Metabolomics analysis for diagnosis and biomarker discovery of transthyretin amyloidosis

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Introduction

Transthyretin (TTR) amyloidosis is either inherited or acquired. Hereditary transthyretin (ATTRv) amyloidosis is an autosomal dominant inherited disease. More than 120 mutations in the TTR gene have been reported, and the majority is associated with the development of systemic amyloidosis. The TTR V30M is the most prevalent variant in Sweden.

The phenotypic expression of ATTRv amyloidosis varies between different mutations, and also within the same mutation. The most common manifestations of the disease are peripheral axonal neuropathy and/or cardiomyopathy [1,2]. However, mutations characterised by central nervous manifestations of the disease, often in combination with ocular symptoms (oculoleptomeningeal) have also been recognised [3].

TTR circulates as a homo-tetramer and functions as a carrier for thyroxine and retinol-binding protein in serum and cerebrospinal fluid. Dissociation of the tetramer into monomers is believed to lead to misfolding and reassembly of the misfolded monomers into insoluble TTR amyloid (ATTR) fibrils that are deposited in different tissues [4,5].

Current diagnostic tools for ATTR amyloidosis are usually not sensitive enough to capture early disease manifestations. There are no biomarkers available to help in the diagnosing of the disease. Since metabolomics is increasingly being utilised to identify new biomarkers in different areas such as neurodegenerative diseases [6] and cardiovascular diseases [7], one of the objectives of this study is to identify novel candidate metabolite biomarkers as a diagnostic tool for the disease.
Untargeted metabolomics, or global metabolome analysis, is a powerful approach that aims to discover and identify a wide range of both unknown and known metabolites in a biological sample [8]. Therefore, untargeted metabolomics can discover novel metabolite species and analyse pathways and metabolism in biological and complex systems. Furthermore, metabolic profiling is a powerful approach for studying the low molecular weight biochemicals presented in the metabolome of an organism [9]. It is the final downstream product of gene expression, which enables the provision of a high-resolution multifactorial phenotypic signature of disease aetiology, manifestation, or pathophysiology [10–12].

This study aimed to identify candidate biomarkers by a metabolomics approach using mass spectrometry (MS)-based methods, gas chromatography (GC/MS) and liquid chromatography (LC)/MS, in ATTRV30M amyloidosis patients for more objective phenotyping and potential candidates for early diagnosis of the disease.

Material and methods

Participants

Twenty-seven patients (13 female, 14 male), diagnosed with ATTRV30M amyloidosis by positive genetic testing (all heterozygous) and detection of amyloid deposits in adipose tissue with Congo red staining and immunohistochemistry, were selected. Additionally, 26 heterozygous carriers (12 female, 14 male) of the TTR V30M mutation with no signs of the disease, according to the individuals’ medical records or that they had not sought medical care as described by Olsson et al. [13], at the time of sampling, denoted asymptomatic TTRV30M, as well as 26 control individuals (12 female, 14 male) from the same geographic area, were included in the study (Table 1). Most patients and asymptomatic TTRV30M carriers belonged to different families. All samples were matched for age at sampling, sex, storage time in the freezer, and the number of freeze-thaw cycles. The three status groups (patients, asymptomatic and controls) consisted of a combination of non-fasting and fasting samples. All ATTRV30M patient samples and a proportion of the samples in both the asymptomatic TTRV30M and control groups were obtained from the Amyloidosis Centre collection (sample collection ID 472-20-024) at Umeå University hospital, and the remaining samples were obtained from Northern Sweden Health and Disease Study (NSHDS) at Northern Sweden Medical Biobank Umeå, registration number 472. The Amyloidosis Centre collection had no record of fasting and non-fasting data on already collected samples and in the Norhen Sweden Health and Disease Study (NSHDS) collection all samples were fasting.

All samples in the matched triplet (patient, asymptomatic TTRV30M and control) were given a shared number and kept together in the analysis, thereby minimising variability in platform performance. The run order of the triplets was randomised.

Ethical approval was obtained from The Regional Ethical Review Board at Umeå University, Dnr 2015/118-31.

Sample collection

Blood samples were collected into glass tubes containing EDTA using the Vacutainer system and centrifuged at 1500g for 15 min. Plasma was then removed and stored in aliquots at −80°C.

