

**Advanced non-alcoholic fatty liver disease and adipose tissue fibrosis in patients with Alström syndrome.**

**Abbreviated title: Hepatic and adipose phenotype in Alström syndrome**

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**Abbreviations:**

NAFLD : non-alcoholic fatty liver disease

AS: Alström syndrome

BMI: body mass index

NASH: non-alcoholic steatohepatitis

ELF: Enhanced Liver Fibrosis

LSE: liver stiffness evaluations

PLAT: plasminogen activator

PLG: plasminogen

EDN1: Endothelin-1 (EDN1)

CTGF: connective tissue growth factor

TGF- $\beta$ 3: transforming growth factor- $\beta$ 3 ()

BMP 7: Bone morphogenetic protein 7

CCL2 and CCL3: chemokine (C-C motif) ligand 2 and 3

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### **Abstract**

**Background and Aims:** Alström syndrome (AS) is a recessive monogenic syndrome characterised by obesity, extreme insulin resistance and multi-organ fibrosis. Despite phenotypically being high risk of non-alcoholic fatty liver disease (NAFLD), there is a lack of data on the extent of fibrosis in the liver and its close links to adipose in patients with AS. Our aim is to characterise the hepatic and adipose phenotype in patients with AS.

**Methods:** Observational cohort study with comprehensive assessment of metabolic liver phenotype including liver elastography (Fibroscan®), serum Enhanced Liver Fibrosis (ELF) Panel and liver histology. In addition, abdominal adipose histology and gene expression was assessed. We recruited 30 patients from the UK national AS clinic. A subset of 6 patients underwent adipose biopsies which was compared with control tissue from 9 healthy participants.

Results: Patients were overweight/obese (BMI 29.3 (25.95-34.05) kg/m<sup>2</sup>). 80% (24/30) were diabetic. 74% (20/27) had liver ultrasound scanning suggestive of NAFLD. As judged by the ELF panel, 96 % (24/25) were categorized as having fibrosis and 10/21 (48 %) had liver elastography consistent with advanced liver fibrosis/cirrhosis. In 7/8 selected cases, there was evidence of advanced NAFLD on liver histology. Adipose tissue histology showed marked fibrosis as well as disordered pro-inflammatory and fibrotic gene expression profiles.

Conclusions: NAFLD and adipose dysfunction are common in patients with AS. The severity of liver disease in our cohort supports the need for screening of liver fibrosis in AS.

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**Keywords:** NAFLD, Alström syndrome, Adipocyte biology, Insulin resistance

**Key points:**

- Alström Syndrome is a rare autosomal recessive monogenic ciliopathy
- Liver fibrosis and adipose fibrosis are common in patients with Alström Syndrome
- The liver fibrosis seen is more advanced than would be anticipated given the young age of the patients
- The liver fibrosis in Alström Syndrome can be identified non-invasively

**Introduction:**

Alström syndrome (AS) is a rare (1 per million) autosomal recessive [OMIM 203800] monogenic metabolic syndrome characterised by childhood onset obesity, extreme insulin resistance, diabetes, dyslipidaemia, hypertension and multi-organ fibrosis. Other features include retinal rod-cone dystrophy, hearing loss, and dilated cardio-myopathy [1]. Alström is caused by mutations in ALMS1 gene which encodes an ubiquitously expressed centrosomal protein of the primary cilium [2,3]. Cilia are membrane-bound, microtubular projections emanating from the cell surface and found on almost all vertebrate cells. Cilia sense a variety

of extracellular signals including hormones transducing them into intracellular signals [4]. ALMS1 protein is expressed in key metabolic tissue (liver, skeletal muscle, adipose and pancreas). *In vitro*, ALMS1 deletion is associated with hepatic lipid accumulation [5,6] and impaired adipocyte lipid storage [7,8]. Patients with AS have disordered lipid metabolism with elevated serum free fatty acids not suppressed by insulin [9] and have insulin resistance disproportionate to their BMI [10]. Additionally, we have described premature cardiovascular disease in patients with AS [11].

