**MiR-3156-5p** is downregulated in serum of MEN1 patients and regulates expression of **MORF4L2**.

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Multiple endocrine neoplasia type 1 (MEN1), caused by mutations in the MEN1 gene encoding menin, is an autosomal dominant disorder characterised by the combined occurrence of parathyroid, pituitary and pancreatic neuroendocrine tumours (NETs). Development of these tumours is associated with wide variations in their severity, order and ages (from <5 to >80 years), such that life-long screening is required. To improve tumour surveillance and quality of life, better circulating biomarkers, particularly for pancreatic NETs that are associated with a higher mortality, are required. We therefore examined the expression of circulating microRNAs (miRNAs) in serum of MEN1 patients. Initial profiling analysis followed by qRT-PCR validation studies identified miR-3156-5p to be significantly downregulated (-1.3-5.8-fold, p<0.05-0.0005) in 9 MEN1 patients, compared to matched unaffected relatives. MEN1 knock-down experiments in BON-1 human pancreatic NET cells resulted in reduced MEN1 (49%, p<0.05), menin (54%, p<0.05) and miR-3156-5p expression (20%, p<0.005), compared to control-treated cells, suggesting that miR-3156-5p downregulation is a consequence of loss of MEN1 expression. In silico analysis identified mortality factor 4 like 2 (MOR4FL2) as a potential target of miR-3156-5p, and in vitro functional studies in BON-1 cells transfected with either miR-3156-5p mimic or inhibitors showed that the miR-3156-5p mimic significantly reduced MORF4L2 protein expression (46%, p<0.005), while miR-3156-5p inhibitor significantly increased MORF4L2 expression (1.5-fold, p<0.05), compared to control treated cells, thereby confirming that miR-3156-5p regulates MORF4L2 expression. Thus, the inverse relationship between miR-3156-5p and MORF4L2 expression represents a potential serum biomarker that could facilitate the detection of NET occurrence in MEN1 patients.
Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant disorder characterised by the combined occurrence of parathyroid tumours, and neuroendocrine tumours (NETs) of the pancreas and pituitary. Over 90% of patients with MEN1 have pathogenic mutations in the \textit{MEN1} gene, which leads to loss of its encoded 610 amino acid tumour suppressor protein, menin (Brandi, \textit{et al.} 2021). Pathogenic germline and somatic \textit{MEN1} mutations reported in both familial and sporadic cases of MEN1 are scattered throughout the nine coding exons of the \textit{MEN1} gene and show no genotype-phenotype correlation (Concolino, \textit{et al.} 2016; Frost, \textit{et al.} 2018; Kooblall, \textit{et al.} 2021; Lemos and Thakker 2008). In addition, patients with MEN1 carry a heterozygous mutation in \textit{MEN1}, and tumours arise when a second hit occurs, causing complete loss of functional menin protein. This results in tumours arising at different time points in different organs, even in identical twins and in individuals within the same family carrying identical mutations (Brandi, \textit{et al.} 2021; Kooblall, \textit{et al.} 2021).

Due to this variability of tumour development, \textit{MEN1} mutation carriers are advised to undergo DNA testing, genetic counselling and regular screening for tumours, from as young as 5 years of age. Current clinical guidelines recommend annual surveillance for tumours by: biochemical analyses (e.g. calcium, fasting glucose and hormones such as insulin, and gastrin, and prolactin); and radiological examination including MRI (or CT) scans of the pancreas and pituitary (Thakker, \textit{et al.} 2012). However, the current single secreted biomarkers for pancreatic NETs, such as gastrin, insulin and chromogranin A (CgA), have limited usefulness for diagnostic or prognostic purposes, owing to the complexity and diversity of multiple tumour development and varying responses to different therapies (Oberg, \textit{et al.} 2015). For example, serum CgA, which is constitutively secreted from neuroendocrine cells, is prone to diagnostic inaccuracies due to the current assays having a wide range in sensitivity (60% to 90%), low specificity (50%) and lack of correlation with imaging techniques.
Moreover, circulating CgA levels do not always correlate with tumour mass, especially when smaller
tumours may be hypersecretory and larger tumours may have low secretion (Lawrence, et al. 2011;
Modlin, et al. 2010; Yao, et al. 2011). The importance of screening has also recently been highlighted
by a study of children and adolescent MEN1 patients, which indicated that 70% of patients developed
a tumour before 18 years of age, including metastatic pancreatic NETs (Shariq, et al. 2021).
Therefore, better less-invasive serum/plasma-based biomarkers are required, particularly for
pancreatic NETs.

