miR-199a-5p silencing regulates the unfolded protein response in chronic obstructive pulmonary disease and α1-antitrypsin deficiency.

Tidi Hassan  
Royal College of Surgeons in Ireland

Tomás P. Carroll  
Royal College of Surgeons in Ireland

Patrick G. Buckley  
Beaumont Hospital, Dublin

Robert Cummins  
Royal College of Surgeons in Ireland

Shane J. O’Neill  
Royal College of Surgeons in Ireland

See next page for additional authors

Citation
Authors
Tidi Hassan, Tomás P. Carroll, Patrick G. Buckley, Robert Cummins, Shane J. O'Neill, Noel G. McElvaney,
and Catherine M. Greene
miR-199a-5p silencing regulates the unfolded protein response in COPD and α1 antitrypsin deficiency

Tidi Hassan¹, Tomás P. Carroll¹, Patrick G. Buckley², Robert Cummins³, Shane J. O’Neill¹, Noel G. McElvaney¹*, Catherine M. Greene¹†.

¹Respiratory Research Division, Department of Medicine, Royal College of Surgeons, Dublin, Ireland, ²Department of Neuropathology, Beaumont Hospital, Dublin, Ireland, ³Department of Pathology, Royal College of Surgeons, Dublin, Ireland.

* Joint senior authors
†Corresponding author: Catherine M. Greene, Respiratory Research Division, Department of Medicine, Royal College of Surgeons in Ireland, Education and Research Centre, Beaumont Hospital, Dublin 9, Ireland. Ph +353-1-8093721 cmgreene@rcsi.ie

At-a-glance commentary: The role of miRNAs in regulating the unfolded protein response in monocytes in alpha-1 antitrypsin deficiency (AATD) and COPD is unknown. miRNAs are differentially expressed in healthy ZZ AATD monocytes, with miR-199a-5p expressed 50-fold higher compared to healthy non-AATD controls. miR-199a-5p expression is decreased in monocytes from individuals with COPD due to epigenetic silencing. Modulation of miR-199a-5p can regulate expression and function of the UPR by direct and indirect methods. miR-199a-5p is a key regulator of the UPR in AATD monocytes and epigenetic silencing of its expression regulates this process in AAT sufficient and AATD-related COPD.

Author contributions: TH, NGMcE and CMG were involved in all aspects of the work. SJO’N was involved in the study design. TPC performed some experiments and with PGB and RC provided advice on experimental design and analysed and interpreted data. All authors have read and revised the article.

Sources of support: This work was supported by Science Foundation Ireland via a Technology Innovation Development Award to CMG (12/TIDA/B2265). pGL3-p50 3’UTR was a gift from Prof. Gao, Shandong University Medical School.

Running title: miR-199a promoter methylation and UPR in monocytes

Descriptor: 3.5 Epigenetics

Total word count: 3464. “This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org”
ABSTRACT

RATIONALE: Retention of abnormal alpha-1 antitrypsin activates the unfolded protein response in alpha-1 antitrypsin deficient monocytes. The regulatory role of microRNAs in unfolded protein responses and chronic obstructive pulmonary disease pathogenesis has not been investigated.

OBJECTIVE: To investigate microRNA expression and function in MM and ZZ monocytes and identify microRNA(s) regulating the unfolded protein response.

METHODS: Peripheral blood monocytes were isolated from asymptomatic and symptomatic MM and ZZ individuals for microRNA expression profiling and pyrosequencing analysis. microRNA/gene and protein expression was measured with quantitative PCR and Western blotting. Overexpression and inhibition studies were performed with pre-miR or anti-miR respectively. Luciferase reporter genes were used to elucidate direct microRNA-target interactions. Inflammatory cytokines were detected using the Meso Scale Discovery Plex assays.

MEASUREMENTS AND MAIN RESULTS: Forty three microRNAs were differentially expressed, with miR-199a-5p most highly upregulated in asymptomatic ZZ versus MM monocytes. miR-199a-2 promoter hypermethylation inhibits miR-199a-5p expression and was increased in symptomatic MM and ZZ monocytes compared to asymptomatic counterparts. GRP78, ATF6, p50 and p65 were increased in symptomatic versus asymptomatic ZZ monocytes. Reciprocal down- or upregulation of these markers was observed after microRNA modulation. Direct miR-199a-5p targeting of ATF6, p50 and p65 by miR-199a-5p was demonstrated using luciferase reporter systems. Overexpression of miR-199a-5p also decreased other arms of the UPR and expression of cytokines that are not putative targets.

CONCLUSION: miR-199a-5p is a key regulator of the unfolded protein response in alpha-1 antitrypsin deficient monocytes and epigenetic silencing of its expression regulates this process in chronic obstructive pulmonary disease.

