.91 | 0.508 | 0.012 | 3/13 | 6.06 | 0.495 | 0.0112 |
| Phenylalanine and Tyrosine Metabolism | 4/28 | 3.67 | 1.09 | 0.0207 | 3/28 | 2.80 | 1.07 | 0.0867 |
| Methionine Metabolism | 5/43 | 2.98 | 1.68 | 0.0227 | 6/43 | 3.66 | 1.64 | 0.00443 |
| Glutamate Metabolism | 5/49 | 2.62 | 1.91 | 0.0378 | 6/49 | 3.21 | 1.87 | 0.00856 |
| Beta-Alanine Metabolism | 4/34 | 3.01 | 1.33 | 0.0397 | 4/34 | 3.10 | 1.29 | 0.0365 |
| Fatty Acid Biosynthesis | 4/35 | 2.92 | 1.37 | 0.0435 | 3/35 | 2.26 | 1.33 | 0.145 |
| Homocysteine Degradation | 2/9 | 5.68 | 0.352 | 0.045 | 2/9 | 5.83 | 0.343 | 0.043 |
| Glutathione Metabolism | 3/21 | 3.66 | 0.82 | 0.0451 | 4/21 | 5.00 | 0.8 | 0.00674 |
| Betaine Metabolism | 3/21 | 3.66 | 0.82 | 0.0451 | 3/21 | 3.75 | 0.8 | 0.0422 |
| Arginine and Proline Metabolism | 5/53 | 2.42 | 2.07 | 0.0506 | 6/53 | 2.97 | 2.02 | 0.0126 |
| Alanine Metabolism | 2/17 | 3.01 | 0.664 | 0.14 | 3/17 | 4.64 | 0.647 | 0.024 |
| Carnitine Synthesis | 2/22 | 2.33 | 0.859 | 0.211 | 3/22 | 3.58 | 0.838 | 0.0477 |

### 6.4.7 Patient characteristics for study of relationship between urinary metabolite profile and FACIT-F

A similar approach was utilised to investigate the relationship between FACIT-F and the urinary metabolite profile. The diagnoses and characteristics of this cohort are described in Table 5-16, Table 5-18, Table 5-19 and Table 5-17. The median FACIT-F value for the entire population was 32 (IQR 21-41). Figure 6-16 shows the distribution of FACIT-F scores across the whole cohort and in the four largest diagnostic subgroups used in urinary metabolomics analysis.

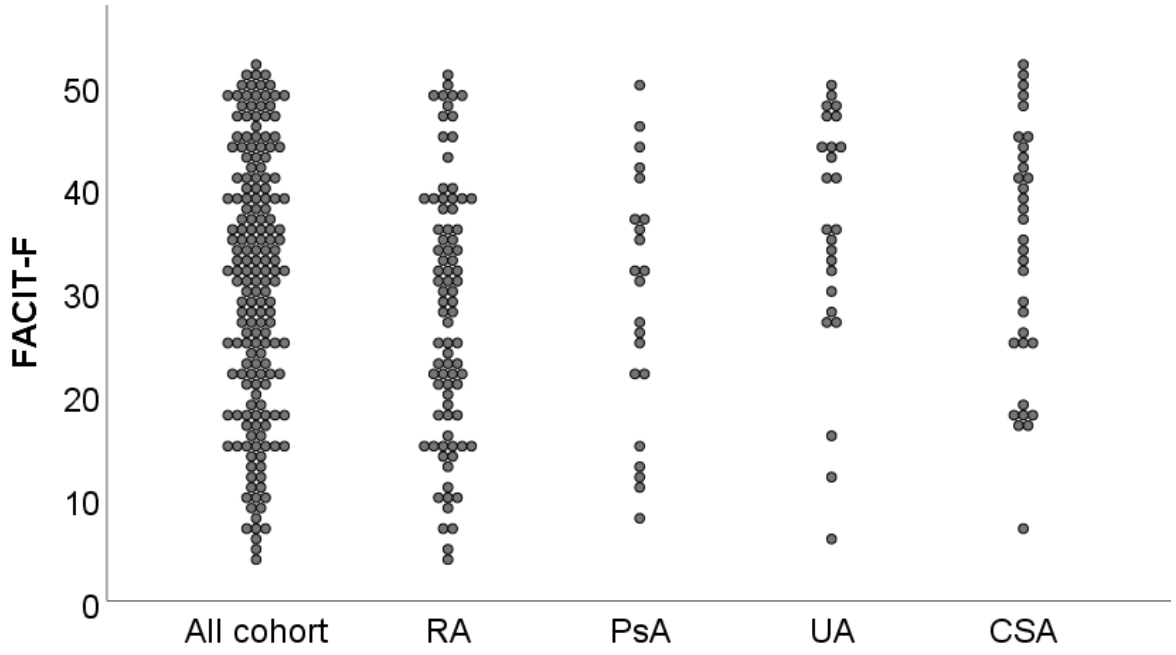



**Figure 6-16 Scatter plot showing the distribution of FACIT-F values across the whole population and diagnostic subgroups used in the urinary metabolomics analysis**

### 6.4.8 Relationship between urinary metabolite profile and FACIT-F in the whole cohort

A PCA and OPLS-DA analysis showed no separation between those patients who scored in the highest and lowest FACIT-F tertile (Figure 6-17). PLS-R analysis, however, revealed a correlation between urinary metabolite profile and fatigue measured by FACIT-F. Figure 6-18A shows a post forward selection PLS-R analysis with an $r^2$ value of 0.2660 (4 LV and $p<0.001$) using all urinary NMR bins. Similarly, Figure 6-18B shows a post forward selection PLS-R analysis with an $r^2$ value of 0.1967 (10 LV and $p<0.001$) using urinary metabolome without the urinary NMR bins which correlate with CRP. Table 6-20 show the potential biomarkers for these analyses.

Pathway and enrichment analyses for the biomarkers implicated in the PLS-R analysis between FACIT-F and all patients' urine metabolome and the urine metabolome without the bins that strongly correlate with CRP are shown in Figure 6-19, Table 6-21 and Figure 6-20, Table 6-22 respectively. Enrichment analyses showed methylhistidine metabolism, urea cycle and beta-alanine metabolism

were the most overrepresented pathways. Alanine, aspartate and glutamate metabolism was the most impacted pathway across both pathway analyses.

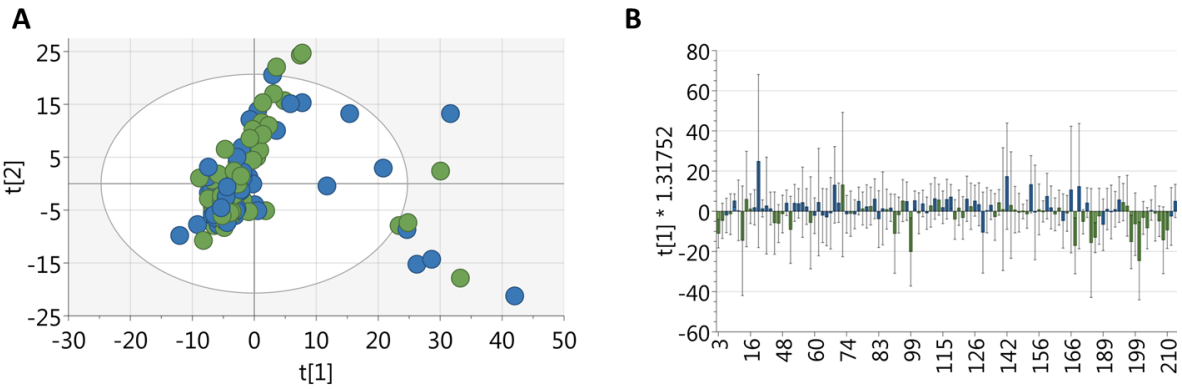



**Figure 6-17 Multivariate analysis of urinary metabolite profile by FACIT-F tertiles with data shown for the highest and lowest tertiles**

**(A)** PCA plot of metabolic data derived from all patients' (n=178) urine (green= FACIT-F<25 and blue=FACIT-F>37; 18 PC, $r^2$=0.664) showing no separation between the two groups. **(B)** OPLS-DA plot of metabolic data derived from all patients' (n=178) urine (green= FACIT-F<25 and blue=FACIT-F>37; 1+0+0 LV, P value=1) showing no separation between highest FACIT-F tertile and the lowest FACIT-F tertile.

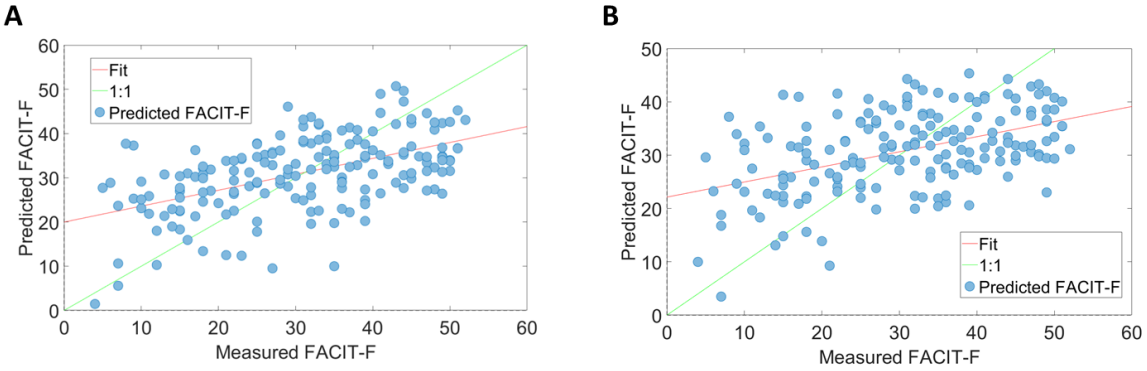



**Figure 6-18 PLS-R analysis of urinary metabolites and FACIT-F**

All models shown are post forward selection. PLS-R for all patients' (n=178) urinary metabolite profile and FACIT-F. Using the full 900 urinary metabolite binned data (**A**) there was a correlation between FACIT-F and urinary metabolite profile (59 NMR bins post forward selection, $r^2$=0.2660, 4 LV, p<0.001). A PLS-R undertaken on 681 NMR bins urinary metabolite binned data (**B**), that is with the 219 bins correlated with CRP removed, showed a correlation between FACIT-F and urinary metabolite profile (21 NMR bins post forward selection, $r^2$=0.1967, 10 LV, p<0.001). Indicating that the correlation between fatigue and urinary metabolite profile may be independent from the relationship between inflammation (measured by CRP) and metabolism.

**Table 6-20 Metabolites responsible for the relationship between FACIT-F and urinary metabolite profile in all patients**

Results from PLS-R models using the complete metabolite dataset and the urinary metabolome devoid of the inflammatory signal in PLS-R analysis. The following metabolites have been ranked by the magnitude of the regression coefficient. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of regression coefficient. The regression coefficient field indicates the nature of correlation (↑ indicating a positive relationship with FACIT-F and ↓ indicating a negative relationship with FACIT-F)

| | | | PLS-R model using all serum metabolome | | PLS-R model using serum metabolome devoid of the inflammatory signal | |
|---|---|---|---|---|---|---|
| Order in PLS-R using all serum metabolome | Order in PLS-R using serum metabolome devoid of the inflammatory signal | Metabolite | Chemical shift of peak (ppm) implicated in PLS-R Model | Regression coefficient | Chemical shift of peak (ppm) implicated in PLS-R Model | Regression coefficient |
| 1 | 2 | Cystine | 3.3733 | ↓ | 3.3733, 3.3967, 3.145 | ↓↑↓ |
| 1 | 2 | 4-Hydroxyproline | 3.3733, 2.4016 | ↓↑ | 3.3733, 2.4016, 3.3967, 2.3314 | ↓↑↑↓ |
| 2 | 7 | Histidine | 7.8707, 7.8727, 7.2074, 7.9301, 7.2132 | ↑↑↓↑↓ | 7.2015, 7.9301 | ↓↑ |
| 2 | 1 | Benzoic acid | 7.8707, 7.8619, 7.8571, 7.8727, 7.5176, 7.9301, 7.5645 | ↑↑↑↑↓↑↑ | 7.5645, 7.8619, 7.8571, 7.9301, 7.5703, 7.5176 | ↑↑↑↑↑↓ |
| 2 | 3 | 7-Methylxanthine | 7.8707, 7.8619, 7.8571, 7.6874, 7.8727, 7.7284, 7.9301, 7.7518 | ↑↑↑↑↑↑↑↓ | 7.8619, 7.6874, 7.8571, 7.9301 | ↑↑↑↑ |
| 2 | 1 | Hippuric acid | 7.8707, 7.8619, 7.8571, 7.6874, 7.8727, 7.5176, 7.7284, 7.9301, 7.5645, 7.7986, 7.7518 | ↑↑↑↑↑↓↑↑↑↑↓ | 7.5645, 7.8619, 7.6874, 7.8571, 7.9301, 7.5703, 7.5176 | ↑↑↑↑↑↑↓ |
| 3 | 4 | Alanine | 1.4369, 1.4416, 1.4235 | ↓↓↓ | 1.4369, 1.4416, 1.4235, 1.4475 | ↓↓↓↓ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 3 | 4 | Lysine | 1.4369, 1.4416, 1.4235, 1.9392, 1.4109, 1.9275, 1.9216, 1.8455, 1.4003, 1.6582 | ↓↓↓↑↓↑↑↓↓↓ | 1.4369, 1.4416, 1.4109, 1.4235, 1.9216, 1.9392, 1.4003, 1.4475 | ↓↓↓↓↑↑↓↓ |
| 3 | 4 | 3-Methyl-2-oxovaleric acid | 1.4369, 1.127, 1.4416, 1.4235, 1.1303 | ↓↓↓↓↓ | 1.4369, 1.4416, 1.4235, 1.0904, 0.89724, 1.4475 | ↓↓↓↓↓↓ |
| 3 | 4 | Isoleucine | 1.4369, 1.9684, 1.9626, 1.4416, 1.4235, 1.986, 1.9802 | ↓↓↓↓↓↑↑ | 1.4369, 1.9626, 1.4416, 1.986, 1.4235, 1.225, 0.89724, 1.4475 | ↓↓↓↑↓↑↓↓ |
| 4 | N/A | Ethanol | 1.127, 1.1303, 1.1468 | ↓↓↑ | N/A | N/A |
| 4 | 12 | 3-Aminoisobutanoic acid | 1.127, 2.6416, 2.6475, 1.1303, 2.665, 1.2075, 1.1468, 2.6358 | ↓↓↓↓↓↑↑↓ | 1.2075, 2.5655, 2.665, 1.225, 1.2133, 1.0904 | ↑↑↓↑↑↓ |
| 4 | N/A | Propylene glycol | 1.127, 1.1303, 1.1468 | ↓↓↑ | N/A | N/A |
| 5 | 9 | L-Kynurenine | 7.8571, 7.5176, 6.6337, 7.9301, 6.8854 | ↑↓↑↑↑ | 7.8571, 7.4006 | ↑↑ |
| 5 | 1 | Tryptophan | 7.6874, 7.5176, 7.7284, 7.2952, 7.2191, 7.2074, 7.5645, 7.2132, 7.3069, 7.225, 7.7518 | ↑↓↑↑↓↓↑↓↑↓↓ | 7.5645, 7.6874, 7.2015, 7.5703, 7.5176 | ↑↑↓↑↓ |
| 5 | 6 | Indoxyl sulfate | 7.6874, 7.5176, 7.7284, 7.2952, 7.2191, 7.2074, 7.2132, 7.225 | ↑↓↑↑↓↓↓↓ | 7.6874, 7.2015, 7.5176 | ↑↓↓ |
| 5 | 1 | Urea | 7.6874, 7.5176, 7.7284, 7.2952, 6.6337, 7.2191, 7.2074, 7.9301, 7.5645, 7.2132, 7.3069, 6.6385, 6.8854, 7.225, 5.3986, 7.7518, 5.4045 | ↑↓↑↑↑↓↓↑↑↓↑↑↑↑↓↓↓ | 7.5645, 7.6874, 7.2015, 6.6337, 7.9301, 7.9684, 5.4045, 7.5703, 5.3986, 5.4103, 7.5176 | ↑↑↓↑↑↓↓↑↓↓↓ |
| 5 | 6 | 3-methylhistidine | 7.6874, 7.7284, 7.7518 | ↑↑↓ | 7.6874, 3.145 | ↑↓ |
| 5 | 6 | Phenylglyoxylic acid | 7.6874, 7.7285, 7.9301, 7.7518 | ↑↑↑↓ | 7.6874, 7.9301, 7.9684, 7.5703 | ↑↑↓↑ |
| 6 | 5 | Glutamate | 1.9684, 1.9626, 2.4016, 1.9392, 1.9275, 1.986, 1.9802 | ↓↓↑↑↑↑↑ | 1.9626, 2.4016, 1.986, 1.9392, 2.3314 | ↓↑↑↑↓ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 6 | 5 | Citraconic acid | 1.9684, 1.9626, 1.9392, 1.9275 | ↓↓↑↑ | 1.9626, 1.9392 | ↓↑ |
| 6 | 5 | L-2-Hydroxyglutaric acid | 1.9684, 1.9626, 1.9392, 1.9275, 1.986, 1.9216, 1.8455, 1.9802 | ↓↓↑↑↑↑↓↑ | 1.9626, 1.986, 1.9216, 1.9392, 0.89724, 2.3314 | ↓↑↑↑↓↓ |
| 6 | 5 | Methylglutaric acid | 1.9684, 1.9626, 1.9392, 1.986, 1.9802 | ↓↓↑↑↑ | 1.9626, 1.986, 1.9392, 0.89724 | ↓↑↑↓ |
| 6 | 14 | Isovalerylglycine | 1.9684, 1.986, 1.9802 | ↓↑↑ | 1.986, 0.89724 | ↑↓ |
| 6 | 5 | N-Acetylputrescine | 1.9684, 1.9626, 1.986, 1.9802, 1.6582 | ↓↓↑↑↓ | 1.9626, 1.986 | ↓↑ |
| 6 | 5 | Arginine | 1.9684, 1.9626, 1.9392, 1.9275, 1.9216, 1.8455, 1.9802, 1.6582 | ↓↓↑↑↑↓↑↓ | 1.9626, 1.986, 1.9216, 1.9392 | ↓↑↑↑ |
| 7 | 1 | Vanillic acid | 7.5176, 7.5645 | ↓↑ | 7.5645, 7.5703, 7.5176 | ↑↑↓ |
| 7 | 1 | Indoleacetate | 7.5176, 7.2952, 7.2191, 7.2074, 7.5645, 7.2132, 7.3069, 7.225 | ↓↑↓↓↑↓↑↑ | 7.5645, 7.2015, 7.5703, 7.5176 | ↑↓↑↓ |
| 7 | 1 | Uracil | 7.5176, 7.5645 | ↓↑ | 7.5645, 7.5703, 7.5176 | ↑↑↓ |
| 7 | 24 | 4-Pyridoxic acid | 7.5176 | ↓ | 2.3314, 7.5176 | ↓↓ |
| 7 | 1 | Phenylalanine | 7.5176, 7.2952, 7.2191, 7.2074, 7.5645, 7.2132, 7.3069, 7.225 | ↓↑↓↓↑↓↑↓ | 7.5645, 7.2015, 7.4006, 7.5703, 7.5176, 3.145 | ↑↓↑↑↓↓ |
| 7 | 25 | Cytosine | 7.5176 | ↓ | 7.5176 | ↓ |
| 7 | 1 | Picolinic acid | 7.5176, 7.9301, 7.5645 | ↓↑↑ | 7.5645, 7.9301, 7.9684, 7.5703, 7.5176 | ↑↑↓↑↓ |
| 7 | 1 | Cinnamic acid | 7.5176, 7.5645 | ↓↑ | 7.5645, 7.5703, 7.5176 | ↑↑↓ |
| 8 | 11 | Pyroglutamic acid | 2.4016, 1.986, 1.9802 | ↑↑↑ | 2.4016, 1.986 | ↑↑ |
| 8 | 11 | Succinylacetone | 2.4016 | ↑ | 2.4016 | ↑ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 8 | 11 | Monomethyl glutaric acid | 2.4016, 1.8455 | ↑↓ | 2.4016 | ↑ |
| 8 | 8 | Levulinic acid | 2.4016, 2.7704 | ↑↑ | 2.7704, 2.4016 | ↑↑ |
| 8 | 10 | 3-Hydroxymethylglutaric acid | 2.4016, 1.2777, 1.348 | ↑↓↓ | 1.2777, 2.4016 | ↓↑ |

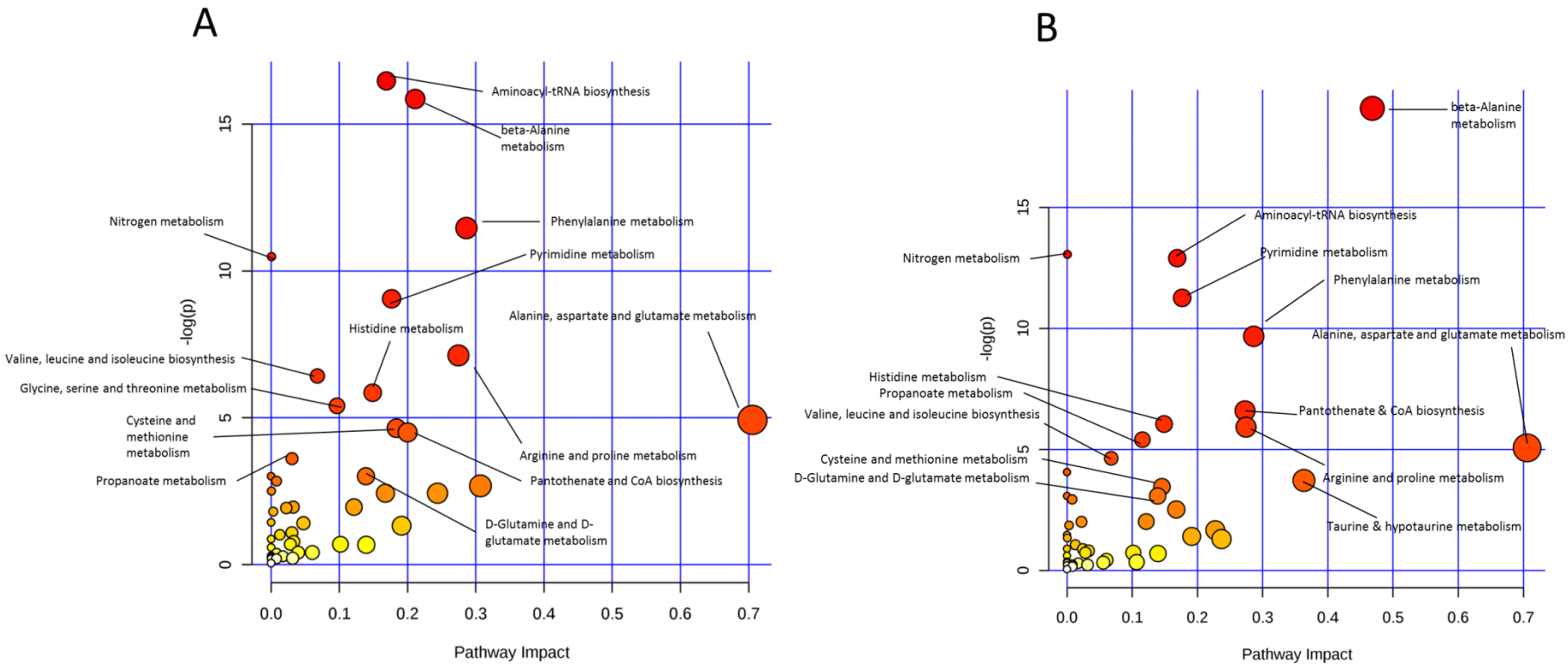



**Figure 6-19 Pathway analysis of potential biomarkers implicated by PLS-R analysis of FACIT-F and all patients' urinary metabolites**
Pathway analysis of potential biomarkers implicated by the PLS-R of (A) all the urinary metabolome and (B) the urinary metabolome devoid of the inflammatory signal.