One of the possible causes of the variability of MEN1 tumour development, could be the influence
of epigenetic changes which can act as cofactors in driving individual MEN1 phenotypes. Thus,
alteration of one or more tissue-specific epigenetic mechanisms, such as DNA methylation, histone
modifications and noncoding RNAs, could affect gene expression and trigger tumour development
and disease occurrence. This makes epigenetic factors suitable molecular markers for diagnostic and
prognostic purposes as well as possible therapeutic targets in human diseases (Frost, et al. 2018;
Hackl, et al. 2016). MicroRNAs (miRNAs) represent one type of epigenetic factor that is commonly
misregulated in tumours (Donati, et al. 2020; Frost, et al. 2018). MiRNAs are small non-coding RNAs
that bind to target mRNAs to regulate gene expression, which can be released from tumour cells into
the circulation. Previous studies have shown that specific miRNA profiles can help distinguish
normal, benign and malignant tissues, and miRNAs are therefore promising diagnostic and prognostic
circulating biomarkers (Ardekani and Naeini 2010). Specifically, miRNAs have been reported as
misregulated in MEN1-associated tumours of the parathyroids (Verdelli and Corbetta 2017), pituitary

Thus, the identification of specific circulating miRNAs in MEN1 patients could lead to potential
tumour biomarkers and possible molecular targets for therapies. We therefore examined the
expression of miRNAs in the serum of MEN1 patients.
Methods

Patient information and serum collection

Serum samples, stored at -80°C, and clinical information from 9 MEN1 patients (4 males and 5 females, age range 27-60 years) with a proven MEN1 mutation, and their sex-matched unaffected relatives who were proven not to have MEN1 mutations, or MEN1-associated tumours, were ascertained (Table 1). All patient and unaffected individual serum samples were processed using our standard protocol. Briefly, 5ml blood was collected using tubes containing no anti-coagulant. Blood samples were allowed to clot and then centrifuged at 2000g for 10 min. The resulting serum was then removed and stored at -80°C. MEN1 patient samples were divided into 2 cohorts, a test cohort and a validation cohort. The test cohort consisted of 2 males and 2 females (age range 35-60 years) who had at least 2 MEN1-tumour manifestations at the time of blood sampling. Clinical information on all known tumours at the time of blood sampling is shown in Table 1. Thus, all test cohort patients had a parathyroid adenoma and pancreatic NET (2 with gastrinomas and 2 with insulinomas), with one female patient also having a prolactinoma. The validation cohort consisted of 5 patients (2 male and 3 female, age range 27-56 years) who had at least one tumour manifestation at the time of blood sampling (Table 1). Thus, all validation cohort patients had a parathyroid adenoma, 2 patients had a pancreatic NET (1 gastrinoma and 1 insulinoma) and 1 female patient had an additional prolactinoma. In both cohorts the sex-matched unaffected relative sample was used as a control. Due to the historical nature of the samples obtained no data on tumour size was available. Informed consent was obtained from patients and relatives using protocols approved by a UK research ethics committee (MREC/02/2/93).

MiRNA sequencing and analysis
Total RNA, including miRNAs were extracted from 600 µl serum using the *Mir*Vana Paris Kit (Ambion). From this the miRNA libraries were prepared using the NEBNext smallRNA kit for Illumina (E7330L) following the manufacturer’s instructions. Size selection was carried out using Blue Pippin. Individual libraries were QC’d using Tapestation (Agilent) before being pooled and sequenced on HiSeq2500 (Illumina) at the Oxford Genomics Centre (Wellcome Centre for Human Genetics, University of Oxford). For analysis, read1 of the FASTQ was trimmed using fastx_clipper (https://github.com/agordon/fastx_toolkit) and aligned using Bowtie2 (Langmead and Salzberg 2012) to GRCh37, and miRNA counts were obtained using htseq-count (Anders, *et al*. 2015) against the annotation from miRBase v20. The raw gene count matrix was imported into the R/BioConductor environment (Huber, *et al*. 2015; Team 2020) for further processing and analysis with the edgeR package (McCarthy, *et al*. 2012; Robinson, *et al*. 2010). Genes with very low expression (i.e. those with ≤10 reads, after normalising for library size, in the 4-paired samples of the test set) were excluded. Multiple testing correction was performed by using edgeR's default Benjamini-Hochburg method for controlling the false discovery rate.