Abstract word count: 250

Key words (3-5): Alpha-1 antitrypsin, chronic obstructive pulmonary disease, unfolded protein response, miR-199a-5p, promoter hypermethylation
INTRODUCTION

Alpha-1 antitrypsin (AAT) is a serine protease inhibitor implicated in the lung and liver manifestations of AAT deficiency (AATD), a disorder characterized by decreased levels of AAT in the circulation (1). AAT is primarily produced by hepatocytes however, other cells including epithelial cells and monocytes can also produce smaller quantities of AAT (2–4). Although, chronic obstructive pulmonary disease (COPD) in AATD is mainly characterized by decreased serum levels of AAT, it can also be classified as an endoplasmic reticulum (ER) stress-related disease resulting in retention of abnormal AAT in the ER (5). This occurs due to the Z mutation (Glu342Lys) which causes the protein to fold aberrantly and accumulate in the ER. Eukaryotic cells undergo ER stress if there is imbalance between the load of correctly folded versus unfolded and/or misfolded proteins (6).

During ER stress, the ER transduces intracellular signals to the nucleus and cytosol (7). One component of ER stress, the unfolded protein response (UPR), is a coordinated tripartite management system involving three integral ER-resident transmembrane sensors; the protein kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol requiring kinase 1 (IRE-1). These receptors are activated by dissociation of the ER chaperone GRP78, a heat shock protein also known as BiP. The next immediate step in UPR engagement is translational attenuation which reduces the load of host proteins in the ER and prevents accumulation of unfolded proteins. This is caused by PERK via phosphorylation of eukaryotic initiation factor 2 α (eIF2-α) and promotes the translation of ATF4, a basic leucine zipper transcription factor (8, 9). IRE1 and ATF6 regulate chaperone induction, ER-associated degradation (ERAD) and expansion of the ER. IRE-1 oligomerizes and activates its RNAse domain which targets X-box binding protein-1 (XBP-1) (10). ATF6 generates a basic leucine zipper transcription factor ATF6p50 that migrates to the nucleus and activates transcription of genes under the control of ER stress-response elements including XBP-1. The ER-stress-induced spliced form of XBP-1 (sXBP-1) translocates into the nucleus and binds to ER and UPR stress response elements, activating transcription of ER chaperone genes and folding enzymes. NF-κB is a family of dimer forming subunits that become transcription factors including the canonical p65/p50 heterodimer. IRE1α can complex
with IκB kinase via the adaptor protein TNF-receptor activating factor 2, leading to degradation of IκBα, an inhibitor of NF-κB. This provides a direct link between ER stress and NF-κB activation (11). Indeed, it has been shown that ER accumulation of ZAAT leads to activation of the transcription factors NF-κB (12-14).

Monocytes from individuals with AATD have been shown to demonstrate an exaggerated inflammatory response generated by the accumulation of aberrantly folded ZAAT (4). This can have important consequences when these cells migrate into the lung or liver and develop into tissue macrophages. In non-AATD related COPD acute and chronic exposure to cigarette smoke adversely affects protein metabolism in the lung despite the production of the native MAAT protein. Cigarette smoke causes misfolding of nascent proteins in the lumen of ER leading to the activation of the UPR (15-17). However, to date there have been no studies examining the UPR in monocytes in non-AATD related COPD.

MicroRNAs (miRNAs) are non-coding RNA molecules that post-transcriptionally and mostly negatively regulate a variety of cellular processes by binding to complementary seed-sequences at the 3’untranslated region (UTR) of target mRNA (18). Recently, the role of miRNAs in the pathogenesis of respiratory diseases has been examined including COPD, lung cancer, idiopathic pulmonary fibrosis and cystic fibrosis (19). Studies have also shown that ER stress modifies the expression of many miRNAs, and a miRNA network that constitutes an additional layer in the regulation of the UPR exists in a variety of physiological and pathophysiological contexts (20-22).

Epigenetic regulation of miRNA expression can be associated with cigarette smoking leading to DNA modification events such as promoter hypermethylation and global hypomethylation (23-25). However, the role of miRNAs in the pathogenesis of AATD- and non-AATD-related COPD associated with ER stress and the UPR has not yet been investigated. Here, we demonstrate that miRNAs are differentially expressed in healthy, asymptomatic AATD ZZ monocytes compared to non-AATD MM monocytes. We show that miR-199a-5p is differentially expressed in MM and ZZ monocytes from individuals with and without COPD and that this may be related to promoter-specific hypermethylation. We also validate several markers of the UPR including ATF6, p50 and p65 as direct targets of miR-199a-5p and demonstrate that miR-199a-5p overexpression
can dampen the UPR and cytokine expression in ZZ monocytes.

