Circulating polymers are associated with COPD in α_1-antitrypsin deficiency

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Abstract

Question of the study. The severe Z deficiency allele of α_1-antitrypsin (Glu342Lys) results in the formation of polymers that are retained within hepatocytes. It is unknown whether they are present in the plasma of individuals with α_1-antitrypsin deficiency and if they are associated with any clinical phenotype.

Methods. We have used the anti-α_1-antitrypsin polymer monoclonal antibody (2C1) to assess the presence of polymers in the plasma of: (i) 293 individuals with a mix of α_1-antitrypsin genotypes, (ii) an individual with α_1-antitrypsin deficiency who underwent liver transplantation and (iii) a cohort of 244 individuals with PiZZ α_1-antitrypsin deficiency.

Results. The presence of circulating polymers was 100% sensitive and 89% specific in identifying 20 PiZZ α_1-antitrypsin homozygotes in a mix of 293 α_1-antitrypsin genotypes. They clear following liver transplantation with a half-life estimated to be 30 hours. Circulating polymers were present in every one of 244 individuals with PiZZ α_1-antitrypsin deficiency with higher levels in men, individuals with airflow obstruction (-0.411 SE 0.116; $p<0.0005$) and those with COPD (adjusted OR 3.6, 95% confidence interval 1.4-9.1).

Answer to the question. Circulating polymers of α_1-antitrypsin are present in all individuals with PiZZ α_1-antitrypsin deficiency and are associated with COPD.

195 words

Keyword: biomarker, COPD, liver, serpin

Introduction

Most individuals carry two wild-type M alleles of the *SERPINA1* gene that encodes α_1-antitrypsin [1, 2]. Ninety-five percent of severe deficiency of α_1-antitrypsin results from the Z allele (Glu342Lys; denoted PiZZ in the homozygote) that causes newly synthesised protein to polymerise and be retained within hepatocytes [3, 4]. These polymers form the Periodic Acid Schiff (PAS) positive, diastase-resistant inclusions that are present in all individuals with a Z allele and are associated with neonatal hepatitis, cirrhosis and hepatocellular carcinoma. The concomitant lack of circulating α_1-antitrypsin predisposes the Z α_1-antitrypsin homozygote to early onset emphysema. Polymers of α_1-antitrypsin form within the lung as a result of local inflammation and exposure to cigarette smoke [5-8]. They have also been identified in the skin of an individual with α_1-antitrypsin deficiency and panniculitis [9] and in a renal biopsy of an individual with α_1-antitrypsin deficiency and vasculitis [10]. We have assessed whether polymers of α_1-antitrypsin are present within serum and whether they are associated with clinical features in individuals with PiZZ α_1-antitrypsin deficiency.

Materials and Methods

Subjects and cohorts

Identification of circulating polymers in individuals with a mix of α_1-antitrypsin genotypes (cohort 1)

This cohort was used to establish whether the presence of circulating polymers could identify individuals with α_1-antitrypsin deficiency. 293 individuals with a mix of *SERPINA1* genotypes were identified from the Alpha-1 Foundation DNA and Tissue Bank. These included the following α_1-antitrypsin genotypes (number): MM (200), MZ (20), ZZ (20), SZ (20), FZ (5), SS (3), MS (20), ZMheerlen (3) and ZMmalton (2). No individual was receiving α_1-antitrypsin augmentation therapy.

Clearance of polymers following liver transplantation

A 54 year old man who was not part of either of the two cohorts presented with a 12 month history of peripheral oedema and was found to have a cirrhotic liver, portal hypertension and gastro-oesophageal varices. Investigation showed him to be a PiZZ α_1-antitrypsin homozygote. He had normal lung function. His condition deteriorated, he developed episodes of encephalopathy and was admitted for orthotopic liver transplantation. The procedure was prolonged by a large portal vein thrombus in the recipient and he developed reperfusion injury with hyperkalaemia that necessitated 3 hours of intra-operative hemofiltration. His post-operative care was subsequently uneventful and he was discharged home.

