

## ORIGINAL ARTICLE

# Impaired hepcidin expression in alpha-1-antitrypsin deficiency associated with iron overload and progressive liver disease

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## Abstract

Liver disease due to alpha-1-antitrypsin deficiency (A1ATD) is associated with hepatic iron overload in a subgroup of patients. The underlying cause for this association is unknown. The aim of the present study was to define the genetics of this correlation and the effect of alpha-1-antitrypsin (A1AT) on the expression of the iron hormone hepcidin. Full exome and candidate gene sequencing were carried out in a family with A1ATD and hepatic iron overload. Regulation of hepcidin expression by A1AT was studied in primary murine hepatocytes. Cells co-transfected with hemojuvelin (HJV) and matriptase-2 (MT-2) were used as a model to investigate the molecular mechanism of this regulation. Observed familial clustering of hepatic iron overload with A1ATD suggests a genetic cause, but genotypes known to be associated with hemochromatosis were absent. Individuals homozygous for the A1AT Z-allele with environmental or genetic risk factors such as steatosis or heterozygosity for the HAMP non-sense mutation p.Arg59\* presented with severe hepatic siderosis. In hepatocytes, A1AT induced hepcidin mRNA expression in a dose-dependent manner. Experiments in overexpressing cells show that A1AT reduces cleavage of the hepcidin inducing bone morphogenetic protein co-receptor HJV via inhibition of the membrane-bound serine protease MT-2. The acute-phase protein A1AT is an inducer of hepcidin expression. Through this mechanism, A1ATD could be a trigger of hepatic iron overload in genetically predisposed individuals or patients with environmental risk factors for hepatic siderosis.

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## Introduction

Genetic liver diseases encompass a heterogeneous group of disorders, including alpha-1-antitrypsin deficiency (A1ATD) and hemochromatosis as the most common entities (1,2). A1ATD-related liver disease is caused by hepatocellular accumulation of the mutant protein, which aggregates in the endoplasmic reticulum (ER) (3,4). The most frequent genetic defect associated with A1ATD-related liver disease is homozygosity for the Z-allele of the *SERPINA1* gene (NM\_000295.4: c.1096G>A), which causes a change from glutamic acid to lysine in position 342 of the secreted protein (5). The mutant protein exhibits altered mobility upon isoelectric focusing and is referred to as protease inhibitor (Pi) Z (6). The normal gene product is known as Pi M and is secreted into the plasma, where it is the most abundant serine Pi (7). Inactivation of neutrophil elastase is the main function of alpha-1-antitrypsin (A1AT), which prevents tissue damage from excess proteases released from granulocytes.

Clinical disease manifestations of A1ATD are highly variable, where lung disease is a consequence of reduced protein secretion and function (8). A1ATD-related liver disease has a lower penetrance and is only caused by mutations that induce protein aggregation such as Pi Z (6). Aggregated polymers of mutant A1AT can accumulate in hepatocytes especially when ER-associated degradation is impaired (3,9). Stimulation of autophagy was recently found to revert A1AT accumulation in a mouse model of liver disease associated with A1ATD (10). Although the molecular details of hepatic A1AT accumulation are well understood, it is unknown why only a minority of adult patients with A1ATD Pi ZZ develop liver cirrhosis (11).

The coincidence of A1ATD and hemochromatosis has been repeatedly reported in liver explant series and case reports, suggesting that iron accumulation is an aggravating factor in A1ATD-related liver disease (12,13). In the absence of A1ATD, the C282Y mutation of the *HFE* gene is present in the majority of patients with hemochromatosis. Although no genetic association between A1ATD and classical *HFE* mutations has been found (14,15), A1ATD appears to increase expressivity of hemochromatosis (13,16). In addition to common *HFE* gene mutations, other genetic causes of hemochromatosis include *HAMP*, *TFR2* and *HFE2* mutations. As a recessive trait, hemochromatosis is caused by homozygous or compound heterozygous mutations in any of these genes, where additional heterozygosity for mutations in other hemochromatosis genes can modify disease manifestation (17–19). In particular, heterozygous *HAMP* mutations have been reported to increase disease severity.

A1ATD and hemochromatosis can therefore both be considered polygenic diseases with strong environmental factors that modify disease manifestation (20). Known environmental disease modifiers include hepatitis C virus infection, alcohol intake and obesity, each of which is known to suppress hepcidin expression (21,22). In addition, common *SERPINA1* variants have also been found to determine the severity of iron storage in patients with non-alcoholic fatty liver disease, which suggests a direct functional interaction of A1AT with iron metabolism (23).