**Table 6-21 Pathway analysis of potential biomarkers implicated by PLS-R analysis of FACIT-F and all patients' urinary metabolites**
Results from PLS-R models using all the urinary metabolome and the urinary metabolome devoid of the inflammatory signal.
**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.
**The impact score**: Each pathway has a total importance score of 1. The importance measure of each metabolite node (a point where pathways intersect within a larger network) is expressed as a fraction with regards to the total pathway importance, and the pathway impact score is the cumulative score of matched metabolite nodes.
* p value calculated using hypergeometric test

| | PLS-R model using all urinary metabolome | | | PLS-R model using urinary metabolome devoid of the inflammatory signal | | |
|---|---|---|---|---|---|---|
| Pathway name | Match status | P value* | Impact score | Match status | P value* | Impact score |
| Alanine, aspartate and glutamate metabolism | 4/24 | 0.007243 | 0.70561 | 4/24 | 0.006324 | 0.70561 |
| Phenylalanine metabolism | 9/45 | 1.05E-05 | 0.28632 | 8/45 | 6.30E-05 | 0.28632 |
| Arginine and proline metabolism | 9/77 | 8.06E-04 | 0.27458 | 8/77 | 0.002684 | 0.27458 |
| beta-Alanine metabolism | 9/28 | 1.30E-07 | 0.21128 | 10/28 | 5.13E-09 | 0.46822 |
| Pantothenate and CoA biosynthesis | 4/27 | 0.011093 | 0.20016 | 5/27 | 0.001367 | 0.27302 |
| Cysteine and methionine metabolism | 6/56 | 0.009615 | 0.18372 | 5/56 | 0.031452 | 0.14566 |
| Pyrimidine metabolism | 9/60 | 1.17E-04 | 0.17665 | 10/60 | 1.29E-05 | 0.17665 |
| Aminoacyl-tRNA biosynthesis | 14/75 | 7.03E-08 | 0.16902 | 12/75 | 2.50E-06 | 0.16902 |
| Histidine metabolism | 6/44 | 0.002871 | 0.14893 | 6/44 | 0.00236 | 0.14893 |
| D-Glutamine and D-glutamate metabolism | 2/11 | 0.0494 | 0.13904 | 11/19 | 0.046086 | 0.13904 |
| Glycine, serine and threonine metabolism | 6/48 | 0.004489 | 0.09661 | 5/48 | 0.01718 | 0 |
| Valine, leucine and isoleucine biosynthesis | 5/27 | 0.001625 | 0.06784 | 4/27 | 0.009713 | 0.06784 |
| Propanoate metabolism | 4/35 | 0.027085 | 0.03068 | 5/35 | 0.0045 | 0.11568 |
| Nitrogen metabolism | 8/39 | 2.79E-05 | 6.70E-04 | 9/39 | 2.14E-06 | 6.70E-04 |
| Taurine and hypotaurine metabolism | 2/20 | 0.14111 | 0.03237 | 3/20 | 0.024252 | 0.36331 |

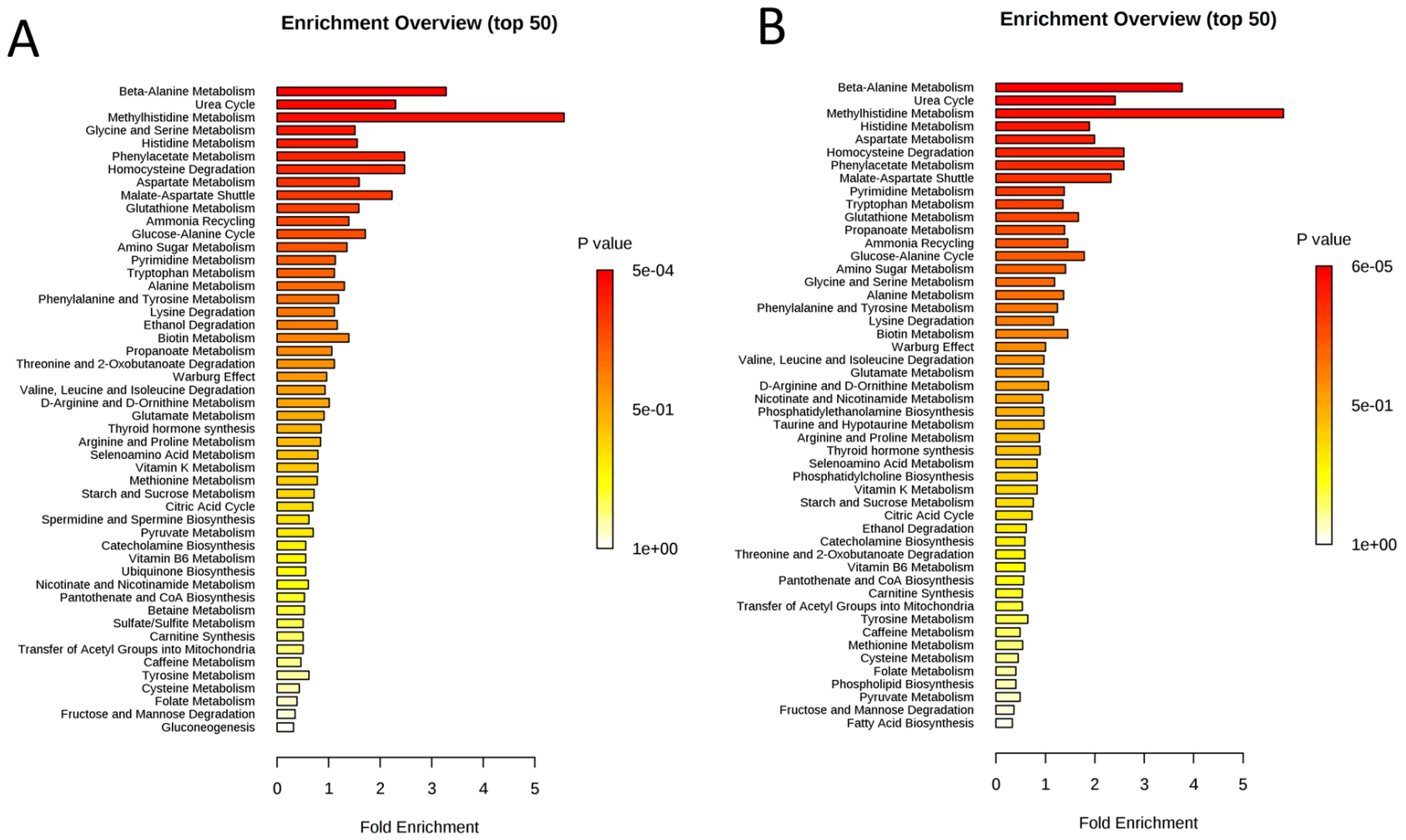



**Figure 6-20 Enrichment analysis of key metabolites in urine implicated as potential biomarkers by the PLS-R analysis of FACIT-F and all patients' urinary metabolites**

Enrichment analysis of potential biomarkers implicated by the PLS-R of (A) all the urinary metabolome and (B) the urinary metabolome devoid of the inflammatory signal

**Table 6-22 Enrichment analysis of key metabolites in urine implicated as potential biomarkers by the PLS-R analysis of FACIT-F and all patients' urinary metabolites**

Results from PLS-R models using all the urinary metabolome and the urinary metabolome devoid of the inflammatory signal

**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.

**The fold enrichment value**: the value is generated by the number of matched metabolites divided by the number of metabolites expected to be present in the pathway as per the reference metabolome provided by KEGG.

* p value calculated using hypergeometric test

| | PLS-R model using all urinary metabolome | | | | PLS-R model using urinary metabolome devoid of the inflammatory signal | | | |
|---|---|---|---|---|---|---|---|---|
| Metabolite pathway | Match status | Fold enrichment | Expected | P value* | Match status | Fold enrichment | Expected | P value* |
| Beta-Alanine Metabolism | 10/34 | 3.28 | 3.05 | 4.51E-04 | 11/34 | 3.77 | 2.92 | 5.61E-05 |
| Urea Cycle | 6/29 | 2.30 | 2.61 | 0.0389 | 6/29 | 2.41 | 2.49 | 0.032 |
| Methylhistidine Metabolism | 2/4 | 5.57 | 0.359 | 0.0425 | 2/4 | 5.81 | 0.344 | 0.0391 |

### 6.4.9 Relationship between urinary metabolite profile and FACIT-F in the RA patients

PCA and OPLS-DA were undertaken for RA (Figure 6-21A), PsA (Figure 6-21B) , UA (Figure 6-21C) and CSA (Figure 6-21D) which showed no statistically significant separation between those patients whose FACIT-F score was in the lowest tertile and those who score was in the highest tertile.

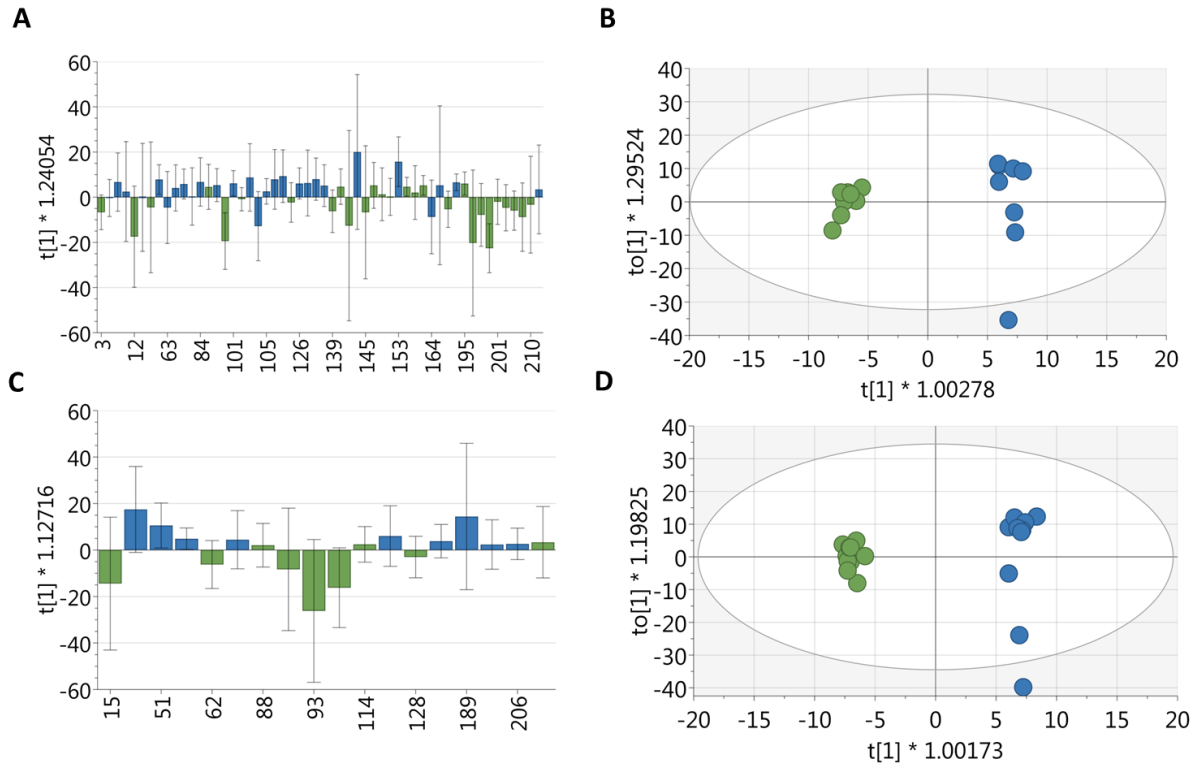



**Figure 6-21 Multivariate analysis of urinary metabolite profile by FACIT-F tertiles and diagnosis with data shown for the highest and lowest tertiles by diagnosis**
Across all diagnoses OPLS-DA showed a separation between patients in the highest FACIT-F tertile compared to patients in the lowest FACIT-F tertile. These relationships were not statistically significant. **(A)** OPLS-DA plot of metabolic data derived from RA patients' (n=83) urine (green= FACIT-F<22 and blue=FACIT-F>34; 1+0+0 LV P value= 1). **(B)** OPLS-DA plot of metabolic data derived from PsA patients' (n=22) urine (green= FACIT-F<26 and blue= FACIT-F>35; 1+3+0 LV P value= 0.38352). **(C)** OPLS-DA plot of metabolic data derived from UA patients' (n=26) (green= FACIT-F<33 and blue= FACIT-F>43; 1+0+0 LV P value= 0.57366). **(D)** OPLS-DA plot of metabolic data derived from CSA patients' (n=32) urine (green= FACIT-F<27 and blue= FACIT-F>41; 1+4+0 LV P value=0.07893).

The relationship between urinary metabolome and FACIT-F was investigated further with PLS-R (Figure 6-22). For each diagnosis there are two PLS-R's, one using all the urinary metabolome (sub labelled 1) before applying forward selection and the other using only urinary metabolome devoid of the NMR bins which strongly correlate with CRP (sub labelled 2) before forward selection was applied.

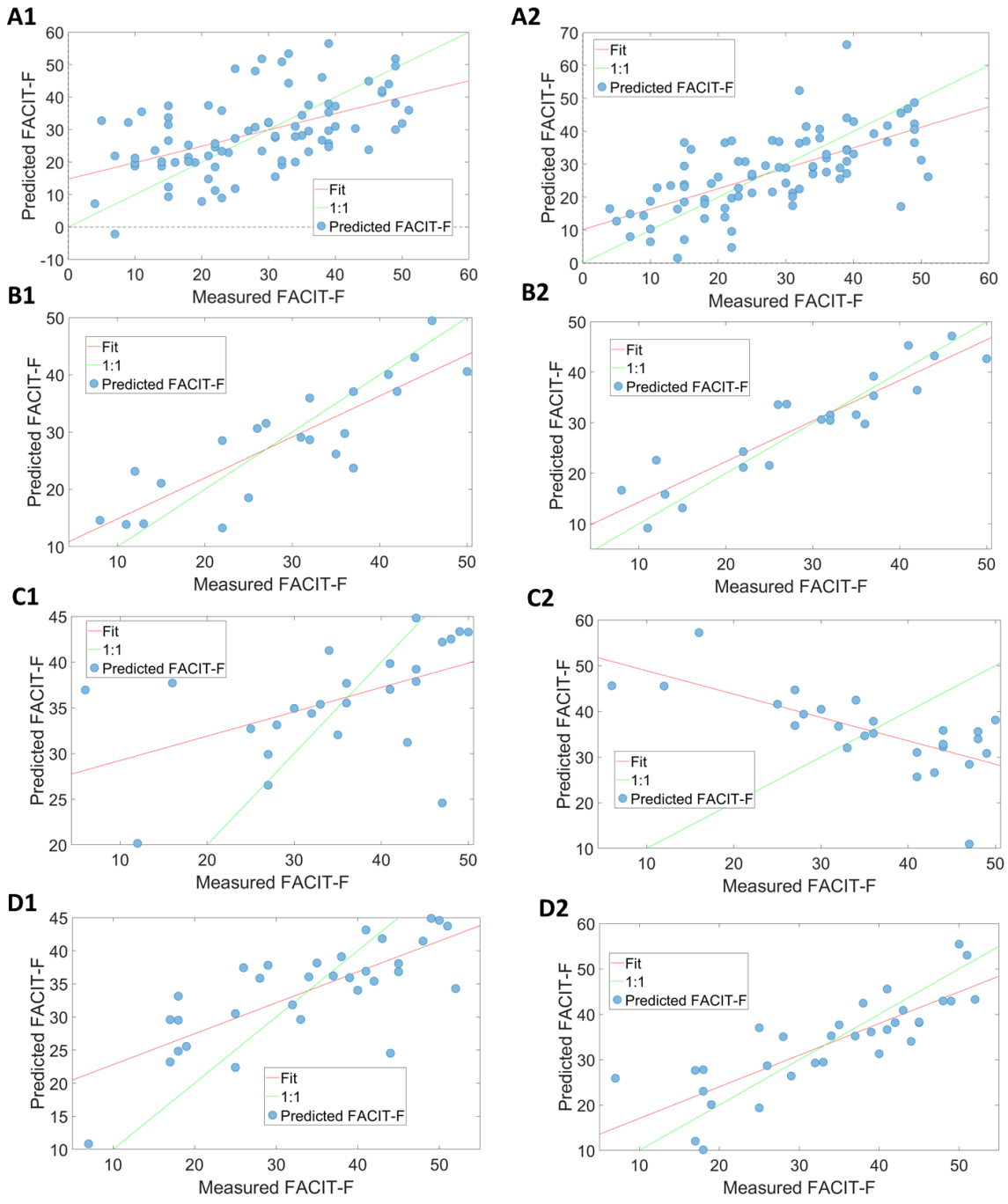



**Figure 6-22 PLS-R analysis of urinary metabolites and FACIT-F by diagnosis**

All PLS-R models shown are post forward selection. All figures sub labelled "1" show PLS-R analyses using the full 900 NMR bins urinary metabolite data before forward selection. All figures sub labelled "2" show PLS-R analyses using 681 bins (the urinary metabolite NMR bins which correlate with CRP removed) before forward selection application. A correlation between FACIT-F and all urinary metabolite profile and urinary metabolite profile with the CRP signal removed existed for RA, CSA and PsA patients. **(A1)** PLS-R of RA patients (n=83) showed a correlation between FACIT-F and the urinary metabolite profile (101 NMR bins post forward selection, $r^2$=0.3207, 7 LV, p<0.001) and the **(A2)** urinary metabolite profile with CRP signal removed (93 NMR bins post forward selection, $r^2$=0.5124, 10 LV, p<0.001). **(B1)** PLS-R of PsA patients (n=22) showed a correlation between FACIT-F and the urinary metabolite profile (46 NMR bins post forward selection, $r^2$=0.6922, 8 LV, p=0.003) and **(B2)** the urinary metabolite profile with CRP signal removed

(16 NMR bins post forward selection, $r^2$=0.7954, 5 LV, p=0.001). **(C1)** PLS-R of UA patients (n=26) showed no statistically significant correlation between FACIT-F and the urinary metabolite profile (137 NMR bins post forward selection, $r^2$=0.3111, 6 LV, p=0.087). **(C2)** Furthermore, there was no correlation between FACIT-F and the urinary metabolite profile with the CRP signal removed in UA patients (670 NMR bins post forward selection, $r^2$=0.3531, 2 LV, p=0.070). **(D1)** PLS-R of CSA patients (n=32) showed a correlation between FACIT-F and the urinary metabolite profile (58 NMR bins post forward selection, $r^2$=0.5527, 2 LV, p=0.004) and **(D2)** the urinary metabolite profile with CRP signal removed (36 NMR bins post forward selection, $r^2$=0.6147, 4 LV, p=0.002).

There was a statistically significant correlation between FACIT-F and the complete urinary metabolite dataset (Figure 6-22A1- 101 NMR bins, $r^2$=0.3207, 7 LV, p<0.001) in RA patients. A relationship was also seen between the urinary metabolite dataset devoid of the NMR bins that correlate strongly with CRP and FACIT-F in RA patients (Figure 6-22A2- 93 NMR bins, $r^2$=0.5124, 10 LV, p<0.001). Table 6-23 shows the potential biomarkers for these correlations.

Pathway and enrichment analyses for the biomarkers implicated in the PLS-R analysis between FACIT-F and RA patients' urine metabolome and the urine metabolome without the bins that strongly correlate with CRP are shown in Figure 6-24, Table 6-24 and Figure 6-25, Table 6-25 respectively. Alanine, aspartate and glutamate metabolism was the most impacted pathway across both pathway analyses. Methylhistidine metabolism was the most overrepresented pathway in the enrichment analyses. Although methylhistidine metabolism was the only pathway which was overrepresented in the analysis of all the urinary metabolome of RA patients, in the analysis using the urinary metabolome devoid of CRP revealed glucose alanine, urea cycle and beta-alanine metabolism, amongst others, which have been overrepresented.

**Table 6-23 Metabolites responsible for the relationship between FACIT-F and urinary metabolite profile in RA patients**

Results from PLS-R models using the complete metabolite dataset and the urinary metabolome devoid of the inflammatory signal in PLS-R analysis. The following metabolites have been ranked by the magnitude of the regression coefficient. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of regression coefficient. The regression coefficient field indicates the nature of correlation (↑ indicating a positive relationship with FACIT-F and ↓ indicating a negative relationship with FACIT-F)

| | | | PLS-R model using all serum metabolome | | PLS-R model using serum metabolome devoid of the inflammatory signal | |
|---|---|---|---|---|---|---|
| Order in PLS-R using all serum metabolome | Order in PLS-R using serum metabolome devoid of the inflammatory signal | Metabolite | Chemical shift of peak (ppm) implicated in PLS-R Model | Regression coefficient | Chemical shift of peak (ppm) implicated in PLS-R Model | Regression coefficient |
| 1 | 3 | Alanine | 1.4235, 1.4202, 1.4369 | ↓↓↓ | 1.4235, 1.4202 | ↓↓ |
| 1 | 3 | Lysine | 1.4235, 1.9275, 1.9392, 1.4202, 1.4109, 1.945, 1.8514, 1.4369 | ↓↑↑↓↓↑↑↓ | 1.4235, 1.9392, 1.4202, 1.9275, 3.0162 | ↓↑↓↑↓ |
| 1 | 3 | 3-Methyl-2-oxovaleric acid | 1.4235, 1.4202, 0.90621, 1.0588, 1.0703, 1.4369 | ↓↓↑↑↑↓ | 1.4235, 1.4202, 0.90621 | ↓↓↑ |
| 1 | 2 | Isoleucine | 1.4235, 1.9684, 1.9626, 0.90621, 1.986, 1.945, 1.9509, 1.4369 | ↓↓↓↑↑↑↑↓ | 1.9626, 1.4235, 0.90621, 1.9509, 1.9919 | ↓↓↑↑↓ |
| 2 | 2 | Glutamate | 1.9684, 1.9275, 1.9392, 2.3958, 1.9626, 1.986, 1.945, 1.9509, 2.0387 | ↓↑↑↓↓↑↑↑↑ | 1.9626, 1.9392, 2.3958, 1.9509, 1.9919 | ↓↑↑↑↓ |
| 2 | 2 | Citraconic acid | 1.9684, 1.9275, 1.9392, 1.9626, 1.945, 1.9509 | ↓↑↑↓↑↑ | 1.9626, 1.9392, 1.9275, 1.9509 | ↓↑↑↑ |
| 2 | 2 | L-2-Hydroxyglutaric acid | 1.9684, 1.9275, 1.9392, 1.9626, 1.986, 1.945, 1.9509, 1.8514, 2.0387 | ↓↑↑↓↑↑↑↑↑ | 1.9626, 1.9392, 1.9275, 2.0094, 1.9509, 1.9919 | ↓↑↑↓↑↓ |
| 2 | 2 | Methylglutaric acid | 1.9684, 1.9392, 1.9626, 0.90621, 1.986, 1.945, 1.9509 | ↓↑↓↑↑↑↑ | 1.9626, 1.9392, 2.0094, 0.90621, 1.9509, 1.9919 | ↓↑↓↑↑↓ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 2 | 9 | Isovalerylglycine | 1.9684, 0.90621, 1.986 | ↓↑↑ | 2.0094, 0.90621, 1.9919 | ↓↑↓ |
| 2 | 2 | N-Acetylputrescine | 1.9684, 1.9626, 1.986, 1.945, 1.9509 | ↓↓↑↑↑ | 1.9626, 2.0094, 1.9509, 1.9919 | ↓↓↑↓ |
| 2 | 2 | Arginine | 1.9684, 1.9275, 1.9392, 1.9626, 1.986, 1.945, 1.9509, 1.8514 | ↓↑↑↓↑↑↑↑ | 1.9626, 1.9392, 1.9275, 1.9509, 1.9919 | ↓↑↑↑↓ |
| 3 | 1 | Urea | 7.9859, 7.7518, 6.9967, 7.5176, 7.7284, 7.4825, 7.9631, 6.9908, 7.7576, 7.143, 7.1489, 7.1547, 7.9801, 7.0318, 7.947, 7.6874, 7.1606, 7.3947, 7.4884, 6.7962, 6.6337 | ↑↓↑↓↑↑↑↑↓↓↓↓↑↓↓↑↓↑↑↑↑ | 7.9859, 7.9801, 7.6874, 7.947, 7.9301, 7.0318, 7.9332, 7.2015, 6.7962, 7.9631, 7.5176 | ↑↑↑↓↑↓↑↓↑↑↓ |
| 3 | 1 | Quinolinic acid | 7.9859, 7.9631, 7.9801 | ↑↑↑ | 7.9859, 7.9801, 7.9631 | ↑↑↑ |
| 3 | 1 | Phenylglyoxylic acid | 7.9859, 7.7518, 7.7284, 7.9631, 7.7576, 7.9801, 7.947, 7.6874 | ↑↓↑↑↓↑↓↑ | 7.9859, 7.9801, 7.6874, 7.947, 7.9301, 7.9332, 7.9631 | ↑↑↑↓↑↑↑ |
| 3 | 1 | Hippuric acid | 7.9859, 7.7518, 7.5176, 7.7284, 7.4825, 7.7576, 7.8619, 7.8707, 7.7928, 7.9801, 7.6874, 7.4884, 7.8727 | ↑↓↓↑↑↓↑↑↑↑↑↑↑ | 7.9859, 7.9801, 7.6874, 7.7928, 7.9301, 7.8619, 7.9332, 7.5176 | ↑↑↑↑↑↑↑↓ |
| 3 | 1 | Carnosine | 7.9859, 2.6592, 2.665, 7.143, 2.6884, 7.1489, 2.6533, 7.1547, 7.9801, 2.6475, 7.1606, 2.7236, 2.6123 | ↑↓↓↓↓↓↓↓↑↓↓↑↑ | 7.9859, 7.9801 | ↑↑ |
| 3 | 1 | Picolinic acid | 7.9859, 7.5176, 7.9631, 7.9801, 7.947 | ↑↓↑↑↓ | 7.9859, 7.9801, 7.947, 7.9301, 7.9332, 7.9631, 7.5176 | ↑↑↓↑↑↑↓ |
| 3 | 1 | Histidine | 7.9859, 6.9967, 6.9908, 7.143, 7.1489, 7.8707, 7.1547, 7.9801, 7.0318, 7.1606, 7.8888, 7.8727 | ↑↑↑↓↓↑↓↑↓↓↑↑ | 7.9859, 7.9801, 7.9301, 7.0318, 7.9332, 7.2015 | ↑↑↑↓↑↓ |
| 3 | 1 | 7-Methylxanthine | 7.9859, 7.7518, 7.7284, 7.7576, 7.8619, 7.8707, 7.9801, 7.6874, 7.8727 | ↑↓↑↓↑↑↑↑↑ | 7.9859, 7.9801, 7.6874, 7.9301, 7.8619, 7.9332 | ↑↑↑↑↑↑ |
| 3 | 1 | Benzoic acid | 7.9859, 7.5176, 7.4825, 7.8619, 7.8707, 7.9801, 7.4884, 7.8888, 7.8727 | ↑↓↑↑↑↑↑↑↑ | 7.9859, 7.9801, 7.9301, 7.8619, 7.9332, 7.5176 | ↑↑↑↑↑↓ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 3 | 1 | Kynurenic acid | 7.9859, 7.5176, 7.9801, 6.6337 | ↑↓↑↑ | 7.9859, 7.9801, 7.9301, 7.9332, 7.5176 | ↑↑↑↑↓ |
| 4 | 4 | Acetate | 1.9275, 1.9392, 1.945, 1.9509 | ↑↑↑↑ | 1.9392, 1.9275, 1.9509 | ↑↑↑ |
| 4 | 5 | Symmetric dimethylarginine | 1.9275, 6.7918, 6.7962 | ↑↑↑ | 1.9275, 6.7962 | ↑↑ |
| 4 | 5 | 3-Methyladipic acid | 1.9275, 2.3958 | ↑↓ | 1.9275, 2.3958 | ↑↓ |
| 4 | 4 | Thymidine | 1.9275, 1.9392, 2.3958, 1.8514, 3.8357 | ↑↑↓↑↑ | 1.9392, 1.9275, 2.3958 | ↑↑↓ |
| 4 | 5 | Aminoadipic acid | 1.9275, 2.3958, 1.8514, 2.0387 | ↑↓↑↑ | 1.9275, 2.3958 | ↑↓ |
| 5 | 7 | 4-Hydroxyproline | 2.3958, 3.3733 | ↓↓ | 2.3958, 2.4719, 3.3616, 2.4894 | ↓↑↓↑ |
| 5 | 7 | Pyroglutamic acid | 2.3958, 1.986, 2.0387 | ↓↑↑ | 2.3958, 2.0094, 2.4719, 2.4894, 1.9919 | ↓↓↑↑↓ |
| 5 | 7 | Succinylacetone | 2.3958 | ↓ | 2.3958 | ↓ |
| 5 | 7 | Monomethyl glutaric acid | 2.3958, 1.8514 | ↓↑ | 2.3958 | ↓ |
| 5 | 7 | Levulinic acid | 2.3958, 2.7762, 2.747 | ↓↑↑ | 2.3958, 2.7645, 2.7411, 2.747 | ↓↑↑↑ |
| 5 | 7 | 3-Hydroxymethylglutaric acid | 2.3958 | ↓ | 2.3958, 2.4719 | ↓↑ |
| 5 | 7 | Glutamine | 2.3958 | ↓ | 2.3958, 2.0094, 2.4719, 2.4894 | ↓↓↑↑ |
| 5 | 7 | Pantothenic acid | 2.3958, 0.90621 | ↓↑ | 2.3958, 0.90621 | ↓↑ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 5 | 7 | Isocitrate | 2.3958, 2.5421, 2.548 | ↓↓↓ | 2.3958, 2.4719, 2.4894, 2.9753 | ↓↑↑↓ |
| 5 | 7 | Citrate | 2.3958, 2.6592, 2.7762, 2.5421, 2.665, 2.6884, 2.747, 2.6533, 2.6475, 2.548, 2.7236, 2.6123 | ↓↓↑↓↓↓↑↓↓↓↑↑ | 2.3958, 2.4719, 2.7645, 2.4894, 2.7411, 2.747 | ↓↑↑↑↑↑ |
| 5 | 5 | Glutaric acid | 2.3958, 1.8514 | ↓↑ | 1.9275, 2.3958 | ↑↓ |
| 5 | 7 | Succinate | 2.3958 | ↓ | 2.3958 | ↓ |
| 6 | 6 | Tryptophan | 7.7518, 7.5176, 7.7284, 7.7576, 7.1489, 7.1547, 7.6874, 7.1606 | ↓↓↑↓↓↓↑↓ | 7.6874, 7.2015, 7.5176 | ↑↓↓ |
| 6 | 6 | 3-methylhistidine | 7.7518, 6.9967, 7.7284, 6.9908, 7.7576, 7.0318, 7.6874 | ↓↑↑↑↓↓↑ | 7.6874, 7.0318 | ↑↓ |
| 7 | N/A | Aspartate | 2.6592, 2.665, 2.6884, 2.6533, 2.6475 | ↓↓↓↓↓ | N/A | N/A |
| 7 | N/A | Dihydrouracil | 2.6592, 2.665, 2.6884, 2.6533, 2.6475 | ↓↓↓↓↓ | N/A | N/A |
| 7 | N/A | Anserine | 2.6592, 2.665, 7.143, 2.6884, 2.6533, 2.6475, 2.7236 | ↓↓↓↓↓↓↑ | N/A | N/A |
| 7 | 8 | Creatinine | 2.6592, 2.7762, 2.665, 2.6884, 2.747, 2.6533, 2.6475, 2.7236, 3.0221, 2.6123 | ↓↑↓↓↑↓↓↑↓↑ | 3.0162, 3.0279, 2.8523, 2.7645, 2.7411, 2.747, 2.9753 | ↓↓↓↑↑↑↓ |
| 7 | N/A | 3-Aminoisobutanoic acid | 2.6592, 2.5421, 2.665, 1.1376, 2.6533, 2.6475, 2.548, 2.6123 | ↓↓↓↓↓↓↓↑ | N/A | N/A |
| 7 | N/A | Hydroxypropionic acid | 2.6592, 2.5421, 2.665, 2.6533, 2.6475, 2.548, 3.8357, 2.6123 | ↓↓↓↓↓↓↑↑ | N/A | N/A |
| 7 | 18 | L-Cystathionine | 2.6592, 2.7762, 2.665, 2.6884, 2.747, 2.6533, 2.6475, 2.7236, 3.8357 | ↓↑↓↓↑↓↓↑↑ | 2.7645, 2.7411, 2.747 | ↑↑↑ |
| 8 | 6 | 3-Methylhistidine | 6.9967, 7.0318 | ↑↓ | 7.6874, 7.9301, 7.0318, 7.9332 | ↑↑↓↑ |
| 8 | 21 | Vanillic acid | 6.9967, 7.5176, 7.4825, 6.9908, 7.4884 | ↑↓↑↑↑ | 7.5176 | ↓ |
| 8 | 12 | Phenol | 6.9967, 6.9908, 7.0318 | ↑↑↓ | 7.0318 | ↓ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 9 | 15 | Indoleacetate | 7.5176, 7.4825, 7.143, 7.1489, 7.1547, 7.1606, 7.4884 | ↓↑↓↓↓↓↑ | 7.2015, 7.5176 | ↓↓ |
| 9 | 21 | Uracil | 7.5176, 7.4884 | ↓↑ | 7.5176 | ↓ |
| 9 | 21 | 4-Pyridoxic acid | 7.5176 | ↓ | 7.5176 | ↓ |
| 9 | 12 | Phenylalanine | 7.5176, 7.4825, 7.143, 7.1489, 7.1547, 7.0318, 7.1606, 7.3947, 7.4884 | ↓↑↓↓↓↓↓↑↑ | 7.0318, 7.2015, 7.5176 | ↓↓↓ |
| 9 | 21 | Cytosine | 7.5176, 7.4825, 7.4884 | ↓↑↑ | 7.5176 | ↓ |
| 9 | 6 | Indoxyl sulfate | 7.5176, 7.7284, 7.4825, 7.6874, 7.4884 | ↓↑↑↑↑ | 7.6874, 7.2015, 7.5176 | ↑↓↓ |
| 9 | 21 | Cinnamic acid | 7.5176, 7.4825, 7.3947, 7.4884 | ↓↑↑↑ | 7.5176 | ↓ |
| 10 | 18 | Dihydrothymine | 2.7762, 2.747 | ↑↑ | 2.7645, 2.7411, 2.747 | ↑↑↑ |
| 10 | 14 | 5-Aminolevulinic acid | 2.7762, 2.747 | ↑↑ | 2.4719, 2.7645, 2.4894, 2.7411, 2.747 | ↑↑↑↑↑ |
| 10 | 18 | 2-Ketobutyric acid | 2.7762, 2.747, 1.0588, 1.0703 | ↑↑↑↑ | 2.7645, 2.7411, 2.747 | ↑↑↑ |
| 10 | 19 | Citramalic acid | 2.7762, 2.747 | ↑↑ | 2.7645, 2.4894, 2.7411, 2.747 | ↑↑↑↑ |
| 11 | 16 | L-Kynurenine | 6.7918, 7.3947, 6.7962 | ↑↑↑ | 6.7962 | ↑ |
| 11 | 16 | Homogentisic acid | 6.7918, 6.7962 | ↑↑ | 6.7962 | ↑ |
| 11 | 16 | 3-Hydroxyphenylacetate | 6.7918, 6.7962 | ↑↑ | 6.7962 | ↑ |
| 12 | N/A | trans-Aconitic acid | 6.9908 | ↑ | N/A | N/A |