The correlation of circulating polymers with clinical phenotypes (cohort 2)

The Alpha-1 Antitrypsin Genetic Modifier Study is a multicentre study performed in collaboration with the Alpha-1 Foundation. The design of this study has been described previously [11]. This cohort was used to determine whether circulating polymers are associated with any demographic or clinical phenotype in individuals with α_1-antitrypsin deficiency.

ELISA assay to detect polymers of α_1-antitrypsin

The ELISA assay used the 2C1 monoclonal antibody that detects only the polymeric conformer of α_1-antitrypsin. It does not detect monomeric M or Z α_1-antitrypsin [12-14]. Briefly the plates were coated overnight with antigen purified rabbit polyclonal α_1-antitrypsin antibody. Standard (plasma purified Z α_1-antitrypsin heated to form polymers [15]) and subject samples were diluted in blocking buffer and incubated overnight at 4°C. The wells were washed and incubated with the 2C1 monoclonal antibody diluted in blocking buffer. Bound 2C1 antibody was detected with rabbit anti-mouse IgG horseradish peroxidase antibody. Horseradish peroxidase activity was subsequently measured in a plate reader at 450 nm. The assays were performed by investigators who were blind to the genotypes and clinical features of the subjects. The inter-assay coefficient of variation was 11.7%

and polymer concentrations are reported in micrograms/millilitre. Values were log transformed for regression analysis. The specificity of the antibody was confirmed by using it to immunoprecipitate polymers from the plasma of individuals with and without a positive signal on ELISA. Sepharose G beads were incubated with the 2C1 monoclonal antibody and then with 1 mL of a plasma sample containing α_1-antitrypsin polymer (PiZZ genotype) or a plasma sample containing no polymer (PiMM). The precipitated protein was eluted by DTT, and analysed with SDS-PAGE followed by Western blot using rabbit polyclonal antibody to α_1-antitrypsin.

Statistical analysis

The α_1-antitrypsin Genetic Modifiers Study cohort (cohort 2) was used to establish whether the levels of circulating polymers correlated with any demographic or clinical phenotype in individuals with PiZZ α_1-antitrypsin deficiency. In this analysis linear regression was performed to identify factors that predicted polymer levels. A multivariate analysis included predictors at the $p<0.1$ level in the univariate analysis. All computations were performed using the SAS statistical package (SAS Statistical Institute, North Carolina, USA).

Ethical approval

The study was approved by local Institutional Review Boards and all subjects provided informed consent.

Results

Identification of circulating polymers in individuals with a mix of α_1-antitrypsin genotypes (cohort 1)

The presence of circulating polymers was evaluated in cohort 1 for its ability to identify individuals with α_1-antitrypsin deficiency. It was 100% sensitive and 89% specific in detecting 20 PiZZ α_1-antitrypsin homozygotes in a mix of 293 genotypes (Fig. 1a). It was 70% sensitive and 99% specific

in identifying the Z allele (PiXZ where X is any allele other than Z). A low signal was detected in 1 of 200 individuals with a normal α_1-antitrypsin phenotype (PiMM) but in none of the 3 SS homozygotes who have mild α_1-antitrypsin deficiency. The antibody only recognised α_1-antitrypsin polymers in plasma, and not other proteins, as immunoprecipitation yielded a single band of α_1-antitrypsin from plasma containing polymer but no protein from plasma with no polymers (Fig. 1b).

Clearance of polymers following liver transplantation

Plasma levels of α_1-antitrypsin were low in the PiZZ α_1-antitrypsin homozygote described in the case report but rose from 0.2 mg/mL to 2.1 mg/mL following hepatic transplantation. Circulating α_1-antitrypsin polymers were detected in this individual prior to transplantation but fell rapidly following the procedure (Fig. 2A). This may in part relate to the requirement for intra-operative haemodialysis. The levels continued to fall in the post-operative phase. Fitting the post-operative decline to an exponential decay function allowed an estimate for the half-life of circulating polymers as 30 hours (Fig. 2B). The level of circulating polymers became undetectable after 4 days (lower limit of quantification of polymers 0.4 μg/mL) demonstrating that they arise from Z α_1-antitrypsin synthesised within the liver.