Reduced expression of the iron hormone hepcidin is considered the unifying pathogenic mechanism of different types of genetic hemochromatosis (19,24). Genetic defects in the hepcidin gene *HAMP* and genetic defects in *HFE2* are both associated with the most severe and early onset form of genetic iron overload disorder—juvenile hemochromatosis (25,26). Hemojuvelin (HJV), which is the gene product of *HFE2*, is an inducer of hepcidin expression by acting as a co-receptor for bone morphogenetic protein-6 (BMP-6) (27,28). BMP-6 is known to act through binding to

Type I and Type II BMP receptors (29). Mayeur *et al.* (30) have shown that the *BMPR1A* (*Alk6*) is important in interleukin-6-mediated induction of hepcidin expression. An orally administered *BMPR1A* inhibitor was found to successfully inhibit hepatic BMP signaling and may be a novel therapeutic option for patients with anemia of inflammation (31). Furthermore, *BMPR1A* seems to be stabilized by HFE on the cell surface by preventing proteasomal degradation of the receptor (32). In contrast, genetic defects in *BMPR1B* (*Alk6*) are reported not to be associated with apparent changes in baseline iron status in mice (30). However, this does not preclude that *BMPR1B* has a specific role in BMP signaling and the control of hepcidin transcription during inflammation or perturbation of iron metabolism. The apparent redundancy of the Type II BMP receptors *BMPR2* and *ActR2a* in maintaining iron homeostasis was recently investigated. Mice lacking either *BMPR2* or *ActR2a* showed no changes in hepcidin expression and iron status, whereas deficiency of both receptors caused iron overload and markedly reduced hepcidin mRNA expression (33). BMP-induced hepcidin expression is inhibited by the membrane-bound serine protease matriptase-2 (MT-2), which is encoded by *TMPRSS6*. MT-2 is thought to suppress hepcidin expression by cleaving the BMP co-receptor HJV (34).

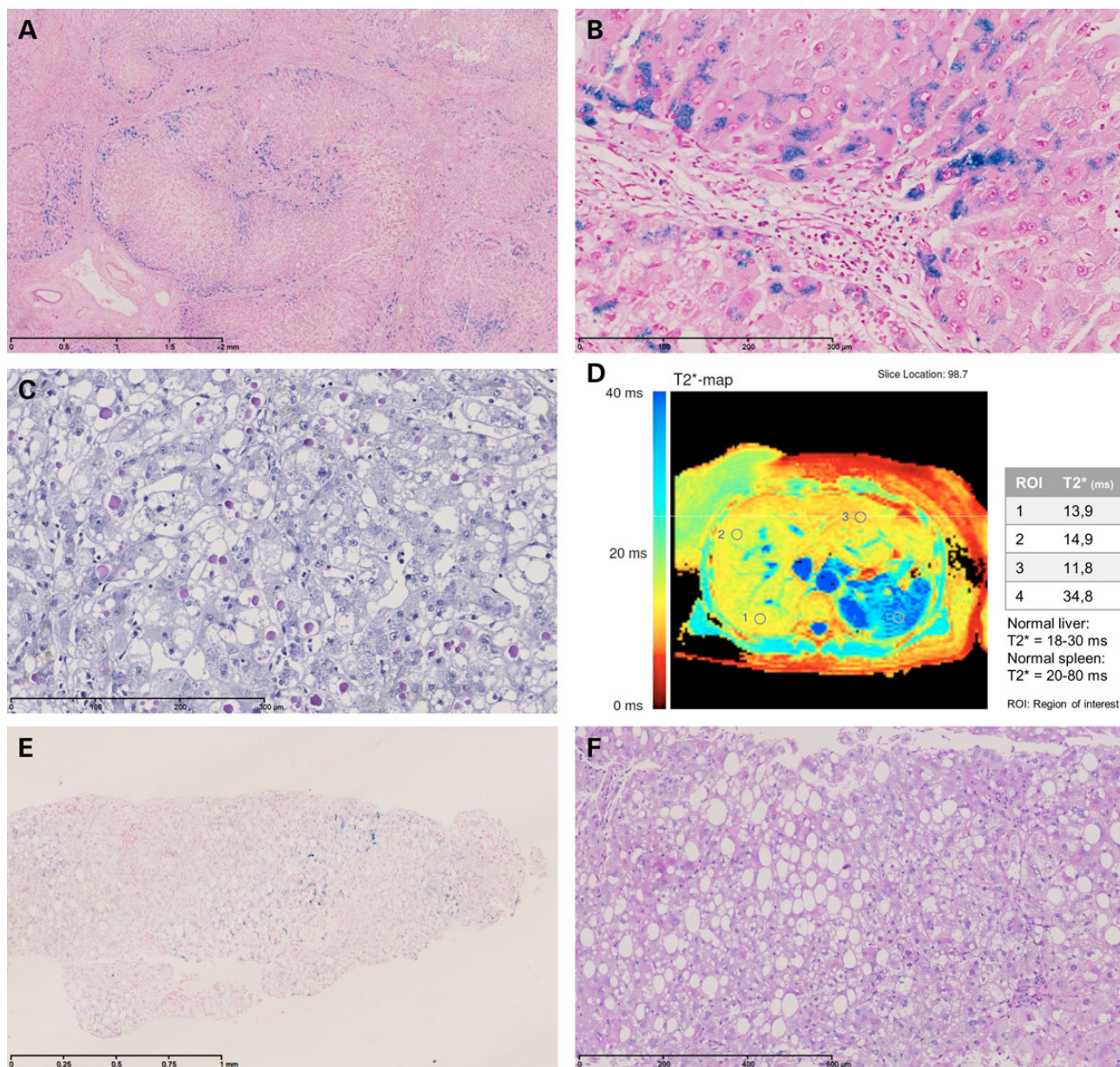
To explore a potential role of A1AT in controlling hepcidin expression by inhibition of MT-2, we have investigated the effect of A1AT on hepcidin expression and HJV cleavage. Our findings show that A1AT induces the expression of hepcidin in hepatocytes probably via inhibition of MT-2. This pathway could contribute to the association of A1ATD and iron overload. Studies in a family with A1ATD and hemochromatosis indicate a putative disease-causing effect of A1ATD in heterozygous carriers of mutations in the gene encoding hepcidin *HAMP* or individuals with environmental risk factors.