Sample preparation

Metabolite extraction was performed according to A et al. [14]. Briefly, extraction buffer (methanol:water) including internal standards were added to 100 μL of plasma. The sample was shaken and proteins were precipitated on ice. Following extraction, quality control (QC) samples were prepared by pooling equal aliquots from all samples. Thereafter, samples were centrifuged for 10 min and 200 μL of supernatant were transferred to GC, LC positive mode (ESI+) and LC negative mode (ESI−) micro vial respectively and solvents were evaporated and stored at −80°C until analysis.

GC/MS analysis

Prior GC/MS analysis samples were derivatized, according to Jylie et al. [14]. In short, 30 μL of methoxamine in pyridine (15 μg/μL) were added to the dry sample and shaken for 10 min. Then, heating the sample to 70°C for 1 h to start the reaction before letting the reaction proceed at room temperature for 16 h. N-methyl-N-(trimethylsilyl)triﬂuoroacetamide (MSTFA) in 1% Trimethylchlorosilane (TMCS) were thenceforth added for silylation, and incubated for 1 h in room temperature. Finally, methyl stearate (15 ng/μL in heptane) was added before analysis.

Samples were analysed on an Agilent 6890 gas chromatograph (Santa Clara, USA) equipped with a 10 m × 0.18 mm fused silica capillary column with a chemically bonded 0.18 μm DB 5-MS UI stationary phase (J&W Scientific). One μL sample was injected in splitless mode by a CTC Combi Pal autosampler (CTC Analytics AG, Zwingen,

### Table 1. Summary of the participant’s details.

|                      | Controls | ATTRV30M | Asymptomatic TTRV30M | Total |
|----------------------|----------|----------|----------------------|-------|
| **Gender, male number (%)** | 14 (53.8%) | 14 (51.8%) | 14 (53.8%) | 42 (53.2%) |
| **Mean age at sampling, years (±SD)** | 60.1 (±7.4) | 59.9 (±7.1) | 59.5 (±7.3) | 59.6 (±7.3) |
| **Mean sample storage time, years (±SD)** | 10.5 (±8.8) | 8.9 (±5.5) | 9.3 (±7.5) | 9.5 (±7.4) |
| **Mean disease duration at sampling, years (±SD)** | n/a | 4.3 (±2.7) | n/a | n/a |

SD: standard deviation; n/a, not applicable.
Switzerland) at an injector temperature of 270 °C. The purge flow rate was 20 mL/min and the purge was turned on after 60 s. The gas flow rate through the column was 1 mL/min, the column temperature was held at 70 °C for 2 min, then increased by 40 °C/min to 320 °C, and held there for 2 min.

The GC system was coupled to a Pegasus III time-of-flight mass spectrometer (TOF-MS) (Leco Corp., St Joseph, MI, USA). The transfer line and the ion source temperatures were 250 °C and 200 °C, respectively. The detector voltage was set to 1.5–2.0 kV. Ions were generated by a 70 eV electron beam (2.0 mA), and 30 spectra/s were recorded in the mass range m/z 50–800. The acceleration voltage was turned on after a solvent delay of 150 s. Pooled QC samples were run in periodic order throughout the analysis.

**LC/MS (ESI±) analysis**

Deproteinated LC/MS samples were dissolved in a methanol/water mixture (1:1), before LC/MS analysis. The separation was performed on an Agilent 1290 Infinity ultra-high-performance liquid chromatography (UHPLC) system, equipped with Acquity UPLC HSS T3 C18 1.8 μm, 2.1 × 50 mm column in combination with a 2.1 mm × 5 mm, 1.8 μm VanGuard pre-column (Waters Corporation, Milford, MA, USA). The columns were preheated to 4 °C and 2 μL extracted samples were injected. Compounds were eluted from the reverse phase columns by a gradient elution at 0.5 mL/min flow rate in 11 min, from 99% mobile phase MilliQ water (0.1% formic acid) to 99% acetonitrile:2-propanol (75:25 v/v, 0.1% formic acid).

The UHPLC system was coupled to an Agilent 6550 accurate-mass quadrupole-time of flight (Q-TOF) tandem MS equipped with a Jetstream ESI source (Agilent), operating in positive (ESI+) or negative (ESI−) ion mode, for compound detection. MS parameters were kept identical between the modes.