METHODS

Study populations

Asymptomatic MM (n=8, 5 males, 3 females) and ZZ (n=11, 5 males, 6 females) individuals, and MM (n=7, 5 males, 2 females) and ZZ (n=11, 6 males, 5 females) individuals with COPD were recruited in this study (Table 1). In this study “symptomatic” ZZ AATD patients were categorized on the basis of lung disease rather than liver disease (an exclusion criterion for the study), which is more classically thought of as resulting from protein misfolding and aggregation. Asymptomatic MM individuals were control volunteers with no evidence of disease or respiratory symptoms. The mean±SEM forced expiratory volume in one second (FEV1) for symptomatic MM and ZZ individuals were 54.1±9.2% and 44±11.5% predicted respectively. Exclusion criteria included current smokers, liver disease, vasculitis, bronchiectasis on high-resolution CT scan and/or other extrapulmonary diseases. All MM individuals had a confirmed MM phenotype as diagnosed by isoelectric focusing, with serum AAT concentrations within the normal range (25-50 µM). ZZ individuals not receiving augmentation therapy were recruited from the Irish AATD Registry with previously confirmed AAT levels and Z allele-specific PCR. Full informed written consent was obtained and approved by the research ethics committee of Beaumont Hospital, Dublin.

Isolation, culture and treatment of peripheral blood monocytes

Mononuclear cells were isolated from heparinized venous peripheral blood as outlined in the online data supplement. MM monocytes were treated with DMSO (vehicle control) or Thapsigargin (TG) (Sigma-Aldrich) at 10, 50 or 100nM for 1 hour.

Quantitative assessment of mRNA and miRNA levels

RNA was isolated and quantified as outlined in the online data supplement. Relative expression of mRNA and miRNA was determined using the 2-ΔΔCt method (26).
miRNA expression profiling using the nCounter miRNA Expression Assay

miRNAs were profiled from asymptomatic MM and ZZ monocytes (n=3 in each group) with the nCounter miRNA Expression Assay (Nanostring Technologies) as outlined in the online data supplement.

DNA isolation, sodium bisulfite conversion and pyrosequencing analysis

Genomic DNA was isolated from the monocytes of asymptomatic and symptomatic MM and ZZ individuals and bisulfite converted using the EZ DNA Methylation Direct kit (ZymoResearch). Non-methylated, methylated and bisulfite converted DNA were used as quality controls. Conditions for PCR amplification and quantitative pyrosequencing analysis for the miR-199a-5p promoter and LINE-1 retrotransposable elements are provided in the online data supplement.

Luciferase reporter plasmid transfection

Briefly, HEK293 cells were transfected with a luciferase reporter vector containing either the full-length wild type (WT) 3’UTR of ATF6, NF-κB1 for p50 or RELA for p65, or the same plasmids in which the miR-199a-5p recognition elements were mutated, and a reference Renilla luciferase reporter plasmid pRLSV40 (Promega). The SERPINA1 3’UTR plasmid vector (Origene) was used as a negative control. Full details are provided in the online data supplement.

Transfection of pre-miRs and anti-miRs for miRNA overexpression/inhibition, western blot analyses and analysis of XBP-1 mRNA splicing and cytokine expression

These methods are described in the online data supplement. XBP-1 mRNA splicing was analyzed using an assay described by Calfon et al. (27). For inflammatory cytokine studies, symptomatic ZZ monocytes were treated with Pseudomonas aeruginosa LPS (2 µg/ml) (Sigma-Aldrich) for 4 hours after miRNA modulation.

IL-8, IL-10, GM-CSF and MCP-1α detection using the Meso Scale Discovery (MSD) Custom V-Plex assays.

Custom V-Plex assays were used to measure four inflammatory cytokines (IL-8, IL-10, GM-CSF and MCP-1α) using a multi-array electrochemiluminescence platform (MSD, Gaithersburg, MD). Equal volumes of supernatants were diluted 1 in 4 for IL-10, GM-CSF
and MCP-1α and 1 in 50 for IL-8 in the relevant assay diluent in a polypropylene plate. Samples (50 µl) were added into wells of the MSD plates and incubated for 2 hours with shaking at room temperature. After 3 washes with PBS-Tween, 25 µl of the 1X Detection Antibody Blend Solution was added into each well. After 2 hours of incubation at room temperature, 150 µl of 2X Read Buffer T was added into each well. The plate was immediately read using the Sector Imager 6000 instrument (MSD). Standard curves were performed and limits of detection for each assay were measured.

**Statistical analysis**

All analyses were performed using GraphPad PRISM 4.0 (San Diego, CA). Results are expressed as the mean ± SEM and were compared by Student t test or ANOVA as appropriate. Differences were considered significant at \( p \leq 0.05 \).

**RESULTS**

**miRNA expression is altered and miR-199a-5p is increased in asymptomatic ZZ compared to MM monocytes**

RNA isolated from asymptomatic MM and ZZ monocytes was profiled to examine the expression of 731 different human miRNAs. Appreciable target detection (normalized data after probe and background correction >1) occurred for 246 miRNAs across all samples. miRNAs expressed with an average difference of greater and less than 1.5 fold were identified for 37 and 6 miRNAs respectively in ZZ monocytes (Figure 1A). Using bioinformatics analysis with DIANA miRPath ([http://diana.cslab.ece.ntua.gr/pathways](http://diana.cslab.ece.ntua.gr/pathways)), these 43 differentially expressed miRNAs were overrepresented in the ER protein folding response pathway amongst others (Figure E1 in the online data supplement). miR-199a-5p was increased by more than 40-fold in asymptomatic ZZ versus MM monocytes.