The correlation of circulating polymers with clinical phenotypes (cohort 2)

Cohort 2 was used to establish whether there was a correlation between circulating polymers of α_1-antitrypsin and various clinical phenotypes in individuals with α_1-antitrypsin deficiency. Polymer levels were measured in blood samples from 244 confirmed PiZZ α_1-antitrypsin homozygote subjects who had never received augmentation therapy. No subjects had undergone liver or lung transplantation. Demographic details and smoking history were recorded at the time of enrolment to the study (Table 1A). Pre-bronchodilator spirometric data were available on 239 subjects with 90 subjects being defined as having COPD based on a pre-bronchodilator $FEV_1/FVC<70\%$ and $FEV_1<80\%$ predicted (Table 1B). Post-bronchodilator spirometry was available on 230 of the overall

subjects and 87 of the subjects with COPD; of the COPD subjects the mean pre and post bronchodilator FEV_1 were 53.4 (SD 19.1) and 57 (20.4) % predicted respectively and the mean pre and post bronchodilator FEV_1/FVC were 0.52 (0.16) and 0.53 (0.17). The pre-bronchodilator spirometry values were used in order to maximise the number of subjects in the analysis. Circulating polymers were detected in all 244 individuals with PiZZ α_1-antitrypsin deficiency with concentrations ranging from 8.2 to 230.2 μg/mL. The mean concentration of α_1-antitrypsin polymers in the 244 subjects was 36.3 μg/mL (SD 33.3). Levels were higher in males (mean 42.8 μg/mL, range 8.2-230.2) compared to females (mean 32.2 μg/mL, range 8.2-183.0; p=0.02) and were higher in subjects with COPD (42.6 μg/mL versus 32.5 μg/mL, p=0.02). Univariate analysis revealed an association between polymer concentration and FEV_1/FVC (-0.411 SE 0.116, $p<0.0005$; Table 2) but no association between ever-smoking history, pack-years of smoking or age started smoking. A multivariate model with and without adjustment for FEV_1 was evaluated including sex and age. Sex remained a robust predictor of polymer level after adjustment for age, ever-smoking and FEV_1 ($p\leq0.01$ for sex for all models). The total R^2 of the full model with age, sex and FEV_1 was 0.19 suggesting that other unmeasured covariates likely impact on the circulating level of α_1-antitrypsin polymers. There was no significant age by sex interaction ($p>0.10$, data not shown). In an unadjusted logistic model predicting COPD, each unit increase in log transformed polymer level was associated with higher odds for COPD (OR 3.8, 95% confidence interval 1.6-9.3); once adjusted for smoking there still remained an association between each unit increase in log transformed polymer level and COPD (OR 3.6, 95% confidence interval 1.4-9.1).

The Alpha-1 Antitrypsin Genetic Modifier Study Cohort was not ascertained to assess liver disease and therefore analysis of the liver specific questions must be considered to be exploratory. Polymer levels were evaluated in data parsed by responses to self-reported histories of abnormal liver function tests, physician-confirmed cirrhosis, physician-confirmed liver disease, hepatitis, neonatal jaundice and any jaundice. Approximately 15.6% of subjects answered ‘yes’ to a question about ever having abnormal liver function tests, 9% reported neonatal jaundice and 3% had physician-confirmed liver disease or cirrhosis. In all cases, a self-report of ‘yes’ to any of these questions was associated with

higher polymer levels in the 244 PiZZ α_1-antitrypsin subjects (Table 3). 6.4% of men self-reported a physician-confirmed history of liver disease compared with 1.3% of women; 5.3% of men and 2.7% of women reported physician-confirmed cirrhosis. Males had higher circulating levels of α_1-antitrypsin polymers than females in all instances of "no" answers to liver disease questions. Interestingly, although the numbers are small and the results not statistically significant, the mean polymer level for individuals with physician-confirmed cirrhosis and liver disease and reports of abnormal liver tests were higher in women than men. This was the only instance in which women had higher polymer levels than men (Table 3).