**Cell culture, transfections and functional assays**

BON-1 cells, isolated from a lymph node metastasis from a pancreatic NET patient (Avniel-Polak, *et al*. 2015), were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM)-F12 (Gibco), supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich), and HEPG2, hepatocellular carcinoma cells, were cultured in DMEM (Gibco) with 10% FCS. Both cell lines were maintained at 37°C, 5% (vol/vol) CO₂. For menin knock-down experiments 2x10⁵ cells were seeded into each well of 6-well plates and transfected with 25 nM of either control, non-targeting (NT) small interfering RNA (siRNA), or ON-TARGETplus SMARTpool of siRNAs against human *MEN1*, using Dharmafect 1 transfection reagent (all Thermo Scientific) prepared in serum free DMEM, as described (Lines, *et al*. 2018). After the addition of siRNAs, cells were incubated for 48h and RNA (mRNA and miRNA) or protein harvested for further analysis. For miRNA mimic and inhibitor
protein and RNA experiments, 2x10^5 cells were seeded into each well of 6-well plates, and transfected with either 5 nM hsa-miR-3156-5p mimic (Qiagen) or 50 nM hsa-miR-3156-5p inhibitor (Qiagen), using Dharmafect 1 transfection reagent (Thermo Scientific). For controls, 5 nM of NT siRNA (Thermo Scientific) (mimic), and 50 nM control inhibitor (Qiagen) were used. After 48h of transfection cells were harvested for miRNA and protein analysis. For viability assays cells were seeded at a density of 5x10^4 cells/ml into black walled 96-well plates and transfected with miR-3156-5p mimic and inhibitor, or controls for 48h. After 5 days 20μl Cell Titer Blue (Promega) was added to each well at 5% (vol/vol), incubated for 1 hour at 37°C before fluorescent output being read on a Pherastar Microplate reader (BMG Labtech). For apoptosis assays cells were seeded at a density of 5x10^4 cells/ml into white opaque 96-well plates and transfected with miR-3156-5p mimic and inhibitor, or controls for 48h. After 5 days 75 μl of Caspase 3/7 Glo reagent (Promega) was added per well, incubated for 1h at room temperature, and the luminescent outputs read on a Pherastar Microplate reader (BMG Labtech). For wound healing migration assays cells were seeded at a density of 5x10^4 cells/ml into 24-well plates and transfected with miR-3156-5p mimic and inhibitor, or controls for 48h. Wounds were made in the cell monolayer using a pipette tip and pictures taken using a light microscopes, x10 magnification, after 3 days images were taken again for comparison. Migration was assessed by measuring the size of the wound using Image J software and subtracting the size of the day 3 wound from the day 0 wound.

Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNA was extracted from patient serum or BON-1 cells using the miRVana Paris kit (Ambion), and 1μg used to generate cDNA using the MiScript Reverse Transcription kit (Qiagen). Quantitect primers (Qiagen) were used for mRNA qRT-PCR reactions, and miScript primers (Qiagen) for miRNA reactions, which utilised the Quantitect SYBR green kit (Qiagen), on a RotorGene 5, as described (Lines, et al. 2017). Each test sample was normalized to the geometric mean of reference genes Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and α-tubulin (TUBA1A) (for mRNA),
or RNA, U6 Small Nuclear 6 (RNU6B) and Small Nucleolar RNA, C/D Box 95 (SNORD95) (for miRNA’s). Serum samples were spiked with 20 fmol cel-miR-39-3p (Qiagen) prior to miRNA extraction as a reference control. Analysis of serum samples were performed on miRNAs extracted on a minimum of 3 separate occasions from different frozen aliquots, and cell line studies were performed in n=4 biological replicates. The relative expression of target cDNA in all qRT-PCR studies was determined using the Pfaffl method (Pfaffl 2001).