**ER stress up regulates miR-199a-5p expression in MM monocytes**

In order to link this observation to ER stress healthy MM monocytes were treated
with TG to induce ER stress. It is well established that treatment with TG induces the UPR by depleting Ca\(^{2+}\) stores from the ER and inhibiting the activity of resident Ca\(^{2+}\) dependent chaperones. Increasing doses of TG lead to a dose-dependent increase in miR-199a-5p expression (p<0.001) (Figure 1B).

**miR-199a-5p is down regulated and the miR-199a-2 promoter is hypermethylated in symptomatic MM and ZZ monocytes**

We examined the expression of miR-199a-5p in symptomatic MM and ZZ monocytes compared to their asymptomatic counterparts by qRT-PCR. Consistent with the miRNA profile, the asymptomatic ZZ monocytes demonstrated a higher expression of miR-199a-5p by almost 50-fold. However miR-199a-5p expression was decreased in both symptomatic MM and ZZ monocytes compared to their asymptomatic counterparts (p<0.01, p<0.001 respectively) (Figure 2A).

We then determined if differential expression of miR-199a-5p was related to hypermethylation of its promoter region. Two genes can potentially encode pri-miR-199a, the primary precursor of hsa-miR-199a; miR-199a-1 on chromosome 19 (NCBI GeneID 406976) and miR-199a-2 human on chromosome 1 (NCBI GeneID 406977). miR-199a-2 has been implicated in diseases involving the UPR (28-30). Its promoter is embedded within an intron of Dynamin (DNM3) and is the authentic origin that produces the primary transcript harboring miR-199a-3p, miR-199a-5p and miR-214, a miRNA in the same intronic cluster (31). Genomic analysis revealed multiple CpG sites in the upstream region of miR-199a-5p (+833). Three CpG sites situated upstream of the transcription start site (+193) were analyzed for aberrant methylation using bisulfite conversion and sequencing (Figure 2D) (n=3 in each MM group, n=6 and 7 in asymptomatic and symptomatic ZZ group respectively) (FEV1 54.1±9.2 and 44±11.5% predicted in symptomatic MM and ZZ groups, respectively). The CpG sites of interest were observed to be inherently hypermethylated by 49.1±12.7% (mean ± SEM) in asymptomatic MM monocytes however, this was decreased in the ZZ counterparts (39.8±7.2%, p<0.05). There was an increase in the average DNA methylation in both the symptomatic MM (65.1±8.3%) and ZZ monocytes (71.5±11.2%) compared to their asymptomatic counterparts (p<0.01 for both) (Figure 2B). Inhibition of methylation using 5-aza-2’deoxycytidine increased relative
miR-199a-5p expression in THP-1 cells (Figure E2).

**miR-199a-2 promoter methylation does not correlate with genome-wide methylation using LINE-1 repetitive elements**

To determine the degree of global DNA methylation using repetitive elements widely distributed in the genome, we analyzed the methylation status of three CpG sites in LINE-1. An inverse observation was seen for the asymptomatic and symptomatic MM monocytes (43.2±3.7 vs. 27.9±3.0, p<0.01) compared to the miR-199a-2 promoter methylation pattern. However, similar to the miR-199a-2 promoter methylation, the asymptomatic ZZ monocytes demonstrated reduced average methylation (24.9±2.3%) compared to their MM counterparts (p<0.05). There was no difference between the average LINE-1 methylation in symptomatic (24.9±1.3%) versus asymptomatic ZZ monocytes (Figure 2C).

**UPR activation is increased in symptomatic versus asymptomatic ZZ monocytes**

The UPR has been shown to be induced *in vitro* in a variety of cell types in response to cigarette smoke exposure (15-17), in experimental animals (32) and in the lungs of smokers (33). We sought to determine if this phenomenon occurs in monocytes of ZZ individuals with COPD. The expression of GRP78, ATF6, p50 and p65 was measured by qRT-PCR and western blot analysis. The relative mRNA and protein expression levels of all four markers were significantly increased in symptomatic compared to asymptomatic ZZ monocytes (Figure 3 p<0.05 to 0.01).

**miR-199a-5p directly targets the 3’UTR of ATF6, NF-κB1 and RELA mRNAs**

The UPR markers GRP78, IRE-1, ATF6, p50 and p65 were predicted to be targeted by miR-199a-5p (microRNA.org). Figure E3 in the online data supplement shows the locations of the predicted miR-199a-5p miRNA recognition elements (MREs) in the 3’UTR of these transcripts and their corresponding miR-SVR scores. We sought to validate the interaction between miR-199a-5p and ATF6, NF-κB1(p50) and RELA (p65) mRNAs using luciferase reporter plasmids. GRP78 and IRE-1 are validated miR-199a-5p targets (34, 35). HEK293 cells were transfected with reporter vectors containing the full-length wild-type (WT) 3’UTR or vectors with mutations in specific miR-199a-5p MREs (Figure 4
Co-transfection with pre-miR-199a-5p resulted in significant decreases in luciferase expression from the WT vectors compared to scrambled control (p<0.01) (Figure 4 D-F). This effect was lost when co-transfections were performed with the MUT-gene specific 3’UTR plasmid reporters. A SERPINA1-3’UTR reporter plasmid which is not predicted to be targeted by miR-199a-5p was used as a negative control.