## Results

### Coincidence of A1ATD and non-HFE hemochromatosis

The female index patient first presented at the age of 60 years for further evaluation of a reduced A1AT plasma concentration of 28 mg/dl (reference range 90–200 mg/dl), hyperferritinemia of 1033 µg/l (reference range 30–200 µg/l) and high transferrin saturation of 98% (reference range 15–45%). These abnormalities were identified during investigation for an elevated gamma-glutamyl transferase activity and a family history of liver disease. At the time of first presentation, the patient had no evidence for lung disease. A1AT phenotyping by isoelectric focusing showed an A1AT Pi Z phenotype, and subsequent genotyping revealed that the patient was homozygous for the Z-allele in rs28929474 of the *SERPINA1* gene (NM\_000295.4: c.1096G>A), which confirmed the diagnosis of A1ATD. Genotyping for the p.Cys282Tyr and p.His63Asp polymorphism in the hemochromatosis gene *HFE* showed that the patient was homozygous for the normal alleles [rs1800562 (NM\_000410.3: c.845G) and rs1799945 (NM\_000410.3: c.187C)]. Histological evaluation of the liver biopsy showed mild portal hepatitis with fibrosis grade 4 (Metavir) and marked accumulation of stainable iron in hepatocytes. Based on these findings, the diagnoses A1ATD Pi ZZ and non-HFE hemochromatosis were made. After 12 months of fortnightly phlebotomies, serum ferritin concentration was 59 µg/l and remained low during 3-monthly maintenance phlebotomies.

Six years after initial presentation, the patient developed decompensated cirrhosis and underwent liver transplantation. Explant histology is shown in Figure 1, where microvesicular steatosis in a cirrhotic liver was found in addition to accumulation of

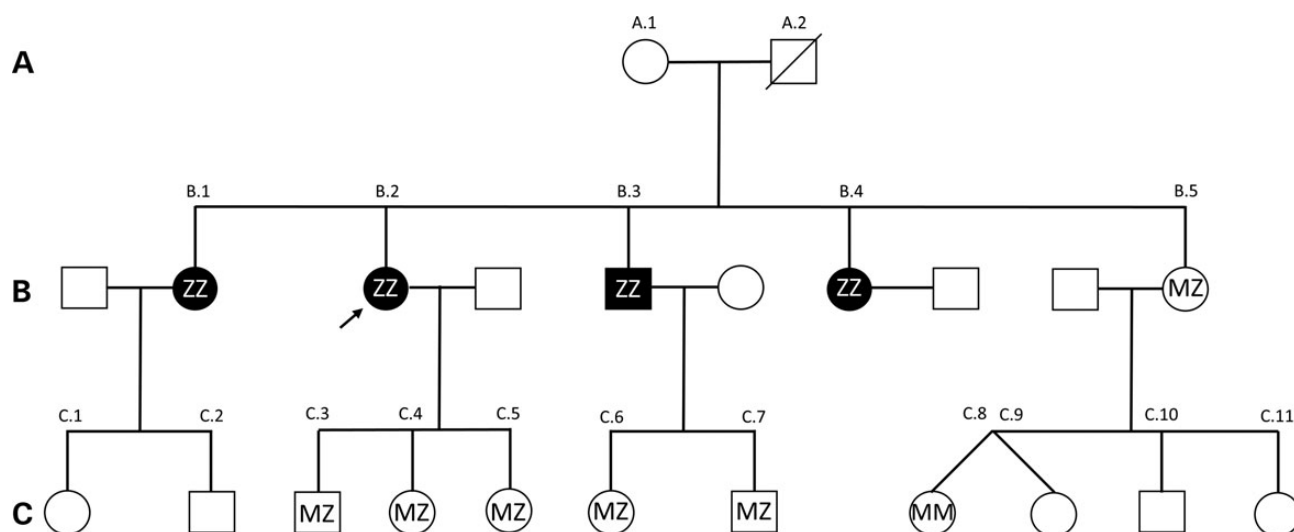


**Figure 1.** Hepatic phenotype of iron overload associated with A1AT: (A) Perls' Prussian blue-stained section of the explanted cirrhotic liver from the index patient shows periportal iron accumulation. (B) Predominantly, hepatocellular iron deposition is found in fine granules (200× magnification). (C) Diastase-resistant periodic acid-Schiff-positive intrahepatocellular inclusions corresponding to A1AT protein (Pi Z) aggregates. (D) T2\* relaxation time represented as T2\* maps in abdominal MRI of Patient B.4 showing markedly reduced T2\* relaxation times compatible with hepatic iron overload (35). Splenic T2\* relaxation times are within the normal range indicating the absence of splenic (macrophage) iron overload (36). (E) Perls' Prussian blue-stained section of a biopsy core taken from the index patient's brother (B.3) showing hepatic iron accumulation with different lobular distribution. (F) Hematoxylin–eosin-stained section of a biopsy core taken from the index patient's brother showing advanced fibrosis and diffusely distributed micro- and macrovesicular steatosis.

stainable iron in periportal hepatocytes despite previous phlebotomy. Six years after liver transplantation, the patient has an excellent graft function without evidence of re-accumulation of hepatic iron with a serum ferritin concentration of 82 µg/l and transferrin saturation within the normal range.