To enable accurate mass measurements a reference ion was connected, reference ions purine and HP-0921 (Agilent) were infused to the MS, the flow rate of 0.05 mL/min, for internal calibration. The monitored masses for purine in ESI+ and ESI− were m/z 121.05 and 119.03632, respectively; those for HP-0921 were m/z 922.0098 and 966.000725, respectively.

The MS was operated with the gas temperature set to 150 °C, the drying gas flow to 16 L min⁻¹ and the nebuliser pressure 35 psi. The sheath gas temp was set to 350 °C and the sheath gas flow 11 L/min. The capillary voltage was set to 4000 V in positive ion mode, and to 4000 V in negative ion mode. The nozzle voltage was 300 V. The fragmentor voltage was 380 V, the skimmer 45 V and the OCT 1 RF Vpp 750 V. The collision energy was set to 0 V. The m/z range was 70–1700, and data were collected in centroid mode with an acquisition rate of 4 scans/s (1977 transients/spectrum). During analysis, QC samples were distributed along the run order.

**Data preprocessing**

Throughout this article, the term ‘compound’ refers to detected analytical features and the term ‘metabolite’ refers to a compound with successful identification.

Sample files from the GC/MS analysis were exported to MATLAB R2011b (Mathworks, Natick, MA, USA), where all data pre-treatment procedures, such as using hierarchical multivariate curve resolution (H-MCR) scripts, were performed according to Jonsson et al. [15]. The extracted mass spectra were identified by comparisons of their retention index and mass spectra with libraries (Swedish Metabolomics Centre SMC, in-house database) of retention time indices and mass spectra [16]. For GC/MS, both identified and unidentified metabolites were included in the statistical analysis. Unknown metabolites were presented by their resolved retention index (RI) value.

The LC/MS data processing was performed both in a target and an untargeted fashion using Agilent Masshunter Profinder v. B.08.00 (Agilent). For untargeted processing, all QC samples were processed using an algorithm within Masshunter Profinder the Batch Recursive Feature Extraction (BRFE). Then the extracted features were matched using Agilent Mass Profiler Professional v. 13.0, resulting in a combined recursion file.

For target processing, the recursion file and a predefined list containing data on compounds commonly found in plasma were used for Batch Target Feature Extraction (BTFE) in Masshunter Profinder on all LC/MS samples. An in-house reference library (Swedish Metabolomics Centre), built up by authentic standards run on the same system with the same chromatographic and mass-spec settings, was also used for the targeted processing. Metabolite identification was based on MS, MS/MS and retention time information. However, some metabolites were considered as identified with high probability, despite not being confirmed by the metabolite identification methods. Percentage relative standard deviation (RSD%) was calculated on QC samples to assess variability from extraction. Metabolites were excluded before data analysis if RSD% >40 on the QC samples. Some metabolites appeared in more than one analysis and to determine by which analysis (GC/MS, LC/MS ESI+, LC/MS ESI−) the metabolite was most stably detected, RSD% was also used. The analysis generating the lowest RSD% for a metabolite was used in the data analysis.

**Statistical analysis**

A variety of statistical techniques were used to investigate the significance of each identified metabolite, including univariate and multivariate statistical analyses.

Processed data acquired from GC/MS (peak areas) and LC/MS (peak areas) were imported separately into the web-based application MetaboAnalyst 4.0 [17,18] for multivariate statistical analyses (MVA). After data scaling and normalisation (log transformation and/or autoscaling), principal component analysis (PCA), an unsupervised method, was used to observe trends/clusters and detect possible outliers in the
data before further analysis. Partial least squares-discriminant analysis (PLS-DA) was applied as a supervised method to identify discriminating metabolites between ATTRV30M patients, controls, and asymptomatic. Cross-validation (7-fold) was used for PLS-DA model validation. The $R^2$ value is an estimation of the goodness of fit and $Q^2$ value is an estimation of the goodness of prediction.

The variable importance in projection (VIP) generated by PLS-DA represents the contribution to the discrimination of each metabolite between groups. Metabolites with a VIP $>1.0$ and a non-zero Jack-Knife confidence interval (CI, 95%) was considered significant in a model. For additional metabolite validation, univariate analysis was performed on the normalised peak area data by Student’s $t$-test with a significance level set to $p<.05$, using SPSS v25 software (IBM).