**Modulation of miR-199a-5p levels affects GRP78, ATF6, p50 and p65 expression**

Next the effect of miR-199a-5p overexpression or inhibition was evaluated. In order to examine transfection efficiencies of pre-miRs in difficult-to-transfect primary monocytes, we first determined the optimal transfection efficiency using a fluorescently labeled non-targeting miRIDIAN miRNA mimic at multiple concentrations including 30, 60, 90 and 120 nM using healthy MM monocytes. Using a dose of 60 nM of miRNA mimic and a transfection time period of 24h, 70% transfection efficiency was consistently achieved (Figure 5A). Transfection with 60 nM pre-miR-199a-5p led to ~700-fold increase in pre-miR 199a-5p expression (Figure 5A) with >80% cell viability assessed using the trypan blue dye exclusion test. Conversely, 24-hour transfection with anti-miR-199a-5p led to a 15-fold decrease in miR-199a-5p expression (Figure 5B).

Asymptomatic ZZ monocytes were transfected with 60 nM synthetic pre-miR-199a-5p. A scrambled RNA was included as a control. Overexpression of pre-miR-199a-5p significantly decreased GRP78, ATF6, p50 and p65 mRNA levels (Figure 5C, p<0.05 to p<0.01). Protein expression was also decreased for GRP78 (p<0.05), ATF6 (p<0.01) and p65 (p<0.05) however, the difference in p50 protein expression was not significant (Figure 5D). Conversely, knockdown of miR-199a-5p with 60 nM of anti-miR led to significant reciprocal increases in GRP78, ATF60, p50 and p65 mRNA and protein expression (Figure 5E and F, p<0.05 to p<0.001).

**Pre-miR-199a-5p overexpression decreased other arms of the unfolded protein response that do not have predictive binding sites for miR-199a-5p**

To examine if pre-miR-199a-5p overexpression has functional effects on the UPR, we measured the expression of UPR-inducible genes that are not predicted to be regulated by miR-199a-5p. ATF4, CHOP and GADD34 are inducible genes that are activated by PERK.
In asymptomatic ZZ monocytes transfected with synthetic pre-miR-199a-5p compared to cells transfected with a scrambled control, the relative mRNA expression of ATF4 mRNA was slightly reduced whilst CHOP and GADD34 mRNAs were significantly decreased (Figure 6A, p<0.05 and p<0.001). GRP58 and GRP94 are regulated by ATF6; expression of both of these transcripts was decreased by pre-miR-199a-5p, and significantly so for GRP58 (Figure 6B, p<0.01). We explored the activation of the IRE-1 arm of the UPR by investigating the expression and splicing of XBP-1. Spliced (s)XBP-1 mRNA levels were decreased in ZZ monocytes in pre-miR transfected cells compared with scrambled controls (Figure 6C, D).

**Modulation of pro-inflammatory cytokines in symptomatic ZZ monocytes treated with anti- and pre-miR-199a-5p**

Finally, to examine if inhibition or overexpression of miR-199a-5p has functional effects on inflammatory cytokine expression that has been previously related to the UPR in monocytes (4), we measured the secretion of IL-8, IL-10, GM-CSF and MCP-1α in supernatants. A dose-dependent treatment showed that 15nM of pre-miR resulted in a 100-fold overexpression of miR-199a-5p which was closely related to the 50-fold induction observed in vivo (Figure 7A). In symptomatic ZZ monocytes, LPS treatment led to an induction of all cytokines of interest compared with scrambled controls (Figure 7B-E). Transfection with 60 nM anti-miR resulted in increased levels of IL-8, IL-10 and MCP-1α which were significant (p<0.05) albeit for IL-10. Conversely, 15 and 30 nM of pre-miR-199a-5p led to significant decreases in detection of four cytokines (p<0.05 to p<0.001) albeit for IL-8 at 15 nM pre-miR transfection which was non-significant.

**DISCUSSION**

We have previously reported that the UPR is activated in monocytes from AATD individuals and that this affects monocyte function and inflammatory gene expression in response to bacterial challenge (4). In this study, we demonstrate that miRNAs are differentially expressed in asymptomatic ZZ compared to asymptomatic MM monocytes. miR-199a-5p which was overexpressed in ZZ monocytes showed a similar expression
profile in MM monocytes treated with thapsigargin, implicating a link between miR-199a-5p expression and ER stress in this context. Using bioinformatic tools, we observed that putative targets of miR-199a-5p are enriched in the ER protein folding pathway. We validated that miR-199a-5p directly targets ATF6, p50 (NF-κB1) and p65 (RELA) 3’UTRs and using gain- and loss-of-function approaches demonstrated that miR-199a-5p directly modulates expression of components of the UPR. We also showed that miR-199a-5p overexpression can dampen the UPR in ZZ monocytes by decreasing levels of downstream targets that are not directly regulated by miR-199a-5p. We also provide the first evidence that expression of key components of the UPR are higher in monocytes from symptomatic versus asymptomatic ZZ individuals, and that miR-199a-5p expression is lower in MM and ZZ monocytes isolated from individuals with COPD compared to asymptomatic MM or ZZ individuals. Based on these studies we propose a mechanism linking aberrant methylation of upstream CpG sites to differential miR-199a-5p expression and UPR activation in individuals with AATD and non-AATD-related COPD.