NAFLD is a spectrum ranging from simple steatosis, through to non-alcoholic steatohepatitis (NASH), fibrosis and an increased risk of cirrhosis and hepatocellular carcinoma. NASH, including cirrhosis and hepatocellular carcinoma, are becoming increasingly prevalent mirroring the obesity epidemic [12]. The relationship between obesity and NASH is well recognised at a population level, but the mechanism linking obesity to the development of NASH remains mostly speculative, partly due to a lack of well-defined human disease models. Patients with lipodystrophy, where mutations in several genes result in marked loss of adipose tissue mass and perturbed adipocyte function, develop profound insulin resistance and accelerated liver disease [13]. These severe hepatic consequences of frank anatomical deficiency of adipose tissue have been conceptually linked to the consequences of obesity by the notion of "adipose expandability" [14]. It follows that the ability of adipose tissue to store excess energy is finite, and when this limit is reached, whether at low absolute levels in lipodystrophy or at high absolute levels as in common obesity, lipotoxicity of distant organs results. Plausibly the adipose tissue dysfunction seen in AS may contribute to the liver phenotype although as ALMS1 is expressed within the liver and adipose it may also be via a direct effect of ALMS1 mutation within the liver.

Case reports and series [1,15–17] as well as our experience within the national AS clinic, reveals unexpectedly high incidence of liver cirrhosis and associated morbidity at a young

age. We have hypothesised that accelerated NAFLD in AS may relate to extreme insulin resistance and be driven (at least in part) by the inability of adipose tissue to provide a healthy adipose buffer. We have therefore undertaken the most detailed metabolic phenotyping of patients with AS published to date, and performed in-depth analysis of metabolic liver disease, incorporating adipose tissue morphology and gene expression profiles.

## **Patients and Methods**

### ***Patients and volunteers***

Patients with AS were recruited from the UK National centre for AS service, based at Queen Elizabeth Hospital, Birmingham, UK. The diagnosis of AS was confirmed on the basis of clinical features and genetic sequencing of ALMS1 gene (**Supplementary table S1**). Informed consent was obtained and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in ethical approval by the Cornwall and Plymouth NRES (UKCRN 9044, REC approval 10/H0203/33). Data collected included patient demographics anthropometric measurements including body mass index (BMI) and blood pressure. Clinical phenotypes were recorded as well as details of diabetes status and management. A subset of patients consented for adipose tissue biopsy to examine the morphology of the adipose tissue and the expression of profibrotic genes. Control adipose tissue biopsies were taken from healthy individuals with ethical approval from West Midlands-Edgbaston NRES (LREC: 12/WM/0206). The liver biopsies included in this study were obtained for clinical reasons.

### ***Serum analysis***

Liver biochemistry, electrolytes, urea, creatinine, cholesterol, triglycerides, glycated haemoglobin, and full blood counts were measured using standard laboratory methods (Roche Modular system, Roche Ltd, Lewis, UK). Blood tests were taken in the non-fasting state.

### ***Liver fibrosis***

Patients underwent an abdominal ultrasound to looking for hepatic steatosis and for structural abnormalities associated with more advanced liver disease (splenomegaly, irregular portal vein blood flow and an irregular or nodular liver). Serum samples from AS patients were analysed for the Enhanced Liver Fibrosis panel (ELF), a well validated non-invasive biomarker to identify fibrotic liver disease [18]. Transient elastography was performed using Fibroscan® (Echosens, France). Only valid liver stiffness evaluations (LSEs) were recorded as per manufacturers guidance (10 readings, IQR <30% of the median LSE, success rate >60%). The cut-off of >7.9kPa was chosen as predictive for fibrosis as this has been shown to correlate well with histological findings in NAFLD [19].