Results

A summary of the participants’ details is shown in Table 1. 85% of the ATTRV30M patients had polyneuropathy, 52% cardiomyopathy, 27% gastrointestinal symptoms, 1% vitreous opacity and 11% had carpal tunnel syndrome.

Two samples were excluded, due to incorrect identity and with a weak signal, before any statistical analysis was conducted. A total of 78 GC/MS and 79 LC/MS samples were recruited for data analysis. The average age at the sampling of the participants was $59.8 \pm 7.3$ years (range: 33–75) with a gender distribution of 42 males and 37 females.

Seventy compounds were detected by GC/MS (of which 64 were identified), 3400 by LC/MS (95 were identified; 34 in negative ionisation (ESI–) and 61 in positive ionisation (ESI+)), Table 2. The non-identified compound was not included in the data analyses.

Seven drug and central stimulant identified metabolites, which could be a result of the presence of non-fasting samples, and 11 metabolites with RSD% $>40$ on the QC samples were excluded before data analyses. In addition, 10 metabolites with the higher %RDS upon detection in more than one MS analysis were also excluded from prior data analyses. Giving a total of 123 unique metabolites, 48 in GC and 75 in LC (44 ESI+, 31 ESI–) in separate data sets, in the data analyses (Table 2). The mean RSD% for the 123 unique metabolites were 18.0%; 15.5% in GC and 19.5% in LC.

Metabolomics profiling

The GC/MS and LC/MS data were analysed independently in the MVA. Four sample outliers were identified by PCA, three in GC/MS and one in both GC/MS and LC/MS, these outliers were excluded from further data analysis. Partial least squares-discriminant analysis (PLS-DA) was used to determine the ability to differentiate metabolomics profiles in three separate models; model 1 (M1) ATTRV30M vs. controls, model 2 (M2) ATTRV30M patients vs. asymptomatic TTRV30M carriers and model 3 (M3) asymptomatic TTRV30M carriers vs. controls. PLS-DA modelling for each of the data sets, GC/MS and LC/MS, showed clear group separation in M1 and M2. Whereas, no PLS-DA model could be calculated, for either GC or LC, to distinguish asymptomatic TTRV30M from controls. Cross-validation testing was performed in the quality of the models and indicated that the models were not over-fitted (GC/MS M1 $R^2 = 0.46$, $Q^2 = 0.30$; GC/MS M2 $R^2 = 0.67$, $Q^2 = 0.24$; LC/MS M1 $R^2 = 0.71$, $Q^2 = 0.35$; LC/MS M2 $R^2 = 0.64$, $Q^2 = 0.41$).

Univariate analysis and student’s $t$-test were used to determine the significance of the unique metabolites with VIP $>1.0$ (Jack Knife CI not including 0). In total, 24 plasma metabolites (VIP $>1.0$ and $p<.05$) were significantly altered in ATTRV30M patient group (6 increased and 18 decreased). 11 of these distinguished the ATTRV30M group from both controls (M1) and asymptomatic TTRV30M (M2), (the analysis method, RSD%, $t$-test p-value and HMDB number are listed in Table 3). In model M1, there were other 9 significant plasma metabolites.

Table 2. Number of compounds and metabolites detected by untargeted GC/MS and LC/MS.

| Analytical method | Detected compounds | Identified metabolites | Identified metabolites (RSD% $<40$) | Identified unique metabolites (RSD% $<40$) |
|------------------|-------------------|----------------------|------------------------------------|------------------------------------------|
| GCMS             | 70                | 64                   | 61                                 | 48                                       |
| LCM ESI+         | 1992              | 61                   | 53                                 | 44                                       |
| LCM ESI–         | 1408              | 34                   | 34                                 | 31                                       |
| LC total         | 3400              | 95                   | 87                                 | 75                                       |
| Total            | 3470              | 159                  | 148                                | 123                                      |

RSD% is based on quality control (QC) samples measured across the analytical runs.

