Hetero-polymerization of alpha-1-antitrypsin mutants in cell models mimicking heterozygosity - implications for the pathogenesis of liver disease.

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KEY WORDS:
Alpha1-antitrypsin deficiency; 2C1 mAb; Alpha-1-antitrypsin polymers, Liver cirrhosis, Proximity Ligation Assay.

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LIST OF ABBREVIATIONS:
ER, Endoplasmic Reticulum; AAT, Alpha-1-Antitrypsin; AATD, Alpha-1-Antitrypsin Deficiency; COPD, Chronic Obstructive Pulmonary Disease; IP, Immunoprecipitation; PLA, Proximity Ligation Assay.

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Abstract (274/275 words)

The most common genotype associated with severe alpha1-antitrypsin deficiency (AATD) is the Z homozygote. The Z variant (Glu342Lys) of alpha1-antitrypsin (AAT) undergoes a conformational change and is retained within the endoplasmic reticulum (ER) of hepatocytes leading to the formation of ordered polymeric chains and inclusion bodies. Accumulation of mutated protein predisposes to cirrhosis whilst the lack of plasma AAT leads to emphysema. Increased risk of liver and lung disease has also been reported in heterozygous subjects who carry the Z allele in association with the milder S AAT variant (Glu264Val) or even with wild type M AAT. However, it is unknown whether Z AAT can co-polymerize with other AAT variants *in vivo*.

We co-expressed two AAT variants, each modified by a different tag, in cell models that replicate AAT deficiency. We used pull-down assays to investigate the formation of AAT hetero-polymers and confocal microscopy to study the sub-cellular distribution of the co-expressed variants. Z AAT formed heteropolymers with Mmalton (Phe52del), S and Mwurzburg (Pro369Ser) AAT, and to a lesser extent with the wild-type protein. Heteropolymers were recognised by the 2C1 monoclonal antibody that binds to pathological polymers of Z AAT *in vivo*. There was increased intracellular accumulation of both S and M AAT variants when co-expressed with Z AAT, suggesting a dominant negative effect of the Z allele. The molecular interactions between S and Z AAT was confirmed by: (i) confocal microscopy showing their co-localization within dilated ER cisternae and (ii) positivity in Proximity Ligation Assays. *Conclusions*: Our results provide the first evidence of intracellular co-polymerization of AAT mutants and contribute to understanding the risk of liver disease in SZ and MZ AATD heterozygotes.

INTRODUCTION

Alpha1-antitrypsin deficiency (AATD) is a genetic disorder caused by mutations in the *SERPINA1* gene encoding for alpha1-antitrypsin (AAT), which lead to reduced levels of the protein in the circulation. AAT is the most important inhibitor of serine proteases and lack of this protein exposes tissues to uncontrolled proteolytic damage, particularly the lungs. As a result, individuals with AATD have an increased risk of early onset emphysema and chronic obstructive pulmonary disease (COPD) (1). AATD is also associated with liver disease as a result of the deposition of polymers of mutant AAT within the endoplasmic reticulum (ER) of hepatocytes, where AAT is chiefly produced (2).

The most frequent and polymerogenic *SERPINA1* variant associated with severe AATD is the Z allele (Glu342Lys) (3). Z AAT homozygotes have a plasma AAT level that is 10-20% of normal and an increased risk of pulmonary disease (1,4). Most ZZ patients also develop liver disease, which can manifest as severe hepatitis in the neonatal period (4,5), cirrhosis and hepatocellular carcinoma (6). Several models have been proposed to explain the mechanism by which the Z mutation causes the formation of ordered polymeric chains in the ER (1), but it is unclear how these polymers deposit in inclusion bodies and how this process leads to cytotoxicity and liver disease. AATD is a recessive disorder, as heterozygous MZ subjects who carry Z with the wild-type M AAT allele and represent approximately 2% of the US and European population (7), are generally healthy. However, large epidemiological studies have reported an increased risk of COPD in individuals with the MZ genotype (4,7,8). The Z variant in heterozygotes may be a co-factor in the progression of chronic liver disease associated with alcohol abuse, fatty liver, viral infection and other environmental or genetic factors (4,7,9).