### ***Histological analysis of liver and adipose tissues***

Liver histology was available for retrospective, review of the diagnosis, fibrosis stage (Kleiner) and NAFLD Activity Score (NAS) in 5 Alström syndrome patients and an additional 3 more histology reports were available for analysis. Liver biopsies were clinically indicated.

Six (20%) patients with AS and 9 controls had an abdominal subcutaneous adipose tissue biopsy performed under local anesthetic (1-2mL of 1% lidocaine), to obtain approximately 250-500mg of adipose tissue. The patients with AS who had adipose tissue biopsies were representative of the cohort as a whole (**Supplementary table 2**). The samples were divided into two and either placed in RNALater® (Ambion Inc., Austin, TX, USA) (initially for 24h at room temperature and then at -20°C) for subsequent gene expression analysis or placed into formalin for histological analysis.



### ***Adipose tissue histology and gene expression***

Adipose biopsies that were formalin-fixed were embedded in paraffin and cut at a thickness of 4µm on a Leica RM2235 microtome (Leica, Milton Keynes, UK) and stained with H&E and Van Gieson's to assess fibrosis.

RNA was prepared using RNeasy Lipid Tissue (QIAGEN). cDNA was generated from the RNA (QIAGEN RT<sup>2</sup> First Strand Kit) and expression of profibrotic genes was assessed using real-time reverse transcription array (RT<sup>2</sup> Profiler™ Arrays: Human fibrosis).

### ***Statistical analysis***

Unless otherwise stated data expressed is median (interquartile range). Gene expression data were obtained as Ct values (Ct is the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine  $\Delta$ Ct values [ $\Delta$ Ct = (Ct of the target gene) – (Ct of the housekeeping gene)]. Fold change was calculated as  $2^{-\Delta\Delta$ Ct [ $\Delta\Delta$ Ct = ( $\Delta$ Ct of the control group) – ( $\Delta$ Ct of the patient group)]. Gene expression was analysed using available online software (<http://www.sabiosciences.com/pcrarraydataanalysis.php>).

## Results

### *Demographic, genetic and metabolic characteristics*

Demographic, anthropometric and metabolic data are presented in **Table 1** (mutation analysis **Supplementary Table 1**). Patients were predominantly male 70% (21/30) with a median age of 24 (21.5-37) years and a BMI of 29.3 (25.95-34.05) kg/m<sup>2</sup>. The diabetes prevalence within the cohort was 80% with the duration of diabetes 11 (7.5-14.75) years. The prevalence of dyslipidaemia was high (total cholesterol 4.95 (4.2-6.3) mmol/L; high density lipoprotein cholesterol 0.89 (0.75-1.06) mmol/L; mean triglycerides 3.25 (2.1-4.75) mmol/ L). C-peptide to glucose ratios were elevated consistent with the anticipated insulin resistance and preserved  $\beta$ -cell function (4.72 (2.67-8.75) (ng/ml)/(mg/dl)\*100).

### *Hepatic phenotype*

Hepatic phenotypic data for individual patients is provided in **Table 2** with histology presented in **Figure 1** and **Table 3**. Median AST and ALT 32 (26-44) and 50 (33-76) IU/ L, respectively. 27/30 patients underwent abdominal ultrasound scanning. 20 of 27 (74%) had either an echobright liver consistent with hepatic steatosis or features suggestive of more advanced disease; in the absence of excess alcohol intake. Of these, 6 patients had splenomegaly (>13.5 cm) and 6 had ultrasound features suggestive of advanced fibrosis (coarse echotexture, irregular contour). The Enhanced Liver Fibrosis panel was performed in 25 patients, of whom 96 % were categorized as having either moderate or severe fibrosis. 21 patients underwent hepatic elastography (Fibroscan®, Echosens, France) with a valid liver stiffness evaluations (LSE), 48% (10/21) had a LSE of  $\geq 7.9$  kPa suggestive of hepatic fibrosis [20].