| | | | | | | |
|---|---|---|---|---|---|---|
| 13 | N/A | Beta-Alanine | 2.5421, 2.548 | ↓↓ | N/A | N/A |
| 14 | 16 | Cystine | 3.3733 | ↓ | 3.3616 | ↓ |
| 15 | N/A | Ethanol | 1.1376 | ↓ | N/A | N/A |
| 15 | N/A | Propylene glycol | 1.1376 | ↓ | N/A | N/A |
| 16 | 11 | 2-hydroxybutyrate | 0.90621 | ↑ | 0.90621 | ↑ |
| 16 | 11 | Ethylmalonate | 0.90621 | ↑ | 0.90621 | ↑ |
| 16 | 11 | Leucine | 0.90621 | ↑ | 0.90621 | ↑ |
| 17 | 10 | 4-Hydroxybenzoic acid | 7.7576, 7.7928 | ↓↑ | 7.7928 | ↑ |
| 18 | 9 | 1,3-Diaminopropane | 1.986, 2.0387 | ↑↑ | 2.0094, 1.9919 | ↓↓ |
| 19 | 15 | p-Hydroxyphenylacetate | 7.143, 7.1489, 7.1547, 7.1606 | ↓↓↓↓ | 7.2015 | ↓ |
| 19 | N/A | p-Cresol sulfate | 7.143, 7.1489 | ↓↓ | N/A | N/A |
| 19 | N/A | Hydroxyphenyllactate | 7.143, 7.1489, 7.1547, 7.1606 | ↓↓↓↓ | N/A | N/A |
| 19 | 15 | Tyrosine | 7.143, 7.1489, 7.1547, 7.1606 | ↓↓↓↓ | 7.2015 | ↓ |
| 20 | 20 | Sarcosine | 2.747 | ↑ | 2.7411, 2.747 | ↑↑ |
| 21 | 19 | Methylsuccinate | 1.0588, 1.0703, 2.6123 | ↑↑↑ | 2.4894 | ↑ |
| 21 | N/A | Valine | 1.0588, 1.0703 | ↑↑ | N/A | N/A |
| 22 | 12 | Vanillylmandelic acid | 7.0318 | ↓ | 7.0318 | ↓ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 23 | N/A | Mandelic acid | 7.3947, 7.4884 | ↑↑ | N/A | N/A |
| 23 | 15 | Phenylacetate | 7.3947 | ↑ | 7.2015 | ↓ |
| 24 | N/A | Glutaconic acid | 6.6337 | ↑ | N/A | N/A |
| 25 | N/A | N-Acetylneuraminic acid | 2.0387, 3.8357 | ↑↑ | N/A | N/A |
| 26 | N/A | Serine | 3.8357 | ↑ | N/A | N/A |
| 26 | N/A | Sorbitol | 3.8357 | ↑ | N/A | N/A |
| 26 | N/A | D-Maltose | 3.8357 | ↑ | N/A | N/A |
| 26 | N/A | Glyceric acid | 3.8357 | ↑ | N/A | N/A |
| 26 | N/A | L-Arabitol | 3.8357 | ↑ | N/A | N/A |
| 26 | N/A | D-Galactose | 3.8357 | ↑ | N/A | N/A |
| 26 | N/A | Ethanolamine | 3.8357 | ↑ | N/A | N/A |
| 26 | N/A | D-Xylitol | 3.8357 | ↑ | N/A | N/A |
| 26 | N/A | Gluconic acid | 3.8357 | ↑ | N/A | N/A |
| 26 | N/A | L-Arabinose | 3.8357 | ↑ | N/A | N/A |
| 26 | N/A | Pseudouridine | 3.8357 | ↑ | N/A | N/A |
| 26 | N/A | Sucrose | 3.8357 | ↑ | N/A | N/A |
| 26 | N/A | Inosine | 3.8357 | ↑ | N/A | N/A |
| 26 | N/A | Dehydroascorbic acid | 3.8357 | ↑ | N/A | N/A |
| 27 | N/A | Methionine | 2.6123 | ↑ | N/A | N/A |

| | | | | | | |
|---|---|---|---|---|---|---|
| 27 | N/A | Methylamine | 2.6123 | ↑ | N/A | N/A |
| N/A | 13 | Asparagine | N/A | N/A | 2.8523 | ↓ |
| N/A | 14 | 5-Aminolevulinic acid | N/A | N/A | 2.4719, 2.7645, 2.4894, 2.7411, 2.747 | ↑↑↑↑↑ |
| N/A | 14 | Pyruvate | N/A | N/A | 2.4719, 2.4894 | ↑↑ |
| N/A | 14 | Carnitine | N/A | N/A | 2.4719 | ↑ |
| N/A | 14 | Oxoglutaric acid | N/A | N/A | 2.4719, 2.9753 | ↑↓ |
| N/A | 15 | Ortho-Hydroxyphenylacetate | N/A | N/A | 7.2015 | ↓ |
| N/A | 15 | Thymol | N/A | N/A | 7.2015 | ↓ |
| N/A | 17 | Methanol | N/A | N/A | 3.3616 | ↓ |
| N/A | 19 | Dimethylamine | N/A | N/A | 2.4894 | ↑ |

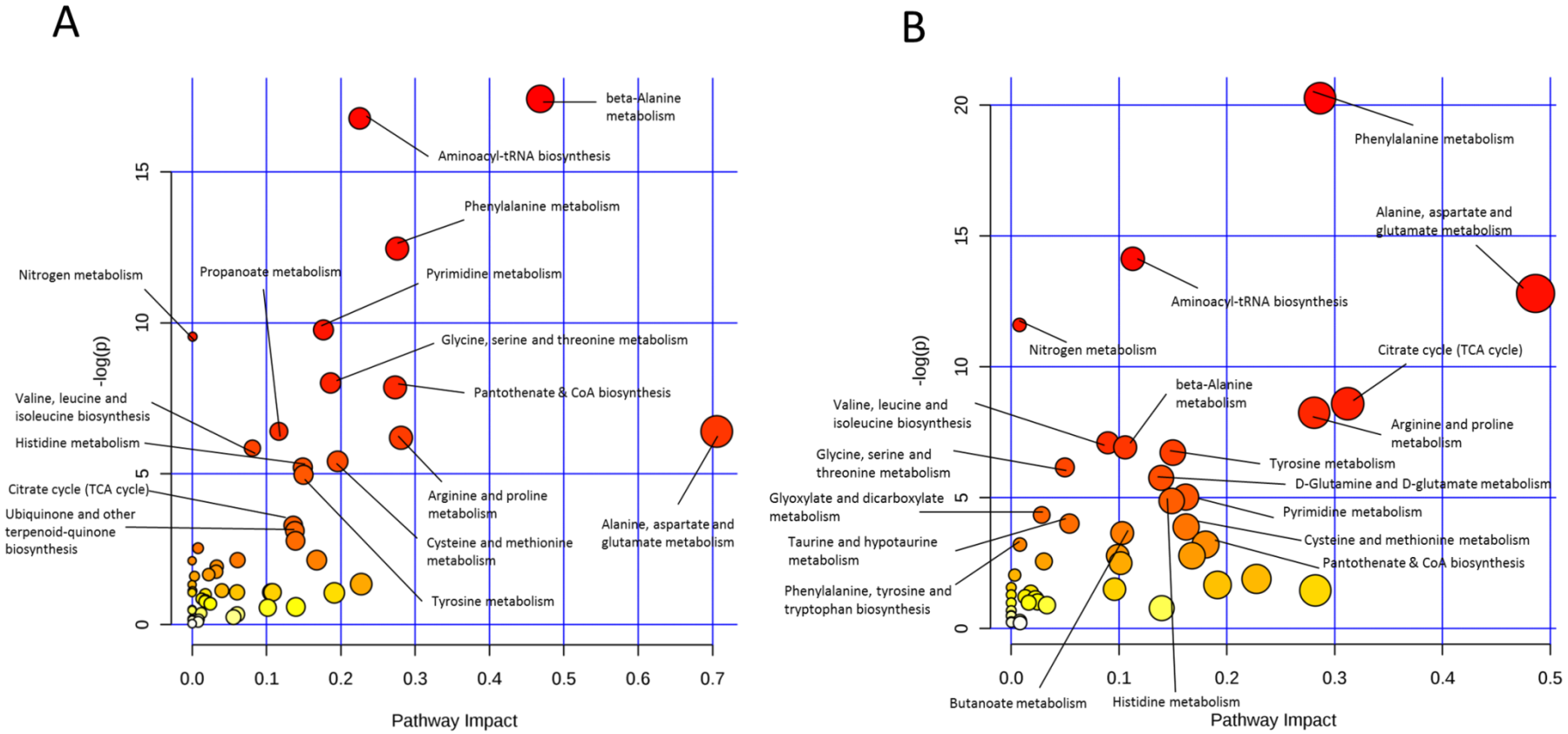



**Figure 6-23 Pathway analysis of potential biomarkers implicated by PLS-R analysis of FACIT-F and RA patients' urinary metabolites**
Pathway analysis of potential biomarkers implicated by the PLS-R of (A) all the urinary metabolome and (B) the urinary metabolome devoid of the inflammatory signal.

**Table 6-24 Pathway analysis of potential biomarkers implicated by PLS-R analysis of FACIT-F and RA patients' urinary metabolites**
Results from PLS-R models using all the urinary metabolome and the urinary metabolome devoid of the inflammatory signal
**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.
**The impact score**: Each pathway has a total importance score of 1. The importance measure of each metabolite node (a point where pathways intersect within a larger network) is expressed as a fraction with regards to the total pathway importance, and the pathway impact score is the cumulative score of matched metabolite nodes.
* p value calculated using hypergeometric test

| | PLS-R model using all urinary metabolome | | | PLS-R model using urinary metabolome devoid of the inflammatory signal | | |
|---|---|---|---|---|---|---|
| Pathway name | Match status | P value* | Impact score | Match status | P value* | Impact score |
| Alanine, aspartate and glutamate metabolism | 5/24 | 0.001663 | 0.70561 | 7/24 | 2.75E-06 | 0.48623 |
| beta-Alanine metabolism | 10/28 | 2.72E-08 | 0.46822 | 5/28 | 9.85E-04 | 0.10564 |
| Arginine and proline metabolism | 9/77 | 0.002048 | 0.28094 | 9/77 | 2.62E-04 | 0.28094 |
| Phenylalanine metabolism | 10/45 | 3.89E-06 | 0.27587 | 12/45 | 1.58E-09 | 0.28632 |
| Pantothenate and CoA biosynthesis | 6/27 | 3.86E-04 | 0.27302 | 3/27 | 0.040334 | 0.18014 |
| Aminoacyl-tRNA biosynthesis | 15/75 | 5.20E-08 | 0.22536 | 12/75 | 7.32E-07 | 0.11268 |
| Cysteine and methionine metabolism | 7/56 | 0.004473 | 0.19569 | 5/56 | 0.020586 | 0.16215 |
| Glycine, serine and threonine metabolism | 8/48 | 3.33E-04 | 0.18624 | 6/48 | 0.002115 | 0.04973 |
| Pyrimidine metabolism | 10/60 | 5.75E-05 | 0.17665 | 6/60 | 0.006582 | 0.16173 |
| Tyrosine metabolism | 8/76 | 0.006979 | 0.14985 | 8/76 | 0.001203 | 0.14985 |
| Histidine metabolism | 6/44 | 0.005478 | 0.14893 | 5/44 | 0.007627 | 0.14879 |
| Ubiquinone and other terpenoid-quinone biosynthesis | 4/36 | 0.044715 | 0.13819 | 3/36 | 0.08216 | 0.10139 |
| Citrate cycle | 3/20 | 0.0374 | 0.13599 | 5/20 | 1.86E-04 | 0.312 |
| Propanoate metabolism | 6/35 | 0.001656 | 0.11672 | 3/35 | 0.076834 | 0.03027 |
| Valine, leucine and isoleucine biosynthesis | 5/27 | 0.002887 | 0.08109 | 5/27 | 8.27E-04 | 0.08957 |
| Nitrogen metabolism | 8/39 | 7.19E-05 | 6.70E-04 | 8/39 | 9.16E-06 | 0.00763 |
| D-Glutamine and D-glutamate metabolism | 2/11 | 0.062285 | 0.13904 | 3/11 | 0.003155 | 0.13904 |
| Butanoate metabolism | 2/40 | 0.45091 | 0.01774 | 4/40 | 0.025996 | 0.1029 |
| Taurine and hypotaurine metabolism | 2/20 | 0.17336 | 0.03237 | 3/20 | 0.018123 | 0.05395 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Glyoxylate and dicarboxylate metabolism | 4/50 | 0.11791 | 0.06111 | 5/50 | 0.013032 | 0.0282 |
| Phenylalanine, tyrosine and tryptophan biosynthesis | 3/27 | 0.0795 | 0.008 | 3/27 | 0.040334 | 0.008 |

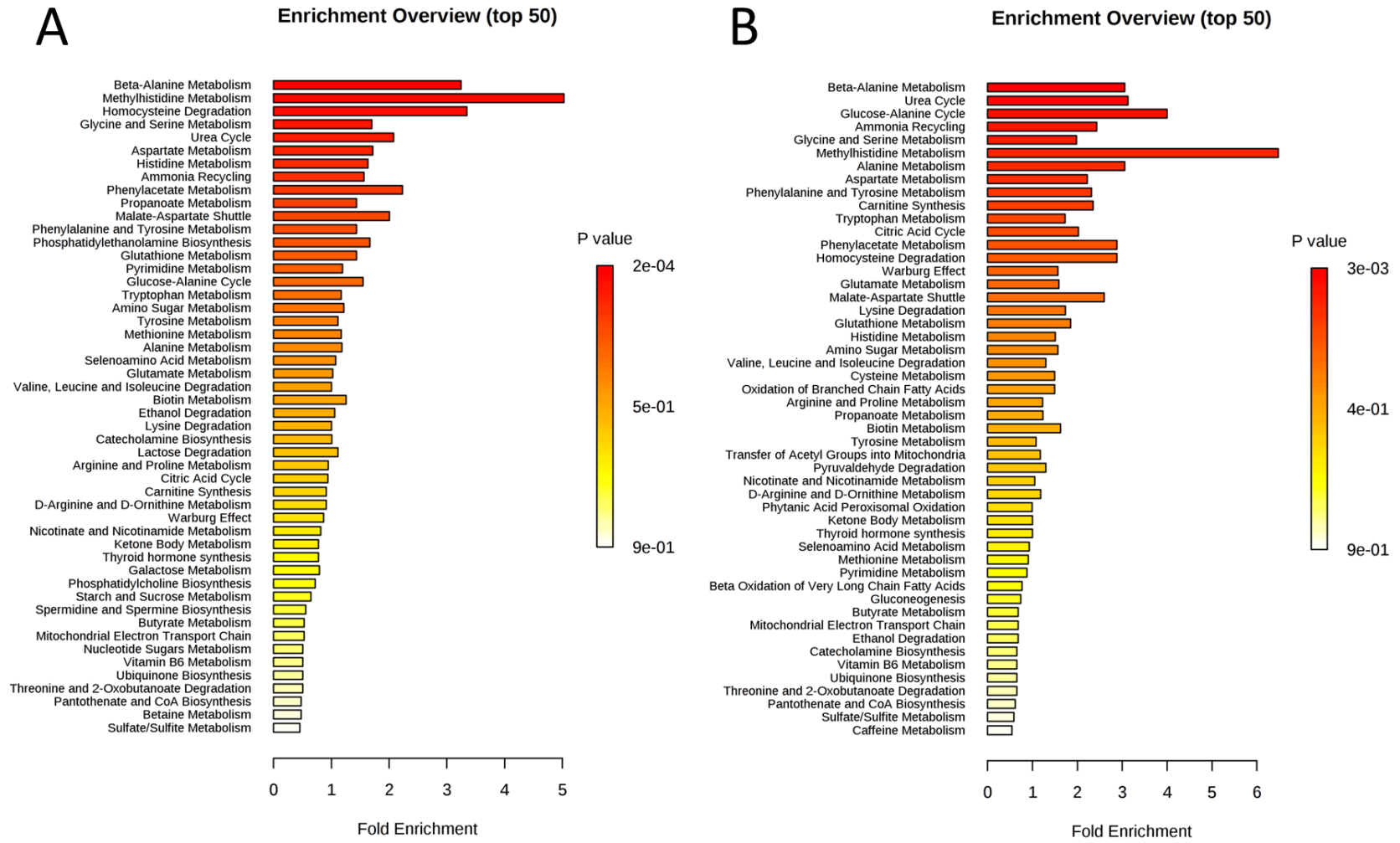



**Figure 6-24 Enrichment analysis of key metabolites in urine implicated as potential biomarkers by the PLS-R analysis of FACIT-F and RA patients' urinary metabolites**

Enrichment analysis of potential biomarkers implicated by the PLS-R of (A) all the urinary metabolome and (B) the urinary metabolome devoid of the inflammatory signal

**Table 6-25 Enrichment analysis of key metabolites in urine implicated as potential biomarkers by the PLS-R analysis of FACIT-F and RA patients' urinary metabolites**

Results from PLS-R models using all the urinary metabolome and the urinary metabolome devoid of the inflammatory signal

**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.

**The fold enrichment value**: the value is generated by the number of matched metabolites divided by the number of metabolites expected to be present in the pathway as per the reference metabolome provided by KEGG.

* p value calculated using hypergeometric test

| | PLS-R model using all urinary metabolome | | | | PLS-R model using urinary metabolome devoid of the inflammatory signal | | | |
|---|---|---|---|---|---|---|---|---|
| Metabolite pathway | Match status | Fold enrichment | Expected | P value* | Match status | Fold enrichment | Expected | P value* |
| Beta-Alanine Metabolism | 11/34 | 3.24 | 3.39 | 2.28E-04 | 8/34 | 3.05 | 2.62 | 0.00307 |
| Urea Cycle | 6/29 | 2.08 | 2.89 | 0.06 | 7/29 | 3.13 | 2.24 | 0.00485 |
| Glucose-Alanine Cycle | 2/13 | 1.55 | 1.29 | 0.377 | 4/13 | 4.00 | 1 | 0.0137 |
| Ammonia Recycling | 5/32 | 1.57 | 3.19 | 0.207 | 6/32 | 2.43 | 2.47 | 0.0311 |
| Glycine and Serine Metabolism | 10/59 | 1.70 | 5.88 | 0.0595 | 9/59 | 1.98 | 4.55 | 0.0317 |
| Methylhistidine Metabolism | 2/4 | 5.03 | 0.398 | 0.0516 | 4/19 | 6.47 | 0.309 | 0.0318 |
| Alanine Metabolism | 2/17 | 1.18 | 1.69 | 0.518 | 4/17 | 3.05 | 1.31 | 0.036 |
| Aspartate Metabolism | 6/35 | 1.72 | 3.49 | 0.126 | 6/35 | 2.22 | 2.7 | 0.0463 |

### 6.4.10 Relationship between urinary metabolite profile and FACIT-F in the PsA patients

Similarly, PsA patients showed a statistically significant correlation between FACIT-F and the entire urinary metabolite dataset (Figure 6-22B1- 46 NMR bins, $r^2$=0.6922, 8 LV, p=0.003). A relationship was also seen between FACIT-F and the urinary metabolite dataset devoid of the urinary NMR bins that correlate with CRP in PsA patients (Figure 6-22B2- 16 NMR bins, $r^2$=0.7954, 5 LV, p=0.001). Table 6-26 shows the potential biomarkers for these correlations.

Pathway and enrichment analyses for the biomarkers implicated in the PLS-R analysis between FACIT-F and PsA patients' urine metabolome and the urine metabolome without the bins that strongly correlate with CRP are shown in Figure 6-25, Table 6-27 and Figure 6-26, Table 6-28 respectively. Alanine, aspartate and glutamate metabolism was the most impacted pathway across both pathway analyses. Methylhistidine metabolism, homocysteine degradation and beta-alanine metabolism were the most overrepresented metabolite sets in both enrichment analyses.