The S variant (Glu264Val) is a highly prevalent AAT allele in Southern Europe (3) and is characterised by AAT plasma concentrations of 60% of the wild type allele, sufficient to protect SS homozygotes from lung disease. Moreover, the misfolding defect of the S AAT leads the variant to intracellular degradation rather than accumulation in the ER (10–12), ensuring SS homozygotes not to develop liver disease (4,13). However when the Z and S alleles are inherited together then plasma levels are 25-35% of normal and there is a risk of both liver and lung disease (4,13,14).

Several rare pathological AAT variants have been reported in association with the Z allele. Most of these variants have been described in single cases or with limited genotype-phenotype correlation. Nonetheless, there is evidence that some rare mutants accumulate in hepatocytes as polymers, such as Mmalton (Phe52del), Siiyama (Ser53Phe), King's (His334Asp), and to a lesser extent, Mwurzburg (Pro369Ser) and Pbrescia (Gly225Arg) (15–19). Based on current epidemiological studies, the highest prevalence of rare AATD genotypes are in Southern Europe (13,20).

Although the compound heterozygous condition found in SZ AAT subjects or in rare/Z AAT genotypes is clinically important, the co-expression of two variants has not been investigated in a cellular setting. In this work, we have developed a strategy to reproduce AAT compound heterozygosity in cells and investigated the formation of hetero-polymers between Z and other variants of AAT.

EXPERIMENTAL PROCEDURES

Reagents and antibodies. Unless stated otherwise, reagents and culture media were purchased from Sigma-Aldrich. Antibodies used are listed in Supplementary Table 1.

Expression vectors. The expression vectors encoding for human M1(Val213) and Z AAT are based on pcDNA3.1/Zeo (+) (Life Technologies) (19). Z_{HA}, Z_{myc}, M_{HA} and M_{myc} were cloned in pcDNA3.1(+) vector (Life Technologies) as previously described (21). The vectors encoding for S, Mwurzburg, Mmalton, S_{myc}, $Mmalton_{myc}$ and $Mwurzburg_{myc}$ variants were obtained by the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) with the primers listed in Supplementary Table 2.

Cell culture and transfection. HEK293T (ATCC, CRL-11268), HeLa (ATCC, CCL-2), Hepa 1-6 (ATCC, CRL-1830) and COS-7 (ATCC, CRL-1651) cells were maintained in DMEM/10% v/v FBS. Transfections were performed with Polyethyleneimine "Max" (PEI, Polysciences Inc) as described in (22). To analyse AAT in the cell media, transfected cells were washed and incubated in Optimem (Life Technologies) for 24h at 37°C. Cell media were collected and centrifuged at 800g for 5'. To analyse intracellular AAT, cells were harvested, lysed for 15 min at room temperature (RT) in lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1% v/v NP40, 10 mM NEM and protease inhibitors) and centrifuged for 30' at 800g to pellet nuclei and NP40-insoluble material. When indicated, the insoluble pellets were solubilised in Laemmli buffer/50 mM DTT and sonicated.

Co-immunoprecipitation

Immunoprecipitation (IP) of HA-tagged AAT variants was performed by a rabbit pAb anti-HA. Cell lysates of HEK293T, collected 24h post-transfection, were pre-cleared by recombinant ProteinG Agarose beads (rPGA, Thermo Fisher) overnight (ON) at 4°C and IP with anti-HA pAb followed by rPGA beads. Immunoprecipitated material was then eluted in Laemmli buffer/50 mM DTT for SDS-PAGE analysis. For the IP assay with 2C1 mAb, cell lysates of $1x10^6$ HEK293T transfected cells were pre-cleared as above and immunoprecipitated with 1µg of purified 2C1 mAb; the immunoprecipitated samples were then eluted in Glycine 0.2 M pH 3.0 for 10', buffered with an equal volume of 1 M Tris-HCl pH 8.0 and diluted 3-fold with TBS. Once eluted, polymers were immunoprecipitated with anti-HA pAb and eluted in Laemmli buffer for SDS-PAGE analysis.

SDS-PAGE, non-denaturating PAGE and immunoblot.

NP40-soluble or NP40-insoluble cellular fractions, total cell media or IP samples were resolved by 7.5% or 10% SDS-PAGE, as indicated in the legends. For non-denaturing analysis, samples were run on 7.5% native PAGE as previously described (23). The gels were blotted to PVDF membranes by wet transfer and probed with the indicated primary antibodies, revealed with HRP-conjugated secondary antibodies and detected by ECL Clarity (Biorad) and exposure to Hyperfilm ECL (Amersham).