Results from clinical, biochemical and genetic evaluation of family members (Fig. 2) are shown in Table 1. Hepatic iron accumulation was assessed non-invasively by determining T2\* relaxation times in siblings with hyperferritinemia, which showed evidence of increased liver iron in individuals B.1, B.3 and B.4 (Table 1, Fig. 1D), where splenic T2\* relaxation times were within

the normal range. Non-invasive quantification of hepatic fat accumulation using T2\*- and T1-corrected chemical shift magnetic resonance imaging (MRI) sequences revealed hepatic steatosis in the range of 7.3–19.3%, where individuals with more severe steatosis had more severe iron overload (37,38). Further disease staging by transient elastography and liver biopsy showed increased liver stiffness (18 ± 3 kPa) and mild accumulation of hepatocellular iron in the index patient's brother (Individual B.3, Fig. 1E). Serum iron parameters and hepatic T2\* relaxation times were also compatible with hepatic iron overload in the index patient's sister (Patient B.4, Fig. 1D). As shown in Table 1,



**Figure 2.** Pedigree of the affected family: familial clustering without the characteristics of simple Mendelian disease inheritance. A1ATD and iron overload coincide exclusively in one generation.

**Table 1.** Demographic, genetic, radiological and biochemical parameters of the index patient's family

Case	Sex	Age	Iron ( $\mu\text{M}$ )	Transferrin (g/l)	Ferritin ( $\mu\text{g/l}$ )	Tf saturation (%)	A1AT (Pi)	A1AT (mg/dl)	Hepcidin (genotype)	BMPR1B (genotype)	Steatosis T2*/T1-corrected chemical shift	T2* relaxation (ms)
B.1	f	77	16,8	0.222	150	30	ZZ	26	WT	p.Arg31His	7.5–8.3%	15,4
B.2	f	73	39,5	0.161	1033	98	ZZ	28	p.Arg59*	p.Arg31His	n.a.	n.a.
B.3	m	66	44,8	0.222	1109	80	ZZ	18	WT	p.Arg31His	17.9–19.3%	10,4
B.4	f	65	24,2	0.242	444	40	ZZ	23	p.Arg59*	p.Arg31His	11–12.6%	13,5
B.5	f	60	18,8	0.277	53	27	MZ	87	p.Arg59*	WT	n.a.	n.a.
C.3	m	40	18,6	0.203	318	36	MZ	83	p.Arg59*	WT	n.a.	n.a.
C.4	f	52	16,6	0.315	20	21	MZ	104	p.Arg59*	p.Arg31His	n.a.	n.a.
C.5	f	50	12,8	0.281	82	18	MZ	91	WT	WT	n.a.	n.a.
C.6	f	40	17,2	0.239	105	29	MZ	90	WT	p.Arg31His	n.a.	n.a.
C.7	m	38	35,7	0.253	158	56	MZ	90	WT	p.Arg31His	n.a.	n.a.
C.8	f	36	10,7	0.249	99	17	MM	248	p.Arg59*	WT	n.a.	n.a.

Reference ranges: iron: 11–28  $\mu\text{M}$ ; ferritin: 30–300  $\mu\text{g/l}$  males, 30–200  $\mu\text{g/l}$  females; transferrin: 0.2–0.36 g/l; transferrin (Tf) saturation: 16–45%; T2\*/T1-corrected chemical shift <5%; A1AT 90–200 mg/dl; liver T2\*: 18–30 ms.

The italicized values are single measurements without a significance value.

serum iron parameters were normal in all descendants in Generation C.

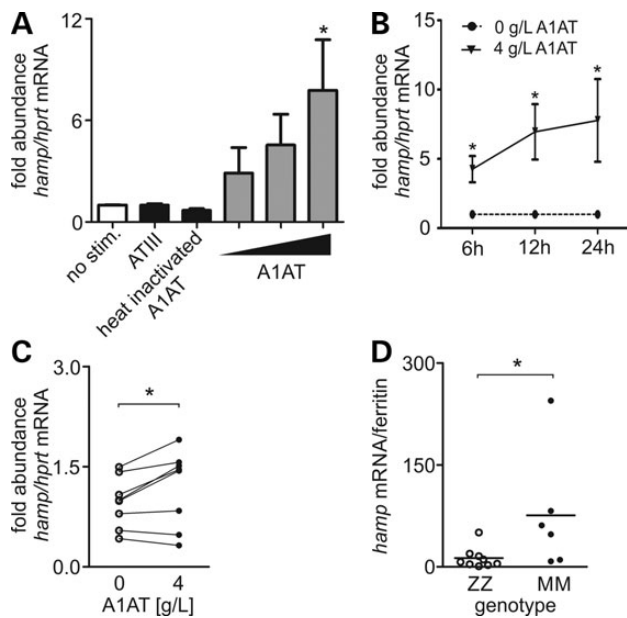
### Complex genetic cause of iron overload in A1ATD