Formal histological analysis was available in 5 patients (17% study subjects) including the Kleiner fibrosis score and NAFLD activity score (**Table 3**). Additionally 2 post mortems and 1 liver biopsy report were available for inclusion although the tissue was not available for central reporting with Kleiner fibrosis score. These 2 post mortems confirmed cirrhosis of the liver with varices in these patients and the biopsy showed extensive fibrosis. In addition, subject 10 who is 19 years old had a liver biopsy at the age of 8 with extensive steatohepatitis predating diabetes by 9 years, indicating early onset severe NAFLD.

### ***Adipose tissue gene expression and histology***

The sub-group of patients who underwent adipose tissue biopsy (n=6, male/female=4/2) were representative of the AS cohort (**Supplementary Table S2**). The control volunteers (n=9) were exclusively female but were BMI- and age-matched to the patients.

### ***Histology***

Adipose tissue architecture and morphology were disrupted in patients with AS. Throughout the biopsies, there was extensive fibrosis evident on H & E staining (**Figure 2b.**) and confirmed using a Van Gieson stain (**Figure 2c**).

### ***Gene expression***

The abnormal adipose histology was reflected in the pattern of gene expression. The expression of multiple groups of genes involved in fibrosis was altered (**Figure 2d**). Genes important for fibrin degradation were reduced including tissue plasminogen activator (PLAT) and plasminogen (PLG). The expression of pro-inflammatory cytokines varied including reduction in IL-4 and IL-13 that are involved in promoting a pro-inflammatory macrophage population, as well as reductions in Endothelin-1 (EDN1) and connective tissue growth factor (CTGF) (fibroblast/myofibroblast activators). Furthermore, mRNA expression of

transforming growth factor- $\beta$ 3 (TGF- $\beta$ 3) was also decreased. In contrast, the expression of the genes encoding the chemotactic proteins, CCL2 and CCL3 (chemokine (C-C motif) ligand 2 and 3) increased.

## Discussion

We have carried out the largest and most detailed metabolic and hepatic phenotypic description of patients with AS to date. Our study highlights that patients with AS are at an increased risk of advanced NAFLD and cirrhosis, which seems disproportionate to age, BMI and duration of diabetes. Our results indicate that their extreme insulin resistance and its attendant complications such as advanced NAFLD and cardiovascular events occur at a very young age in the presence of disorganised and dysfunctional adipocytes.

Consistent with previous data we have observed high levels of obesity and insulin resistance in AS patients. There are similarities between the clinical and biochemical profiles of individuals with AS and those with common obesity [1]. In contrast to common obesity, patients with AS invariably have childhood onset obesity, severe insulin resistance and very early occurrence of coronary heart disease before the age of 40 [11].

NAFLD ranges from relatively benign steatosis to cirrhosis. Our data revealed that a large proportion of patients with AS have evidence of NAFLD and advanced fibrosis at an early age. This supports published case reports and series that reveal unexpectedly high incidence of liver cirrhosis and associated mortality in Alström patients [1,15,16,21,22]. Our data, supports the need for early screening of liver fibrosis in patients with AS. Due to the rarity of the condition it is very difficult to validate the use of non-invasive markers of fibrosis in this setting. However, given the consistent accuracy and reproducibility of liver elastography (Fibroscan) in other liver diseases, we would advocate its use in AS to identify those in need of closer monitoring (i.e. for portal hypertension, liver cancer) and intensive metabolic optimisation.

Accelerated liver disease has also been reported in patients with lipodystrophies [13], and indeed this has been reported to be a major contributor to premature mortality in these

disorders. Like AS lipodystrophies are characterized by severe dyslipidaemic insulin resistance with attendant complications, and are exquisitely sensitive to variations in caloric and fat intake [23]. In contrast to AS, lipodystrophies are defined by partial or complete absence of adipose tissue, and often, though not always, feature low levels of adipocyte-derived hormones such as leptin. Lipodystrophies have thus been cited as monogenic evidence of the "adipose expandability" hypothesis [13,14,24]. Importantly, in some lipodystrophies, severe NAFLD arises even though the functional defect is restricted to adipose tissue, proving that NAFLD is not necessarily a liver autonomous process.