Cycloheximide treatment

16h after transfection, HeLa cells were incubated in culture medium supplemented with 50 µg/ml cycloheximide (CHX) for 8h at 37°C. Cells were lysed at the indicated time points and the NP40-soluble fractions were analysed by SDS-PAGE followed by immunoblot.

Sandwich ELISA

Quantification of AAT in cell media was performed by sandwich ELISA as previously described (23) using rabbit anti-AAT pAb (DAKO) for capture and HRP-conjugated sheep anti-AAT pAb for detection. AAT concentrations were calculated for each experiment using a standard curve of purified AAT (Millipore) and expressed as percentages of M AAT concentration.

Confocal microscopy and Proximity ligation assay

For immunofluorescence, HeLa cells were grown on glass coverslips, transfected and, 24h later, fixed and permeabilized as described previously (19). Cells were then stained with anti-HA, anti-myc, anti-calreticulin or anti-GM130 antibodies, followed by Alexa®-conjugated secondary antibodies. Confocal imaging was performed by the LSM510META confocal microscope and Zen2009 software (Zeiss) at 488nm and 543nm excitation wavelengths in multi-track mode. Sub-cellular co-localization analysis was based on Mander's coefficient calculated by ImageJ Plugin JACoP (24).

For Proximity Ligation Assay (PLA, Duolink), cells, transfected on coverslips as above, were co-stained with mouse anti-HA mAb and rabbit anti-myc pAb. Cells were further incubated with the PLA reagents according to the manufacturer's protocol and nuclei stained with DAPI. Cells were imaged by the LSM510META confocal microscope at 405nm and 543 nm. PLA signals were quantified using Blobfinder Software (Centre for Image Analysis, Uppsala University).

Statistical analysis

All the statistical analyses were performed by software Prism5 (GraphPad software Inc, San Diego, CA, USA) as detailed in the figure legends.

RESULTS

Development of a cellular model to study heterozygosity in AATD

To study the molecular interactions between AAT variants in cells, we established an experimental model in which we co-expressed two AAT variants, respectively modified by the influenza hemagglutinin (HA) tag or the myc tag at the C-terminus of the protein. To test whether addition of tags affected the overall behaviour of the variants, we expressed in HEK293T cells either the untagged or the tagged variants and compared them by assessing their secretion efficiency as well as their ability to form polymers (Figure 1). After transfection, HEK293T cells were grown in the absence of serum for 24 hours. Cell media were then collected, and the cells lysed in a buffer containing the mild NP40 detergent, followed by centrifugation, to separate the soluble cytoplasmic material from the NP40-insoluble fraction containing nuclei and large protein aggregates. The different fractions were analysed by SDS-PAGE and immunoblot with anti-AAT antibodies (Figure 1A). Even when modified by tags, the wild-type M and the AAT mutants showed the expected profile: M was secreted efficiently, showed low levels in the NP40-soluble fraction, and was undetectable in the insoluble material; in comparison Z, S, Mmalton (Mal) and Mwurzburg (W) mutants were deficient in secretion and accumulated in both the NP40-soluble and –insoluble intracellular fractions. In our expression conditions, after loading equivalent amounts of the two intracellular fractions, the percentage of NP40-insoluble S and Z were 15% and 30%, respectively (Figure 1B). AAT concentrations in cell media were quantified by ELISA (Figure 1C). Compared to the level of M, set as 100%, both Z and Mmalton were severely deficient (respectively 15% and 10%), while S and Mwurzburg showed milder secretory defects (respectively 40% and 45%). The extracellular levels of the tagged variants, expressed as percentages of the M_{myc} control, behaved similarly to the corresponding unmodified variants.

As demonstrated previously (18,23,25), polymers accumulate within the cells but can also be detected in the cell media with a polymer/monomer ratio that reflects the intrinsic tendency of each mutant to polymerise. We therefore analysed the cell supernatants of transfected HEK293T by non-denaturing PAGE and immunoblot analysis (Figure 1D). While the normal M AAT and its tagged counterparts are exclusively in the monomeric form, the mutants show a typical monomer to polymer proportion, independent of the presence of tags. Taken together these data rule out major effects of C-terminal tag addition on AAT variant handling by cells.