**Table 6-26 Metabolites responsible for the relationship between FACIT-F and urinary metabolite profile in PsA patients**

Results from PLS-R models using the complete metabolite dataset and the urinary metabolome devoid of the inflammatory signal in PLS-R analysis. The following metabolites have been ranked by the magnitude of the regression coefficient. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of regression coefficient. The regression coefficient field indicates the nature of correlation (↑ indicating a positive relationship with FACIT-F and ↓ indicating a negative relationship with FACIT-F)

| | | | PLS-R model using all serum metabolome | | PLS-R model using serum metabolome devoid of the inflammatory signal | |
|---|---|---|---|---|---|---|
| Order in PLS-R using all serum metabolome | Order in PLS-R using serum metabolome devoid of the inflammatory signal | Metabolite | Chemical shift of peak (ppm) implicated in PLS-R Model | Regression coefficient | Chemical shift of peak (ppm) implicated in PLS-R Model | Regression coefficient |
| 1 | 3 | trans-Aconitic acid | 6.9506, 6.9733, 6.9908 | ↓↓↓ | 6.9733, 6.9674, 6.9557 | ↓↓↓ |
| 1 | 3 | Phenol | 6.9506, 6.9733, 6.9908, 7.0376 | ↓↓↓↓ | 6.9733, 6.9138, 6.9674, 7.0376, 7.0084, 6.9557 | ↓↓↓↑↓↓ |
| 1 | 3 | Pyrocatechol | 6.9506, 6.9733 | ↓↓ | 6.9733, 6.9138, 6.9674, 6.9557 | ↓↓↓↓ |
| 1 | 3 | Vanillic acid | 6.9506, 6.9733, 7.4767, 7.5052, 7.4884, 6.9908, 7.5001, 7.4298 | ↓↓↑↑↑↓↑↑ | 6.9733, 7.4767, 7.4298, 7.5052, 6.9138, 6.9674, 7.0084, 6.9557 | ↓↑↑↑↓↓↓↓ |
| 1 | 25 | Sumiki's acid | 6.9506 | ↓ | 6.9557 | ↓ |
| 1 | 1 | Urea | 6.9506, 7.184, 7.9684, 7.7518, 4.3333, 6.9733, 7.9801, 7.7869, 7.1254, 7.4767, 7.7576, 7.9497, 7.5052, 7.4884, 6.9908, 7.9859, 7.0376, 7.5001, 8.0018, 7.9631 | ↓↑↓↑↑↓↓↑↑↑↑↓↑↑↓↑↑↑↓ | 7.184, 7.9684, 6.9733, 7.7869, 4.3333, 7.4767, 7.7518, 7.9497, 7.9801, 7.5052, 7.9859, 6.9138, 6.9674, 7.0376, 8.0018, 7.9332, 7.0084, 7.7635, 7.9631, 6.9557, 7.9301 | ↑↓↓↑↑↑↑↓↓↑↓↓↓↑↑↑↓↑↓↓↑ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 1 | Ortho-Hydroxyphenylacetate | 6.9506, 7.184 | ↓↑ | 7.184, 6.9138, 6.9674, 6.9557 | ↑↓↓↓ |
| 1 | 16 | Homovanillic acid | 6.9506 | ↓ | 6.9138, 6.9557 | ↓↓ |
| 1 | 5 | 4-Hydroxybenzoic acid | 6.9506, 7.7869, 7.7576 | ↓↑↑ | 7.7869, 6.9138, 7.7635, 7.7928, 6.9557 | ↑↓↑↑↓ |
| 1 | 3 | 3-methylhistidine | 6.9506, 7.7518, 6.9733, 7.7576, 3.0865, 6.9908, 7.0376, 8.0018 | ↓↑↓↑↑↓↑↑ | 6.9733, 7.7518, 6.9674, 7.0376, 7.9332, 7.0084, 7.9301, 3.0865, 6.9557 | ↓↑↓↑↑↓↑↑↓ |
| 1 | 12 | L-Kynurenine | 6.9506, 7.4298 | ↓↑ | 7.4298, 6.9138, 7.8571 | ↑↓↑ |
| 1 | 1 | Tyrosine | 6.9506, 7.184 | ↓↑ | 7.184, 6.9138 | ↑↓ |
| 2 | 1 | Indoxyl sulfate | 7.184, 7.5052, 7.4884, 7.5001 | ↑↑↑↑ | 7.184, 7.5052 | ↑↑ |
| 2 | 1 | Tryptophan | 7.184, 7.7518, 7.7576, 7.5052, 7.5001 | ↑↑↑↑↑ | 7.184, 7.7518, 7.5052, 7.7635 | ↑↑↑↑ |
| 2 | 1 | Indoleacetate | 7.184, 7.1254, 7.4767, 7.5052, 7.4884, 7.0376, 7.5001 | ↑↑↑↑↑↑↑ | 7.184, 7.4767, 7.5052, 7.0376 | ↑↑↑↑ |
| 2 | 1 | Phenylalanine | 7.184, 7.1254, 7.4767, 7.5052, 7.4884, 3.0865, 7.0376, 7.5001, 7.4298, 3.0982 | ↑↑↑↑↑↑↑↑↑↑ | 7.184, 7.4767, 7.4298, 7.5052, 7.0376, 7.0084, 3.0865 | ↑↑↑↑↑↓↑ |
| 2 | 1 | Histidine | 7.184, 7.9801, 7.1254, 7.8727, 6.9908, 7.9859, 7.0376, 7.8707, 8.0018 | ↑↓↑↑↓↓↑↑↑ | 7.184, 7.9801, 7.9859, 7.0376, 8.0018, 7.9332, 7.0084, 7.9301 | ↑↓↓↓↑↑↓↑ |
| 2 | 1 | p-Hydroxyphenylacetate | 7.184, 7.1254 | ↑↑ | 7.184 | ↑ |
| 2 | 1 | Hydroxyphenyllactate | 7.184, 4.3333, 2.8992, 2.864 | ↑↑↓↓ | 7.184, 4.3333, 2.8992, 2.864 | ↑↑↓↓ |
| 3 | 2 | Quinolinic acid | 7.9684, 7.9801, 7.9859, 8.0018, 7.4298, 7.9631 | ↓↓↓↑↑↓ | 7.9684, 7.9801, 7.4298, 7.9859, 8.0018, 7.9631 | ↓↓↑↓↑↓ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 3 | 2 | Phenylglyoxylic acid | 7.9684, 7.7518, 7.9801, 7.7576, 7.9497, 7.9859, 8.0018, 7.9631 | ↓↑↓↑↓↓↑↓ | 7.9684, 7.7518, 7.9497, 7.9801, 7.9859, 8.0018, 7.9332, 7.7635, 7.9631, 7.9301 | ↓↑↓↓↓↑↑↑↓↑ |
| 3 | 2 | Picolinic acid | 7.9684, 7.9801, 7.9497, 7.5052, 7.9859, 8.0018, 7.9631 | ↓↓↓↑↓↑↓ | 7.9684, 7.9497, 7.9801, 7.5052, 7.9859, 8.0018, 7.9332, 7.9631, 7.9301 | ↓↓↓↑↓↑↑↓↑ |
| 4 | 21 | Adipic acid | 1.506, 1.4943, 1.4475, 2.1499 | ↑↑↓↑ | 1.4475 | ↓ |
| 4 | 14 | Lysine | 1.506, 1.4943, 1.372, 1.4416, 1.4475, 1.9158, 1.6699 | ↑↑↓↓↓↓↑ | 1.6699, 1.4416, 1.9158, 1.4475, 1.8045 | ↑↓↓↓↑ |
| 4 | 24 | Azelaic acid | 1.506, 1.4943, 2.1499, 1.365 | ↑↑↑↓ | 1.365 | ↓ |
| 4 | N/A | Sebacic acid | 1.506, 1.4943, 1.225, 2.1499 | ↑↑↑↑ | N/A | N/A |
| 4 | 14 | 3-Methyladipic acid | 1.506, 1.4943, 1.9158, 2.1499, 1.6699 | ↑↑↓↑↑ | 1.6699, 1.9158 | ↑↓ |
| 4 | N/A | Suberic acid | 1.506, 1.4943, 2.1499 | ↑↑↑ | N/A | N/A |
| 4 | 17 | Alanine | 1.506, 1.4943, 1.4416, 1.4475 | ↑↑↑↓ | 1.4416, 1.4475 | ↓↓ |
| 5 | 5 | Hippuric acid | 7.7518, 7.9801, 7.7869, 7.8727, 7.4767, 7.7576, 7.8619, 7.5052, 7.4884, 7.9859, 7.5001, 7.8707, 8.0018 | ↑↓↑↑↑↑↑↑↑↓↑↑↑ | 7.7869, 7.4767, 7.7518, 7.8619, 7.9801, 7.5052, 7.9859, 8.0018, 7.9332, 7.7635, 7.8571, 7.7928, 7.9301 | ↑↑↑↑↓↑↓↑↑↑↑↑↑ |
| 5 | 5 | 7-Methylxanthine | 7.7518, 7.9801, 7.7869, 7.8727, 7.7576, 7.8619, 7.9859, 7.8707, 8.0018 | ↑↓↑↑↑↑↓↑↑ | 7.7869, 7.7518, 7.8619, 7.9801, 7.9859, 8.0018, 7.9332, 7.7635, 7.8571, 7.9301 | ↑↑↑↓↓↑↑↑↑↑ |
| 6 | 6 | 4-Hydroxyproline | 4.3333, 2.1499 | ↑↑ | 4.3333 | ↑ |
| 6 | 6 | D-Tartaric acid | 4.3333 | ↑ | 4.3333 | ↑ |
| 6 | 4 | Creatinine | 4.3333, 2.7587, 2.8348, 2.8992, 2.7645, 2.7294, 2.6592, 2.7528, 3.0865, 2.7879, 2.8406, 2.8055, 2.864, 2.8465, 2.7821, 3.0982, 2.8816, 2.6884 | ↑↓↓↓↓↓↑↓↑↓↓↓↓↓↓↑↓↑ | 2.8348, 4.3333, 2.8992, 2.7645, 2.7821, 2.864, 2.7528, 2.8055, 3.0865 | ↓↑↓↓↓↓↓↓↑ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 6 | 6 | Gluconic acid | 4.3333 | ↑ | 4.3333 | ↑ |
| 6 | 6 | Adenosine | 4.3333 | ↑ | 4.3333 | ↑ |
| 6 | 6 | Pseudouridine | 4.3333 | ↑ | 4.3333 | ↑ |
| 7 | 4 | Citrate | 2.7587, 2.8348, 2.7645, 2.7294, 2.6592, 2.7528, 2.7879, 2.8406, 2.8055, 2.7821, 2.6884 | ↓↓↓↓↓↓↓↓↓↓↑ | 2.8348, 2.7645, 2.7821, 2.7528, 2.8055 | ↓↓↓↓↓ |
| 7 | 9 | Dihydrothymine | 2.7587, 2.7645, 2.7528, 2.7879, 2.8055, 2.7821 | ↓↓↓↓↓↓ | 2.7645, 2.7821, 2.7528, 2.8055 | ↓↓↓↓ |
| 7 | 9 | 5-Aminolevulinic acid | 2.7587, 2.7645, 2.7528, 2.7879, 2.8055, 2.7821 | ↓↓↓↓↓↓ | 2.7645, 2.7821, 2.7528, 2.8055 | ↓↓↓↓ |
| 7 | 9 | 2-Ketobutyric acid | 2.7587, 2.7645, 2.7528, 2.7879, 1.0871, 2.7821, 1.0904 | ↓↓↓↓↓↓↓ | 2.7645, 2.7821, 1.0871, 2.7528, 1.0904 | ↓↓↓↓↓ |
| 7 | 9 | Levulinic acid | 2.7587, 2.7645, 2.7528, 2.7879, 2.7821 | ↓↓↓↓↓ | 2.7645, 2.7821, 2.7528 | ↓↓↓ |
| 7 | 9 | L-Cystathionine | 2.7587, 2.7645, 2.7294, 2.6592, 2.7528, 3.0865, 2.1499, 2.7821, 3.0982, 2.6884 | ↓↓↓↑↓↑↑↓↑↑ | 2.7645, 2.7821, 2.7528, 3.0865 | ↓↓↓↑ |
| 7 | 9 | Citramalic acid | 2.7587, 2.7645, 2.7528, 1.365 | ↓↓↓↓ | 2.7645, 2.7528, 1.365 | ↓↓↓ |
| 7 | 15 | Sarcosine | 2.7587, 2.7645, 2.7528 | ↓↓↓ | 2.7528 | ↓ |
| 8 | 11 | Carnosine | 7.9801, 7.1254, 2.7294, 2.6592, 7.9859, 8.0018 | ↓↑↓↑↓↑ | 7.9801, 7.9859, 8.0018 | ↓↓↑ |
| 8 | 7 | Benzoic acid | 7.9801, 7.8727, 7.4767, 7.8619, 7.5052, 7.4884, 7.9859, 7.5001, 7.8707 | ↓↑↑↑↑↑↓↑↑ | 7.4767, 7.8619, 7.9801, 7.5052, 7.9859, 7.9332, 7.8571, 7.9301 | ↑↑↓↑↓↑↑↑ |
| 8 | 11 | Kynurenic acid | 7.9801, 7.5052, 7.9859, 7.5001 | ↓↑↓↑ | 7.9801, 7.5052, 7.9859, 7.9332, 7.9301 | ↓↑↓↑↑ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 9 | 10 | 3-Methyl-2-oxovaleric acid | 1.4943, 1.4416, 1.0871, 1.4475, 1.6699, 1.0904 | ↑↓↓↓↑↓ | 1.0871, 1.6699, 1.4416, 1.0904, 1.4475 | ↓↑↓↓↓ |
| 10 | 4 | Asparagine | 2.8348, 2.8992, 2.8406, 2.864, 2.8465, 2.8816 | ↓↓↓↓↓↓ | 2.8348, 2.8992, 2.864, 6.9138 | ↓↓↓↓ |
| 10 | 4 | Succinylacetone | 2.8348, 2.7879, 2.8406, 2.8055, 2.7821 | ↓↓↓↓↓ | 2.8348, 2.7821, 2.8055 | ↓↓↓ |
| 10 | 4 | Aspartate | 2.8348, 2.6592, 2.7879, 2.8406, 2.8055, 2.7821, 2.6884 | ↓↑↓↓↓↓↑ | 2.8348, 2.7821, 2.8055 | ↓↓↓ |
| 10 | 4 | Symmetric dimethylarginine | 2.8348, 2.8055, 1.9158, 1.6699 | ↓↓↓↑ | 2.8348, 1.6699, 2.8055, 1.9158 | ↓↑↓↓ |
| 10 | 4 | Methylguanidine | 2.8348, 2.7879, 2.8055 | ↓↓↓ | 2.8348, 2.8055 | ↓↓ |
| 11 | 8 | 3-Aminoisobutanoic acid | 2.8992, 2.6592, 3.0865, 1.0871, 1.225, 3.0982, 1.0904 | ↓↑↑↓↑↑↓ | 2.8992, 1.0871, 1.0904, 3.0865 | ↓↓↓↑ |
| 11 | 8 | Trimethylamine | 2.8992, 2.864, 2.8816 | ↓↓↓ | 2.8992, 2.864 | ↓↓ |
| 11 | 8 | Dimethylglycine | 2.8992 | ↓ | 2.8992 | ↓ |
| 12 | 24 | Lactate | 1.372, 1.365 | ↓↓ | 1.365 | ↓ |
| 12 | 24 | Alpha-Hydroxyisobutyric acid | 1.372, 1.365 | ↓↓ | 1.365 | ↓ |
| 13 | N/A | p-Cresol sulfate | 7.1254 | ↑ | N/A | N/A |
| 13 | N/A | Anserine | 7.1254, 2.7294, 2.6592, 2.6884 | ↑↓↑↑ | N/A | N/A |
| 13 | 16 | trans-Ferulic acid | 7.1254 | ↑ | 6.9138 | ↓ |
| 14 | 7 | Cytosine | 7.4767, 7.5052, 7.4884, 7.5001 | ↑↑↑↑ | 7.4767, 7.5052 | ↑↑ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 14 | 7 | Cinnamic acid | 7.4767, 7.5052, 7.4884, 7.5001, 7.4298 | ↑↑↑↑↑ | 7.4767, 7.4298, 7.5052 | ↑↑↑ |
| 15 | 17 | Isoleucine | 1.4416, 1.4475, 1.9684, 1.225 | ↓↓↓↑ | 1.4416, 1.4475 | ↓↓ |
| 16 | 13 | Uracil | 7.5052, 7.4884, 7.5001 | ↑↑↑ | 7.5052 | ↑ |
| 16 | 13 | 4-Pyridoxic acid | 7.5052, 7.5001 | ↑↑ | 7.5052 | ↑ |
| 17 | N/A | Dihydrouracil | 2.6592, 2.6884 | ↑↑ | N/A | N/A |
| 17 | N/A | Hydroxypropionic acid | 2.6592 | ↑ | N/A | N/A |
| 18 | 12 | Mandelic acid | 2.6592, 7.4298 | ↑↑ | 7.4298 | ↑ |
| 19 | 22 | 1,3-Diaminopropane | 3.0865, 3.0982 | ↑↑ | 3.0865 | ↑ |
| 20 | 22 | Cysteine | 3.0865, 3.0982 | ↑↑ | 3.0865 | ↑ |
| 21 | 10 | Methylsuccinate | 1.0871, 1.0904 | ↓↓ | 1.0871, 1.0904 | ↓↓ |
| 22 | N/A | Glutamate | 1.9684, 2.1499 | ↓↑ | N/A | N/A |
| 22 | N/A | Citraconic acid | 1.9684 | ↓ | N/A | N/A |
| 22 | 20 | L-2-Hydroxyglutaric acid | 1.9684, 1.9158, 2.1499 | ↓↓↑ | 1.9158, 1.8045 | ↓↑ |
| 22 | N/A | Methylglutaric acid | 1.9684 | ↓ | N/A | N/A |
| 22 | N/A | Isovalerylglycine | 1.9684, 2.1499 | ↓↑ | N/A | N/A |
| 22 | 14 | N-Acetylputrescine | 1.9684, 1.6699 | ↓↑ | 1.6699 | ↑ |
| 22 | 14 | Arginine | 1.9684, 1.9158, 1.6699 | ↓↓↑ | 1.6699, 1.9158, 1.8045 | ↑↓↑ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 23 | 19 | Acetate | 1.9158 | ↓ | 1.9158 | ↓ |
| 23 | 20 | Thymidine | 1.9158 | ↓ | 1.9158 | ↓ |
| 23 | 14 | Aminoadipic acid | 1.9158, 1.6699 | ↓↑ | 1.6699, 1.9158, 1.8045 | ↑↓↑ |
| 23 | 20 | N-Acetylneuraminic acid | 1.9158 | ↓ | 1.9158, 1.8045 | ↓↑ |
| 23 | 20 | D-Alpha-aminobutyric acid | 1.9158 | ↓ | 1.9158 | ↓ |
| 24 | 16 | Vanillylmandelic acid | 7.0376 | ↑ | 6.9138, 7.0376 | ↓↑ |
| 25 | N/A | 3-Hydroxyisovaleric acid | 1.225 | ↑ | N/A | N/A |
| 25 | N/A | Methylmalonate | 1.225 | ↑ | N/A | N/A |
| 25 | N/A | L-Fucose | 1.225 | ↑ | N/A | N/A |
| 26 | N/A | Methionine | 2.1499 | ↑ | N/A | N/A |
| 26 | N/A | Pimelic acid | 2.1499 | ↑ | N/A | N/A |
| 26 | 23 | Monomethyl glutaric acid | 2.1499 | ↑ | 1.8045 | ↑ |
| 26 | N/A | Glutamine | 2.1499 | ↑ | N/A | N/A |
| 27 | 14 | 5-Aminopentanoic acid | 1.6699 | ↑ | 1.6699 | ↑ |
| 27 | 14 | Leucine | 1.6699 | ↑ | 1.6699 | ↑ |
| 28 | 24 | Threonine | 1.365 | ↓ | 1.365 | ↓ |
| 29 | N/A | Malonate | 3.0982 | ↑ | N/A | N/A |

| | | | | | | |
|---|---|---|---|---|---|---|
| N/A | 14 | N-Acetylputrescine | N/A | N/A | 1.6699 | ↑ |
| N/A | 16 | 4-Aminohippuric acid | N/A | N/A | 6.9138 | ↓ |
| N/A | 18 | 3-Methylxanthine | N/A | N/A | 8.0018 | ↑ |

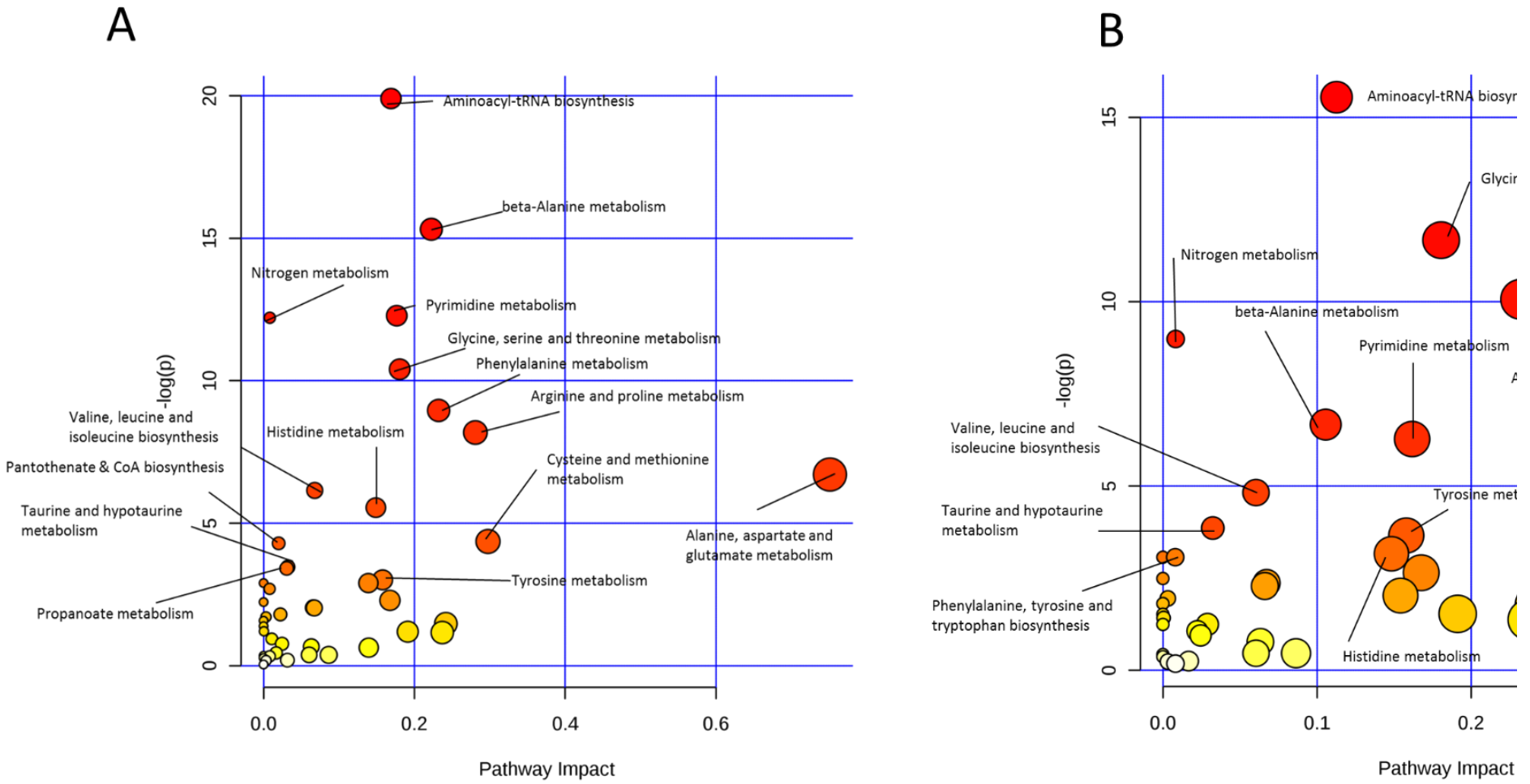



**Figure 6-25 Pathway analysis of potential biomarkers implicated by PLS-R analysis of FACIT-F and PsA patients' urinary metabolites**
Pathway analysis of potential biomarkers implicated by the PLS-R of (A) all the urinary metabolome and (B) the urinary metabolome devoid of the inflammatory signal.

**Table 6-27 Pathway analysis of potential biomarkers implicated by PLS-R analysis of FACIT-F and PsA patients' urinary metabolites**
Results from PLS-R models using all the urinary metabolome and the urinary metabolome devoid of the inflammatory signal
**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.
**The impact score**: Each pathway has a total importance score of 1. The importance measure of each metabolite node (a point where pathways intersect within a larger network) is expressed as a fraction with regards to the total pathway importance, and the pathway impact score is the cumulative score of matched metabolite nodes.
* p value calculated using hypergeometric test

| | PLS-R model using all urinary metabolome | | | PLS-R model using urinary metabolome devoid of the inflammatory signal | | |
|---|---|---|---|---|---|---|
| Pathway name | Match status | P value* | Impact score | Match status | P value* | Impact score |
| Alanine, aspartate and glutamate metabolism | 5/24 | 0.001221 | 0.75119 | 3/24 | 0.034304 | 0.36752 |
| Cysteine and methionine metabolism | 6/56 | 0.012797 | 0.29768 | 5/56 | 0.025647 | 0.25962 |
| Arginine and proline metabolism | 10/77 | 2.79E-04 | 0.28094 | 8/77 | 0.001902 | 0.24512 |
| Phenylalanine metabolism | 8/45 | 1.29E-04 | 0.2322 | 8/45 | 4.25E-05 | 0.2322 |
| beta-Alanine metabolism | 9/28 | 2.23E-07 | 0.22247 | 5/28 | 0.001274 | 0.10564 |
| Glycine, serine and threonine metabolism | 9/48 | 3.03E-05 | 0.18054 | 9/48 | 8.55E-06 | 0.18054 |
| Pyrimidine metabolism | 11/60 | 4.63E-06 | 0.17665 | 7/60 | 0.001884 | 0.16173 |
| Aminoacyl-tRNA biosynthesis | 16/75 | 2.26E-09 | 0.16902 | 13/75 | 1.77E-07 | 0.11268 |
| Tyrosine metabolism | 6/76 | 0.049271 | 0.15793 | 6/76 | 0.025805 | 0.15793 |
| Histidine metabolism | 6/44 | 0.003902 | 0.14893 | 4/44 | 0.04236 | 0.14828 |
| Valine, leucine and isoleucine biosynthesis | 5/27 | 0.002133 | 0.06784 | 4/27 | 0.008056 | 0.06042 |
| Taurine and hypotaurine metabolism | 3/20 | 0.031388 | 0.03237 | 3/20 | 0.021062 | 0.03237 |
| Propanoate metabolism | 4/35 | 0.032942 | 0.03068 | 2/35 | 0.28725 | 0.02893 |
| Pantothenate and CoA biosynthesis | 4/27 | 0.013666 | 0.02002 | 3/27 | 0.046483 | 0 |
| Nitrogen metabolism | 9/39 | 4.97E-06 | 0.0083 | 7/39 | 1.25E-04 | 0.0083 |
| Phenylalanine, tyrosine and tryptophan biosynthesis | 3/27 | 0.067556 | 0.008 | 3/27 | 0.046483 | 0.008 |

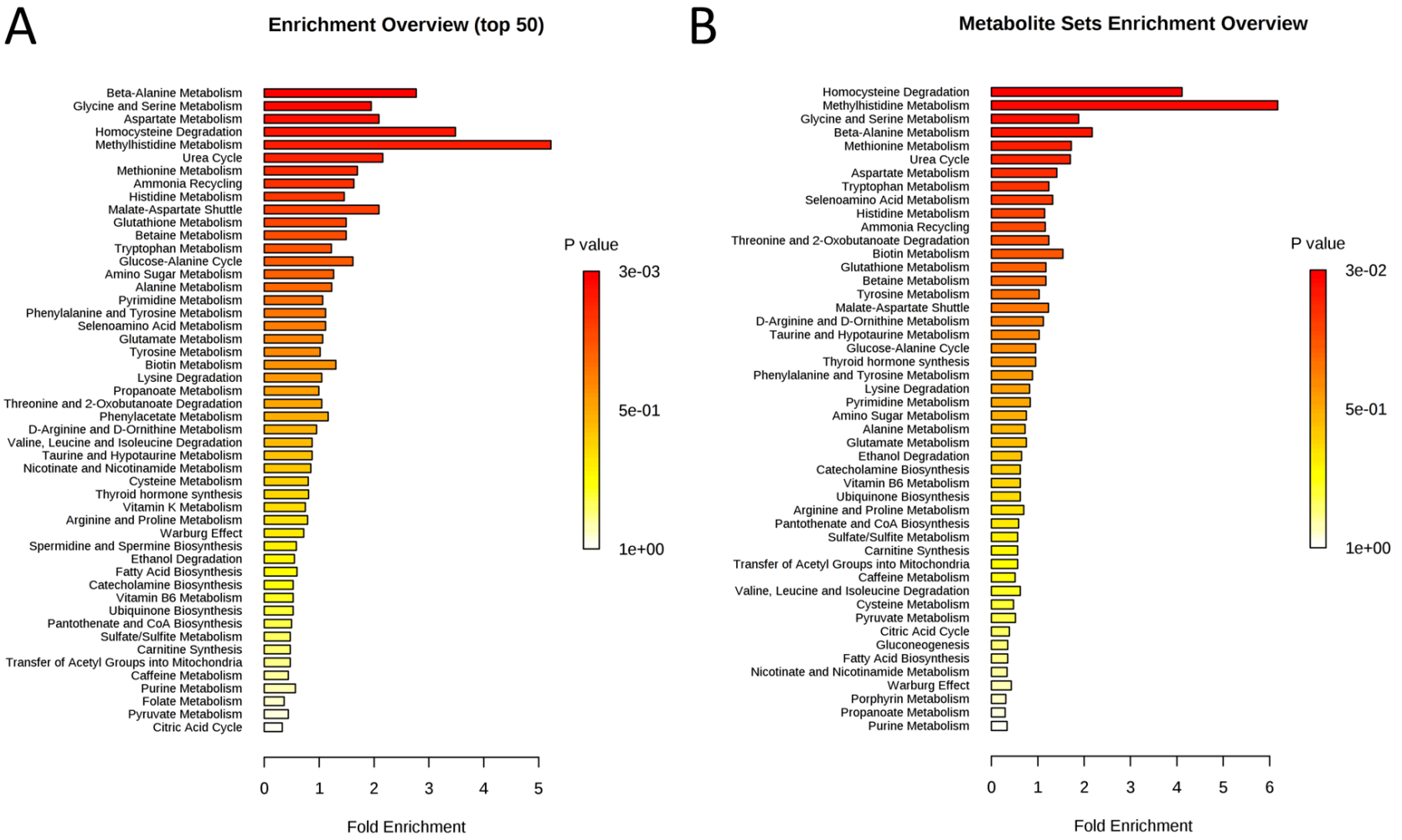



**Figure 6-26 Enrichment analysis of key metabolites in urine implicated as potential biomarkers by the PLS-R analysis of FACIT-F and PsA patients' urinary metabolites**

Enrichment analysis of potential biomarkers implicated by the PLS-R of (A) all the urinary metabolome and (B) the urinary metabolome devoid of the inflammatory signal.