A number of studies have reported the possible role of miR-199a-5p in ER stress. miR-199a-5p has been implicated in the regulation of cell-proliferation, autophagy and angiogenesis (31, 36, 37-35). Dai et al. identified that miR-199a-5p is necessary for the modulation of hepatic ER stress progression by negatively regulating the IRE-1α-related pathway (35). In another study, miR-199a-5p was shown to suppress GRP78 and induce apoptosis in prostate cancer cells (34). Contrary to our study, Duan et al. demonstrated that activation of the UPR suppressed the expression of the miR-199a-5p/miR-214 cluster in hepatocellular cancer cell lines (30). Together these suggest that miR-199a-5p differentially mediates ER stress in a tissue and/or disease-specific manner. Indeed, we have previously reported that different miRNAs functionally target AAT mRNA in a cell-specific manner (38). As overexpression of miR-199a-5p also regulates expression of components of the three arms of the UPR that are not predicted to be direct targets of miR-199a-5p, we conclude that miR-199a-5p is a potent and key regulator of ER stress in ZZ monocytes. Previously we demonstrated that UPR activation in asymptomatic ZZ monocytes can amplify the host cell immune response (4). Based on the idea that UPR can mediate the induction of cytokine expression, all cytokines tested displayed the expected
expression pattern following miR-199a-5p modulation. The lack of an increase in GM-CSF expression in LPS-treated symptomatic ZZ monocytes transfected with antimiR-199a-5p may be due to an already maximal induction of GM-CSF following LPS treatment. Collectively the data show that modulation of miR-199a-5p levels reciprocally affects expression of inflammatory cytokines, an observation that has functional consequences which relate to the pathogenesis of ZZ AATD lung disease.

Emerging studies into the epigenetic regulation of miRNA have reported that miRNA expression can be regulated by DNA methylation and/or histone deacetylation (39). Hypermethylated promoters have been observed in clinically cancer-free and cancer-bearing smokers in sputum, BAL, alveolar macrophages and lymphoblasts (24, 40, 41). Current smokers in general have a higher methylation index than ex-smokers (42). A recent study reported that miR-199a-5p promoter hypermethylation correlated with its down regulation in malignant testicular tumors and that restoration of miR-199a-5p expression suppressed tumour invasiveness (29).

Conversely, LINE-1 repetitive transposable elements, which are widely distributed in the human genome with ~500,000 copies, showed hypomethylation in symptomatic MM and ZZ monocytes, compared to asymptomatic MM monocytes. LINE-1 elements were also hypomethylated in asymptomatic ZZ monocytes. Hypomethylation of LINE-1, which reflects global hypomethylation, has been correlated significantly with tumor phenotype, progression and prognosis in various cancers (43, 44). One study found minimal reduction of LINE-1 methylation in respiratory epithelium under high dosage cigarette smoke condensate treatment (45), another reported increased LINE-1 methylation with higher pack-years of smoking (46) whilst others have reported no change in LINE-1 methylation in response to cigarette smoke (47, 48). Recently it has been suggested that LINE-1 methylation is not sufficiently sensitive or accurate to determine global methylation changes as opposed to locus-specific methylation (49). This may apply to the current study in which miR-199a-5p methylation was a locus-specific hypermethylation event as opposed to a wider genomic phenomenon.

In non-AATD related COPD, studies have indicated that acute and chronic exposure to cigarette smoke adversely affects protein metabolism in the lung. It has been
shown that in cultured cells, activation of the UPR by cigarette smoke is rapid and dose dependent (17, 33). In chronic cigarette smokers, activated UPR is reflected by the upregulation at the protein level of UPR chaperones including GRP78 in small airway epithelial cells and type II pneumocytes. The mechanism by which cigarette smoke induces UPR is not entirely established although direct oxidation of target proteins by reactive oxygen/nitrogen species has been proposed to cause ER stress and protein misfolding. Cigarette smoke also increases cytosolic calcium suggesting that ER calcium depletion may contribute (50). We have shown that miR-199a-5p is downregulated in monocytes of MM individuals with COPD which may relate to hypermethylation of the miR-199a-2 promoter. This suggests an epigenetic-mediated regulation of miR-199a-5p, leading to modulation of the UPR which occurs in both AAT sufficient and AATD COPD. To date, no study has explored the potential role of miRNAs in regulating the ER stress in these diseases.