To determine if the iron overload trait was due to a genetic defect, full exome sequencing was carried out in the index patient and two additionally affected family members (B.3 and B.4). The results of full exome sequencing are shown in the Supplementary Material. When filtered for the presence of mutations in genes known to be associated with hemochromatosis, heterozygosity for the disease-associated non-sense mutation p.Arg59\* (ENST00000222304: c.175C>T) in the HAMP gene was identified in the index patient and one sib (B.4). As shown in Table 1, both patients homozygous for A1AT Pi ZZ and heterozygous for p.Arg59\* exhibited an iron overload phenotype. In addition, Patient B.3 presented with hepatic iron overload despite normal HAMP gene sequencing. Of all variants identified by full exome

sequencing, 167 rare non-synonymous single-nucleotide variants were present in all three affected family members. A manual search for mutations in genes potentially involved in iron regulation revealed a heterozygous mutation in BMPR1B p.Arg31His (rs200035802, NM\_001203.2 c.92G>A). Sanger sequencing confirmed that this variant segregated with hepatic iron overload.

### A1AT induces hepatocellular hepcidin expression in vitro

Family studies showed that the iron overload phenotype was present in three patients with homozygous A1ATD. To explore the effect of A1AT on hepcidin expression, isolated murine hepatocytes were used for *in vitro* studies. As shown in Figure 3A and B, addition of purified human A1AT to the serum-free incubation medium resulted in a time- and dose-dependent induction of hepcidin mRNA expression. The observation that neither heat-inactivated A1AT nor the plasma serine Pi antithrombin III induces hepcidin mRNA expression shows specificity of native



**Figure 3.** A1AT induces hepcidin mRNA expression: (A) hepcidin mRNA expression in isolated murine hepatocytes incubated in serum-free medium, with 4 g/l heat-inactivated A1AT, 4 g/l antithrombin III as controls and increasing concentrations of active A1AT (1, 2 and 4 g/l). (B) Hepcidin mRNA expression in isolated murine hepatocytes after incubation with or without 4 g/l A1AT for various durations as indicated in the abscissa. (C) Hepcidin mRNA expression in isolated murine hepatocytes incubated with 10% serum from patients ( $n=8$ ) with A1ATD (Pi ZZ) or with 10% serum of the same individuals supplemented with 4 g/l A1AT. (D) Hepcidin mRNA expression in isolated murine hepatocytes incubated with serum from A1ATD patients (Pi ZZ) or from control patients (Pi MM). To correct for the effect of different degrees of iron loading in A1AT-deficient patients on hepcidin expression, hepcidin mRNA/serum ferritin ratios are shown. \* $P < 0.05$ .

A1AT. Additional control experiments were carried out by siRNA silencing of endogenous hepatocellular SERPINA1 transcription and with anti-A1AT antibody inactivation in sera, which further support a specific effect of A1AT on hepcidin transcription (see Supplementary Material, Fig. S1). To further determine if the effect was specific to the anti-protease activity of A1AT, hepcidin mRNA concentration was measured in hepatocytes incubated with medium containing 10% serum from patients with A1ATD or in hepatocytes incubated in medium containing the same sera supplemented with 4 g/l A1AT. As shown in Figure 3C, supplementation of patient sera with A1AT results in a significant induction of hepcidin mRNA in hepatocytes. In addition, primary hepatocytes were also incubated with medium containing 10% human serum from patients with A1ATD or with 10% serum from control patients without A1ATD. In order to minimize the effect of different degrees of iron loading on hepcidin expression, matched control subjects were selected from a group of liver disease patients with comparable serum ferritin concentrations and normal A1AT. Hepcidin mRNA concentration was then expressed as ratio over serum ferritin. As shown in Figure 3D, mean hepcidin/ferritin ratio was significantly lower in patients with A1ATD than in control patients with iron overload unrelated to A1ATD. Taken together our findings show that extracellular A1AT induces hepcidin mRNA transcription in primary murine hepatocytes.

#### A1AT inhibits MT-2-mediated cleavage of HJV

In order to investigate if the inhibition of the serine protease MT-2 is required for A1AT-mediated induction of hepcidin expression,

the effect of A1AT on the cleavage of HJV was measured. For this aim, FLAG-tagged HJV and MT-2 were overexpressed in HEK293T cells. Figure 4A shows that the anti-FLAG immunoreactive band in the supernatant, which corresponds to soluble HJV, is reduced by incubation of HJV and MT-2 overexpressing cells with 0.4 or 4 g/l purified human A1AT. The effect of A1AT on MT-2 protease activity was further studied with an artificial substrate by incubating membrane fractions from hepatocyte cell lysates with or without A1AT. As shown in Figure 4B, A1AT significantly reduces the proteolytic activity of MT-2 in a dose-dependent manner. In conclusion, the serine Pi A1AT inhibits MT-2-mediated cleavage of HJV at the cell surface as illustrated in Figure 5.