**Table 6-28 Enrichment analysis of key metabolites in urine implicated as potential biomarkers by the PLS-R analysis of FACIT-F and PsA patients' urinary metabolites**

Results from PLS-R models using all the urinary metabolome and the urinary metabolome devoid of the inflammatory signal.

**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.

**The fold enrichment value**: the value is generated by the number of matched metabolites divided by the number of metabolites expected to be present in the pathway as per the reference metabolome provided by KEGG.

* p value calculated using hypergeometric test

| | PLS-R model using all urinary metabolome | | | | PLS-R model using urinary metabolome devoid of the inflammatory signal | | | |
|---|---|---|---|---|---|---|---|---|
| Metabolite pathway | Match status | Fold enrichment | Expected | P value* | Match status | Fold enrichment | Expected | P value* |
| Beta-Alanine Metabolism | 9/34 | 2.77 | 3.25 | 0.00322 | 6/34 | 2.17 | 2.76 | 0.0503 |
| Glycine and Serine Metabolism | 11/59 | 1.95 | 5.65 | 0.0196 | 9/59 | 1.88 | 4.78 | 0.0422 |
| Aspartate Metabolism | 7/35 | 2.09 | 3.35 | 0.0423 | 4/35 | 1.41 | 2.84 | 0.314 |
| Homocysteine Degradation | 3/9 | 3.48 | 0.861 | 0.0466 | 3/9 | 4.12 | 0.729 | 0.0302 |
| Methylhistidine Metabolism | 2/4 | 5.22 | 0.383 | 0.0479 | 2/4 | 6.17 | 0.324 | 0.035 |

For UA patients both the entire urinary metabolite dataset (Figure 6-22C1- 137 NMR bins, $r^2$=0.3111, 6 LV, p=0.087) and the urinary metabolite dataset devoid of the NMR bins that correlate with CRP showed no correlation with FACIT-F (Figure 6-22C2- 670 NMR bins, $r^2$=0.3531, 2 LV, p=0.070).

### 6.4.11 Relationship between urinary metabolite profile and FACIT-F in the CSA patients

CSA patients showed a statistically significant correlation between FACIT-F and the complete urinary metabolite dataset (Figure 6-22D1- 58 NMR bins, $r^2$=0.5527, 2 LV, p=0.004). A relationship was also seen between FACIT-F and the urinary metabolite dataset devoid of the urinary NMR bins that correlate with CRP in CSA patients (Figure 6-22D2- 36 NMR bins, $r^2$=0.6147, 4 LV, p=0.002). Table 6-29 shows the potential biomarkers for these correlations.

Pathway and enrichment analyses for the biomarkers implicated in the PLS-R analysis between FACIT-F and CSA patients' urine metabolome and the urine metabolome without the bins that strongly correlate with CRP are shown in Figure 6-27, Table 6-30 and Figure 6-28, Table 6-31 respectively. Alanine, aspartate and glutamate metabolism, in addition to cysteine and methionine metabolism were the most impacted metabolite pathways in both pathway analyses. Glycine and serine metabolism, aspartate metabolism and beta-alanine metabolism were the most overrepresented metabolic pathways in both enrichment analyses.

**Table 6-29 Metabolites responsible for the relationship between FACIT-F and urinary metabolite profile in CSA patients**

Results from PLS-R models using the complete metabolite dataset and the urinary metabolome devoid of the inflammatory signal in PLS-R analysis. The following metabolites have been ranked by the magnitude of the regression coefficient. The bins that each metabolite was implicated as a biomarker were also listed by descending order of magnitude of regression coefficient. The regression coefficient field indicates the nature of correlation (↑ indicating a positive relationship with FACIT-F and ↓ indicating a negative relationship with FACIT-F)

| | | | PLS-R model using all serum metabolome | | PLS-R model using serum metabolome devoid of the inflammatory signal | |
|---|---|---|---|---|---|---|
| Order in PLS-R using all serum metabolome | Order in PLS-R using serum metabolome devoid of the inflammatory signal | Metabolite | Chemical shift of peak (ppm) implicated in PLS-R Model | Regression coefficient | Chemical shift of peak (ppm) implicated in PLS-R Model | Regression coefficient |
| 1 | N/A | Adipic acid | 1.506, 1.348, 1.4943, 1.5111, 1.5002 | ↓↓↓↓↓ | N/A | N/A |
| 1 | 9 | Lysine | 1.506, 1.4943, 1.5111, 1.5002, 3.7421, 3.7362, 1.9216 | ↓↓↓↓↓↓↑ | 1.4003, 1.3831, 3.7362 | ↓↓↓ |
| 1 | 6 | Azelaic acid | 1.506, 1.4943, 1.5111, 1.5002, 1.3538, 1.358, 1.3128, 1.2777 | ↓↓↓↓↓↓↓↓ | 1.3538, 1.2777, 1.3011 | ↓↓↓ |
| 1 | 11 | Sebacic acid | 1.506, 1.4943, 1.5111, 1.5002, 1.3128, 1.2777 | ↓↓↓↓↓↓ | 1.2777, 1.3011 | ↓↓ |
| 1 | 17 | 3-Methyladipic acid | 1.506, 1.4943, 1.5111, 1.5002, 1.9216, 2.3372 | ↓↓↓↓↑↓ | 2.3197 | ↓ |
| 1 | 11 | Suberic acid | 1.506, 1.348, 1.4943, 1.5111, 1.5002, 1.3128, 1.2777 | ↓↓↓↓↓↓↓ | 1.2777, 1.3011 | ↓↓ |
| 1 | N/A | Alanine | 1.506, 1.4943, 1.5111, 1.5002 | ↓↓↓↓ | N/A | N/A |
| 2 | 6 | Lactate | 1.348, 1.3538, 1.358, 1.3128, 1.2777 | ↓↓↓↓↓ | 1.3538, 1.2777, 1.3831, 1.3011, 4.1343 | ↓↓↓↓↑ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 2 | 6 | Alpha-Hydroxyisobutyric acid | 1.348, 1.3538, 1.358, 1.3128 | ↓↓↓↓ | 1.3538, 1.3831 | ↓↓ |
| 2 | 6 | Citramalic acid | 1.348, 1.3538, 1.358, 2.2553, 1.3128 | ↓↓↓↓↓ | 1.3538, 2.2553 | ↓↓ |
| 2 | 6 | Threonine | 1.348, 1.3538, 1.358, 1.3128 | ↓↓↓↓ | 1.3538, 1.3011, 4.1343 | ↓↓↑ |
| 2 | 6 | 3-Hydroxymethylglutaric acid | 1.348, 1.3538, 1.358, 1.3128, 1.2777 | ↓↓↓↓↓ | 1.3538, 1.2777, 1.3011 | ↓↓↓ |
| 3 | 10 | Asparagine | 2.8465, 2.8406, 2.8523, 2.8816, 2.8582, 2.8699 | ↑↑↑↑↑↑ | 2.8816, 2.8699 | ↑↑ |
| 3 | 3 | Creatinine | 2.8465, 2.8406, 2.8523, 2.8816, 2.6416, 2.8582, 3.1509, 2.6358, 2.8699, 3.1157, 2.7997, 3.1392, 3.1333, 3.8709, 3.145 | ↑↑↑↑↓↑ ↓↓↑↓↑↓ ↓↓↓ | 3.145, 3.1392, 3.1157, 3.1333, 2.8816, 2.6358, 3.1275, 4.6377, 2.8699, 4.6318, 4.1343 | ↓↓↓↓↑ ↓↓↓↑↓ ↑ |
| 4 | 15 | 3-Methyl-2-oxovaleric acid | 1.4943, 1.5002 | ↓↓ | 1.0588 | ↓ |
| 5 | 11 | Pimelic acid | 1.5111, 1.3128, 1.2777 | ↓↓↓ | 1.2777, 1.3011 | ↓↓ |
| 6 | 1 | Cystine | 3.3792, 3.3733, 3.1509, 3.1392, 3.145 | ↓↓↓↓↓ | 3.3733, 3.3792, 3.145, 3.1392, 3.3616, 4.1343 | ↓↓↓↓↓ ↑ |
| 6 | 1 | 4-Hydroxyproline | 3.3792, 3.3733, 3.3557, 3.3206, 2.3372 | ↓↓↓↑↓ | 3.3733, 3.3792, 3.3557, 3.3206, 3.3616 | ↓↓↓↑↓ |
| 6 | 2 | Pantothenic acid | 3.3792 | ↓ | 3.3792 | ↓ |
| 7 | 18 | Succinylacetone | 2.8406, 3.8533, 2.7997, 2.2553, 3.8709, 3.8474 | ↑↓↑↓↓↓ | 2.2553 | ↓ |
| 7 | 12 | Citrate | 2.8406, 2.6416, 2.6358, 2.7997 | ↑↓↓↑ | 2.6358 | ↓ |
| 7 | 12 | Aspartate | 2.8406, 2.6416, 2.6358, 2.7997, 3.8709 | ↑↓↓↑↓ | 2.6358 | ↓ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 8 | N/A | Trimethylamine | 2.8816, 3.3206 | ↑↑ | N/A | N/A |
| 9 | 16 | D-Threitol | 3.6016, 3.5957, 3.7362, 3.7187 | ↓↓↓↓ | 3.5957, 3.7362 | ↓↓ |
| 9 | 16 | myo-Inositol | 3.6016, 3.5957 | ↓↓ | 3.5957, 4.1343 | ↓↑ |
| 9 | N/A | Threonic acid | 3.6016 | ↓ | N/A | N/A |
| 9 | N/A | Sarcosine | 3.6016 | ↓ | N/A | N/A |
| 9 | 14 | D-Maltose | 3.6016, 3.7421, 3.5957, 3.8533, 3.8709, 3.7187, 3.8474 | ↓↓↓↓↓↓↓ | 4.6377, 3.5957, 4.6318 | ↓↓↓ |
| 9 | 16 | Alpha-Lactose | 3.6016, 3.7421, 3.5957, 3.8533, 3.8709, 3.7187, 3.8474 | ↓↓↓↓↓↓↓ | 3.5957 | ↓ |
| 10 | 12 | Carnosine | 2.6416, 3.1509, 2.6358, 7.143 | ↓↓↓↑ | 2.6358 | ↓ |
| 10 | 12 | Methylsuccinate | 2.6416, 2.6358 | ↓↓ | 2.6358, 1.0588 | ↓↓ |
| 10 | 12 | Anserine | 2.6416, 2.6358, 7.143 | ↓↓↑ | 2.6358 | ↓ |
| 10 | 5 | 3-Aminoisobutanoic acid | 2.6416, 2.6358, 1.1958, 3.1157, 1.2016 | ↓↓↑↓↑ | 3.1157, 2.6358, 1.1899, 1.1958, 1.1841 | ↓↓↑↑↑ |
| 10 | 12 | Hydroxypropionic acid | 2.6416, 2.6358, 3.8533, 3.8474 | ↓↓↓↓ | 2.6358 | ↓ |
| 10 | 12 | Methionine | 2.6416, 2.6358, 3.8533, 3.8474 | ↓↓↓↓ | 2.6358 | ↓ |
| 11 | 3 | 3-methylhistidine | 7.6757, 3.1509, 7.0201, 6.9908, 3.1392, 3.1333, 3.145 | ↑↓↓↓↓↓↓ | 3.145, 3.1392, 3.1333, 7.0259 | ↓↓↓↓ |
| 11 | 16 | Pseudouridine | 7.6757, 3.7362, 3.8533, 3.7187, 3.8474 | ↑↓↓↓↓ | 3.7362, 4.1343 | ↓↑ |
| 11 | 23 | Hippuric acid | 7.6757, 7.8045 | ↑↓ | 4.1343 | ↑ |
| 11 | N/A | 4-Aminohippuric acid | 7.6757 | ↑ | N/A | N/A |

| | | | | | | |
|---|---|---|---|---|---|---|
| 11 | 14 | Urea | 7.6757, 7.143, 7.0201, 6.9908 | ↑↑↓↓ | 4.6377, 4.6318, 6.5686, 7.0259, 6.6678, 4.1343 | ↓↓↓↓↓↑ |
| 11 | N/A | Indoleacetate | 7.6757, 7.143 | ↑↑ | N/A | N/A |
| 11 | N/A | Cinnamic acid | 7.6757, 7.424 | ↑↓ | N/A | N/A |
| 11 | 3 | Phenylalanine | 7.6757, 7.143, 7.0201, 3.1157, 3.1392, 3.1333, 3.145, 7.424 | ↑↑↓↓↓↓↓↓ | 3.145, 3.1392, 3.1157, 3.1333, 3.1275, 7.0259 | ↓↓↓↓↓↓ |
| 11 | 17 | Thymidine | 7.6757, 1.9216, 2.3372, 3.8533, 3.8474 | ↑↑↓↓↓ | 2.3197 | ↓ |
| 12 | 7 | Methanol | 3.3557 | ↓ | 3.3557, 3.3616 | ↓↓ |
| 13 | N/A | Hydroxyphenyllactate | 2.8582, 7.143 | ↑↑ | N/A | N/A |
| 14 | 3 | Ethanolamine | 3.1509, 3.1157, 3.8533, 3.1392, 3.1333, 3.145, 3.8474 | ↓↓↓↓↓↓↓ | 3.145, 3.1392, 3.1157, 3.1333, 3.1275 | ↓↓↓↓↓ |
| 14 | 3 | L-Cystathionine | 3.1509, 3.1157, 3.8533, 3.1392, 3.1333, 3.8709, 3.145, 3.8474 | ↓↓↓↓↓↓↓↓ | 3.145, 3.1392, 3.1157, 3.1333, 3.1275 | ↓↓↓↓↓ |
| 14 | 20 | Histidine | 3.1509, 7.143, 7.0201, 6.9908 | ↓↑↓↓ | 7.0259 | ↓ |
| 15 | 16 | D-Xylitol | 3.7421, 3.7362, 3.8533, 3.8709, 3.7187, 3.8474 | ↓↓↓↓↓↓ | 3.7362 | ↓ |
| 15 | 16 | L-Arabitol | 3.7421, 3.7362, 3.8533, 3.8709, 3.8474 | ↓↓↓↓↓ | 3.7362, 4.1343 | ↓↑ |
| 15 | 16 | Glutamate | 3.7421, 3.7362, 2.3372, 2.2553 | ↓↓↓↓ | 3.7362, 2.3197, 2.2553, 1.9626 | ↓↓↓↑ |
| 15 | 16 | D-Galactose | 3.7421, 3.7362, 3.8533, 3.7187, 3.8474 | ↓↓↓↓↓ | 3.7362 | ↓ |
| 15 | N/A | Isovalerylglycine | 3.7421 | ↓ | N/A | N/A |
| 15 | N/A | Glutamine | 3.7421 | ↓ | N/A | N/A |
| 15 | 16 | Glyceric acid | 3.7421, 3.7362, 3.8533, 3.8474 | ↓↓↓↓ | 3.7362, 4.1343 | ↓↑ |
| 15 | N/A | Sorbitol | 3.7421, 3.8533, 3.8709, 3.8474 | ↓↓↓↓ | N/A | N/A |

| | | | | | | |
|---|---|---|---|---|---|---|
| 15 | 16 | N-Acetylneuraminic acid | 3.7421, 3.5957, 1.9216, 3.8533, 3.8709, 3.8474 | ↓↓↓↓↓↓ | 3.5957 | ↓ |
| 15 | 16 | trans-Aconitic acid | 3.7421, 3.7362, 6.9908 | ↓↓↓ | 3.7362 | ↓ |
| 16 | 16 | Phosphorylcholine | 3.5957 | ↓ | 3.5957, 4.1343 | ↓↑ |
| 16 | 8 | D-Xylose | 3.5957, 3.3206 | ↓↑ | 3.3206, 3.5957 | ↑↓ |
| 16 | 16 | Erythritol | 3.5957 | ↓ | 3.5957 | ↓ |
| 17 | N/A | p-Hydroxyphenylacetate | 7.143 | ↑ | N/A | N/A |
| 18 | 18 | p-Cresol sulfate | 7.143, 2.2553 | ↑↓ | 2.2553 | ↓ |
| 18 | N/A | Tyrosine | 7.143 | ↑ | N/A | N/A |
| 19 | 13 | Ethanol | 1.1958, 1.2016 | ↑↑ | 1.1899, 1.1841, 1.1958 | ↑↑↑ |
| 19 | 13 | L-Fucose | 1.1958, 1.2016 | ↑↑ | 1.1899, 1.1958, 1.1841 | ↑↑↑ |
| 19 | 13 | Dihydrothymine | 1.1958, 2.7997, 1.2016 | ↑↑↑ | 1.1899, 1.1958, 1.1841 | ↑↑↑ |
| 20 | 16 | L-Kynurenine | 3.7362, 3.7187, 7.424 | ↓↓↓ | 3.7362, 4.1343 | ↓↑ |
| 21 | 22 | Arginine | 1.9216 | ↑ | 1.9626 | ↑ |
| 21 | N/A | Acetate | 1.9216 | ↑ | N/A | N/A |
| 21 | N/A | Symmetric dimethylarginine | 1.9216, 2.7997 | ↑↑ | N/A | N/A |
| 21 | 17 | L-2-Hydroxyglutaric acid | 1.9216, 2.3372, 2.2553 | ↑↓↓ | 2.3197, 2.2553, 1.9626 | ↓↓↑ |
| 21 | 17 | Aminoadipic acid | 1.9216, 2.3372, 2.2553, 3.7187 | ↑↓↓↓ | 2.3197, 2.2553 | ↓↓ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 22 | N/A | 4-Hydroxybenzoic acid | 7.8045 | ↓ | N/A | N/A |
| 22 | 23 | 7-Methylxanthine | 7.8045, 3.8533, 3.8709, 3.8474 | ↓↓↓↓ | 4.1343 | ↑ |
| 23 | 11 | 3-Hydroxyisovaleric acid | 2.3372, 1.3128, 1.2777 | ↓↓↓ | 1.2777, 1.3011 | ↓↓ |
| 23 | 17 | 4-Pyridoxic acid | 2.3372 | ↓ | 2.3197 | ↓ |
| 23 | N/A | Glutaric acid | 2.3372 | ↓ | N/A | N/A |
| 23 | 20 | Vanillic acid | 7.0201, 6.9908, 3.8709, 7.424 | ↓↓↓↓ | 7.0259 | ↓ |
| 23 | 20 | Phenol | 7.0201, 6.9908 | ↓↓ | 7.0259 | ↓ |
| 24 | 5 | 1,3-Diaminopropane | 3.1157 | ↓ | 3.1157, 3.1275 | ↓↓ |
| 24 | 5 | Malonate | 3.1157 | ↓ | 3.1157 | ↓ |
| 24 | 5 | Cysteine | 3.1157 | ↓ | 3.1157 | ↓ |
| 24 | N/A | Homovanillic acid | 3.8533, 3.8474 | ↓↓ | N/A | N/A |
| 24 | 23 | Serine | 3.8533, 3.8709, 3.8474 | ↓↓↓ | 4.1343 | ↑ |
| 24 | N/A | Inosine | 3.8533 | ↓ | N/A | N/A |
| 24 | 23 | Gluconic acid | 3.8533, 3.8474 | ↓↓ | 4.1343 | ↑ |
| 24 | N/A | Adenosine | 3.8533, 3.8709, 3.8474 | ↓↓↓ | N/A | N/A |
| 25 | N/A | Methylguanidine | 2.7997 | ↑ | N/A | N/A |
| 25 | N/A | 5-Aminolevulinic acid | 2.7997 | ↑ | N/A | N/A |

| | | | | | | |
|---|---|---|---|---|---|---|
| 26 | 11 | Methylmalonate | 1.2016, 1.2777 | ↑↓ | 1.2777 | ↓ |
| 27 | 4 | Dimethyl sulfone | 3.1392, 3.1333 | ↓↓ | 3.1392, 3.1333, 3.1275 | ↓↓↓ |
| 28 | 13 | Thymol | 2.2553 | ↓ | 1.1899, 2.2553, 1.1841 | ↑↓↑ |
| 28 | 18 | Acetoacetate | 2.2553 | ↓ | 2.2553 | ↓ |
| 28 | 15 | Valine | 2.2553 | ↓ | 1.0588, 2.2553 | ↓↓ |
| 29 | N/A | Sucrose | 3.8709, 3.8474 | ↓↓ | N/A | N/A |
| 29 | N/A | Betaine | 3.8709 | ↓ | N/A | N/A |
| 29 | N/A | Propylene glycol | 3.8709 | ↓ | N/A | N/A |
| 30 | N/A | L-Arabinose | 3.8474 | ↓ | N/A | N/A |
| 30 | N/A | Dehydroascorbic acid | 3.8474 | ↓ | N/A | N/A |
| 30 | N/A | 1-Methyladenosine | 3.8474 | ↓ | N/A | N/A |
| 31 | N/A | Quinolinic acid | 7.424 | ↓ | N/A | N/A |
| 31 | N/A | Mandelic acid | 7.424 | ↓ | N/A | N/A |
| N/A | 8 | Trimethylamine N-oxide | N/A | N/A | 3.3206, 2.8816 | ↑↑ |
| N/A | 14 | D-Glucuronic acid | N/A | N/A | 4.6377, 4.6318 | ↓↓ |
| N/A | 15 | 2-Ketobutyric acid | N/A | N/A | 1.0588 | ↓ |

| | | | | | | |
|---|---|---|---|---|---|---|
| N/A | 16 | Alpha-Lactose | N/A | N/A | 3.5957 | ↓ |
| N/A | 19 | cis-Aconitic acid | N/A | N/A | 6.5686 | ↓ |
| N/A | 20 | Histidine | N/A | N/A | 7.0259 | ↓ |
| N/A | 21 | Glutaconic acid | N/A | N/A | 6.6678 | ↓ |
| N/A | 21 | Kynurenic acid | N/A | N/A | 6.6678 | ↓ |
| N/A | 22 | Citraconic acid | N/A | N/A | 1.9626 | ↑ |
| N/A | 22 | Methylglutaric acid | N/A | N/A | 1.9626 | ↑ |
| N/A | 22 | N-Acetylputrescine | N/A | N/A | 1.9626 | ↑ |
| N/A | 22 | Isoleucine | N/A | N/A | 1.9626 | ↑ |
| N/A | 23 | Glucaric acid | N/A | N/A | 4.1343 | ↑ |
| N/A | 23 | Pyroglutamic acid | N/A | N/A | 4.1343 | ↑ |
| N/A | 23 | Glycolic acid | N/A | N/A | 4.1343 | ↑ |

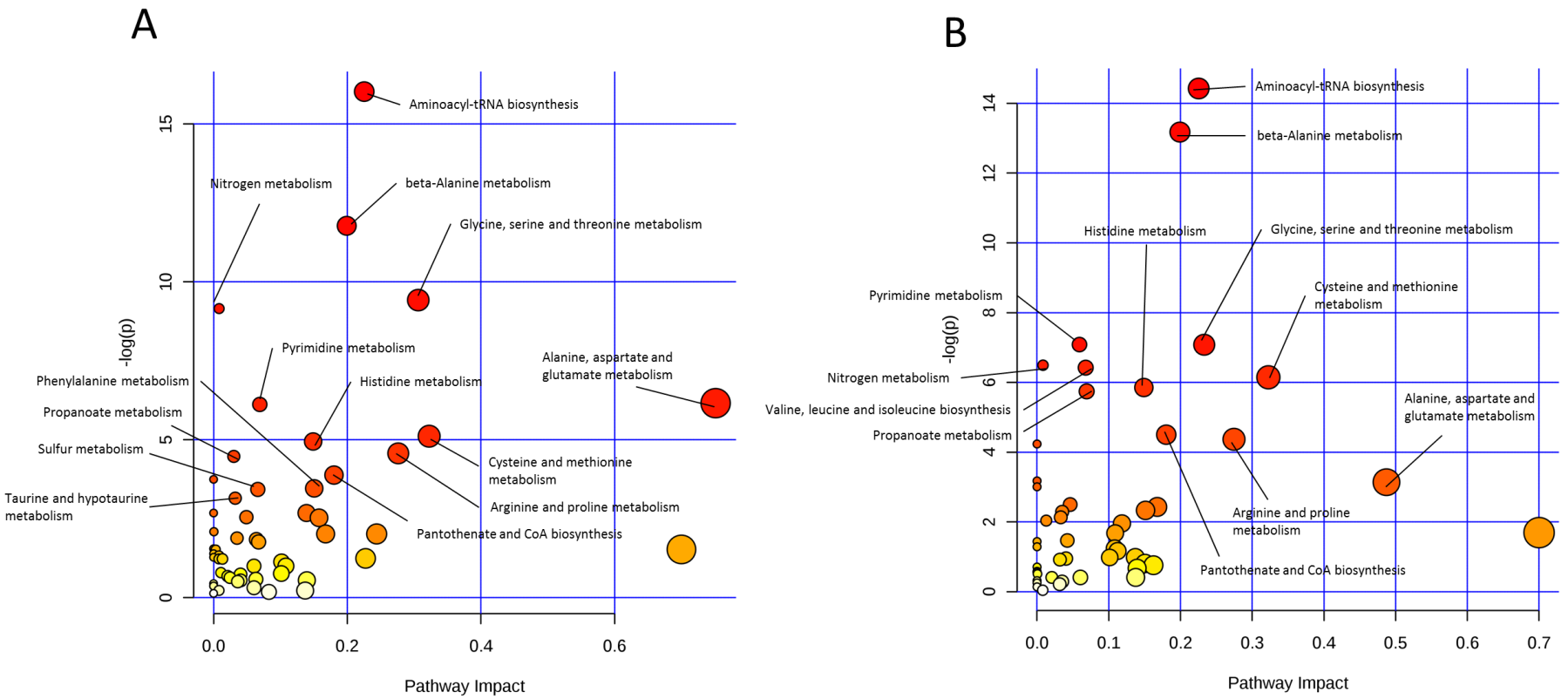



**Figure 6-27 Pathway analysis of potential biomarkers implicated by PLS-R analysis of FACIT-F and CSA patients' urinary metabolites**
Pathway analysis of potential biomarkers implicated by the PLS-R of (A) all the urinary metabolome and (B) the urinary metabolome devoid of the inflammatory signal.