miR-199a-5p has been linked to non-ER stress studies of COPD and other lung diseases. Mizuno et al. reported an increase in miR-199a-5p expression in lung tissues from 55 COPD patients and suggested a pathogenic contribution of this miRNA in COPD (51). In another study, miR-199a-5p was implicated as part of an early-response miRNA signature associated with pulmonary tumorigenesis (52). In idiopathic pulmonary fibrosis, miR-199a-5p is significantly increased, induced upon TGFβ exposure and regulates CAV1, a critical mediator of pulmonary fibrosis (53). Here, we propose that the altered expression of miR-199a-5p in ZZ monocytes is specific to ER stress due to ZAAT accumulation observed in healthy, asymptomatic ZZ monocytes, however the pathophysiological role of miR-199a-5p may not be isolated to UPR.

In conclusion, we show that miR-199a-5p is increased in asymptomatic ZZ monocytes likely in response to the activation of ER stress. miR-199a-5p directly targets multiple components of the UPR including GRP78, IRE1, ATF6, p50 and p65 and overexpression and inhibition of this miRNA modulates the levels of both direct and indirect targets of the UPR and proinflammatory cytokine expression. Compared to asymptomatic counterparts, miR-199a-5p was decreased in symptomatic MM and ZZ monocytes, an event which is likely to be mediated by promoter-specific hypermethylation (Figure 8). This knowledge adds another layer of complexity in miRNA regulation of the UPR, which may have
potential for the development of future therapies for both AATD-related COPD, and other ER-stress related diseases including AAT sufficient COPD.
REFERENCES

12. Lawless MW, Greene CM, Mulgrew A, Taggart CC, O’Neill SJ, McElvaney NG. Activation of endoplasmic reticulum-specific stress responses associated with the...


**FIGURE LEGENDS**

**Figure 1.** Altered miRNA expression in asymptomatic ZZ versus MM monocytes. *A*, miRNAs expressed with an average difference of greater and less than 1.5 fold (37, 6 miRNAs respectively) in asymptomatic ZZ monocytes (n=3) compared to asymptomatic MM monocytes (n=3) were profiled using the nCounter miRNA Expression Assay. *B*, Relative expression of miR-199a-5p normalized to U6 snRNA in MM monocytes (1 x 10^5 cells, n=3, separate cultures in triplicate) treated with increasing dose of thapsigargin (10, 50, 100nM) for 1 hour using TaqMan miRNA assays. Data are represented as mean ± SEM and were compared by ANOVA (***p<0.001).

**Figure 2.** MiR-199a-5p expression, miR-199a-2 promoter methylation and global methylation in asymptomatic and symptomatic MM and ZZ monocytes. *A*, Relative expression of miR-199a-5p normalized to U6 snRNA in asymptomatic and symptomatic MM (n=7 in each group) and ZZ monocytes (n=10 asymptomatic and n=11 symptomatic) using Taqman miRNA assays. Values presented in y-logarithmic 10. *B*, Average methylation percentage of three CpG sites on the miR-199a-2 promoter in asymptomatic and symptomatic MM (n=3 in each group) and ZZ monocytes (n=6 and 7 in each group respectively) using pyrosequencing assays. *C*, Average percentage LINE-1 methylation in asymptomatic and symptomatic MM and ZZ monocytes (n=3 in each group). Data are represented as mean ± SEM and were compared by student *t*-test (non-parametric, one-tailed) (*p<0.05, **p<0.01, ***p<0.001). *D*, A schematic illustration of the miR-199a-2 gene embedded in the antisense strand within an intron of Dynamin in human chromosome 1. Three CpG sites (indicated as black pin needles) closest to the transcription start site in the miR-199a-2 promoter were analyzed.

**Figure 3.** Markers of the unfolded protein response are increased in symptomatic ZZ monocytes. Relative expression of GRP78, ATF6, p50 and p65 *A*, mRNA and *B*, protein in symptomatic ZZ monocytes (n=7) compared with asymptomatic ZZ monocytes (n=6). mRNA and protein relative expression were analyzed in triplicates by qRT-PCR and Western blot respectively and normalized to GAPDH. Data are represented as mean ± SEM and were compared by student *t*-test (non-parametric, one-tailed) (*p<0.05,
**p<0.01).

**Figure 4.** MiR-199a-5p target validation in ATF6, p50 and p65. The miR-199a-5p binding site predictions for A, ATF6, B, p50 and C, p65 3’UTR by microRNA.org. Vertical lines represent Watson-Crick base pairs whilst colons represent wobble base pairs. The underlined letters indicate the mutations inserted by site-directed mutagenesis. Relative luciferase activities in transiently transfected HEK293 cells (1x10^5 cells in triplicate) were measured using D, ATF6, E, p50 and F, p65 luciferase reporters and constitutive Renilla luciferase reporter pRLSV40 in HEK293 cells analyzed 48 hours after co-transfection with no pre-miR (No PM), scrambled (ScR) and pre-miR-199a-5p (PM). SERPINA1 3’UTR luciferase reporter was used as a negative control. Firefly luciferase activity was normalized to the Renilla luciferase activity. Data are represented as mean ± SEM and were compared by student t-test (non-parametric, one-tailed) (**p<0.01).