#### Decreased serum hepcidin in patients with A1ATD and iron overload

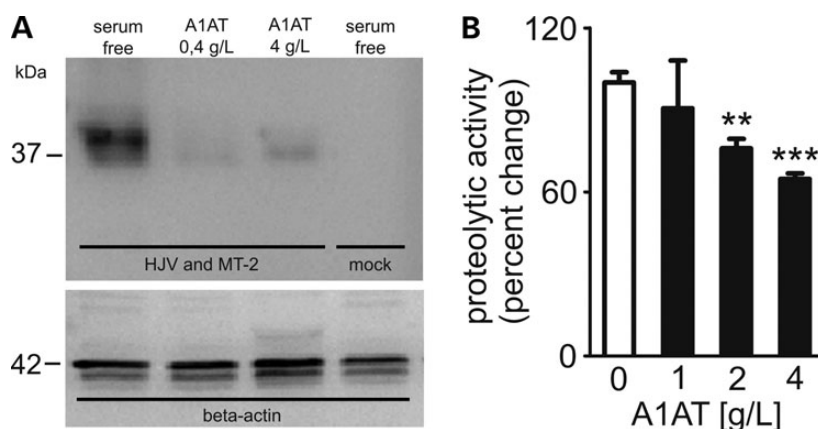
To further study the effect of A1ATD in combination with identified environmental and genetic risk factors on hepcidin expression, serum hepcidin concentrations were determined in archival serum samples from patients, unaffected family members and controls by enzyme-linked immunosorbent assay (ELISA). As shown in Figure 6, mean serum hepcidin concentration was significantly lower in affected family members with A1ATD Pi ZZ and iron overload when compared with Pi MM control subjects. Figure 6 further shows that mean serum hepcidin concentration is also lower in unrelated Pi ZZ patients without iron overload and in second-generation Pi MZ family members without iron overload, although these differences did not reach statistical significance when compared with Pi MM controls.

#### Discussion

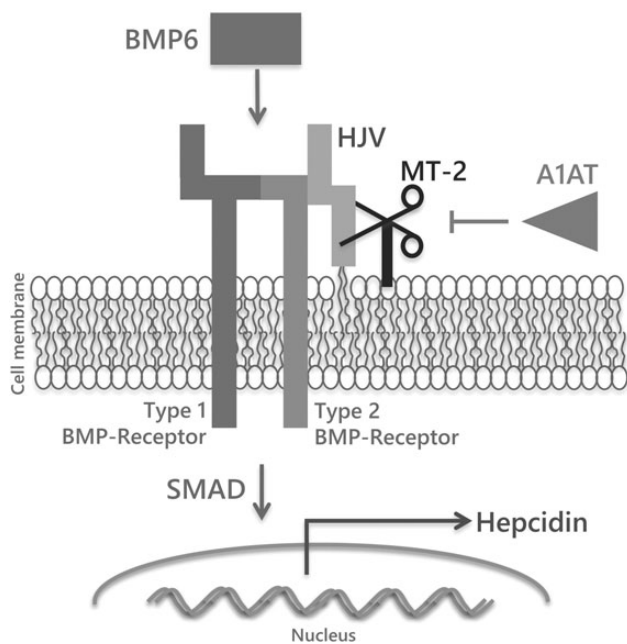
Here, we present a pedigree where several family members had severe hepatocellular iron overload and liver disease related to A1ATD. This disease presentation was associated with progressive liver fibrosis in affected family members and end-stage liver disease requiring liver transplantation in the index patient. Familial clustering suggests a genetic link between A1ATD and iron overload. Findings reported in this study support the concept that coinheritance of a heterozygous HAMP mutation with homozygosity for the Pi ZZ allele could cause the observed phenotype. Studies in unaffected family members further show that a heterozygous HAMP mutation is insufficient to cause iron overload in individuals with heterozygous A1ATD Pi MZ or Pi MM. Homozygous A1ATD can thus be considered a novel genetic risk factor for the development of iron overload in genetically predisposed individuals.

In line with this notion, previous studies have shown that A1ATD is a risk factor for significantly higher serum ferritin concentrations and liver siderosis (23,39). Nevertheless, full-blown hemochromatosis is rarely associated with A1ATD. This could be due to the fact that accumulation of Pi Z protein induces ER stress, which induces upregulation of hepatic hepcidin expression (40).

Independently of the genetic risk factor of a heterozygous HAMP mutation, alcohol could be an environmental risk factor that causes hepatic iron overload in patients with A1ATD Pi ZZ (41). This notion is supported by distinct histological findings in the index patient's brother (B.3) who also showed advanced fibrosis and mild hepatic iron overload, but diffusely distributed, micro- and macrovesicular steatosis and a different histological distribution of stainable iron in the liver. In search of a potential genetic cause for the hepatic iron overload in this patient, a manual screen for mutations in genes and pathways known to be associated with iron regulation was carried out (42). This revealed