Z forms mixed polymers with S and rarer AAT variants

To investigate the interactions between Z and other AAT variants in cells, Z_{HA} was co-transfected in HEK293T cells with the myc-tagged versions of M, Z, S, Mmalton or Mwurzburg. As a control,

M_{myc}, Z_{HA}, Z_{myc} and S_{myc} were transfected alone. In order to evaluate the expression levels of each variant and verify the specificity of anti-tag antibodies, the NP40-soluble cell extracts were analysed by immunoblot with antibodies against total AAT, myc or HA tag (Figure 2A). To investigate the formation of hetero-complexes between Z_{HA} and the co-expressed myc-tagged variants, the same samples were subjected to a pull-down assay with anti-HA antibodies (Figure 2B). The immunoprecipitated material was first probed with anti-AAT antibodies showing comparable levels of immunoprecipitation (IP) except for cells expressing Z_{myc} only (Figure 2B, upper panel). The same membrane was stripped and probed by anti-myc 9E10 mAb in order to detect AAT variants pulled-down along with Z_{HA} (Figure 2B, lower panel). As expected, Z_{myc} was efficiently co-immunoprecipitated from cells expressing both Z_{HA} and Z_{myc}. S_{myc} and the other mutants were similarly co-immunoprecipitated, showing the formation of hetero-complexes with Z_{HA}. Interestingly, also a small fraction of the wild-type M was found in association with Z_{HA}.

To clarify whether the co-IP shown in panel B was due to formation of mixed polymers, we took advantage of the 2C1 mAb, a conformational antibody that specifically recognises AAT polymers in liver biopsies from ZZ patients (17) and in the plasma (25). We used the 2C1 mAb to set up a novel biochemical assay to immunopurify polymers from the cell extracts. The efficiency of IP with 2C1 mAb was first tested on cells expressing Z_{HA}, showing that, in our experimental conditions, about 10% of Z AAT present in the NP40-soluble cell extract was precipitated by 2C1 and that the precipitated polymers could be eluted from beads by mild acid treatment (Figure 2C).

Polymers were immunopurified with 2C1 mAb from HEK293T cells co-transfected with $Z_{HA}Z_{myc}$, $S_{myc}Z_{HA}$ and $M_{myc}Z_{HA}$, then re-immunoprecitated with anti-HA antibodies and analysed by SDS-PAGE and immunoblot. Membranes were probed with both anti-HA and anti-myc antibodies (Figure 2D), showing that, when co-expressed, S_{myc} and Z_{HA} are associated in 2C1-positive polymers. In order to exclude that acidic elution may artificially induce association of S and Z AAT, a control IP was performed on a sample obtained by mixing the NP40-soluble extracts of cells expressing either S_{myc} or Z_{HA}, treated with acid (Z_{HA}+S_{myc}). Similar results were obtained co-expressing S_{myc} and Z_{HA} in Hepa cells (data not shown).

In summary our results demonstrate that the S variant, and to a lesser extent the wild-type M, form 2C1-positive hetero-polymers when co-expressed with Z AAT.

Co-polymerization with Z increases intracellular accumulation of S and M AAT

Hetero-complex formation with Z AAT would be expected to affect the intracellular accumulation of the co-expressed AAT variants. We therefore analysed the intracellular decay of M_{myc}, S_{myc} and Z_{myc} expressed alone or in the presence of Z_{HA}. Transfected HeLa cells were treated with

cycloheximide (CHX, 50 µg/ml) in a time course of 8 hours. The immunoblots of intracellular AAT at the different time points and their densitometric analysis (Figure 3) showed that M_{myc} quickly cleared from the cell as a result of rapid secretion, while a large proportion of Z_{myc} persisted over the 8h of CHX treatment. S_{myc} showed an intermediate behaviour, in agreement with previously published data (12). When co-expressed with Z_{HA}, both M_{myc} and S_{myc} exhibited a slower decay. Under the same co-expression conditions, we did not observe major effects of Z on the secretion of either S or M AAT (Supplementary Figure 1A) and only minor changes in the accumulation of these variants in the NP40-insoluble cellular fractions (Supplementary Figure 1B). Comparable results were obtained in the Hepa 1-6 cellular model (not shown).