Western blot analysis

NET cells were lysed in NP40 lysis buffer and prepared in 4x Laemmlı loading dye (BioRad) boiled at 95°C for 5 min, resolved using 6% or 10% SDS-PAGE gel electrophoresis, and transferred to polyvinylidene difluoride membrane, as described (Lines, et al. 2017). Membranes were probed with the primary antibodies rabbit anti-menin, rabbit-anti mortality factor 4 like 2 (MORF4L2), rabbit-anti GAPDH or rabbit-anti calnexin (all Abcam), and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology), as described (Lines, et al. 2017). Blots were visualised using Pierce Enhanced chemiluminescence (ECL) Western blotting substrate (Thermo Fisher Scientific), as described (Lines, et al. 2017). GAPDH or calnexin protein expression was determined as a loading control. Densitometry analysis was performed by calculating the number of pixels per band using ImageJ software. Data was represented as the number of pixels of the protein band, relative to the number of pixels of the corresponding GAPDH or calnexin band.

In silico analysis

The miRNA target prediction database (MiRDB) (http://mirdb.org) (Chen and Wang 2019; Liu and Wang 2019) was examined for potential targets of the miRNA miR-3156-5p. Targets were searched using the term ‘miR-3156-5p' and limited to human targets only. The database was last accessed on 5th August 2021.
Statistical Analysis

Data were analysed using Students t-test where there were only 2 groups, or one-way ANOVA using a Bonferroni correction for multiple comparisons where there were >2 groups.

Results

miRNAs are dysregulated in the serum of MEN1 patients.

Profiling analysis of the test cohort of 4 MEN1 patients (2 females and 2 males) identified 5 miRNAs that were upregulated, and 6 miRNAs that were downregulated in the serum of all MEN1 patients, compared to their matched control relative (Table 2). These miRNAs were all dysregulated by greater than 2-fold and p<0.05. The two most highly upregulated miRNAs were miR-125a-3p (4.38-fold, p=0.03) and miR-582-3p (4.06-fold, p=0.04), and the two most highly downregulated miRNAs were miR-3156-5p (-11.62-fold, p=0.02) and miR-3168 (-3.66-fold, p=0.01) (Table 2).

MiR-3156-5p is downregulated in the serum of MEN1 patients

The observed up- and down-regulation of the miRNAs from our sequencing analysis (Table 2) was confirmed using qRT-PCR analysis in samples from a validation cohort of an additional 5 MEN1 patients (3 females and 2 males) and sex matched unaffected relatives (Table 1). This showed that miR-3156-5p was significantly downregulated by 2.4-fold (p<0.005) in the serum of MEN1 patients, when compared to the unaffected control relatives (Figure 1A). Moreover, all 5 MEN1 patients also demonstrated a significant decrease in miR-3156-5p, when compared to their unaffected control relative, with a range of 1.3-fold (p<0.05) to 5.8-fold (p<0.0005) (Figure 1B). Analysis of miR-3156-5p expression in a MEN1 patient who had undergone significant treatment to remove their tumours, including parathyroidectomy, pancreatectomy and gastrectomy, indicated there to be no significant difference when compared to their unaffected control relative (P10, Figure 1B).
alterations were not consistently observed in the expression of miRNAs miR-125a-3p, miR-582-3p, or miR-3168 (Supplementary Figure 1).

MiR-3156-5p expression is downregulated after MEN1 knock-down

To determine whether the downregulation of miR-3156-5p was a consequence of loss of MEN1 expression we undertook MEN1 knock-down experiments in the menin-expressing pancreatic NET cell line, BON-1. Knock-down after 48h was confirmed by: qRT-PCR, which showed a decrease in MEN1 mRNA by 49% (p<0.05), compared to control NT siRNA controls (Figure 2A); and Western blot analysis, which showed a 54% reduction (p<0.05) in the expression of menin (Figure 2B and C). This menin knock-down was associated with a 20% (p<0.005) decrease in miR-3156-5p expression when compared to control treated cells (Figure 2D).

MORF4L2 is a target of miR-3156-5p.