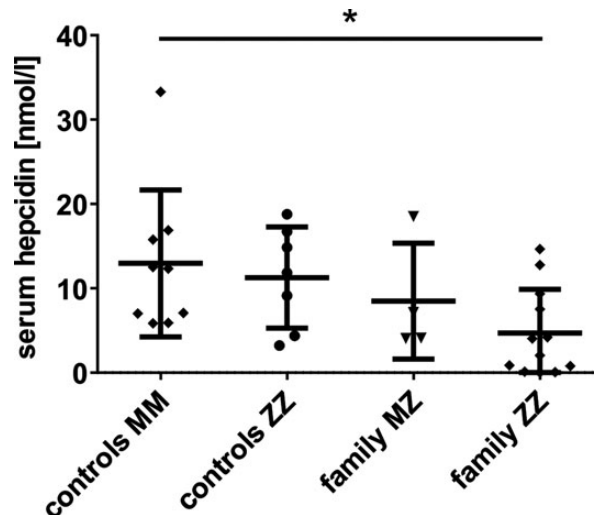
**Figure 4.** A1AT inhibits HJV cleavage and MT-2: (A) immunoblot of supernatant from HEK293T cells overexpressing FLAG-tagged HJV and MT-2. Incubation of transiently transfected cells in the presence of 0.4 or 4 g/l purified human A1AT causes a marked decrease in the FLAG immunoreactive band at ~37 kDa in the supernatant when compared with the supernatant from cells incubated in serum-free medium alone. Beta-actin immunoreactivity is shown in the corresponding cell lysates in the lower panel as a loading control. (B) Proteolytic activity of cell extracts from isolated murine hepatocytes for an artificial substrate mimicking the MT-2 specific cleavage site is inhibited by A1AT in a dose-dependent manner. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .



**Figure 5.** Decreased serum hepcidin in patients with A1ATD and iron overload: serum hepcidin concentrations were determined in archival serum samples from affected (A1ATD Pi ZZ) and unaffected (Pi MM) family members and unrelated controls with Pi MM and Pi ZZ by ELISA.

that all individuals with A1ATD and iron overload were also heterozygous carriers of the BMPR1B mutation p.Arg31His. The deleterious effect of this rare mutation (minor allele frequency 0.0006) (43) on the function of BMPR1B has been shown in a previous study, which predicted a discrete structural change in BMPR1B and showed a moderate loss of function on growth differentiation factor-induced BMP signaling despite correct subcellular localization (44). One could, therefore, speculate that the heterozygous BMPR1B mutation p.Arg31His further contributes to reduced BMP signaling and hepcidin expression in this family.

Hepcidin is considered a key factor in the disease pathogenesis of hepatic iron overload and is under control of a range of



**Figure 6.** Proposed molecular mechanism of A1AT's effect on hepcidin expression via MT-2 inhibition: HJV is a membrane-bound co-receptor for the heterodimeric BMP Type I (e.g. BMPR1B) receptor and BMP Type II receptor complex and is essential for BMP-6-induced activation of SMAD proteins that increase hepcidin expression. The serine protease MT-2 cleaves HJV and subsequently inhibits hepcidin expression. A1AT-mediated inhibition of MT-2 thus stimulates hepcidin expression via reduced HJV cleavage. \* $P < 0.05$ .

regulatory factors in the circulation, which include erythropoietin, growth differentiation factor-15, interleukin-6, platelet-derived growth factor and BMP-6 (24,28,45–48). To determine the functional interaction between A1AT and hepcidin, *in vitro* studies on hepcidin regulation were carried out. Our investigations show that A1AT induces hepcidin expression in primary murine hepatocytes. This effect was observed at concentrations that are present in the circulation of patients with inflammatory conditions (49).

The serine protease inhibitors hepatocyte growth factor activator inhibitors (HAI-1 and HAI-2) have also been recently identified as inhibitors of MT-2 (50). However, the substrate specificity of HAI-1 and HAI-2 in relation to A1AT has not yet been determined. Given the relatively high concentrations of A1AT reaching

up to 4 g/l during acute inflammation, A1AT could well act as an important inhibitor of MT-2 even if substrate specificity of A1ATD was lower than that of HAI-1 or HAI-2 (49). This is also supported by our finding that the proteolytic activity of membrane fractions from murine hepatocytes on an artificial MT-2 substrate can be inhibited by A1AT.

MT-2 is thought to exert its effect on hepcidin regulation through HJV cleavage and elimination of BMP signaling in hepatocytes. In accordance with the findings from studies in primary hepatocytes and biochemical studies on MT-2 activity, *in vitro* studies show that incubation of HJV overexpressing cells in the presence of A1AT can inhibit HJV shedding. This finding supports the hypothesis that induction of hepcidin expression by A1AT is mediated via MT-2 inhibition. Although furin can cleave HJV, this protease is an improbable target for A1AT for two reasons (51). First, extracellular A1AT is unlikely to inhibit intracellular furin-mediated HJV processing. Second, overexpression of MT-2 has been shown to inhibit furin-mediated HJV processing in a previous study (52).

In conclusion, A1AT inhibits hepcidin expression probably through inhibition of MT-2-mediated HJV cleavage *in vitro*. *In vivo*, this mechanism could contribute to the induction of hepcidin expression during acute-phase responses. Deficiency of the serine proteinase inhibitor A1AT due to genetic defects can be associated with hepatic iron overload and progressive liver disease. The present study shows that A1ATD could trigger hemochromatosis in individuals with different environmental and genetic risk factors. In this family, hepatic steatosis, heterozygous HAMP and BMPR1B mutations could have predisposed to hepatic siderosis. These findings elucidate the molecular pathogenesis of a complex genetic disease and the transition of genomic medicine from simple monogenic disorders to polygenic maladies.