Discussion

Our previous work has shown that mutant Z α_1-antitrypsin forms polymers that accumulate as Periodic Acid Schiff (PAS) positive inclusions within hepatocytes [3]. These polymers are also present within the skin [9], kidney [10] and lung [5-8, 16]. We report here the use of a novel anti-α_1-antitrypsin polymer monoclonal antibody (2C1) to evaluate the presence of polymers in the plasma of individuals with α_1-antitrypsin deficiency. Circulating polymers were present in all individuals with PiZZ α_1-antitrypsin deficiency. The presence of polymers was 100% sensitive in identifying PiZZ α_1-antitrypsin homozygotes in a mix of other genotypes. Polymers were also detected in individuals who were heterozygous for Z and another allele (M, S, Mmalton and Mheerlen). One PiMM α_1-antitrypsin individual had a low signal for circulating polymers but they were not detected in the other 199 individuals with a normal α_1-antitrypsin phenotype. We have shown that severe deficiency alleles such as: Mmalton (52Phe del) [17], Siiyama (Ser53Phe) [18] and King's (His334Asp) [12] α_1-antitrypsin also form polymers. The mild deficiency alleles S (Glu264Val) [19] and I (Arg39Cys) [20] α_1-antitrypsin similarly form polymers but at a slower rate in keeping with less retention in the liver, a lack of inclusions and milder plasma deficiency. This is in keeping with the absence of

circulating polymers in the 3 PiSS α_1-antitrypsin individuals in this study. The epitope recognised by the 2C1 anti-polymer antibody is unknown but *in vitro* studies have shown it to be polymer specific irrespective of the underlying mutation in the α_1-antitrypsin gene [12]. It will therefore detect circulating polymers generated from other polymer forming alleles. This may represent a useful biomarker for α_1-antitrypsin deficiency. However the specificity is reduced as one individual with a normal PiMM α_1-antitrypsin phenotype also had low circulating level of polymers. Moreover individuals with rare Null alleles will not have circulating polymers as there is no secreted gene product.

Polymers of α_1-antitrypsin are present within the lungs of individuals with α_1-antitrypsin deficiency [5-7]. They are pro-inflammatory for human neutrophils *in vitro* [6, 16] and following their instillation into the lungs of mice [7]. Moreover cigarette smoke induces both intrapulmonary polymer formation and neutrophil influx in transgenic mice that express Z, but not M α_1-antitrypsin [8]. It was therefore important to assess whether the circulating levels of polymers correlated with any demographic factor or clinical outcome in α_1-antitrypsin deficiency. Our data show that there was a relationship between circulating levels of polymer and male gender. There were also associations between FEV_1/FVC (which correlates closely with emphysema [21]) and with the presence of COPD. Thus circulating polymers may be causal in the pathogenic pathway of lung function decline and emphysema in individuals with α_1-antitrypsin deficiency. The association between sex and levels of polymers was seen in all analyses with the possible exception of individuals with liver disease. The cause of this is unclear but previous studies have shown that treatment with the synthetic androgen, Danazol, raised levels of α_1-antitrypsin in individuals with α_1-antitrypsin deficiency [22]. Thus sex hormones may also be important in driving the production of circulating polymers.

Alpha-1-antitrypsin is largely secreted from the liver. It is also synthesized in the bronchial epithelial cells where it contributes to the local production of polymers [6]. α_1-antitrypsin is also synthesized in the gut [23], in circulating monocytes [24] and tissue macrophages [25]. In all cases α_1-antitrypsin from PiZZ homozygotes has the propensity to form polymers and so may contribute to

the circulating levels detected in these individuals. However the observation that circulating polymers become undetectable following hepatic, but not lung transplantation (data not shown), demonstrates that they arise from α_1-antitrypsin that is synthesised in the liver. The estimate of the half-life of polymer clearance of 30 hours should be interpreted with caution as the individual undergoing transplantation required intraoperative haemodialysis and will have received blood products containing α_1-antitrypsin. The association of circulating polymers with liver disease in cohort 2 can only be considered as exploratory and was not significant. Nevertheless our findings provide support for further studies to evaluate this biomarker in longitudinal studies of liver disease in individuals with PiZZ α_1-antitrypsin deficiency.

Taken together, our data show that circulating polymers are present in all individuals with PiZZ α_1-antitrypsin deficiency and that they are associated with lung, and possibly liver disease. Further studies are now required to establish the temporal stability of circulating polymers and whether this biomarker has utility in predicting clinically important outcomes in individuals with PiZZ α_1-antitrypsin deficiency. However, this polymer-based biomarker will be useful to monitor the efficacy of small molecules designed to block polymerisation should the current lead molecules progress to clinical trials [26].