Table 3. PLS-DA and univariate significant metabolites in ATTRV30M amyloidosis patients compared to both matched controls and asymptomatic TTRV30M carriers.

| Metabolites      | HMDB   | QC, RSD% | Analysis | ATTRV30M vs. Controls | Increased group | p-Value ($t$-test) | ATTRV30M vs. AS | Increased group | p-Value ($t$-test) |
|-----------------|--------|----------|----------|-----------------------|-----------------|-------------------|-----------------|-----------------|-------------------|
| *Ketoleucine    | HMDB00695 | 5.8 | GC/MS    | Controls               | .0003           | AS                 | .006            | V30M            | .0009             |
| *Leucine        | HMDB00867 | 16.1 | LC/MS ESI+ | Controls               | .033            | AS                 | .008            | V30M            | .002              |
| *Kynurenine     | HMDB00684 | 21.2 | LC/MS ESI+ | Controls               | .039            | AS                 | .016            | V30M            | .002              |
| *Gamma-Glu-Leu  | HMDB11171 | 22.4 | LC/MS ESI+ | Controls               | .008            | AS                 | .001            | V30M            | .004              |
| *Glutarol carnitine (AC) | HMDB13130 | 24.9 | LC/MS ESI+ | Controls               | .017            | AS                 | .042            | V30M            | .002              |
| *Phenylalanine  | HMDB00159 | 9.0  | LC/MS ESI+ | Controls               | 5.9–5           | AS                 | 5.4–5           | V30M            | .008              |
| *Tryptophan     | HMDB00929 | 6.7  | LC/MS ESI– | Controls               | .002            | AS                 | 4.4–6           | V30M            | .0004             |
| *Indoxylsulfuric acid | HMDB00682 | 8.8  | LC/MS ESI+ | Controls               | .008            | AS                 | .0004           | V30M            | .0005             |
| *Malic acid     | HMDB00156 | 12.8 | GC/MS    | V30M                  | .0009           | V30M              | .0005           |
| *Mallose        | HMDB00163 | 8.1  | GC/MS    | V30M                  | .0003           | V30M              | .005            |
| *Niacinamide (NAM) | HMDB01406 | 24.3 | LC/MS ESI+ | V30M                  | .002            | V30M              | .0043           |

AC: acylcarnitines; HMDB: Human Metabolomics Data Base; QC: quality control; V30M: ATTRV30M amyloidosis patients; AS: asymptomatic TTRV30M carrier.
differentiating ATTRV30M patients from matched controls (analysis method, RSD%, t-test p-value and HMDB number are listed in Table 4). The variable importance in projection (VIP) plot with Jack-Knife CI are shown for 20 significant metabolites in model M1 (5 GC/MS and 15 LC/MS) according to the PLS-DA and the univariate test, between ATTRV30M and controls (Figure 1). Four additional significant plasma metabolites differentiating ATTRV30M patients from asymptomatic TTRV30M in model M2 (4 GC/MS and 11 LC/MS).

The MVA sub-analysis, between the two sample collections: Amyloidosis Centre collection versus Northern Sweden Health and Disease Study (NSHDS), were performed in the same way as in M1 and M2 to validate the significant metabolites separating ATTRV30M patients from TTRV30M carriers and controls. Four significantly altered metabolites, not reported, were also significant between the two different sample collections and therefore removed.

**Discussion**

Biomarkers are strongly linked to underlying pathological conditions and such metabolites can compose objective measures for disease diagnosis and prevention.

### Table 4. Metabolites significantly altered only between ATTRV30M amyloidosis patients compared to matched controls.

| Metabolites                  | HMDB    | QC, RSD% | Analysis | ATTRV30M vs Controls |
|------------------------------|---------|----------|----------|----------------------|
| Glucose                     | HMDB00122 | 6.0      | GC/MS    | Increased group      |
| Valine                      | HMDB00883 | 18.6     | GC/MS    | Controls             |
| L-Carnitine                 | HMDB00062 | 17.6     | LC/MS ESI+ | Controls             |
| Decenoyl carnitine (AC)     | HMDB13205 | 28.7     | LC/MS ESI+ | Controls             |
| Decanoyl carnitine (AC)     | HMDB00651 | 20.8     | LC/MS ESI+ | Controls             |
| LysoPC203/00                | HMDB10393 | 16.1     | LC/MS ESI+ | Controls             |
| Thyroxine                    | HMDB00248 | 29.8     | LC/MS ESI+ | Controls             |
| Xanthine                    | HMDB00292 | 12.6     | LC/MS ESI-- | V30M               |
| Sphingosine-1-phosphate (S1P) | HMDB00277 | 8.2      | LC/MS ESI-- | V30M               |

AC: acylcarnitines; HMDB: Human Metabolomics Data Base; QC: quality control; V30M: ATTRV30M amyloidosis patients.