**Table 6-30 Pathway analysis of potential biomarkers implicated by PLS-R analysis of FACIT-F and CSA patients' urinary metabolites**
Results from PLS-R models using all the urinary metabolome and the urinary metabolome devoid of the inflammatory signal.
**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.
**The impact score**: Each pathway has a total importance score of 1. The importance measure of each metabolite node (a point where pathways intersect within a larger network) is expressed as a fraction with regards to the total pathway importance, and the pathway impact score is the cumulative score of matched metabolite nodes.
* p value calculated using hypergeometric test

| | PLS-R model using all urinary metabolome | | | PLS-R model using urinary metabolome devoid of the inflammatory signal | | |
|---|---|---|---|---|---|---|
| Pathway name | Match status | P value* | Impact score | Match status | P value* | Impact score |
| Alanine, aspartate and glutamate metabolism | 5/24 | 0.002114 | 0.75119 | 3/24 | 0.043299 | 0.48718 |
| Cysteine and methionine metabolism | 7/56 | 0.006018 | 0.32254 | 7/56 | 0.002141 | 0.32254 |
| Glycine, serine and threonine metabolism | 9/48 | 8.09E-05 | 0.30622 | 7/48 | 8.43E-04 | 0.23312 |
| Arginine and proline metabolism | 8/77 | 0.010356 | 0.27629 | 7/77 | 0.012667 | 0.27458 |
| Aminoacyl-tRNA biosynthesis | 15/75 | 1.10E-07 | 0.22536 | 13/75 | 5.44E-07 | 0.22536 |
| beta-Alanine metabolism | 8/28 | 7.69E-06 | 0.19919 | 8/28 | 1.90E-06 | 0.19919 |
| Pantothenate and CoA biosynthesis | 4/27 | 0.020606 | 0.18014 | 4/27 | 0.011093 | 0.18014 |
| Phenylalanine metabolism | 5/45 | 0.031406 | 0.15056 | 2/45 | 0.44501 | 0.15056 |
| Histidine metabolism | 6/44 | 0.007114 | 0.14893 | 6/44 | 0.002871 | 0.14893 |
| Pyrimidine metabolism | 8/60 | 0.002201 | 0.06914 | 7/60 | 0.003204 | 0.06914 |
| Sulfur metabolism | 3/18 | 0.03247 | 0.06614 | 2/18 | 0.11848 | 0.03307 |
| Taurine and hypotaurine metabolism | 3/20 | 0.042839 | 0.03237 | 1/20 | 0.49275 | 0 |
| Propanoate metabolism | 5/35 | 0.011409 | 0.03023 | 6/35 | 8.38E-04 | 0.05916 |
| Nitrogen metabolism | 8/39 | 1.06E-04 | 0.0083 | 6/39 | 0.001515 | 0.0083 |
| Valine, leucine and isoleucine biosynthesis | 2/27 | 0.2931 | 0.01325 | 5/27 | 0.001625 | 0.06784 |

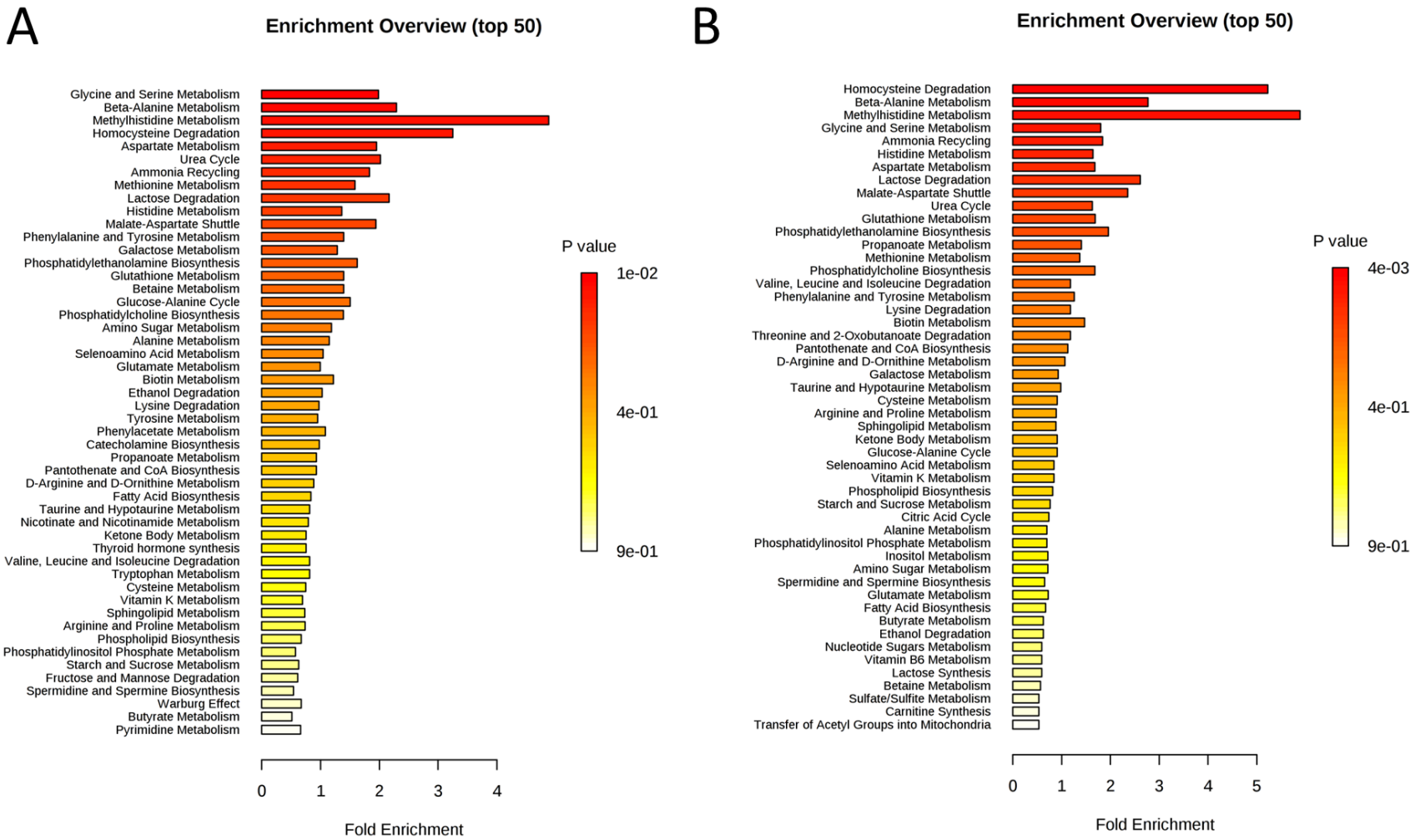



**Figure 6-28 Enrichment analysis of key metabolites in urine implicated as potential biomarkers by the PLS-R analysis of FACIT-F and CSA patients' urinary metabolites**

Enrichment analysis of potential biomarkers implicated by the PLS-R of (A) all the urinary metabolome and (B) the urinary metabolome devoid of the inflammatory signal.

**Table 6-31 Enrichment analysis of key metabolites in urine implicated as potential biomarkers by the PLS-R analysis of FACIT-F and CSA patients' urinary metabolites**

Results from PLS-R models using all the urinary metabolome and the urinary metabolome devoid of the inflammatory signal.

**Match status**: The number of matched metabolites expressed as a fraction of the total metabolites in the pathway.

**The fold enrichment value**: the value is generated by the number of matched metabolites divided by the number of metabolites expected to be present in the pathway as per the reference metabolome provided by KEGG.

* p value calculated using hypergeometric test

| | PLS-R model using all urinary metabolome | | | | PLS-R model using urinary metabolome devoid of the inflammatory signal | | | |
|---|---|---|---|---|---|---|---|---|
| Metabolite pathway | Match status | Fold enrichment | Expected | P value* | Match status | Fold enrichment | Expected | P value* |
| Glycine and Serine Metabolism | 12/59 | 1.98 | 6.05 | 0.0128 | 9/59 | 1.80 | 5.01 | 0.0547 |
| Beta-Alanine Metabolism | 8/34 | 2.29 | 3.49 | 0.0175 | 8/34 | 2.77 | 2.89 | 0.00567 |
| Homocysteine Degradation | 3/9 | 3.25 | 0.923 | 0.0556 | 4/9 | 5.23 | 0.765 | 0.0044 |
| Methylhistidine Metabolism | 2/4 | 4.89 | 0.41 | 0.0545 | 2/4 | 5.88 | 0.34 | 0.0382 |

## 6.5 Discussion

In this study, we applied $^{1}$H-NMR metabolomics to study the relationship between the extent of patient reported fatigue, as assessed by the FACIT-F, and the serum and urinary metabolome in a cohort of DMARD naïve patients with inflammatory arthritis and CSA at presentation. Multi-variate statistical analysis produced serum and urinary metabolite models which were able to predict FACIT-F. NMR analysis of serum and urine samples taken at presentation of all patients demonstrated that down regulation of the urea cycle, oxidative stress, reduced glycolysis and skeletal muscle degradation are associated with worse fatigue (see Figure 6-29 and Figure 6-30).

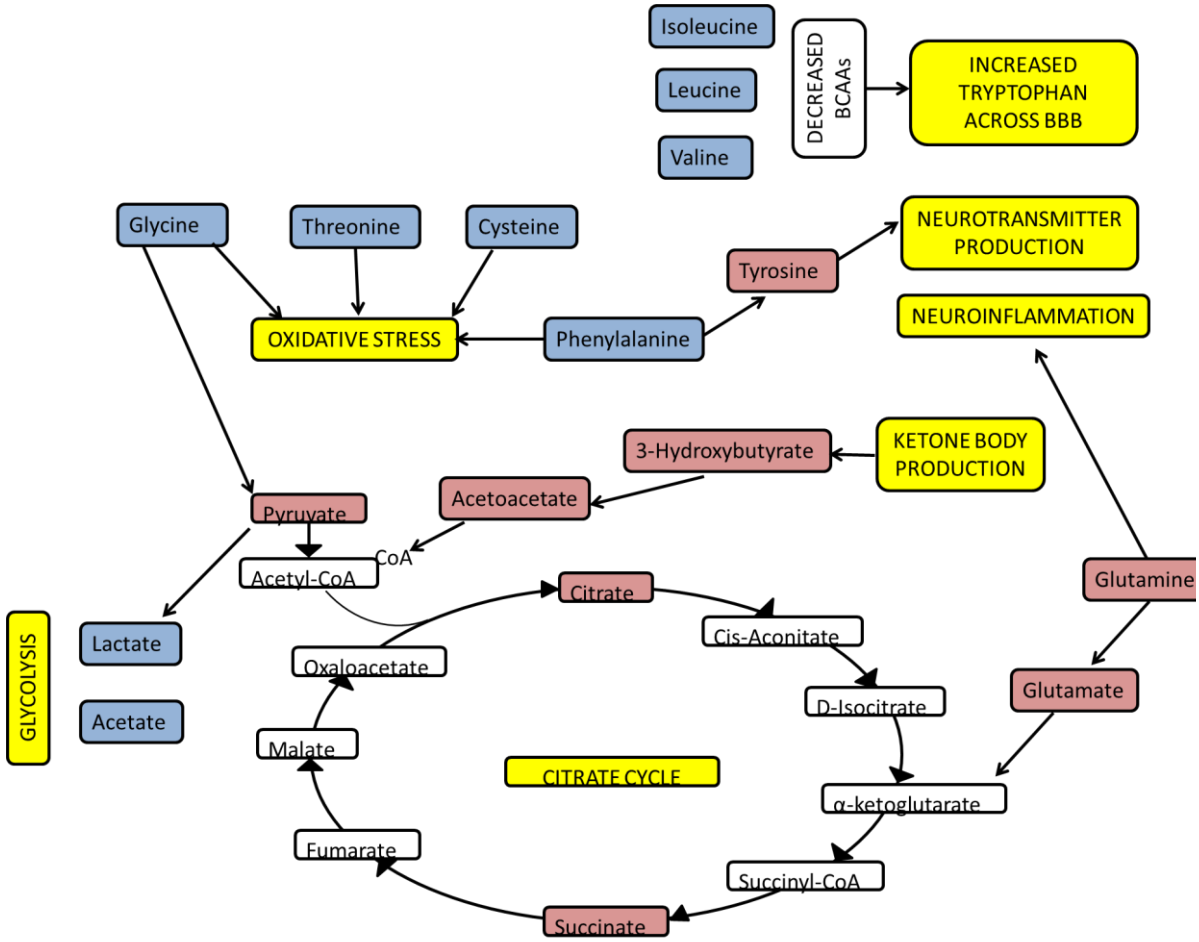



**Figure 6-29 Overview of key pathways and serum metabolites correlating with fatigue.**
A summary of the metabolic permutations shown by the PLS-R analysis between fatigue and the serum metabolome of inflammatory arthritis and CSA patients as assessed by NMR spectroscopy. Red metabolites had a positive correlation with fatigue and blue metabolites had a negative correlation with fatigue.

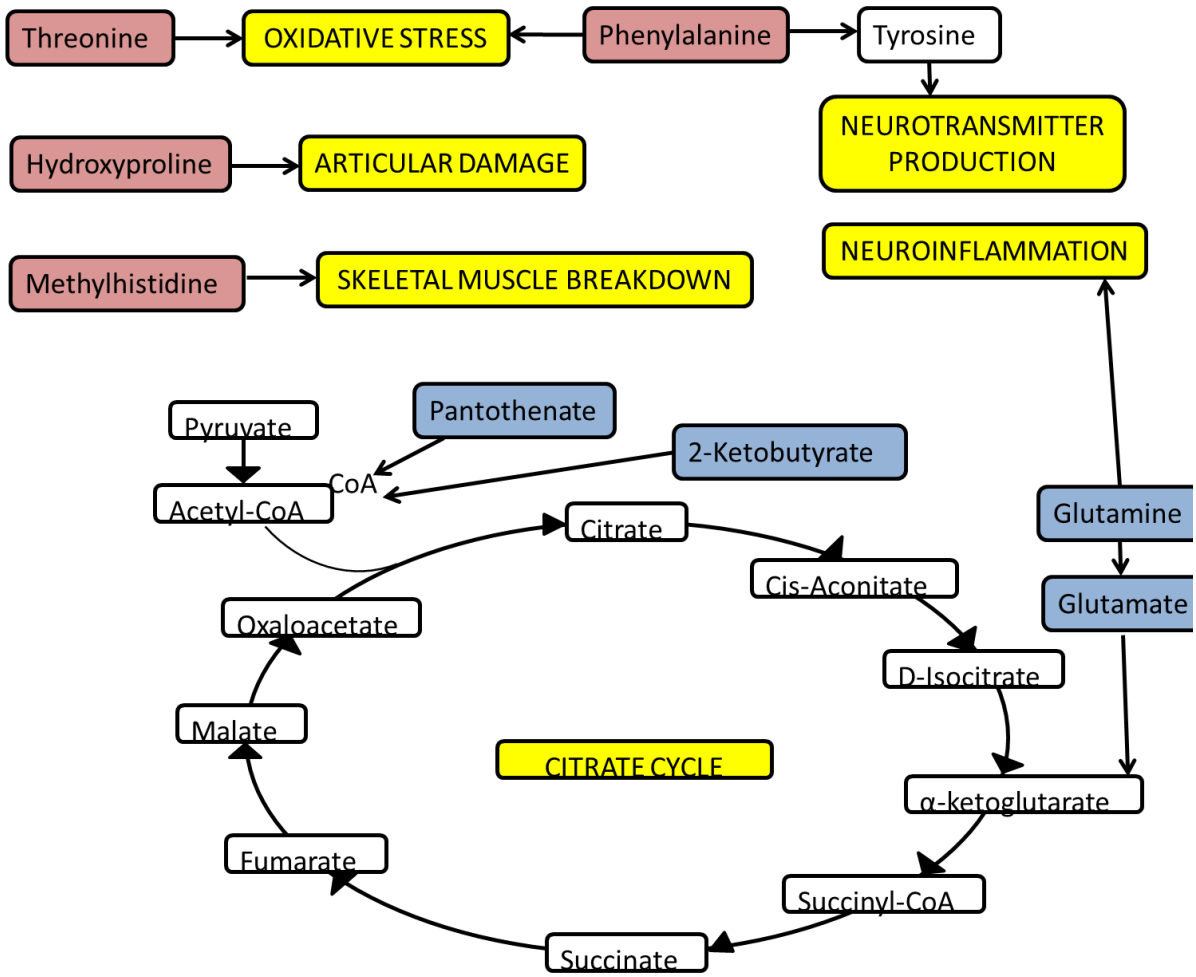



**Figure 6-30 Overview of pathways and urinary metabolites correlating with fatigue.** A summary of the metabolic permutations shown by the PLS-R analysis between fatigue and the urinary metabolome of inflammatory arthritis and CSA patients as assessed by NMR spectroscopy. Red metabolites had a positive correlation with fatigue and blue metabolites had a negative correlation with fatigue.

Two small studies are known to date to have used metabolomics to examine the relationship between fatigue and metabolism using metabolomic approaches to assess plasma in RA. Sixteen bDMARD naïve RA patients, although most patients were on csDAMARDs, showed increased oxidative stress and dysregulated niacin metabolism correlate with fatigue (439). A study of RA patients ≥65 years of age showed a programme of moderate to high intensity exercise was associated with improved patient reported physical fatigue. Plasma metabolomics of 48 subjects, showed changes in physical fatigue were associated with changes in the metabolism of lipids, bile acids, the urea cycle and several sugars (440). Both studies suggest there is a relationship between fatigue and the serum and plasma metabolome respectively in RA patients.

The present study validates these findings and, in addition, shows a relationship between the urinary metabolome and fatigue in RA patients. Metabolic changes were subject to cross referencing using multiple PLS-R analyses to assess the serum and urinary metabolome. This study also examines the relationship between fatigue and metabolism in PsA, UA, CSA and a heterogeneous cohort including various other inflammatory arthopathies (see Table 5-16 and Table 6-1). The PLS-R metabolomics models showed a relationship between fatigue and metabolism across the entire cohort and sub-

analyses of RA, PsA and CSA patients. A relationship between fatigue and metabolism was seen in UA patients using the entire serum metabolome.

### 6.5.1 Alanine, aspartate and glutamate metabolism (including nitrogen metabolism)

PLS-R models of the serum metabolome and FACIT-F revealed a positive correlation between greater fatigue and glutamine and glutamate.

The alanine, aspartate and glutamate metabolism pathway plays a role in nitrogen handling, energy production and managing oxidative stress (309). Glutamate and its precursor glutamine are utilised for numerous purposes including nitrogen metabolism, alpha-ketoglutarate production and neurotransmitter production. Glutamate has been postulated as a cause of fatigue mediated by its effect on the astrocytes. Astrocytes provide nutrients to neurones and rely upon regulated intracellular and extracellular glutamate to function. TNFα, IL-1β, and IL-6 compromise astrocyte glutamate uptake resulting in accumulation of extracellular glutamate postulated to be the foundation of cognitive fatigue (442, 443). Neuroinflammation is also associated with decreased glutamate receptors on the abluminal membranes of the blood brain barrier which results in increased elimination of glutamate from the brain while concurrently preventing entry from the systemic circulation [189]. This could provide a mechanism by which glutamate accumulation is associated with fatigued states.

PLS-R models of serum and urinary metabolome show a positive association between aspartate and fatigue. Aspartate, along with other amino acids, are responsible for transporting nitrogen to hepatocytes in the form ammonia (Figure 6-31) before it enters the urea cycle (Figure 5-28) and the resulting urea is excreted in urine (444).

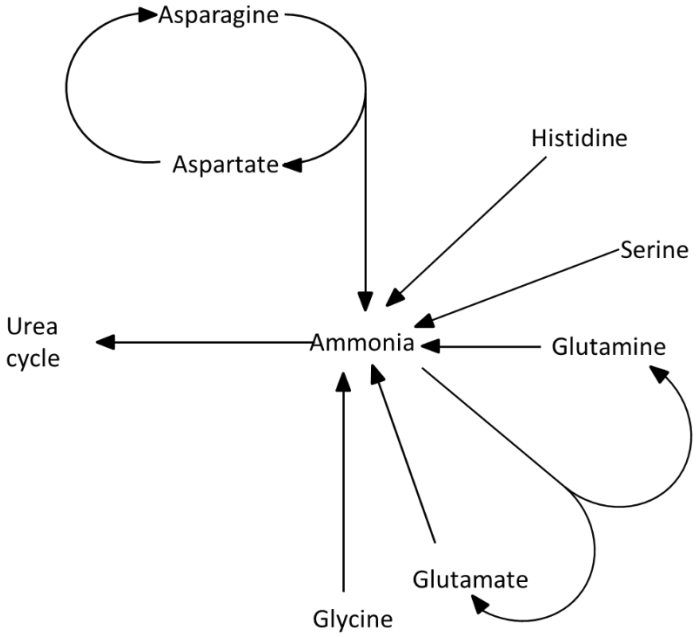



**Figure 6-31 Nitrogen metabolism**

The nitrogenous waste product ammonia can cross the blood brain barrier and impair cerebral metabolism and neurotransmission which could result in the perception of fatigue (445, 446). Studies

have shown reduced ammonia is associated with elevated aspartate (447). Our findings suggest that elevated fatigue is associated with decreased urea cycle activity. As evidenced by a positive correlation between fatigued states and urea cycle precursors serum glutamine, glutamate and alanine. Furthermore, there is a negative correlation between fatigued states and urea cycle products such as ornithine, arginine and urea. Urea cycle down regulation has been seen in fatigued states in RA (439) and CFS patients (429) in previous metabolomics studies.

The alanine, aspartate and glutamate pathway was identified as a pathway of importance in both the CRP and FACIT-F functional analysis of serum and urinary metabolomics. Enrichment analysis showed metabolites related to glutamate and aspartate metabolism, ammonia recycling and the urea cycle were overrepresented to a greater extent of the functional analysis of the biomarkers identified by the PLS-R models of all patients' serum metabolome and FACIT-F compared to the PLS-R model of all patients' serum metabolome and CRP. This suggests that whilst alterations in these pathways are associated with inflammatory states, they appear to be associated with fatigued states to a greater degree.

### 6.5.2 Glycine, serine & threonine metabolism

Both serum threonine and glycine had a negative association with fatigue according to PLS-R models of the serum metabolome and FACIT-F. Threonine is an essential amino acid. It is absorbed in the small intestine and metabolised in mitochondria into glycine, which can then be utilised for glutathione production. Glutathione is essential for combating ROS and oxidative stress and has been shown to correlate with fatigued states (448).

Metabolites belonging to the glycine and serine pathway were overrepresented in the enrichment analysis of the biomarkers identified by the PLS-R of the serum metabolome and FACIT-F. This finding is applicable in both the analysis of all the serum metabolome and serum metabolome with inflammatory signal removed. Metabolites belonging to the glycine and serine pathway were not overrepresented in the enrichment analysis of the biomarkers identified by the PLS-R of the serum metabolome and CRP. This suggests that in this metabolic pathway there are greater perturbations associated with fatigue than inflammation.

### 6.5.3 Arginine and proline metabolism

Arginine and proline are made from glutamate. The pathways linking arginine, glutamate, and proline are bidirectional. Utilization or production of these amino acids is highly dependent on cell type and developmental stage, so the equilibrium may be perturbed in immune cells, neurones or muscle to affect fatigue. Hydroxyproline, has a positive correlation with fatigue in the PLS-R models of CSA and RA patients' urinary metabolome and FACIT-F. Hydroxyproline is an abundant amino acid formed from hydroxylation of proline. It is a major component of collagen and has been suggested as a surrogate marker of collagen degradation (449). As such urinary hydroxyproline has long been thought of as a biomarker for articular damage in RA (450) and has been shown to discriminate between early RA, early OA, early non-RA inflammatory arthritis and individuals with good skeletal health (451). The positive correlation between fatigue and urinary hydroxyproline suggest however there may be a relationship between fatigue and articular damage.

According to PLS-R models of the urinary metabolome and FACIT-F, there is a negative correlation between urinary arginine and fatigue. Arginine, the precursor of nitric oxide, has been associated with improved skeletal and cardiac function due to nitric oxide induced endothelial vasodilatation (452). Dysregulated arginine metabolism has been associated with cognitive impairment in older adults (453). Thus, arginine is important for both muscle and cognitive function.

Enrichment analysis of the entire cohort showed whilst arginine and proline metabolism is overrepresented in the model examining CRP and serum metabolome (Table 5-8 and Table 5-12), it is overrepresented to a greater extent in the model examining FACIT-F and serum metabolome (Table 6-7).

### 6.5.4 Glycolysis, gluconeogenesis & pyruvate metabolism

A positive correlation between serum pyruvate and fatigue is seen across the majority of PLS-R models of the serum metabolome and FACIT-F. This finding may be explained by a reduction in PDH, a finding that has been seen in CFS patients (435). There is a negative correlation between fatigue and both lactate and acetate seen in most PLS-R models of the serum metabolome and FACIT-F. Models of carbohydrate metabolism perturbations in CFS patients provide a potential explanation for the above observations. McGregor and colleagues (454) postulate that a "hypermetabolic event" is evidence of impaired glycolysis in CFS patients. This is characterised by a release of metabolites and inhibition of protein synthesis from skeletal muscle tissue when cytoplasmic or mitochondrial ATP is insufficient to meet demand. A similar phenomenon is seen in lymphocytes when glycolysis is inhibited in vitro (455, 456). Impaired glycolysis may be a result of a reduction in acetate, secondary to reduced acetate production via impaired pyruvate catabolism and or histone deacetylation (454). Thus, reduced glycolysis could account for the positive correlation with pyruvate and the negative correlation with lactate and acetate with fatigue respectively.

### 6.5.5 Cysteine, methionine and glutathione metabolism

Serum cysteine had a negative correlation with fatigue according to the majority of the PLS-R models of serum metabolome and FACIT-F. It is possible that oxidative stress, which has been associated with fatigued states (439, 448, 457, 458), is responsible for this finding. Cysteine may be utilised in replenishing GSH as GSH neutralise oxidative stress.

There was a positive correlation of cysteine precursors, methionine and cystathionine, and fatigue according to the PLS-R of the urinary metabolome and FACIT-F. The finding of urinary methionine positively correlate with fatigue has been also identified in healthy individuals (459). A potential explanation for this is as methionine is an essential amino acid, it could be liberated from proteinolysis a rate which is faster than that it is incorporated into cysteine.

Furthermore, enrichment analysis of the PLS-R models of all patients' serum and urinary metabolome and FACIT-F showed overrepresentation of homocysteine degradation and methionine metabolism for the serum metabolome alone respectively. These findings could represent a metabolic adaptation to replenish cysteine stores for GSH synthesis.

### 6.5.6 Methane metabolism

There is a positive correlation between formate and fatigue in the PLS-R models using all and CSA patients' entire serum metabolome and FACIT-F. Formate is a short chain fatty produced as a product of methane metabolism or as an intermediary of acetate production. Inflammation associated intestinal dysbiosis has been shown to increase gut lumen formate levels (460). Although some of this formate is oxidised for energy purposes by some intestinal bacteria, some increased formate could potentially be absorbed into the systemic circulation and thus could represent a metabolic signature of inflammation associated intestinal dysbiosis. RA has long been associated with inflammatory associated gut dysbiosis in both preclinical and established disease (461, 462). Our finding may indicate an association between fatigue and intestinal inflammation associated dysbiosis.