## Material and Methods

The study was approved by the local ethics research committee at Medical University of Innsbruck (Study No. UN4218), and written informed consent was obtained from all participants.

### Exome sequencing

Exome enrichment for the three samples was carried out using Illumina's TruSeq (San Diego, USA) enrichment kit, and sequencing was performed on the Illumina HiSeq2000 (San Diego, USA). The raw reads were aligned against human genome build hg19 using BWA (53), followed by the removal of polymerase chain reaction duplicates with Picard (<http://picard.sourceforge.net>) and local realignment around InDels as well as quality score recalibration using GATK (54). Variant calling was performed with SAMtools (55) and GATK, and variants were annotated using snpActs ([www.snpackts.ikmb.uni-kiel.de](http://www.snpackts.ikmb.uni-kiel.de)). The variants were then filtered using the exomes of 150 healthy in-house controls and reduced to rare (maximum minor allele frequency of 1% in the 1000 Genomes data) and non-synonymous variants. Further details are given in online Supplementary Material.

### Cell culture

Primary murine hepatocytes were isolated from male 6- to 8-week-old C57BL/6 mice. Further details are given in online Supplementary Material.

HEK293T cells (American Type Culture Collection No. 11268; LGC Standards GmbH, Wesel, Germany) were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and cultivated

in Dulbecco's modified Eagle's medium (Gibco, purchased from LifeTech, Vienna, Austria) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U/ml) and streptomycin (100 µg/ml) (PAA, Pasching, Austria).

### Treatment of isolated murine hepatocytes with patient's sera and serine protease inhibitor

Prior to stimulation experiments, the culture medium was changed to serum-free William's Medium E (Gibco) containing 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were stimulated with medium containing 10% sera of patients and with A1AT (Prolastin, Grifols, Frankfurt am Main, Germany) or antithrombin III (Kybernin P, CSL Behring, Marburg, Germany) purified from human plasma for up to 24 h. For heat inactivation, A1AT was incubated at 95°C for 5 min.

### RNA preparation, reverse transcription and real-time polymerase chain reaction

RNA was extracted from adherent cells using a guanidinium-isothiocyanate-phenol-chloroform-based protocol as previously reported (56). Protein extraction was performed as detailed elsewhere (57). Further details are given in online Supplementary Material.

### Statistical analysis

Results were expressed as mean ± SEM. Statistical tests included unpaired and paired two-tailed Student's t-test or Mann-Whitney U test for not normally distributed data and one-way ANOVA followed by Bonferroni-Holm multiple comparison test. P-values of 0.05 or less were considered to denote significance.

### Transfection of HEK293T cells and treatment with alpha-1-antitrypsin

Attractene Transfection Reagent (Qiagen, Hilden, Germany) was used for transient transfection of HEK293T cells following the manufacturer's protocol. The used p3xFLAG-CMV-9 vector containing cDNA for human HJV had three FLAG tags at the N-terminus of HJV and lacked the signal peptide (27). The pCR3.1 MT-2 expression vector was kindly provided by Dr Muckenthaler (University of Heidelberg, Germany). Twenty-four hours after transfection, the medium was changed, and transfected cells were incubated in Dulbecco's modified Eagle's medium (LifeTech) with or without A1AT at the indicated concentration (Prolastin). After 24 h of incubation, conditioned medium was obtained for immunoblot analysis.

### Western blot analysis

After protein separation by SDS-PAGE, proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Amersham Hybond-P, GE Healthcare, Little Chalfont, UK). Membranes were probed with affinity-purified mouse anti-DYKDDDDK epitope tag monoclonal antibody (1 : 1000; Acris Antibodies GmbH, Herford, Germany). Immunodetection was carried out using a corresponding horseradish peroxidase (HRP)-conjugated secondary antibody at a 1 : 1000 dilution (polyclonal rabbit anti-mouse immunoglobulins/HRP; Dako, Glostrup, Denmark) and chemiluminescence substrate (Super Signal West Dura; Pierce Biotechnology, Rockford, IL, USA). Chemiluminescence was visualized using a CCD camera (Chemidoc XRS, BioRad, Vienna, Austria).

### Determination of MT-2 activity

Proteolytic activity was determined in membrane fractions from isolated murine hepatocytes corresponding to a total amount of 30 µg of protein. The activity assay was carried out with the artificial substrate *N*-Boc-Gln-Ala-Arg-*p*-nitroanilide (Bachem, Bubendorf, Switzerland) at a substrate concentration of 400 µM as previously described (58). Membrane fractions were isolated with a Complete Cell Fractionation Kit (PromoKine, Heidelberg, Germany). The activity of MT-2 was assayed at 37°C by monitoring the release of *para*-nitroaniline from the chromogenic substrate *N*-Boc-Gln-Ala-Arg-*p*-nitroanilide at 405 nm using an Infinite M200 multimode microplate reader (Tecan, Männedorf, Switzerland) for up to 30 min.

### Quantification of serum hepcidin by ELISA

Serum hepcidin was quantified with a human hepcidin-25 peptide enzyme immunoassay (Peninsula Laboratories Int., Inc., San Carlos, CA, USA) following the manufacturer's instructions.

### Supplementary Material

Supplementary Material is available at HMG online.

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