![Figure 1](image)
Identification of biomarkers could promote the early detection of diseases and enhance our understanding of disease progression [19]. Findings from numerous studies on identifying biomarkers could promote disease diagnosis [20]. To our knowledge, this is the first metabolomics analysis investigating the metabolic disturbances in ATTRV30M amyloidosis patients.

The results, based on matched samples from ATTRV30M patients, asymptomatic TTRV30M carriers, and controls using untargeted GC/MS and LC/MS metabolomics techniques, show that ATTRV30M patients have a substantially different metabolomics profile. This untargeted metabolomics platform identified a broad array of totally 24 plasma metabolites that were significantly different between ATTRV30M patients and controls (model M1) and between ATTRV30M patients and asymptomatic TTRV30M carriers (model M2).

Decreased levels of several amino acids were found in the ATTRV30M amyloidosis patients. Amino acids are essential as building materials and as nutrient regulators of critical signalling pathways [21]. The amino acids tryptophan, phenylalanine, and tyrosine have been reported to be altered...
in different diseases, suggesting the potential of using these amino acids as biomarkers.

Tryptophan has been suggested to be involved in numerous neurodegenerative diseases such as Alzheimer’s disease and ALS [22]. Tryptophan is an essential amino acid and is metabolised by at least two major pathways, the serotonin and kynurenine pathways [23]. Serotonin plays a role in gastrointestinal regulation and is a modulator of blood vessel tone. Nyhlin et al. [24] showed a decrease in serotonin-producing cells in the gastrointestinal tract in patients with ATTRV30M. The decrease in tryptophan in this study suggests that tryptophan could be involved in autonomic dysfunction seen in patients with ATTRV30M. Hatano et al. [25] suggested that the decrease in tryptophan levels may be linked to differences in the serotonin pathway, but not in the kynurenine pathway. This study showed that the kynurenine pathway is likewise affected, as a decreased kynurenine levels in ATTRV30M patients was detected. Dysregulation of this pathway may have a neurotoxic effect [26] which could be involved in the neuropathy affecting ATTR patients.

De novo synthesis of the cofactor nicotinamide adenine dinucleotide (NAD) can be made through the kynurenine pathway by degradation of tryptophan, but since NAD levels are unaffected it does not seem to be the case in our ATTR patients. However, NAD can also be synthesised by salvage pathways, where niacinamide (NAM) is one of the precursors that are recycled back to NAD in the mammal body. NAM, which is part of the vitamin B group, was found elevated in ATTRV30M patients compared to controls and TTRV30M carriers. The reason for this could be the decreased gut motility seen in ATTR patients. The low levels of tryptophan for NAD synthesis could suggest that vitamin B supplementation is important for ATTR patients, since NAD is needed as a link between the TCA and oxidative phosphorylation.

One consequence of severe ATTR amyloidosis is decreased muscle mass and weight loss. In Becker muscular dystrophy and Duchenne muscular dystrophy blood glucose levels are increased [27] in contrast to ATTRV30M patients who showed decreased levels of glucose. Increased demand for energy could have lowered the levels of blood glucose in ATTR amyloidosis.

Other decreased amino acids in ATTRV30M disease were leucine, ketoleucine and valine. Valine and leucine are branched-chain amino acids (BCAA). Ketoleucine is a metabolite that originates from the incomplete breakdown of BCAA leucine. BCAA are metabolised to Acetyl-CoA which then enters the TCA. This could reflect that BCAA is being used as an additional source of energy and substrate for gluconeogenesis.

We also found decreased phenylalanine and tyrosine levels in subjects with ATTRV30M amyloidosis. Phenylalanine is a precursor for tyrosine and biogenic amine neurotransmitters dopamine, noradrenaline, and adrenaline, also known as catecholamines [28]. Defects in the catabolic pathways of phenylalanine and tyrosine deplete downstream neurotransmitters which can lead to autonomic dysfunction such as hypotension seen in ATTR patients. The decreased level of tyrosine in ATTRV30M patients additionally imposes effects on tyrosine hormone synthesis resulting in decreased thyroxine, the thyroid hormone T4 which is transported by transthyretin.