### 6.5.7 Citrate cycle

There is a positive correlation between fatigue and serum citrate and succinate respectively across most PLS-R models of the serum metabolome and FACIT-F. In the context of inflammation and cancer metabolism, there appears to be changes in citrate cycle (344, 463). Evidence suggests the citrate cycle has multiple functions in addition to energy generation, including immune signalling (344) and macromolecule synthesis (463). Activated dendritic cells and macrophages have an altered citrate cycle, one consequence of which is the accumulation of both citrate and succinate (344). Accumulation of serum citrate and succinate positively correlates with fatigue in the present study and may reflect changes in immunometabolism. Furthermore, urinary pantothenate had a negative correlation with fatigue according to the PLS-R model of all patients' urinary metabolome and FACIT-F. Pantothenate is a component of CoA. This provides further evidence to implicate upregulation of the citrate cycle as fatigue increases. Our findings suggest glycolysis could be impaired (**section 6.5.4**), however fatty acid catabolism and glutaminolysis can provide alternative fuel for the citrate acid cycle.

### 6.5.8 Phenylalanine, tyrosine & tryptophan biosynthesis

In PLS-R models of the entire cohort and of RA, serum phenylalanine has a negative correlation with fatigue. Phenylalanine, an essential amino acid, is a precursor of tyrosine. It is possible that phenylalanine is being utilised as a result of increased oxidation secondary to oxidative stress (439, 464). Another possibility is that phenylalanine is metabolised for producing substrates for the citrate cycle, especially as glycolysis may be impaired (436). Finally, phenylalanine could be metabolised further to tyrosine. In support of this, the PLS-R model of all patients' serum metabolome and FACIT-F shows a positive correlation between serum tyrosine and fatigue. Tyrosine is a non-essential amino acid that can be converted into biological important metabolites including L-DOPA, which can be further metabolised to dopamine, noradrenaline and adrenaline. Approximately 15% of phenylalanine is converted to tyrosine and this predominantly takes place in hepatocytes by the enzyme PAH (465). PAH is downregulated during inflammation (466) and malignancy (467), so represents a potential novel finding in fatigued states. Phenylalanine and tyrosine metabolism are overrepresented in enrichment analyses of the PLS-R models of all patients' serum metabolome and FACIT-F.

### 6.5.9 Synthesis and degradation of ketone bodies & butanoate metabolism

In the majority of PLS-R models there is a positive correlation between fatigue and 3-hydroxybutyrate and its precursor acetoacetate. Acetoacetate is a ketone body which can spontaneously breakdown

into acetone. Acetoacetate can also be metabolised to 3-hydroxybutyrate. One possible explanation for the positive correlation between fatigue and 3-hydroxybutyrate and its precursor acetoacetate, is increased fatty acid oxidation to fuel the citrate cycle which lends further support to the findings reported in **section 6.5.7**. Increased fatty acid oxidation has been seen in hepatocytes in animal models with adjuvant induced arthritis (468). Fatty acid oxidation has also been postulated to play a role in the development of chronic fatigue syndrome (435, 469).

Beta-alanine metabolism was overrepresented to a greater extent in the PLS-R model of all patients' urinary metabolome and FACIT-F. As beta-alanine metabolism could indicative of fatty acid synthesis, this could by a metabolic adaptation to meet fatty acid demand.

### 6.5.10 Valine, leucine and isoleucine biosynthesis & degradation

There is a negative correlation between fatigue and valine, leucine and isoleucine in the majority of PLS-R models of the serum metabolome and FACIT-F. Valine, leucine and isoleucine are all branched chain amino acids which have important roles in energy metabolism in muscle and cognition (470). Valine, leucine and isoleucine can be metabolised by muscle to provide energy. These BCAA donate their amino group to, predominantly, either glutamine or alanine, which can be used to fuel the citrate cycle. Decrease in branch chain amino acids result in more tryptophan being absorbed across the blood brain barrier as they both compete for the large neutral amino acid transporter. Tryptophan can be metabolised into serotonin. Patients with CFS have been shown to exhibit increased brain serotonin function (471), but diminished serotonin receptor binding (472). It is possible that an increased serotonergic state seen in CFS, may be present in those patients experiencing fatigue with inflammatory arthritis and CSA.

### 6.5.11 Histidine metabolism

Urinary histidine has a positive correlation with fatigue in the majority of PLS-R models of the urinary metabolome and FACIT-F. Similarly, urinary 3-methylhistidine has a positive correlation with fatigue in the majority of PLS-R models of the urinary metabolome and FACIT-F. Histidine is an essential amino acid. 3-methylhistidine is methylated histidine integrated into actin and myosin. Urinary and plasma 3-methylhistidine has been suggested as a marker of skeletal muscle turnover (364-368). CFS patients appear to have increased urinary 3-methylhistidine (427, 454). 3-Methylhistidine metabolism was overrepresented in the enrichment analysis of the PLS-R model all cohorts' urinary metabolome and FACIT-F. However methylhistidine metabolism was not overrepresented in the PLS-R models of CRP and urinary and serum metabolome. This suggests that muscle protein degradation may be associated with fatigue to a greater extent than inflammation.

## 6.6 Limitations

In addition to the limitations stated in **section 5.6**, the cohort used to isolate an inflammatory signal from the serum metabolome (n=270) was different to the final cohort used to investigate the relationship between serum metabolome (n=252) without the inflammatory signal and fatigue due to incomplete data collection. Although such an inflammatory signal could be considered "universal" independent of patients, the likelihood is that the precise NMR bins are likely to vary from cohort to

cohort depending on other variables such as magnetic field drift and pH variation between samples. Similarly, the cohort of patients used to investigate the relationship between serum metabolome and FACIT-F (n=252) was different to the cohort of patients used to investigate the relationship between urinary metabolome and FACIT-F (n=178). This is a confounding factor when comparing findings between biofluids.

Finally, fatigue PROMs may also introduce bias. As fatigue is often experienced at cognitive level, participants may have difficulty understanding questions contained in PROMs and may also be subjected to recall bias when reporting their fatigue. Sequela of inflammatory arthritis related to disease activity or damage, such as sleep disturbance, pain and depression may also be difficult to distinguish from fatigue and may lead to inaccurate patient reporting of fatigue.

## 6.7 Conclusion

The PLS-R models assessing the relationship between fatigue and metabolite profile showed a statistically significant relationship between metabolic permutations and fatigue in a cohort of patients with inflammatory arthritis and CSA. This relationship was present even after control for the impact on inflammation on metabolism had been implemented. Although a relationship between serum metabolome and fatigue in RA patients has been reported (439, 440), the relationship between urinary metabolome and fatigue represents a novel finding. There are number of metabolic changes that occur in fatigued states that overlap with the findings seen with elevated CRP these includes down regulation of the urea cycle, oxidative stress, intestinal dysbiosis and cachexia. However, there are interesting changes observed in carbohydrate metabolism which provide a unique and plausible cause of fatigue. Glycolysis appears to be downregulated and despite this the citrate cycle continues to proceed with evidence to suggest it is fuelled by amino acid and fatty acid catabolism. In contrast, findings associated seen **chapter 5** support aerobic glycolysis and inhibition of certain reactions within the citrate cycle. Furthermore, a positive correlation between fatigue and urinary hydroxyproline suggests a relationship between fatigue and articular damage. This suggests there may be a relationship between disease activity and fatigue.

The relationship between fatigue and metabolites, were seen across multivariate analyses using RA, PsA, UA and CSA patients in addition to the entire cohort. The metabolites implicated as potential biomarkers reveal broadly similar findings across each diagnostic group. Interestingly, however, the PLS-R model of CSA patients' serum metabolome and FACIT-F revealed a negative correlation between fatigue and pyruvate. Whilst PLS-R models of CSA patients' urinary metabolome and FACIT-F revealed a positive correlation between fatigue and lactate. These findings are contrary to the findings seen in the multivariate analyses using metabolic data from the entire cohort and RA patients. It is possible, that in the CSA patients there is increased glycolysis as fatigue increases and this relationship could reverse as the disease progresses from CSA to RA.

These findings provide novel insights into the relationship between serum and urinary metabolite profile and fatigue as measured by FACIT-F, and most notably this relationship persists despite using controlling for the effects of inflammation on the metabolome.

# 7 Conclusion

## 7.1 Introduction

We assessed the extent of fatigue in patients with newly presenting inflammatory arthritis and CSA using the PROMs FACIT-F and FVAS, and examined its relationship with clinical and demographic variables. Our study revealed fatigue was common and levels of fatigue were equivalent in patients with CSA and inflammatory arthritis.

Multivariate analysis revealed that low mood and disability were independently associated with fatigue in inflammatory arthritis and CSA patients, with tender joint count, additionally, independently associated with fatigue in inflammatory arthritis patients at baseline. Multinomial logistic regression modelling revealed greater PHQ-9 at baseline was associated with worse fatigue at follow up ($p=0.002$). Greater PHQ-9 at follow up was associated with a decreased chance of improved fatigue at follow up ($p<0.001$) and a greater a chance of worse fatigue at follow up ($p<0.001$). Finally, failure to achieve an improvement of ≥1.2 in DAS28CRP was associated with worse fatigue at follow up ($p=0.0018$).

We have investigated the effect that varying pre-processing parameters can have on supervised multivariate metabolomics analysis to ensure our analysis is robust. Evidence suggests PQN normalisation for urinary metabolomics has the advantage of accounting for dilutional effects (177). Furthermore, our data showed PQN normalisation of sera metabolomics data results in OPLS-DA models with greater $r^2$ values showing separation between patients whose CRP falls within the lowest tertile and individuals who have a CRP which falls in the highest tertile, compared to TSA normalisation in serum. Furthermore, the data showed greater g-log transformation, also resulted into OPLS-DA models with greater $r^2$ values showing separation between patients whose CRP falls within the lowest tertile and individuals who have a CRP which falls in the highest tertile. The present study demonstrates the impact of the magnitude of logarithmic transformation has on discriminant analysis. The original paper which describes how to apply g-log transformation applied this to a pooled biological sample (161), however, here we describe a technique to select an appropriate g-log transformation in situations where a pooled biological sample is not available.

As seen in multivariate models of fatigue in inflammatory arthritis and CSA patients, conventional clinical and demographic do not fully explain the variability of fatigue ($r^2$=0.666 and 0.539 respectively). We investigated whether metabolic permutations, as assessed by NMR metabolomics of urine and filtered sera, associated with fatigue. Prior to my work there were no publications utilising metabolomics to examine both urine and filtered sera in a diagnostically diverse cohort of newly presenting inflammatory arthritis and CSA patients. Many factors are known to influence metabolomics studies such as genes (473), sex (474), age (475), nutrition (476), body mass index (477), physical activity (478), alcohol (479), smoking (480), stress (481), circadian rhythm (482) and medication (483). Another important factor is inflammation (195). The relationship between inflammation and the metabolome has been previously studied in early inflammatory arthritis patients of ≤3 months duration (152). Young et al used unfiltered sera from two separate groups of early inflammatory arthritis patients to create PLS-R models to assess the relationship between the serum metabolome and CRP, which showed $r^2$ values of 0.671 ($n=89$, $p<0.001$) and 0.4157 ($n=127$, $p<0.001$) respectively, underscoring the importance of the relationship between inflammation and metabolism (152). Our study served not only to validate this relationship between CRP and the blood metabolome in a separate cohort of newly presenting inflammatory arthritis and CSA patients but also confirmed this in urine. Furthermore, I identified metabolic variables which associated with CRP, a metabolic 'inflammatory signal', allowing to control for the effect of inflammation when assessing the relationship between fatigue and metabolism.

PLS-R models of metabolomics data derived from both filtered serum and urine in the present study showed a correlation between CRP and metabolite profiles. PLS-R analysis of the full 590 serum metabolite binned data for all patients (n=270) showed that there was a correlation between metabolite data and CRP ($r^2$=0.2897, 7 LV, p<0.001). Using forward selection, 83 bins were identified which correlated with inflammation and a subsequent PLS-R analysis using these bins showed a strong correlation between serum metabolite profile and CRP ($r^2$=0.4811, 6 LV, p<0.001). PLS-R with the remaining 507 bins showed no correlation between serum metabolite profile and CRP ($r^2$=0.0356, 8 LV, p=0.059). The remaining 507 bins can thus be considered a serum metabolite profile without a significant inflammatory signal. A similar technique was applied to study the relationship between CRP and the urinary metabolome. Using the full 900 NMR urinary metabolite bins for all patients (n=178) there was a correlation between metabolite profile and CRP ($r^2$=0.0947, 1 LV, p=0.008). Using forward selection, 6 bins were identified which correlated with CRP and a subsequent PLS-R using these bins showed a correlation between urinary metabolite profile and CRP ($r^2$=0.1347, 1 LV, p=0.021). Two other additional forward selections were performed to identify 219 binned urinary NMR data which correlated with CRP and a PLS-R of the remaining 681 NMR bins showed no correlation between urinary metabolite profile and CRP ($r^2$=0.0187, 1 LV, p=0.241). Thus the remaining 681 NMR bins can be considered to represent a metabolite profile without a significant inflammatory signal.

PLS-R models of metabolomics data derived from both filtered serum (n=252) and urine (n=178) in the present study showed a correlation between metabolism and fatigue, as assessed by FACIT-F, across all patients. PLS-R analysis of metabolic data derived from all patients' sera and FACIT-F showed a correlation between FACIT-F and serum metabolite profile when the full metabolic profile ($r^2$=0.1809, 7 LVs, p<0.001) was used and also when the metabolite profile devoid of the inflammatory signal was used ($r^2$=0.1227, 4 LV, p<0.001). Furthermore, there was a correlation between FACIT-F and urinary metabolite profile using the entire metabolite profile ($r^2$=0.2660, 4 LV, p<0.001) and the metabolite profile devoid of the inflammatory signal ($r^2$=0.1967, 10 LV, p<0.001). These results not only indicate that the correlations between fatigue and both serum and urinary metabolite profile may be independent from the relationship between inflammation and metabolism, but also represents a novel technique for controlling for the effect of a known confounder for metabolic studies (i.e the signal related to inflammation). The metabolic permutations associated with CRP and FACIT-F respectively, have been summarised in Table 7-1.

**Table 7-1 Table showing summary of altered metabolic processes associated with multivariate metabolomics models correlating with FACIT-F & CRP**
**CRP**- C-reactive protein; **FACIT-F**- Functional assessment of chronic illness and therapy fatigue subscale

| Metabolic process | Fatigue | CRP |
|---|---|---|
| Urea cycle | **Decreased** flux | **Increased** flux |
| Branched chain amino acids | **Negative** correlation | **Positive** correlation |
| Glycolysis | **Decreased** flux | **Increased** flux |
| Ketone body production | **Increased** | -- |
| Citric acid cycle | Altered | Altered |
| Articular damage | Present | Present |
| Protein catabolism | Present | Present |
| Oxidative stress | Present | Present |

Both FACIT-F and CRP correlate with metabolic permutations. These metabolic permutations could merely reflect those associated with the underlying disease. For instance, PLS-R and OPLS-DA models

of the relationship between the serum metabolome and CRP revealed a negative correlation between CRP and citrate. This is a finding that has been seen across multiple chronic inflammatory conditions such RA (151), SLE (484), inflammatory bowel disease (IBD) (485) and PsA (299). Citrate has been proposed as a biomarker for the diagnosis of autoimmune disease and negatively correlates with disease activity (299). PLS-R models of the serum metabolome and CRP revealed a positive correlation between CRP and succinate. Glutamine anaplerosis of α-ketoglutarate (349), increased conversion of isocitrate to succinate (348) and decreased succinate dehydrogenase activity (486) could be responsible for succinate accumulation in inflammatory states. Accumulation of succinate appears to be associated with several proinflammatory effects. Succinate, like lactate (487), stabilizes HIF-1α in activated macrophages independent of the presence of a hypoxic tissue microenvironment (348). Succinate stimulates dendritic cells via its receptor succinate receptor 1, which has been shown to upregulate pro-inflammatory response in macrophages during antigen-induced arthritis (488). Furthermore, succinate has been shown to post-translationally modify proteins by lysine succinylation (349), which could lead to alterations of the citric acid cycle and glycolysis as enzymes which regulate these pathways are targets for lysine succinylation (489).

PLS-R model of all patients' serum metabolome and CRP showed a positive correlation between CRP and 3-methylhistidine. Similarly, urinary methylhistidine has a positive correlation with fatigue in PLS-R models of the urinary metabolome and FACIT-F. These findings support the presence of protein catabolism representing the presence of sarcopenia, which is commonly seen in rheumatic diseases (490). Interestingly, evidence suggests that sarcopenia when assessed by appendicular muscle index, does not strongly correlate with PROMs related to fatigue and physical function in RA patients (491).

PLS-R analysis of serum metabolite data in relation to CRP showed a positive correlation between CRP and proline. Furthermore, hydroxyproline, has a positive correlation with fatigue in the PLS-R models of CSA and RA patients' urinary metabolome and FACIT-F. These metabolites could reflect underlying articular damage as a consequence of disease activity (451). Finally, both analyses examining the relationship between the metabolome and CRP and FACIT-F showed evidence of metabolic changes related to oxidative stress. Not only is oxidative stress associated with chronic inflammatory disease (492), but it appears to be important in the development of RA. ROS may activate the NF-κB pathway in early RA, which increases proinflammatory cytokine production which in turn results in further activation of the NF-κB (493, 494).

Our findings suggest there is a positive correlation between fatigue and pyruvate, and a negative correlation between fatigue and lactate and acetate. Together, these, suggests that high levels of fatigue are associated with low levels of glycolysis. A study using $^{1}$H NMR metabolomics to assess serum and urine from 34 females with CFS and 25 controls showed increased serum glucose and decreased lactate and acetate in the CFS patients (436). Another study investigating the serum and urinary metabolome of CFS patients showed an increased glucose : lactate ratio, suggestive of impaired glycolysis (454). It is possible that impaired PDH activity results in reduced glycolysis. In normal aerobic circumstances, pyruvate is transported into the mitochondria where it is metabolised into acetyl-CoA by the PDH complex. In addition to allosteric regulation, PDH activity is regulated by PDKs and sirtuin 4 that inhibit PDH enzyme (495, 496) and PDH phosphatases that activate the PDH complex (497). PDK1–PDK4 are controlled at the transcriptional level via factors such as AMP-dependent protein kinase (AMPK) (498), PPARs (499), and HIF1 (500). Fluge et al (435) showed functional impairment of PDH, as evidenced by increased mRNA expression of the inhibitory PDH kinases 1, 2, and 4; sirtuin 4; and PPARδ in peripheral blood mononuclear cells from CFS patients (435). This deregulation of glycolysis results in falls of acetate and activation of histone deacetylation (501, 502) as well as deregulation of acetylation of cytoplasmic and mitochondrial enzymes. It is possible

that glycolytic anomalies mediated by inhibited PDH activity could be present in patients reporting fatigue with newly presenting inflammatory arthritis and CSA.

Our findings indicated fatigued states correlated with metabolites indicating reduced glycolysis, downregulation of the urea cycle, and reduced serum BCAAs. These metabolic permutations have been reported previously in CFS patients (430, 436), which may validate a homogenous biological footprint for fatigue across different conditions.

The PLS-R model of all patients' serum metabolome and CRP showed a positive correlation between CRP and lactate, which, in contrast to the relationship between fatigue and glycolysis, suggests that inflammatory states were associated with increased glycolysis. Increased glycolysis may be favourable for perpetuating inflammation and the progression of arthropathy. For instance, increased glycolysis may be utilised to produce glycine to meet collagen biosynthetic demands of TGF-$\beta_1$–activated myofibroblasts' MMP-mediated matrix remodelling (503, 504). Lactate dehydrogenase-A (LDHA) isomer, which is responsible for the conversion of pyruvate into lactate and NAD+, can promote T cell effector functions by increasing acetylation and transcription of interferon- γ (505). Furthermore, inhibiting LDHA in macrophages results in downregulation of pro-inflammatory cytokines and chemokines (506). Furthermore, in addition to being an end-product of glycolysis, lactate also functions as a signalling molecule in inflamed tissues (507). Increased lactate induces pyruvate kinase isozyme M2 (PKM2) translocation to the nucleus of CD4+ T cells, which leads to phosphorylation of signal transducer and activator of transcription 3 (STAT3) and increased expression of IL-17 (508). In the inflamed synovium, lactate regulates immune cell functions in numerous ways, including stopping migration of T-cells away from the site of inflammation (404), switching to pro- inflammatory subsets of T-cells, such as TH1 cells and TH17 cells (509). The differences between the metabolic permutations could provide an explanation as to why inflammation and fatigue do not strongly correlate with one another.

PLS-R models of all patients' serum metabolome and CRP revealed a positive correlation between CRP and the BCAAs valine, leucine, and isoleucine. Increased BCAA concentrations are associated with obesity and insulin resistance (510). Consuming a diet of reduced BCAAs were associated with reduced obesity and improved insulin sensitivity in animal models (511, 512). Furthermore, leucine is known to activate the anabolic signalling molecule mTORC1 (mammalian target of rapamycin complex 1), which is associated with cardiovascular disease (513). It is possible that changes in BCAA metabolism could be contributing either directly or indirectly to sarcopenia, insulin resistance and adverse cardiovascular risk associated with inflammatory arthritis.

A negative correlation appears to be present between fatigue and valine, leucine, and isoleucine in the PLS-R models of the serum metabolome and FACIT-F. BCAAs, as mentioned in **section 6.5.10**, compete with tryptophan to bind the transporter which is responsible for the transport of tryptophan across the blood brain barrier (514). Tryptophan is initially converted into 5-hydroxytryptophan, which is the precursor to serotonin. In animal models of diabetes investigating diabetic cognitive dysfunction, urinary 5-hydroxytryptophan was reduced in those mice with diabetic cognitive dysfunction compared to controls (515). A study assessing brain 5-HT1A receptor binding potential directly using the specific radioligand [11C]WAY-100635 and positron emission tomography showed there was a decrease in 5-HT1A receptor binding or number of receptors in CFS patients relative to control subjects (472). It is possible that those neuroendocrine alterations are, in part, responsible for the development of the symptom of fatigue.

Our study shows a correlation between metabolic permutations and fatigue in patients with new onset inflammatory arthritis and CSA. This finding has potentially far reaching consequences for

patients, clinicians, and researchers. From a patient's perspective, a link between fatigue and metabolism could lend credence to an organic aetiology of the symptom of fatigue, rather than a manifestation of psychological disorder, which could lead to changes in the way fatigue is managed. Patients have difficulty conveying their feelings and experience of living with fatigue to clinicians (6, 413). Part of this difficulty arises from belief that support from health professionals is uncommon and health professionals tend to concentrate on physical symptoms and disease activity rather than fatigue (197). Consequently, patients feel clinicians are unable to help with their fatigue and it is up to them to manage this symptom alone (6, 197, 413, 516, 517). The relationship between fatigue and metabolism may result in a paradigm shift amongst clinicians to routinely screen for and discuss symptoms related to fatigue. Furthermore, it may result in an increased confidence amongst patients to discuss their experience of fatigue to their clinicians.

In addition, clinicians could utilise a relationship between fatigue and metabolism to assist in diagnosis and management of fatigue. Identifying a panel of metabolites which correlate specifically with fatigue would be the first step towards identifying a clinically useful biomarker. Once a distinct metabolite panel has been identified and validated, this could be used as a biomarker to aid diagnosis of fatigue in clinical practice and in selection of a defined cohort of fatigued patients for research purposes. Fatigue PROMs have many limitations which could effect the measurement's reliability (see **section 6.6**), a biomarker could introduce a consistent objective measure of fatigue, providing an objective endpoint to assess treatment efficacy in studies specifically targeting fatigue.

The correlation between fatigue and metabolism in patients with new onset inflammatory arthritis and CSA has consequences for other conditions such as cancer, IBD, parkinsonism and multiple sclerosis. Furthermore, with a growing number of individuals infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes COVID-19, it is increasingly recognised that fatigue is a common presenting complaint in individuals infected with SARS-CoV-2, with as many as 34-46% of patient reporting fatigue at presentation (518-520). "Long COVID" is a term being used to describe illness in individuals who have either recovered from COVID-19 but still report lasting effects of the infection or have had the typical symptoms for longer than usual (521). Of the symptoms associated with long coronavirus (COVID), fatigue has been shown to be burdensome and persist after the acute phase of the COVID-19 illness (522). A unique aspect of COVID-19 related fatigue compared to other chronic inflammatory diseases is the timing of the triggering insult a readily identifiable. Fatigue in Long COVID could thus turn out to be an enlightening model to study the longitudinal course of fatigue in relation to metabolic permutations following a defined inflammatory insult. Finally, if a metabolic fingerprint associated with fatigue can be identified amongst several different conditions, then this could provide hope for a therapeutic target which would have utility for a considerable number of patients. Increasing number of agents are targeting metabolic pathways such as dichloroacetate (inhibits phosphoinositide-dependent kinase-1) (407), Soraphen A (inhibits acetyl-CoA carboxylases) (523) and 2-Deoxy-D-glucose (inhibits hexokinase) (524); the ability to target such pathways with the aim of improving fatigue may open up interesting therapeutic avenues.

## 7.2 Limitations

There are several limitations to consider when assessing this body of work. Firstly, data related to some important clinical variables such as sleep duration and quality were not available. RA patients often have difficulties falling asleep and have unrefreshed sleep, which has been found to be associated with pain (525, 526). Aside from pain being an important factor associated with persistent

fatigue (527), a bidirectional relationship between circadian rhythm disruption and sleep disturbance also occurs which may contribute to fatigue (528). Furthermore, RA patients appear to have a higher prevalence of sleep apnoea (529), which even in isolation, is associated with a proinflammatory response (530). In turn TNF-α and IL-1 have been shown to induce non-rapid eye movement sleep in animal models (531, 532). Toda et al have recently characterised the antimicrobial peptide NEMURI, produced by a fruit fly *Drosophila melanogaster*, which promotes sustained sleep and enhances survival after infection (533). It is possible that fatigue related to sickness behaviour may be mediated through antimicrobial peptides produced by humans (534). Collectively, evidence suggests that proinflammatory cytokines and dysregulated circadian rhythm can lead to sleep disturbance which may exacerbate fatigue.

Furthermore, another limitation is the lack of complementary assessment of brain function with neuroimaging. Fatigue has been postulated to be a manifestation of sickness behaviour (27, 28, 288). Recent studies have investigated this further with neuroimaging to assess brain activity and have shown activation of various brain regions that correlate with sickness behaviour (535-537). Recently, a double-blind crossover trial study of 20 healthy males who were inoculated with *Salmonella typhi* vaccine or placebo, showed increased serum IL-6 and effective connectivity between specific nodes of the insula on resting-state functional MRI scan 3 hours after receiving the *S. typhi* vaccine (538). Furthermore, RA patients with concomitant fibromyalgia show increased functional connectivity on MRI of the insula–left inferior parietal lobe, left inferior parietal lobe–dorsal anterior cingulate, and left inferior parietal lobe–medial prefrontal cortex regions that correlated with higher levels of ESR (539). Furthermore, another study using functional connectivity MRI in RA patients showed a positive correlation between default mode network connectivity to the left mid/posterior insula and fibromyalgianess (540). These findings collectively suggest that symptoms related to fibromyalgia, such as fatigue, may originate from the central nervous system.