In summary, co-expression with Z AAT modifies the behaviour of S and M AAT, favouring their intracellular accumulation.

The S and Z variants colocalize in dilated ER cisternae

In order to study their localization by immunofluorescence, tagged M, S and Z variants were expressed alone or in combinations in HeLa cells for 24h. M_{myc}, S_{myc} and Z_{myc} cells were fixed and stained with anti-myc mAb in combination with antibodies to either GM130, a cis-Golgi marker, or to the ER marker calreticulin (CRT) (Figure 4). Co-localization was analysed by determining Mander's coefficients as represented by histograms (Figure 4, right panels). As expected for an efficiently secreted protein, M_{myc} co-localizes with GM130. Z_{HA} and S_{myc} show weaker co-staining with this marker (Figure 4A). In contrast, these two variants show significant co-localization with CRT, confirming their accumulation in the ER. Interestingly, Z_{HA} is enriched in CRT-positive dilated cisternae around the nuclei and is relatively less abundant in the peripheral ER which preserves its reticular morphology (21,26).

To mimic the heterozygous condition found in AATD patients, we co-expressed Z_{HA} with the myc-tagged versions of M, S or Z AAT and stained the cells with anti-HA and anti-myc antibodies (Figure 4E and F). As positive control of co-localization, we observed a complete staining overlap in the cells co-expressing Z_{myc} and Z_{HA}. A significant co-staining was also observed in $S_{myc}Z_{HA}$ cells. S_{myc} is both co-localised with Z_{HA} in inclusion bodies (IB) and distributed as single staining in the peripheral ER. Interestingly, also M_{myc} and Z_{HA} displayed a remarkable overlap within dilated vesicular structures, although part of M AAT segregates from Z in a peri-nuclear area that likely represents the Golgi apparatus. Similar results were obtained in COS-7 cells (not shown).

Taken together, our results show, within the resolution limits of this confocal technique, co-localization of S and M AAT within Z AAT inclusion bodies.

The Z and S AAT variants are in close proximity in the cells

To further investigate the co-localization of AAT variants within the ER we used the Proximity Ligation Assay (PLA), a technique developed to demonstrate proximity of two epitopes within 4-40 nm (27). HeLa cells expressing $M_{myc}M_{HA}$, $S_{myc}M_{HA}$, $M_{myc}Z_{HA}$, $S_{myc}Z_{HA}$ or $Z_{myc}Z_{HA}$ were treated with anti-tag antibodies and processed with the PLA procedure, to mark adjacent HA and myc epitopes by fluorescence spots.

Micrographs in Figure 5A demonstrate that $M_{myc}M_{HA}$ and $S_{myc}M_{HA}$ co-expressing cells were negative in the PLA, while fluorescent spots were evident in $M_{myc}Z_{HA}$, $S_{myc}Z_{HA}$ and $Z_{myc}Z_{HA}$ cells. Positive cells did not show substantial differences in spot distribution among samples. The efficiency of co-transfection was determined in parallel by conventional immunofluorescence with antibodies to the HA and myc tags and shown to be similar in all different samples (Supplementary Figure 2 and Supplementary Table 3).

We further processed the PLA acquisitions with the software Blobfinder, to determine the red fluorescence intensity per cell, which is represented in a scatter plot (Figure 5B). Both $M_{myc}M_{HA}$ and $S_{myc}M_{HA}$ showed basal values, while cells expressing $M_{myc}Z_{HA}$, $S_{myc}Z_{HA}$ and $Z_{myc}Z_{HA}$ combinations displayed increasing intensities of PLA signal, although with high internal variability. Assuming the average of $M_{myc}M_{HA}$ cells as background, we determined the number of PLA positive cells, regardless of their intensity (Figure 5C). This analysis confirmed that $S_{myc}Z_{HA}$ and $Z_{myc}Z_{HA}$ behave similarly, demonstrating that the two co-expressed variants are in close proximity. Conversely, $M_{myc}Z_{HA}$ positive cells were less abundant and less intense (Figure 5B and C). Similar results were obtained in the COS-7 cellular model (data not shown).