Unlike lipodystrophies, AS has been reported to feature excess obesity with abundant adipose tissue. It is plausible, however, that an adipose autonomous pro-fibrotic tendency in AS leads to a state of "relative" adipose failure more akin to the metabolic disease seen at high levels of "common" obesity. In keeping with this, patients with AS, like "common" obesity patients, can successfully ameliorate metabolic syndrome with lifestyle changes to offload adipose tissue [23]. The histological studies we report in a subset of AS patients may provide tissue-level support for this hypothesis: The increase in adipose tissue fibrosis may impair adipose lipid storage, increasing the likelihood of 'spill-over'. This is supported by increasing evidence of the adverse impact of adipose tissue fibrosis on metabolic flexibility [25], and recently published data suggest that adipose tissue fibrosis contributes to hepatic steatosis [26]. One example of this in a model organism is afforded by collagen VI-null ob/ob mice where reduced adipose tissue extracellular matrix permitted increased adipose depot size and adipocyte hypertrophy in response to high fat diet, and conferred metabolic protection even in the face of hyperphagia driven by leptin deficiency [25].

The pattern of gene expression that we observed in the adipose tissue was complex although several of the altered genes have been implicated in the pathogenesis of fibrosis, adipose dysfunction and global metabolic homeostasis. The TGF- $\beta$  / BMP7 pathway is an important

regulator of fibrogenesis and we observed decreased expression of TGF- $\beta$ 3 and BMP7 in adipose tissue from AS patients. BMP7 inhibits fibrogenesis [27] and, although TGF- $\beta$ s are predominantly profibrotic, TGF- $\beta$ 3 supports tissue repair and limits scar formation [28] . BMP7 also has metabolic effects on the adipose tissue driving adipogenesis in mesenchymal stem cells [29] and increasing mitochondrial activity and fatty acid oxidation in brown adipose tissue [30]. Additionally, it is an anorectic factor with a decrease in food intake [31] as well as increased energy expenditure [32] contributing to weight loss in mice with increased BMP7.

CCL2, whose expression was increased, has been implicated in steatohepatitis and metabolic dysfunction. CCL2 null mice have reduced hepatic fibrosis and markers of oxidative stress on a methionine/choline-deficient diet [33]. CCL2 overexpression leads to adipose inflammation and macrophage accumulation, systemic insulin resistance and hepatic steatosis [34].

The precise role of ALMS1 in adipocytes is not fully understood with expression falling early in differentiation yet unchanged by differentiation modulating agents [35]. Knockdown experiments have shown that reduced ALMS1 expression decreases adipogenesis with preserved insulin action [8]. ALMS1 knockout mice gain weight rapidly on an obesogenic diet (6 weeks) with adipose tissue mass expansion. However, over time, adipose tissue mass fails to expand further, contrasting with observations in wild type animals, and increasing body weight is driven by increased hepatic lipid loading [6]. ALMS1 has also been shown to regulate fibroblast function. In dermal fibroblasts derived from patients with AS, extracellular matrix production including collagen production was increased and the cells resisted apoptosis [36].

Given the available pre-clinical data the adipose phenotype that we have characterized may represent a combination of effects both on adipocyte development, but also modification of

the inflammatory and fibrotic response. The net effect is likely to be an impaired ability of adipocytes to effectively store lipid that may then be delivered to the liver to fuel the development of advanced NAFLD.