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A.

Characteristic	All (N=244)	Male (N=94)	Female (N=150)
Age	50.5 (9.7)	50.2 (9.1)	50.6 (10.1)
FEV_1 % predicted*	83.9 (28.2)	72.8 (30.6)	90.9 (24.2)
FEV_1/FVC*	0.67 (0.17)	0.61 (0.18)	0.70 (0.14)
COPD (%)	90 (36.9)	50 (53.2)	40 (26.7)
Current smokers (%)	8 (3.3)	3 (3.2)	5 (3.3)
Ever-smokers (%)	106 (43.4)	43 (45.7)	63 (42)
Pack-years*	13.4 (13.3)	15.6 (14.3)	11.8 (12.4)
Polymer levels, μg/mL	36.3 (33.3)	42.8 (37.6)	32.2 (29.8)

B.

Characteristic	All (N=90)	Male (N=50)	Female (N=40)
Male (%)	50 (55.6)	50	40
Age	53.1(10.4)	52.2(9.1)	54.1(11.9)
FEV_1 % predicted*	53.4 (19.1)	49.4(20.5)	58.5(16.2)
FEV_1/FVC*	0.52(0.15)	0.50(0.16)	0.55(0.15)
Current smokers (%)	4(4.4)	1(2.0)	3(7.5)
Ever-smokers (%)	51(56.7)	29(58.0)	22(55.0)
Pack-years	16.9(14.2)	18.6(15.5)	14.8(12.4)
Polymer levels (μg/mL)	42.6(37.2)	45.9(37.5)	38.6(36.8)

Table 1. A (above) Characteristics of 244 PiZZ α_1-antitrypsin subjects and B (below) the 90 individuals with COPD. The values are number and percentage or mean and standard deviation. *Spirometric values are pre-bronchodilator; pack-years are reported for ever and current smokers.

Predictor	Regression coefficient (SE)	P value
Sex	0.121 (0.039)	0.0022
Age	0.012 (0.002)	<0.0001
Ever smoking	-0.005 (0.039)	0.9086
Pack-years	0.003 (0.002)	0.2064
FEV_1	-0.003 (0.001)	0.0003
FEV_1/FVC	-0.411 (0.116)	0.0005

Table 2. Univariate models predicting log transformed polymer concentration in the 244 PiZZ α_1-antitrypsin subjects. Females are the reference group for sex, never smoker is the reference group for ever smoking.

Characteristic	All (N=244)	Polymer level (µg/mL)- Yes	Polymer level (µg/mL)- No
Abnormal liver tests	38 (15.6%)	44.4 (35.6)	34.7 (32.8)
Confirmed cirrhosis	9 (3.7%)	61.1 (40.3)	35.3 (32.8)
Confirmed liver disease	8 (3.3%)	51.0 (37.4)	35.8 (33.2)
Hepatitis	12 (4.9%)	62.4 (67.9)	34.9 (30.2)
Neonatal jaundice	22 (9.0%)	43.1 (35.5)	35.6 (33.1)
Any jaundice	23 (9.4%)	54.9(51.0)	34.3 (30.5)