Taken together, the PLA data presented here, and the pull-down assays showed in Figure 2 demonstrate that $S_{myc}Z_{HA}$, and to a lesser extent $M_{myc}Z_{HA}$, physically interact within the cell.

DISCUSSION

Liver disease is common in Z AAT homozygotes (4–6) but is also seen in individuals with SZ and rare/Z genotypes (1,4,9). Moreover, MZ AAT heterozygotes are over represented on liver transplant waiting lists (28) suggesting that the single Z allele is sufficient to cause liver disease or that it is a co-factor with other conditions such as alcohol abuse, fatty liver or viral hepatitis. The principal cause of chronic liver disease in AATD is the accumulation of AAT polymers within the ER of hepatocytes (2). This is clearly the case for Z AAT homozygotes, but it is unknown whether Z forms heteropolymers with other polymerogenic mutants within the hepatocyte and indeed, whether it can form polymers with the wild type M AAT. The only work to date has shown that Z can form heteropolymers with S, but not M AAT, in biochemical assays (29). In this report we have studied the molecular interactions between Z and other AAT mutants in cells to reflect the cellular handling of AAT in individuals who are heterozygous for the Z allele. In particular, we have investigated whether Z AAT can hetero-polymerise with other variants and thereby exert a dominant negative effect on the intracellular retention of milder variants, such as the common S mutant or the wild type M AAT.

Our co-expression experiments show for the first time that molecular interactions and co-polymerization involving two AAT variants can occur in cells. We demonstrated, by pull-down experiments, the formation of intracellular complexes between Z and Mmalton (Phe52del) AAT. This is not surprising as both Z and Mmalton rapidly form polymers *in vitro* and in cell models of disease (12,30). Z AAT also formed heteropolymers with the less polymerogenic S (Glu264Val) and Mwurzburg (Pro369Ser) variants. The SZ AAT hetero-polymers were investigated in more detail and shown to be recognised by the polymer-specific 2C1 monoclonal antibody. This implies that SZ heteropolymers share an epitope with pathological polymers of Z AAT that form within hepatocytes of individuals with AATD-associated liver disease (14) and so are likely to have a similar conformation. An alternative explanation is that the 2C1 mAb is detecting the Z AAT within the SZ heteropolymer. Both Z and S AAT can form polymers independently (1,11) and form heteropolymers when incubated together *in vitro* (29). The ability to form heteropolymers within cells most likely reflects the ability of each protein to form an unstable intermediate conformer (M*) which acts as nucleus for hetero-polymer formation (1). However, it may result from destabilised Z and S molecules binding from a near-native conformation .

Analysis of the intracellular kinetics demonstrated that co-polymerization with Z AAT influenced the intracellular fate of S AAT. Indeed, upon co-expression with Z, there was slower intracellular clearance of S AAT, suggesting that co-polymerization with Z decreases the fraction of S destined

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for degradation (10). The formation of intracellular heteropolymers did not have a significant effect on secretion of the S mutant.

One surprising finding is that Z AAT can also form heteropolymers with wild type M AAT. This increased intracellular retention of M AAT, but similar to the S variant, had no effect on secretion. M AAT folds rapidly and traffics through the secretory pathway. An interaction between M and Z AAT was not seen when the two proteins were incubated together *in vitro* (29). This suggests that heteropolymer formation must occur whilst the M and Z AAT are folding within the cell, rather than from native conformers of protein. However, we cannot exclude alternative AAT polymerization patterns that have been proposed for Z (1) as well as for rare AAT variants (22,23).

It is known that polymerization of Z AAT dramatically changes the architecture of the ER as a result of the formation of inclusion bodies. This may reduce the mobility of cargo proteins in the ER lumen (26,30), as well as their transport in the exocytic pathway. Our data did not reveal a dominant negative effect of Z AAT on secretion of either the normal M or the S variant, in agreement with the concept that plasma levels in MZ and SZ heterozygotes result from independent secretion of the two variants. Indeed, even in the presence of Z aggregates, a large part of the ER network, marked by CRT staining, preserves its architecture and likely its secretory function. Moreover, a previous report showed that, in cells expressing Z AAT, inclusion bodies maintain capabilities of protein synthesis and vesicular exchange (26).