Xanthine was increased in ATTRV30M patients compared to controls. Hypoxanthine is catalysed by xanthine oxidase (XO) to produce Xanthine and these together are involved in the purine metabolism pathway [29]. Choi et al. [30] showed that hypoxanthine and inosine are involved in the oxidative stress generated by haemodialysis. Ando et al. [31] showed that oxidative stress participates in the formation of amyloid fibrils in ATTR amyloidosis. The presence of xanthine in plasma of ATTRV30M patients may be attributed to oxidative stress.

Another biomarker for weight loss is decreased levels of lysophosphatidylycholine (LysoPC) [32]. In model M2, the levels of LysoPC were decreased in ATTRV30M patients compared to asymptomatic TTRV30M. LysoPC is mostly obtained by hydrolysis of phosphatidylycholine (PC) in the circulation. Once formed, LysoPCs can be degraded or promptly transported to target tissue to activate signalling pathways involved in oxidative stress and inflammation [33–36]. Whereas sphingosine-1-phosphate (S1P), also a member of the PC family, showed increased levels in M2. The receptors for S1P are a family of five (S1PR1–5). It has been proposed that S1PR1 regulates inflammatory response [37], that S1PR1 and S1PR5 can promote myelination [38], and that S1PR3 signalling induces bradycardia [39]. All these responses can be associated with the ATTR phenotype.

Malic acid is an intermediate in the tricarboxylic acid cycle (TCA) which is aimed to generate energy (ATP). Alteration in TCA has been correlated with numerous pathologies such as cancer [40], cardiovascular diseases [41] and neurodegenerative disorders [42]. In this study, malic acid was elevated in ATTRV30M amyloidosis compared to matched controls and asymptomatic TTRV30M carriers.

In the energy metabolism, L-carnitine transport fatty acid into the mitochondria for future beta-oxidation and ATP, a process that generates acylcarnitines (AC) [43]. The AC levels in plasma is determined by the nutritional state and tissue contribution. Although, in fasting samples it has also been observed an increased AC concentration due to the fatty acid oxidation rate is increased [44,45]. Therefore, the decreased levels of L-carnitine and AC in ATTRV30M patients may be the result of higher proportion of fasting samples among controls and asymptomatic TTRV30M carriers.

In our material, the metabolites of seven known drugs and central stimulants were identified, probably due to the presence of non-fasting samples. These metabolites were removed before the statistical analysis in order not to affect the results.

In this study, we were not able to detect any metabolic difference between controls and asymptomatic TTRV30M carriers. The two different PLS-DA models, M1 and M2, showed a shared pattern for many of the significant
metabolites, which confirms that the control group and the asymptomatic TTRV30M carrier group have similar metabolic profiles.

Limitations of our study which could have influenced the results are the following: (i) The study was conducted with a limited number of samples. The interesting potential metabolites from our study should be verified in a more coherent and larger sample cohort. (ii) A mixture of fasting and non-fasting samples. Because there was no record of fasting and non-fasting data for the samples from the Amyloidosis Centre collection and the knowledge that all samples from the Northern Sweden Health and Disease Study (NSHDS) were fasting, a sub-analysis was performed to address the potential bias of fasting versus non-fasting. In the sub-analysis, the two different sample collections were compared and only four significant metabolites had to be excluded after the data analysis due to possible sample collection and/or fasting effects, which is consistent with previously published results that the human metabolome shows low variability independent of fasting and non-fasting [46]. (iii) No clinical data, at sampling date, were available for samples from the Northern Sweden Medical Biobank, but all samples were genotyped. These samples were only available to us coded and without any personal id.

In conclusion, we have used well-established techniques platform, gas- and liquid chromatography systems and found dysregulated levels of metabolites possibly involved in the physiological and neurological phenotype of ATTR including the energy metabolism. The potential biomarkers identified in this study are statistically significant but not necessarily biochemically significant. Therefore, the clinical application of the candidate metabolites needs more future studies as well as the inclusion of a validation set to establish biochemical significance in ATTR30M amyloidosis. Healthy TTRV30M carriers could be followed over time when the panel of candidate metabolites is confirmed to have a biochemical impact on the disease and correlated to future clinical symptoms. Early detection of ATTR among carriers would enable medical treatment before severe damage of deposited amyloid fibrils has occurred.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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