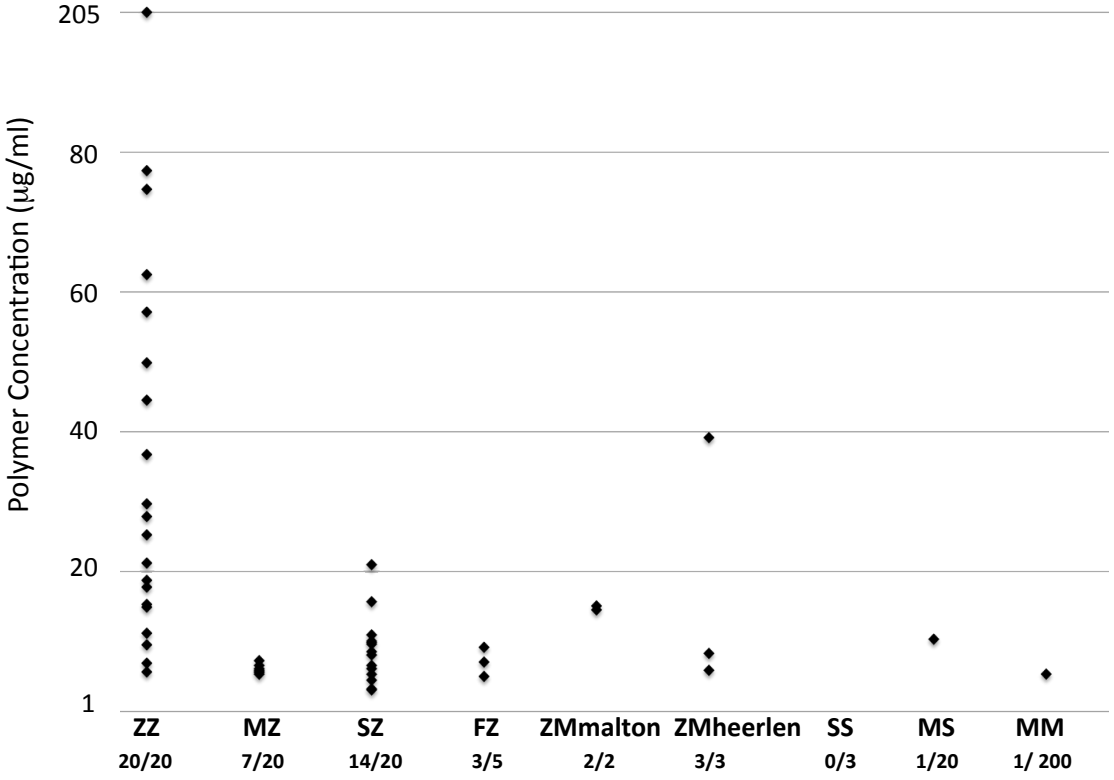
Males

Characteristic	All (N=94)	Polymer level (µg/mL)- Yes	Polymer level (µg/mL)- No
Abnormal liver tests	18 (19.1%)	42.8 (25.9)	42.8 (40.0)
Confirmed cirrhosis	5 (5.3%)	58.2 (43.0)	41.9 (37.4)
Confirmed liver disease	6 (6.4%)	36.9 (26.0)	43.2 (38.3)
Hepatitis	4 (4.3%)	78.8 (102.7)	41.2 (32.6)
Neonatal jaundice	9 (9.6%)	53.6 (38.7)	41.6 (37.5)
Any jaundice	9 (9.6%)	72.0 (67.3)	39.7 (32.1)

Females

Characteristic	All (N=150)	Polymer level (µg/mL)- Yes	Polymer level (µg/mL)- No
Abnormal liver tests	20 (13.3%)	45.9 (43.2)	30.1 (26.8)
Confirmed cirrhosis	4 (2.7%)	64.6 (42.8)	31.3 (29.0)
Confirmed liver disease	2 (1.3%)	93.1 (40.5)	31.3(28.9)
Hepatitis	8 (5.3%)	54.2 (49.9)	30.9 (28.0)
Neonatal jaundice	13 (8.7%)	35.8 (32.7)	31.8 (29.6)
Any jaundice	14 (9.3%)	43.9 (35.8)	31.0 (29.0)

Table 3. Exploratory analysis of liver disease in 244 PiZZ α_1-antitrypsin subjects. The values are number and percentage or mean and standard deviation.



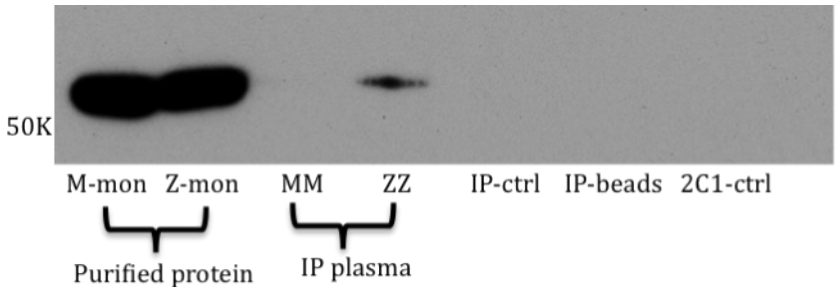


Figure 1. Detection of α_1-antitrypsin polymers in plasma. Fig. 1a. Concentration of serum α_1-antitrypsin polymers in 293 individuals with a mix of α_1-antitrypsin genotypes. The values below the genotypes are the number of subjects with detectable levels of circulating polymers (lower limit of