The biological consequences of the downregulation of miR-3156-5p expression were investigated using an in silico approach to identify potential target genes, and examination of the miRDB (http://mirdb.org) database revealed a total of 353 predicted targets. The top 25 ranked targets (Table 3) comprised genes involved in a wide range of cellular activities including: regulation of gene transcription (e.g. Pleiomorphic adenoma gene 1 zinc finger (PLAG1), Promyelocytic Leukemia nuclear body scaffold (PML), RNA polymerase III subunit G (POLR3G), Zinc finger protein 273 (ZNF273), 5’-3’ exoribonuclease 1 (XRN1), Zinc finger protein 711 (ZNF711), and CCR4-NOT transcription complex subunit 7 (CNOT7)); ion transport (e.g. ChaC Glutathione Specific Gamma-Glutamylcyclotransferase 2 (CHAC2), and Potassium inwardly rectifying channel subfamily J member 16 (KCNJ16)); ras signalling (e.g. Ras-related protein rap-1a, member of ras oncogene family (RAP1A), and Ras-related protein rab-27a, member of ras oncogene family (RAB27A)); and cell cycle regulation (e.g. Cell division cycle 123 (CDC123), and Syndecan 2 (SDC2)). In addition, mortality factor 4 like 2 (MOR4FL2) was identified as a potential target. This was of particular interest...
as this transcript has been described as part of the neuroendocrine neoplasms test (NETest), which consists of 51 transcripts that are upregulated in NETs, and is used as a blood biomarker test for the management and diagnosis of multiple NET subtypes including gastro-enteropancreatic and bronchopulmonary NETs (Modlin, et al. 2014; Modlin, et al. 2018b; Modlin, et al. 2016).

**MORF4L2 expression is regulated by miR-3156-5p**

To investigate the role of miR-3156-5p in regulating the expression of MORF4L2 we transfected BON-1 cells with miRNA mimics and inhibitors and assessed for alterations in MORF4L2 transcripts and protein. Successful transfection of a miR-3156-5p mimic was confirmed using qRT-PCR, which demonstrated a 7566-fold increase (p<0.0001) in miR-3156-5p expression (Figure 3A). Expression of miR-3156-5p was not altered after inhibitor treatment (Figure 3B), but this was to be expected as the inhibitor blocks the activity of the miRNA by complementary binding, thereby removing the ability of miR-3156-5p to bind to its target mRNAs, rather than by reducing its expression. Transfection with the miR-3156-5p mimic significantly reduced MORF4L2 protein expression, assessed by Western blot and densitometry quantification, by 46% (p<0.005), when compared to control treated cells (Figure 3C and D). Treatment with miR-3156-5p inhibitor significantly increased MORF4L2 expression 1.5-fold when compared to control (p<0.05) (Figure 3E and F). To determine whether changes in miR-3156-5p affected BON-1 cellular function we also undertook cell viability (Figure 4A), apoptosis (Figure 4B) and migration assays (Figure 4C) after miR-3156-5p mimic and inhibitor treatment. This showed no significant differences. To confirm these mechanistic insights into the regulation of MORF4L2 we also transfected HEPG2 cells with miR-3156-5p mimic. Successful transfection resulting in a 346-fold increase (p<0.005) in miR-3156-5p expression was confirmed by qRT-PCR (Figure 5A). This increased miR-3156-5p expression caused a 21% (p<0.005) decrease in MORF4L2 expression, as shown by Western blot (Figure 5B and C). Similar to BON-1 cells this change in miR-3156-5p and MORF4L2 expression did not result in changes in cell viability (Figure 5D), apoptosis (Figure 5E) or cell migration (Figure 5F).
Discussion

Our studies have revealed that miR-3156-5p is significantly downregulated in the sera of MEN1 patients (Table 2 and Figure 1) and that this downregulation of miR-3156-5p may be a direct result of reduced MEN1 expression (Figure 2). MiR-3156-5p is a human specific mature miRNA that is processed from the transcribed stem loop miR-3156-2, the sequence for which is located within an intron of the ankyrin repeat domain 30B (ANKRD30B) on chromosome 18. The potential utility of miR-3156-5p as a serum biomarker has been investigated in patients with breast, colorectal, lung, thyroid and melanoma tumours (Ferracin, et al. 2015). For example, miR-3156-5p was reported to be significantly upregulated in the tumour tissue of patients with metastatic colorectal cancer (mCRC), who responded to treatment with bevacizumab/5-flourouracil, leucovorin, oxaliplatin (FOLFOX), when compared to tumour tissue from non-responding patients (Kiss, et al. 2017). Moreover, miR-3156-5p in combination with 3 other predictive miRNAs correctly identified responders to the bevacizumab/FOLFOX therapy with 82% sensitivity and 64% specificity (Kiss, et al. 2017). In breast cancer miR-3156-5p has been reported to target the proapoptotic gene, Caspase 2 (CASP2) and the long non-coding RNA tumour protein translationally controlled 1 (TPT1) antisense RNA 1(TPT1-AS1), thereby facilitating TPT1-AS1 inhibition of cell proliferation and sensitisation of breast cancer cells to chemotherapy (Huang, et al. 2021). Based on these studies, the application of using miR-3156-5p, in combination with other biomarkers, represents a novel approach to evaluate cancer progression, prognosis and sensitivity to treatment.