Taken together our data show that Z AAT can form heteropolymers with other mutants and with wildtype AAT. Heteropolymer formation delays clearance but does not affect secretion of S or M AAT. Heteropolymer formation will increase the load of polymers within the ER and hence the risk of liver disease, particularly where the liver is exposed to a second 'hit' such as alcohol, fat, or viral hepatitis. The formation of heteropolymers helps to explain the presence of liver disease in individuals who are heterozygous for AAT deficiency.

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ML, RB and RR performed and analysed the experiments, AF conceived the study and wrote the paper with ML and DL. All authors critically read and approved the submitted manuscript.

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FIGURE LEGENDS

Figure 1 – Development of a cellular model to study heterozygosity in AATD

HEK293T cells were transfected for 24h with vectors encoding M, Z, S, Mmalton (Mal) and Mwurzburg (W) or their tag-modified versions. (A) Cell media, NP40-soluble and -insoluble cellular fractions were analysed by reducing 7.5% SDS-PAGE and immunoblot with an anti-AAT pAb. (B) Equal volumes of NP40-soluble (SOL) and -insoluble (INS) fractions were resolved by reducing 7.5% w/v acrylamide SDS-PAGE and immunoblot with anti-AAT pAb to evaluate the relative amounts of the variants. The arrowhead indicates AAT. (C) AAT levels in the cell media were measured by ELISA and expressed as percentage of wild-type M (left panel) or M_{myc} (right panel) (n=3, MEAN±SEM, 1way-ANOVA $p<0.0001$). (D) The cell media were analysed by non-reducing 7.5% w/v acrylamide native-PAGE and immunoblot with anti-AAT pAb to evaluate the polymer/monomer ratio.

Figure 2 - Z forms hetero-polymers with S and rare AAT variants

HEK293T cells were transfected for 24h with vectors encoding M_{myc}, Z_{HA}, Z_{myc}, S_{myc} alone or with M_{myc}, Z_{HA}, Z_{myc}, S_{myc}, Mal_{myc} and W_{myc} in combination with Z_{HA}. (A) The NP40-soluble cellular fractions were separated by reducing 7.5% w/v acrylamide SDS-PAGE and immunoblotted with the anti-myc 9E10 mAb, the anti-HA pAb and anti-AAT pAb. Half volume was loaded for single expressing cell extracts. (B) The NP40-soluble cellular fractions were immunoprecipitated with an anti-HA pAb (IP anti-HA), separated on non-reducing 10% w/v acrylamide SDS-PAGE and revealed by immunoblot with either anti-AAT pAb (upper panel) or anti-myc 9E10 mAb (lower panel). (C) A cell lysate of HEK293T expressing Z_{HA} was immunoprecipitated with the 2C1 mAb or by an irrelevant mAb to assess specificity of the 2C1 IP (left panel). Samples were analysed by non-reducing 10% w/v acrylamide SDS-PAGE and immunoblot with anti-AAT pAb. The efficiency of the acidic elution of the 2C1 IP is comparable with elution in Laemmli buffer. (D) To confirm the presence of mixed hetero-polymers made by Z and S AAT, the lysates deriving from cells co-expressing Z_{HA} with $M_{myc,}$ S_{myc} or Z_{myc} were immunopurified by 2C1 mAb as in panel C and the eluted polymers were immunoprecipitated using anti-HA pAb, separated on non-reducing 10% w/v acrylamide SDS-PAGE and revealed by immunoblot with anti-HA pAb or anti-myc-HRP mAb. As controls, we loaded the mixed lysates from Z_{HA} and S_{myc}, treated with acid and pull-down by anti-HA (Z_{HA}+ S_{myc}) and the antibodies used for IP (2C1 mAb and anti-HA pAb). Note that a fraction of Z migrates in non-reducing conditions as a disulphide-mediated dimer (arrowhead), as previously reported (22).

Figure 3 - Co-polymerization with Z increases intracellular accumulation of S and M AAT

HeLa cells expressing M_{myc}, S_{myc} and Z_{myc} alone or in combination with Z_{HA} for 16h were treated with CHX (50µg/ml) in an 8h time course experiment. The NP40-soluble fractions, collected at the indicated time points, as well as the untreated cells (8h-) were analysed by reducing 7.5% w/v acrylamide SDS-PAGE and immunoblot with anti-myc-HRP mAb. The immunoblots from two independent experiments were analysed by densitometry and the data are presented in the graphs (n=2, mean±SEM).