**Figure 5.** Direct targets in the unfolded protein response can be modulated with pre-miR- and anti-miR-199a-5p. A (top), Asymptomatic MM monocytes were nontransfected (NT) and transfected (T) with 60nM pierce dye547-labelled non-targeting miRIDIAN miRNA control to monitor transfection efficiency. Relative expression of miR-199a-5p in asymptomatic MM monocytes after 60 nM of pre-miR- (bottom) and B, anti-miR-199a-5p normalized to U6 snRNA using Taqman assays. Relative expression of GRP78, ATF6, p50 and p65 C, mRNA and D, protein in asymptomatic ZZ monocytes (1x10^5 cells in triplicate, n=3) treated with 60 nM of scrambled RNA (ScR) and pre-miRs (PM). Relative expression of GRP78, ATF6, p50 and p65 E, mRNA and F, protein in asymptomatic ZZ monocytes (n=3) treated with 60 nM of scrambled RNA (ScR) and anti-miRs (AM). Relative expression of miRNA and protein were analyzed in triplicate using qRT-PCR and Western blot respectively, normalized to GAPDH. Data are represented as mean ± SEM and were compared by student t-test (non-parametric, one-tailed) (*p<0.05, **p<0.01,***p<0.001).

**Figure 6.** Non-direct targets in the unfolded protein response can be modulated with
pre-miR-199a-5p. Relative expression of mRNAs in the A, PERK arm, B, ATF6 arm and C, IRE-1 arm of the unfolded protein response in asymptomatic ZZ monocytes (n=3, cultured in triplicate) treated with either 60 nM scrambled RNA (ScR) or pre-miR-199a-5p (PM) using qRT-PCR normalized to GAPDH. D, A representative gel indicating spliced (sXBP-1) and unspliced (uXBP-1) in asymptomatic ZZ monocytes treated with either 60 nM scrambled RNA (ScR) or pre-miR-199a-5p (PM). Data are represented as mean ± SEM and were compared by student t-test (non-parametric, one-tailed) (*p<0.05, **p<0.01, ***p<0.001).

**Figure 7.** Pro-inflammatory functional effects can be modulated with anti- and pre-miR-199a-5p. A, Relative expression of miR-199a-5p normalized to U6 snRNA using Taqman assays in symptomatic ZZ monocytes (n=3) after transfection with 15 nM and 30nM of pre-miR. Levels of B, IL-8 C, IL-10 D, GM-CSF and E, MCP-1α in symptomatic ZZ monocytes (n=3) transfected with 60 nM anti-miR or 15 and 30 nM pre-miR using the Meso Scale Discovery Pro-Inflammatory Custom V-Plex assays. Data are represented as mean ± SEM and were compared by either ANOVA or the student t-test (non-parametric, one-tailed) (*p<0.05, **p<0.01, ***p<0.001).

**Figure 8.** Model for the role and regulation of miR-199a-5p in the unfolded protein response (UPR) in asymptomatic and symptomatic ZZ monocytes. The accumulation of the aberrant ZAAT protein activates the UPR which in turn induces miR-199a-5p expression. In asymptomatic ZZ, elevated miR-199a-5p could negatively regulate ER stress by targeting the 3’UTR mRNAs of GRP78, ATF6, NF-κB1 (p50) and RELA (p65). In symptomatic ZZ monocytes, hypermethylated CpG sites in the miR-199a-5p promoter attenuate the expression of miR-199a-5p which in turn leads to intensification of the UPR.
Table 1. Clinical characteristics of MM and ZZ individuals recruited in this study

<table>
<thead>
<tr>
<th>Patient group</th>
<th>N (M:F)</th>
<th>Age</th>
<th>FEV1 (% predicted)</th>
<th>FEV1/FVC (% predicted)</th>
<th>Pack-years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic MM</td>
<td>8 (5:3)</td>
<td>30.7±11.2</td>
<td>103.3±9.5</td>
<td>83.2±5.0</td>
<td>0</td>
</tr>
<tr>
<td>Symptomatic MM</td>
<td>7 (5:2)</td>
<td>46.7±8.1</td>
<td>54.1±9.2</td>
<td>59.9±19.0</td>
<td>29±13.1</td>
</tr>
<tr>
<td>Asymptomatic ZZ</td>
<td>11 (5:6)</td>
<td>34.3±9.2</td>
<td>97±8.6</td>
<td>96.7±6.4</td>
<td>0</td>
</tr>
<tr>
<td>Symptomatic ZZ</td>
<td>11 (6:5)</td>
<td>40.7±8.7</td>
<td>44±11.5</td>
<td>51.6±12.4</td>
<td>18.6±11.2</td>
</tr>
</tbody>
</table>

Values are mean ± standard error of the mean.