quantification of polymers 0.4 μg/mL). Fig 1b. The 2C1 monoclonal antibody immunoprecipitated only α_1-antitrypsin from the plasma of an individual who had a positive signal on ELISA. Lane 1, Purified M α_1-antitrypsin control; lane 2, purified Z α_1-antitrypsin control; lane 3, α_1-antitrypsin immunoprecipitated with the 2C1 monoclonal antibody from the plasma of a PiMM individual with no polymers on ELISA; lane 4, α_1-antitrypsin immunoprecipitated with the 2C1 monoclonal antibody from the plasma of a PiZZ individual with a positive signal for polymers on ELISA; lane 5, sepharose G beads incubated with 2C1 without adding plasma sample (IP-ctrl); lane 6, sepharose G beads alone (IP-beads); lane 7, 2C1 antibody alone (2C1-ctrl).

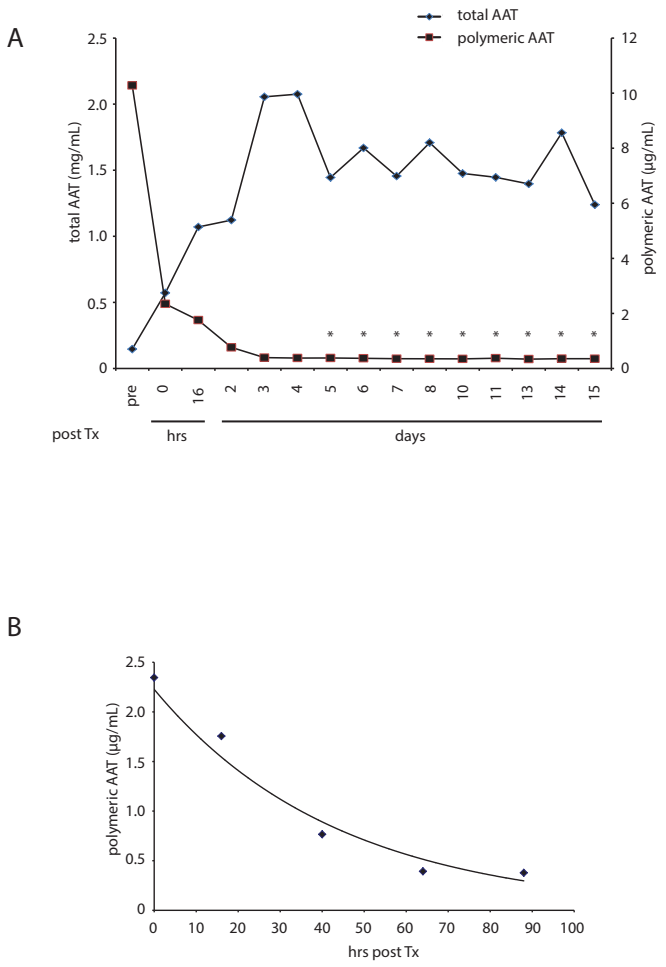


Figure 2. Z α_1-antitrypsin polymers are present in the circulation and are cleared following liver transplantation. (A) Serum α_1-antitrypsin was quantified using sandwich ELISA. Mouse monoclonal antibodies produced by our group that detect all conformers of α_1-antitrypsin, or only polymeric α_1-antitrypsin (2C1 [12, 13]), were used to quantify total α_1-antitrypsin and α_1-antitrypsin polymers respectively. Time points marked * are below the lower limit of quantification (0.4 µg/mL). (B) Estimation of Z α_1-antitrypsin polymer $t_{1/2}$ using $t_{1/2} = \ln 2/\lambda$. The pre-transplant sample was excluded in view of the requirement for intra-operative hemofiltration.