Additionally, in this study I have measured fatigue using two linear PROMs. However in addition to the limitations described in **section 4.2.6**, emerging evidence suggests that fatigue is heterogenous and in fact can be separated into distinct subtypes (157). A study of established RA patients taking either csDMARDs (n=522) or commencing bDMARDs (n=3909) were subjected to hierarchical cluster analysis across clinical variables and PROMs. The results indicated that there were four clusters of patients which may have unique fatigue subtypes. A cluster which had characteristics of severe pain, disability and fatigue, but low inflammation and good mental wellbeing was labelled as 'basic' and comprised 46% of patients. The 'affective' cluster, included 40% of participants who reported severe pain, disability and poor mental health, but low inflammation. Only 4.5% of participants were categorised as having 'global' fatigue, in which the patients reported severe pain, disability, poor mental health and had elevated inflammation. The final cluster encompassed 8.9% of participants, who reported severe pain, disability and high inflammation, but good mental health (157). This study outlines different types of fatigue which may have distinct aetiologies and as such should be accounted for when investigating fatigue.

Metabolomics studies alone have many limitations as described above (**section 5.6** and **section 7.1**). Importantly, in cross sectional studies such as the one described here, it can be difficult to distinguish whether changes are a cause or a consequence of fatigue. Therefore, further work is required to provide mechanistic insights with the aim of identifying therapeutic targets to reduce the personal and social impacts (541), morbidity and mortality (542) associated with fatigue.

## 7.3 Future work

### 7.3.1 Methodological development

For future work, it would be important to conduct pre-processing with a pooled sample. In addition to assessing for wider systematic error, such as inaccuracies during NMR spectroscopy such as drift, the magnitude of transformation can be deduced using a maximum likelihood method. Subsequently, the impact on pre-processing using the magnitudes of transformations used in this study and the calculated transformation using maximum likelihood method can be investigated. One method of doing this could be assessing the impact of pre-processing parameters, such as transformation, on the performance of multivariate discriminant analysis, such as OPLS-DA. When performing multivariate analysis to assess the performance of pre-processing parameters, it would be prudent to choose categorical variables which impact on metabolism but is not the focus of the study itself. For instance, OPLS-DA can be performed using groupings based on gender. In addition to assessing the impact on multivariate analysis performance, it would be worthwhile to investigate the impact of transformation on metabolomics data using a test for normality, as transformation aims to normalise metabolomics data.

### 7.3.2 Functional Metabolomics

Finally, a "functional" metabolomics approach may offer further insights into metabolic permutations associated with fatigue. Traditionally, metabolomics studies focus on identify metabolites of interest using discovery metabolomics, which largely uses existing literature to speculate mechanisms which could account for the findings. However using a functional metabolomics approach, one would use further experimental techniques to improve understanding of the findings from discovery metabolomics, this may include genomics, transcriptomics and proteomics. Whilst the large amount of data generated using this approach would be challenging to interpret, the use of sophisticated artificial intelligence-based programmes, predictive algorithms and biomarker identification could lead to meaningful results (543). This would allow the formulation of a hypothesis which could then be tested further using appropriate experimental models. For instance, a Seahorse Analyzer can be utilised to assess glycolytic flux in vitro and can offer extracellular acidification rates and oxygen consumption rate to provide information on glycolysis and mitochondrial respiration (544).

### 7.3.3 Validation of results

Furthermore, it would be important to **validating this study's findings** in a second cohort of newly presenting inflammatory arthritis, CSA patients and COVID-19 patients with accompanying quantification of metabolites identified as biomarkers for univariate analysis. The focus of this study has been the assessment of metabolic permutations associated with fatigue in newly presenting inflammatory arthritis and CSA patients, however metabolomics can be used to assess longitudinal changes in metabolism and how that correlates with fatigue. The unique aspect of COVID-19 patients will be that the time of onset of the disease will be more discernible compared to patients who present with CSA and inflammatory arthritis. With a precise time of onset, longitudinal assessment of metabolic permutations associated with the disease could be possible.

A validation study may provide novel insights which could be taken forward to inform new pharmacotherapeutic or nutritional interventions to improve patient's fatigue. For instance, our

findings suggest that fatigue is associated with decreased glycolysis. Should this be validated, then this may pave the way to trial agents which increase glycolytic flux. One such agent is metformin. The mode of action of metformin is incompletely understood, but it is thought to inhibit respiratory chain complex 1. The decreased mitochondrial respiration and ATP production is accompanied by a compensatory increase in glycolysis (545, 546). Metformin has shown promise as an emerging treatment for symptoms associated with fibromyalgia, such as pain (547) and fatigue (547, 548). Furthermore, metformin appears to increase glucose delivery to skeletal muscle in CFS patients (549). It is possible, that after further study, metformin may be utilised in the future as a treatment for fatigue associated with inflammatory arthritis and CSA.

A potential study design may involve an assessment of metformin on fatigue via a placebo-controlled trial in individuals with 1) inflammatory arthritis and CSA 2) long COVID-19 and 3) healthy controls. Metformin is an important candidate therapy for several reasons; it is a cheap, widely used with a proven safety profile. Participants would receive comprehensive baseline and follow up assessment, including routine clinical and demographic data collection as performed in the present study. Participants would also have blood and urine taken for mulit-omics assessment- including metabolomics, proteomics, transcriptomics and genomics. Patients would also have their sleep data captured with wearable sleep trackers and a validated PROM to assess sleep, such as the Epworth sleepiness scale (550). Finally, patients would have functional MRI to check for functional connectivity previously seen in RA patients with concomitant fibromyalgia symptoms with higher levels of ESR (539). The inflammatory arthritis cohort would be further substantified into four clusters: basic (characteristics of severe pain, disability and fatigue, but low inflammation and good mental wellbeing), affective (severe pain, disability and poor mental health, but low inflammation), global (severe pain, disability, poor mental health and had elevated inflammation) and inflammatory (severe pain, disability and high inflammation, but good mental health) (157). These clusters could be treated as four distinct subgroups which could facilitate further subanalysis. Once this approach has been used to assess the effectiveness of metformin to treat fatigue, the same study design could be utilised to assess the effectiveness of other treatments including immunomodulatory treatment. This methodology could facilitate personalised medicine by identifying patient characteristics which could benefit from certain mode of actions of immunomodulatory treatment.

Collectively these biological assessments combined with PROMs of fatigue could provide a comprehensive assessment of fatigue before and during treatment. This could provide insight into pathoaetiological mechanisms which lead to the development of fatigue. Furthermore, if these assessments change over time with fatigue, they could provide a method assess fatigue which is sensitive to change.

# 8 Appendix

Search strategy:
Database: PubMed
#1 Construct search
Fatigue
#2 Population search
Rheumatoid arthritis
#3 Measurement properties filter
(instrumentation[sh] OR methods[sh] OR "Validation Studies"[pt] OR "Comparative Study"[pt] OR "psychometrics"[MeSH] OR psychometr*[tiab] OR clinimetr*[tw] OR clinometr*[tw] OR "outcome assessment (health care)"[MeSH] OR "outcome assessment"[tiab] OR "outcome measure*"[tw] OR "observer variation"[MeSH] OR "observer variation"[tiab] OR "Health Status Indicators"[Mesh] OR "reproducibility of results"[MeSH] OR reproducib*[tiab] OR "discriminant analysis"[MeSH] OR reliab*[tiab] OR unreliab*[tiab] OR valid*[tiab] OR "coefficient of variation"[tiab] OR coefficient[tiab] OR homogeneity[tiab] OR homogeneous[tiab] OR "internal consistency"[tiab] OR (cronbach*[tiab] AND (alpha[tiab] OR alphas[tiab])) OR (item[tiab] AND (correlation*[tiab] OR selection*[tiab] OR reduction*[tiab])) OR agreement[tw] OR precision[tw] OR imprecision[tw] OR "precise values"[tw] OR test-retest[tiab] OR (test[tiab] AND retest[tiab]) OR (reliab*[tiab] AND (test[tiab] OR retest[tiab])) OR stability[tiab] OR interrater[tiab] OR inter-rater[tiab] OR intrarater[tiab] OR intra-rater[tiab] OR intertester[tiab] OR inter-tester[tiab] OR intratester[tiab] OR intra-tester[tiab] OR interobserver[tiab] OR inter-observer[tiab] OR intraobserver[tiab] OR intra-observer[tiab] OR intertechnician[tiab] OR inter-technician[tiab] OR intratechnician[tiab] OR intra-technician[tiab] OR interexaminer[tiab] OR inter-examiner[tiab] OR intraexaminer[tiab] OR intra-examiner[tiab] OR interassay[tiab] OR inter-assay[tiab] OR intraassay[tiab] OR intra-assay[tiab] OR interindividual[tiab] OR inter-individual[tiab] OR intraindividual[tiab] OR intra-individual[tiab] OR interparticipant[tiab] OR inter-participant[tiab] OR intraparticipant[tiab] OR intra-participant[tiab] OR kappa[tiab] OR kappa's[tiab] OR kappas[tiab] OR repeatab*[tw] OR ((replicab*[tw] OR repeated[tw]) AND (measure[tw] OR measures[tw] OR findings[tw] OR result[tw] OR results[tw] OR test[tw] OR tests[tw])) OR generaliza*[tiab] OR generalisa*[tiab] OR concordance[tiab] OR (intraclass[tiab] AND correlation*[tiab]) OR discriminative[tiab] OR "known group"[tiab] OR "factor analysis"[tiab] OR "factor analyses"[tiab] OR "factor structure"[tiab] OR "factor structures"[tiab] OR dimension*[tiab] OR subscale*[tiab] OR (multitrait[tiab] AND scaling[tiab] AND (analysis[tiab] OR analyses[tiab])) OR "item discriminant"[tiab] OR "interscale correlation*"[tiab] OR error[tiab] OR errors[tiab] OR "individual variability"[tiab] OR "interval variability"[tiab] OR "rate variability"[tiab] OR (variability[tiab] AND (analysis[tiab] OR values[tiab])) OR (uncertainty[tiab] AND (measurement[tiab] OR measuring[tiab])) OR "standard error of measurement"[tiab] OR sensitiv*[tiab] OR responsive*[tiab] OR (limit[tiab] AND detection[tiab]) OR "minimal detectable concentration"[tiab] OR interpretab*[tiab] OR ((minimal[tiab] OR minimally[tiab] OR clinical[tiab] OR clinically[tiab]) AND (important[tiab] OR significant[tiab] OR detectable[tiab]) AND (change[tiab] OR difference[tiab])) OR (small*[tiab] AND (real[tiab] OR detectable[tiab]) AND (change[tiab] OR difference[tiab])) OR "meaningful change"[tiab] OR "ceiling effect"[tiab] OR "floor effect"[tiab] OR "Item response model"[tiab] OR IRT[tiab] OR Rasch[tiab] OR "Differential item functioning"[tiab] OR DIF[tiab] OR "computer adaptive testing"[tiab] OR "item bank"[tiab] OR "cross-cultural equivalence"[tiab])

#4 (#1 AND #2 AND #3)
Database: EMBASE
#1 Construct search
Fatigue
#2 Population search
Rheumatoid arthritis
#3 Measurement properties filter

'intermethod comparison'/exp OR 'data collection method'/exp OR 'validation study'/exp OR 'feasibility study'/exp OR 'pilot study'/exp OR 'psychometry'/exp OR 'reproducibility'/exp OR reproducib*:ab,ti OR 'audit':ab,ti OR psychometr*:ab,ti OR clinimetr*:ab,ti OR clinometr*:ab,ti OR 'observer variation'/exp OR 'observer variation':ab,ti OR 'discriminant analysis'/exp OR 'validity'/exp OR reliab*:ab,ti OR valid*:ab,ti OR 'coefficient':ab,ti OR 'internal consistency':ab,ti OR (cronbach*:ab,ti AND ('alpha':ab,ti OR 'alphas':ab,ti)) OR 'item correlation':ab,ti OR 'item correlations':ab,ti OR 'item selection':ab,ti OR 'item selections':ab,ti OR 'item reduction':ab,ti OR 'item reductions':ab,ti OR 'agreement':ab,ti OR 'precision':ab,ti OR 'imprecision':ab,ti OR 'precise values':ab,ti OR 'test-retest':ab,ti OR ('test':ab,ti AND 'retest':ab,ti) OR (reliab*:ab,ti AND ('test':ab,ti OR 'retest':ab,ti)) OR 'stability':ab,ti OR 'interrater':ab,ti OR 'inter-rater':ab,ti OR 'intrarater':ab,ti OR 'intra-rater':ab,ti OR 'intertester':ab,ti OR 'inter-tester':ab,ti OR 'intratester':ab,ti OR 'intratester':ab,ti OR 'interobeserver':ab,ti OR 'inter-observer':ab,ti OR 'intraobserver':ab,ti OR 'intraobserver':ab,ti OR 'intertechnician':ab,ti OR 'inter-technician':ab,ti OR 'intratechnician':ab,ti OR 'intratechnician':ab,ti
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#4 (#1 AND #2 AND #3)
Database: CINAHL
#1 Construct search
Fatigue
#2 Population search
Rheumatoid arthritis
#3 Measurement properties filter
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outcome assessment or AB outcome assessment ) or ( TI outcome measure* or AB outcome measure* ) or (MH “Health Status Indicators”) or (MH “Reproducibility of Results”) or (MH “Discriminant Analysis”) or ( ( TI reproducib* or AB reproducib* ) or ( TI reliab* or AB reliab* ) or ( TI unreliab* or AB unreliab* ) ) or ( ( TI valid* or AB valid* ) or ( TI coefficient or AB coefficient ) or ( TI homogeneity or AB homogeneity ) ) or ( TI homogeneous or AB homogeneous ) or ( TI “coefficient of variation” or AB “coefficient of variation” ) or ( TI “internal consistency” or AB “internal consistency” ) or (MH “Internal Consistency+”) or (MH “Reliability+”) or (MH “Measurement Error+”) or (MH “Content Validity+”) or “hypothesis testing” or “structural validity” or “cross-cultural validity” or (MH “Criterion-Related Validity+”) or “responsiveness” or “interpretability” or ( TI reliab* or AB reliab* ) and ( (TI test or AB test) OR (TI retest or AB retest) ) or ( TI stability or AB stability ) or ( TI interrater or AB interrater ) or ( TI inter-rater or AB inter-rater ) or ( TI intrarater or AB intrarater ) or ( TI intra-rater or AB intrarater ) or ( TI intertester or AB intertester) or (TI inter-tester or AB inter-tester) or ( TI intratester or AB intratester) or ( TI intra-tester or AB intra-tester) or ( TI interobserver or AB interobserver) or (TI inter-observer or AB inter-observer ) or ( TI intraobserver or AB intraobserver) or ( TI intra-observer or AB intra-observer) or ( TI intertechnician or AB intertechnician) or (TI inter-technician or AB inter-technician) or ( TI intratechnician or AB intratechnician ) or ( TI intra-technician or AB intra-technician ) or ( TI interexaminer or AB interexaminer ) or (TI inter-examiner or AB inter-examiner) or (TI intraexaminer or AB intraexaminer ) OR (TI intra-examiner or AB intra-examiner ) or (TI intra-examiner or AB intraexaminer ) or (TI interassay or AB interassay ) or ( TI inter-assay or AB inter-assay ) or ( TI intraassay or AB intraassay) or ( TI intra-assay or AB intra-assay ) or (TI interindividual or AB interindividual) or (TI inter-individual or AB inter-individual) OR (TI intraindividual or AB intraindividual) or (TI intra-individual or AB intra-individual) or (TI interparticipant or AB interparticipant) or (TI inter-participant or AB inter-participant ) or (TI intraparticipant or AB intraparticipant) or (TI intra-participant or AB intra-participant ) or (TI kappa or AB kappa) or (TI kappa’s or AB kappa’s ) or (TI kappas or AB kappas) or (TI repeatab* or AB repeatab*) or ( TI responsive* or AB responsive* ) or ( TI interpretab* or AB interpretab* )
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Fatigue
#2 Population search
Rheumatoid arthritis
#3 Measurement properties filter
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8. outcome measure*.tw.
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16. (cronbach* and (alpha or alphas)).ti,ab.
17. (item and (correlation* or selection* or reduction*)).ti,ab.
18. (agreement or precision or imprecision or “precise values” or test-retest).ti,ab.
19. (test and retest).ti,ab.
20. (reliab* and (test or retest)).ti,ab.
21. (stability or interrater or inter-rater or intrarater or intra-rater or intertester or inter-tester or intratester or intra-tester or interobserver or inter-observer or intraobserver or intraobserver or intertechnician or inter-technician or intratechnician or intra-technician or interexaminer or inter-examiner or intraexaminer or intra-examiner or interassay or interassay or intraassay or intra-assay or interindividual or inter-individual or intraindividual or intra-individual or interparticipant or inter-participant or intraparticipant or intra-participant or kappa or kappa’s or kappas or repeatab*).ti,ab.
22. ((replicab* or repeated) and (measure or measures or findings or result or results or test or tests)).ti,ab.
23. (generaliza* or generalisa* or concordance).ti,ab.
24. (intraclass and correlation*).ti,ab.
25. (discriminative or “known group” or factor analysis or factor analyses or dimension* or subscale*).ti,ab.
26. (multitrait and scaling and (analysis or analyses)).ti,ab.
27. (item discriminant or interscale correlation* or error or errors or “individual variability”).ti,ab.
28. (variability and (analysis or values)).ti,ab.
29. (uncertainty and (measurement or measuring)).ti,ab.
30. (“standard error of measurement” or sensitiv* or responsive*).ti,ab.
31. ((minimal or minimally or clinical or clinically) and (important or significant or detectable) and (change or difference)).ti,ab.
32. (small* and (real or detectable) and (change or difference)).ti,ab.
33. (meaningful change or “ceiling effect” or “floor effect” or “Item response model” or IRT or Rasch or “Differential item functioning” or DIF or “computer adaptive testing” or “item bank” or “cross-cultural equivalence”).ti,ab.
34. 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20 or 21 or 22 or 23 or 24 or 25 or 26 or 27 or 28 or 29 or 30 or 31 or 32 or 33
35. (child* or pediatric* or infan* or neonat* or newborn* or teen* or youth*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept, rare disease supplementary concept, unique identifier] 36. 34 and 35
#4 (#1 AND #2 AND #3)
All duplicates are removed.

<u>Inclusion criteria</u>

Scales were included only if they met the following criteria:

1) Self-assessment scales initially validated in patients with rheumatic conditions and/or subsequently broadly used in populations with rheumatic conditions
2) Scales must have been used in a second test population for separate validation of their use in patients with rheumatic conditions.
3) 90% of the participants must be over 18 years.
4) Scales must have achieved a minimum quality score. The paper describing the scale must have included information on at least three of the following: test-retest reliability, known group

validity (discriminant validity), internal consistency, and responsiveness to change or convergent validity (against other scales). As outlined in the table S1 below:

5) Scales must be in English or translated and validated for English language use.

Table S1: Psychometric attributes

| | |
|---|---|
| Internal consistency | Do items within the scale correlate with each other and the total score; as measured by Cronbach's alpha. |
| Test-retest reliability | Assesses reproducibility of results if given to the same population but at a different time. |
| Discriminant validity | Does the group distinguish between cohorts expected to have different results: ie cases versus controls |
| Responsiveness to change | Does the scale measure change during a course of treatment? |
| Convergent validity | Does the scale provide a similar measure to previously validated fatigue tools. |

Exclusion criteria

There were also explicit exclusion criteria:

1) Objective rating scales testing power/strength, etc.
2) Fatigue subscales as part of a broader quality-of-life measure. Unless specific data were available relating to the psychometric properties of the subscale.

Excluded scales:

| ***Name of scale*** | ***Reason for exclusion*** |
|---|---|
| Dutch exertion fatigue inventory | No psychometric data for rheumatic conditions |
| Fatigue assessment questionnaire | No psychometric data for rheumatic conditions |
| Piper fatigue scale | Required an initial screening tool to screen for fatigue. |
| Revised piper fatigue scale | Validated in breast cancer population originally used predominately in the oncology population |
| Four item fatigue scale | No psychometric data for rheumatic conditions |
| Neurological fatigue index (MS) | Used in multiple sclerosis |
| Fatigue severity scale item (HIV) | Used in HIV |
| Somn-Perelli fatigue scale | No psychometric data for rheumatic conditions |
| Fatigue associated with depression questionnaire | No psychometric data for rheumatic conditions |
| Fatigue impact scale | No psychometric data for rheumatic conditions |
| Modified fatigue impact scale | Used in psoriatic arthritis and chronic fatigue syndrome; insufficient psychometric data |
| Multidimensional fatigue symptom inventory | Validated in breast cancer population originally used predominately in the oncology population |
| Modified fatigue severity scale | Used in Psoriatic arthritis; not enough psychometric properties published |

| | |
|---|---|
| Fatigue assessment inventory | Originally validated in patients with chronic illness including SLE; inadequate psychometric properties and not used in rheumatic conditions thereafter |
| Unidimensional fatigue impact scale | No psychometric data for rheumatic conditions |
| Iowa fatigue scale | No psychometric data for rheumatic conditions |
| Parkinson fatigue scale | No psychometric data for rheumatic conditions |
| (Schwartz) Cancer fatigue scale | Used in oncology population |
| Wu cancer fatigue scale | Used in oncology population |
| EORTC QLQ-C30 | Used in oncology population |
| The HIV related fatigue scale | Used in HIV patients |
| Fatigue scale for motor & cognitive function | No psychometric data for rheumatic conditions |
| Quick Piper Scale | No psychometric data for rheumatic conditions |
| Pictorial representation of self & illness measurement (PRISM) | No psychometric data for rheumatic conditions |
| Neurological fatigue index for stroke | Used in stroke patients |
| Clinical global impression scale for fatigue | No psychometric data for rheumatic conditions |
| Fatigue pictogram | No psychometric data for rheumatic conditions |
| Maastrict Questionnaire | No psychometric data for rheumatic conditions |
| Warzburg Gatigue Inventory for MS (WEIMuS) | Used predominately in MS patients |
| Multiple fatigue types questionnaire | No psychometric data for rheumatic conditions |
| Real-time digital fatigue score | No psychometric data for rheumatic conditions |
| The fatigue scale | No psychometric data for rheumatic conditions |
| The Manchester COPD fatigue scale | Used in COPD patients |
| Fibromyalgia Impact Questionnaire-Fatigue | Fatigue subscale used in fibromyalgia; not enough psychometric data in these studies |
| Q1 of BASDAI | Used in Ankylosing Spondylitis; not enough psychometric data in these studies |
| Fatigue numeric rating scales | Used in psoriatic arthritis and rheumatoid arthritis but no psychometric data available |
| Fatigue symptom control checklist | No psychometric data for rheumatic conditions; not validated in English |
| The Swedish occupational fatigue inventory | No psychometric data for rheumatic conditions; has limitations |
| The fatigue management barriers questionnaire | No psychometric data for rheumatic conditions |
| The clinical survey for cancer related fatigue (QFAS) | No psychometric data for rheumatic conditions |
| Cancer-related fatigue distress scale | No psychometric data for rheumatic conditions |
| Chronic fatigue index | used in RA; did not meet minimum quality validation data in a rheumatic condition |
| Composite index fatigue Impairment | used in RA; did not meet minimum quality validation data in a rheumatic condition |
| Feeling tone checklist | used in RA; did not meet minimum quality validation data in a rheumatic condition |
| Fatigue symptom inventory | Validated in breast cancer population originally used predominately in the oncology population |
| Myasthenia gravis fatigue scale | Used in myasthenia gravis |

| | |
|---|---|
| Pearson & Byars fatigue feeling checklist | No psychometric data for rheumatic conditions |
| Rhoten fatigue scale | No psychometric data for rheumatic conditions |
| Schedule of fatigue and anergy | No psychometric data for rheumatic conditions |
| Brief Fatigue Inventory | Used in OA & RA; not enough psychometric data available in these studies |
| Lee VASF | No psychometric data for rheumatic conditions |

**Supplementary table 1- Demographic and clinical data of patients at baseline with comparison of patients who had follow up versus those patients who had incomplete follow up**

**SD**- standard deviation; **IQR**- interquartile range; **DAS28ESR**- disease activity score 28 with erythrocyte sedimentation rate; **DAS28CRP**- disease activity score 28 with C-reactive protein; **FVAS**- fatigue visual analogue scale; **FACIT-F**- functional assessment of chronic illness therapy fatigue subscale; **VAS-P**- visual analogue scale pain; **PHQ-9**- patient health questionnaire-9; **HAQ**- health assessment questionnaire; **ACPA**- anti-citrullinated protein antibody.

| | Patients with complete follow up (n=440) | Patients with no follow up (n=299) |
|---|---|---|
| Age, mean +/- SD (years)<br>Missing (%) | 51.7 +/- 14.6<br>0 (0) | 49.5 +/- 16.4<br>0 (0) |
| | p=0.056* | |
| Sex, female<br>Missing (%) | 307 (69.8)<br>0 (0) | 165 (55.1)<br>0 (0) |
| | p=0.278** | |
| Symptom Duration, median (IQR) (weeks)<br>Missing (%) | 25 (14-53)<br>4 (0.9) | 27.5 (13-59)<br>2 (0.7) |
| | p=0.314* | |
| FVAS, median (IQR)<br>Missing (%) | 61 (32-83)<br>10 (2.3) | 54 (23-77.3)<br>8 (2.7) |
| | p=0.060* | |
| FACIT-F, median (IQR)<br>Missing (%) | 34 (22-41)<br>51 (11.6) | 33 (24.4-42)<br>34 (11.4) |
| | p=0.623* | |
| VAS-P, median (IQR)<br>Missing (%) | 58 (31-78)<br>34 (7.7) | 60 (33-78)<br>23 (7.7) |
| | p=0.891* | |
| PHQ-9, median (IQR)<br>Missing (%) | 7 (3-13)<br>56 (12.7) | 7.4 (3-13.6)<br>20 (6.7) |
| | p=0.737* | |
| HAQ, median (IQR)<br>Missing (%) | 1 (0.5-1.6)<br>50 (11.4) | 0.9 (0.3-1.5)<br>36 (12.0) |
| | p=0.109* | |
| DAS28ESR, mean+/-SD<br>Missing (%) | 4.5 +/- 1.5<br>28 (6.4) | 4.3+/- 1.7<br>13 (6.4) |
| | p=0.051* | |
| DAS28CRP,mean+/-SD<br>Missing (%) | 4.3 +/- 1.4<br>41 (9.3) | 4.2 +/- 1.5<br>27 (9.0) |
| | p=0.305* | |
| Haemoglobin, mean +/- SD (gm/dl) | 132.6 +/- 15.0 | 130.3 +/- 15.8 |

| | | |
|---|---|---|
| Missing (%) | 8 (1.5) | 3 (1.0) |
| | p=0.941* | |
| C-reactive protein, median (IQR)(mg/litre)<br>Missing (%) | 6 (3-15)<br>4 (0.9) | 5 (2-13)<br>3 (1.0) |
| | p=0.147* | |
| Erythrocyte sedimentation rate, median (IQR) (mm/hr)<br>Missing (%) | 19 (9.3-33)<br>28 (6.4) | 16 (8-31)<br>16 (3.0) |
| | p=0.173* | |
| Rheumatoid factor positive<br>Missing (%) | 177 (40.2)<br>10 (3.3) | 93 (31.1)<br>2 (0.7) |
| | p=0.388** | |
| ACPA positive<br>Missing (%) | 162 (36.8)<br>6 (1.4) | 87 (29.1)<br>2 (0.7) |
| | p=0.634** | |

* p value calculated using Kruskal-Wallis test ** p value calculated using chi-squared test

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