Figure 4 - The S and Z AAT variants colocalize in dilated ER cisternae

HeLa cells were transfected with M_{myc}, S_{myc} or Z_{myc} alone or with Z_{HA} and after 24h processed for immunofluorescence with the indicated primary antibodies followed by ALEXA®-conjugated 488nm anti-rabbit and 594nm anti-mouse secondary antibodies and confocal analysis by the LSM510META microscope (100x/1,3 oil objective) (White bars=20µm). (A) HeLa cells expressing M_{myc}, S_{myc} or Z_{myc} were co-stained with anti-myc pAb and mAb anti-GM130 to label the Golgi compartment. (B) The co-localization is shown as Mander's coefficients of the GM130 over the myc signals (n=20, mean±SEM, 1way-ANOVA $p<0.0001$, Bonferroni's Multiple Comparison Test). (C) HeLa cells expressing M_{myc}, S_{myc} or Z_{myc} were co-stained with anti-myc mAb and pAb anti-calreticulin (CRT) to label the ER. (D) The co-localization is shown as Mander's coefficients of myc over the CRT signal (n=20, mean±SEM, 1way-ANOVA $p<0.0001$, Bonferroni's Multiple Comparison Test). (E) HeLa cells co-expressing AAT variants were stained with primary pAb anti-myc and mAb anti-HA. (F) The co-localization is shown as Mander's coefficients of the myc over the HA signal, and vice versa (n=20, mean±SEM, 1way-ANOVA $p<0.0001$, Bonferroni's Multiple Comparison Test).

Figure 5 - Z and S AAT are in close proximity in the cells

HeLa cells were co-transfected for 24h with $Z_{myc}Z_{HA}$, $M_{myc}M_{HA}$, $M_{myc}Z_{HA}$ $S_{myc}M_{HA}$ or $S_{myc}Z_{HA}$. (A) Cells were stained by anti-myc pAb and anti-HA mAb, processed by PLA and visualized by the LSM510META confocal microscope. In red are PLA spots and in blue the nuclei stained by DAPI. (63x/1.3 oil objective) (White bars=50µm). (B) and (C) PLA signals from 10 random fields were quantified using Blobfinder Software and the data are presented in the scatter plot (B) as PLA intensity/cell (mean±SEM, 1way-ANOVA $p<0.0001$) or as number of positive cells (C), with intensity higher than the average of $M_{myc}M_{HA}$ (n=2, mean±SEM, 1way-ANOVA $p<0.0005$, unpaired one tailed t-test).

LEGENDS OF SUPPLEMENTARY FIGURES

Supplementary Figure 1

(A) Extracellular AAT levels of HeLa cells expressing the tagged M, S or Z AAT variants alone or in combination with $Z_{HA.}$ Transfected Hela cells were cultured for 24h in serum-free media and the AAT was quantified in the cell supernatants by ELISA and expressed as percentage of the M_{myc} level. Black bars indicate the predicted (PRED) AAT percentages in the co-expression conditions, calculated by summing up half the levels of the AAT variants expressed alone (n=3; MEAN±SEM, 1way ANOVA $p<0.0001$). (B) Distribution between the NP40-soluble and -insoluble cellular fractions of the myc-tagged AAT variants in co-expression with Z_{HA}. Transfected Hela cells were cultured for 24h and lysed in NP40 buffer. The insoluble material was solubilised with the same volume of Laemmli buffer and sonicated. Equal volumes of the two cellular fractions were loaded on reducing 7.5% w/v acrylamide SDS-PAGE and revealed by immunoblot with either anti-myc-HRP mAb or anti-HA pAb.

Supplementary Figure 2

Efficiency of co-transfection of tagged AAT variants in Hela cells was evaluated by immunofluorescence staining with anti-myc pAb and anti-HA mAb, followed by 488nm ALEXA®-conjugated anti-rabbit and 594nm ALEXA®-conjugated anti-mouse secondary antibodies. Nuclei were stained by DAPI. Cells were imaged by LSM510META confocal microscope (63x/1.3 oil objective). Single and double-stained cells were counted by ImageJ and the results are presented in Supplementary Table 3.