There are several limitations of the present study. Firstly, although our single-centre national service is the largest centre in the world, the ultra-rare nature of the syndrome led to the small numbers of study subjects precluded the ability to analyse clinical variables that may predict NAFLD progression to fibrosis within AS (i.e. multi-variate analysis). Although as in our cohort 28/29 (96.6%) patients had biallelic nonsense or frameshift mutations we have not found evidence that site of mutation dictates the extent of liver disease. Secondly, as this is a cross-sectional study not all patients' data is complete. However, as we were able to undertake several indices (biomarkers, transient elastography and histology) to assess the nature of NAFLD, we are able to offset missing variables. Thirdly, the control adipose biopsies were taken from female volunteers, however they were age and BMI matched. Finally, there is growing interest in genetic variants associated with NAFLD and in particular in patatin like phospholipase domain containing 3 (PNPLA3) which is associated not only with hepatic steatosis but in progression to fibrosis [37]. Additional genetic tests were beyond the scope of this study and would not alter the management of our patients who develop liver fibrosis at a much younger age than those seen in genome wide association studies. In general, its strengths lie: a) in the coherence of the patient group for an extremely rare monogenic metabolic disease; b) the in-depth characterisation of NAFLD and c) the link with adipose tissue structural and gene expression study.

In conclusion, our study highlight the importance of detailed assessment of the liver in patients with AS, as many harbour asymptomatic advanced fibrotic disease. This may have relevance to other related ciliopathies including Bardet Biedl syndrome where similar mechanisms may operate [38]. It is possible that adipose tissue dysfunction is an important



contributor to the severe NAFLD that we have described, but a causal link cannot be conclusively demonstrated from our cohort. Further studies are warranted to define the precise molecular pathways that are responsible for these observations in both liver and adipose tissue, and this may ultimately lead to the identification of regulatory pathways and novel therapeutic targets for the treatment of NAFLD.

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## Figure legends

**Figure 1. Liver histology from a patient with Alström syndrome** Representative histology is shown for patient 15. A. 200 x magnification image of H&E stain showing macrovesicular steatosis and inflammation, inset panel is staining for cytokeratin. B. 200 x magnification image of Sirius Red stain.

**Figure 2. Abdominal subcutaneous adipose tissue gene expression is deregulated in patients with Alström syndrome (n=6).** Representative histological sections are presented in panels A-C (A. Control adipose H & E staining, B. Alström syndrome adipose H & E staining, C. Alström syndrome adipose Van Gieson staining D.) gene expression in panel E. (PLG=plasminogen, PLAT=tissue plasminogen activator, IL1 $\alpha$ = interleukin 1 $\alpha$ , IL4=interleukin 4, IL13=interleukin 13, IL13RA2=interlukin 13 receptor  $\alpha$  2, CCL2= chemokine ligand 2, CCL3= chemokine ligand 3, CCL11=chemokine ligand 11, IFN $\gamma$ =intergeron  $\gamma$ , EDN1= endothelin 1, CTGF=connective tissue growth factor, MMP-1= matrix metallopeptidase 1, MMP-3= matrix metallopeptidase 3, BMP7= bone morphogenetic protein 7, TGF- $\beta$ 3= transforming growth factor  $\beta$  3, LTBP=latent transforming browth factor  $\beta$  binding protein 1, INHBE=inhibin  $\beta$  E, VEGFA=vascular endothelial growth factor A).

**Table 1. The demographic and metabolic characteristics of 30 patients with Alström syndrome** (BMI=body mass index; HbA1c=glycosylated haemoglobin; BP=blood pressure; HDL=high density lipoprotein cholesterol)

**Table 2. The hepatic phenotype of 30 patients with Alström Syndrome.** (M=male; F=female; N=no; Y=yes; Spleen=spleen enlarged; AST= aspartate aminotransferase; ALT=alanine aminotransferase; Plts=platelets; ELF=Enhanced liver fibrosis panel; None=not suggestive of fibrosis; Mod=predictive of moderate fibrosis; Severe=predictive of severe fibrosis; LSE=liver stiffness evaluation; NP=not predictive of fibrosis; Predictive=predictive of fibrosis; IQ=interquartile range; \*Patients with liver histology available).

**Table 3. Histological analysis of livers of patients with Alström syndrome.** (M=male; F=female; IQR=interquartile range; NP=not predictive of fibrosis; NAS=non alcoholic fatty liver disease activity score.)