In our study, we demonstrated miR-3156-5p to be consistently significantly downregulated in the sera of 9 individual MEN1-patients, compared to sex-matched unaffected control relatives (Figure 1). All patients had a parathyroid adenoma, however each patient exhibited different tumour manifestations, ranging from 1 MEN1-associated tumour, to 3 MEN1-associated tumours (Table 1). However our
study consists of a small sample size, and due to the historical nature of the samples collected only limited samples and clinical data was available. Therefore, our data does not take into account tumour size, or ongoing treatments, or miR-3156-5p and MORF4L2 expression levels with the tumours. A larger, prospective study would therefore be required to determine whether miR-3156-5p either alone or in combination (i.e. with existing biomarkers for example chromogranin A, or the neuroendocrine neoplasms test (NETest), or hormone levels e.g. insulin) could be a reliable MEN1-associated NET biomarker, and whether this would correlate with disease burden. This could include recruiting patients with different types of NETs, for example pancreatic versus thoracic NETs, as well as subgroups of MEN1 patients, for example those without NETS, those with small <2cm NETs, and those with metastatic lesions, as well as those with syndromes including Zollinger–Ellison syndrome.

Our in silico analysis (Table 3) identified MORF4L2 as a potential predicted gene target of miR-3156-5p, and our in vitro functional studies in BON-1 and HEPG2 cells confirmed that miR-3156-5p regulates MORF4L2 expression (Figures 3 and 5). MORF4L2 is a component of the NuA4 histone acetyltransferase complex, which catalyses the acetylation of histone H2A and H4 tails. This nucleosomal modification alters the interaction between DNA, histones and other proteins that facilitate the transcriptional activation of selected genes involved in the activation of oncogene and proto-oncogene-mediated growth induction, tumour suppressor mediated growth arrest, replicative senescence, suppressed apoptosis and DNA repair (Kuete, et al. 2012). In our studies miR-3156-5p expression changes did not alter cell viability, apoptosis or cell migration (Figures 4 and 5). This is likely because key oncogenic changes have already occurred in these cell lines to drive these mechanisms. MORF4L2 is also a component of the NETest, which is reported to be of use in the management and the diagnosis of multiple NET subtypes. The NETest, which is a blood biomarker test that comprises 51 transcripts that are upregulated in NETs (Modlin, et al. 2014; Modlin, et al. 2018b; Modlin, et al. 2016), is reported to be the highest predictive assessment method for NET disease status and progression (69%), when compared to other single secreted NET biomarkers.
assays, such as CgA (13%) (Modlin, et al. 2014). For example, the NETest is reported to have a sensitivity of >95% and specificity of >90%, making it more accurate than CgA at monitoring NET disease occurrence, progression and response to therapies (Modlin, et al. 2018a). Although developed for sporadic NETS, this NETest can detect multiple NET subtypes, and therefore may have utility in MEN1 patients. However, MEN1 patients commonly have concurrent tumours, and it seems that modifications to the NETest are likely to be required to improve its diagnostic use in such patients. Moreover, currently the measurement of miRNAs in the circulation remains complex and is not available as a standardised assay in clinical practice (Kidd, et al. 2015; Oberg, et al. 2015). However, the use of matched miRNA and transcript data, such as miR-3156-5p and MORF4L2 respectively, may be a way of refining and improving currently available biomarker tests for monitoring NET disease and progression in MEN1 patients, as it would be expected that miR-3156-5p levels would decrease whilst MORF4L2 levels simultaneously increase with increasing tumour burden. Thus, longitudinal analysis of miR-3156-5p and MORF4L2 within an individual MEN1 patient could provide important information on when tumour development has occurred, and aid in determining the appropriate timing to initiate more invasive screening methods.

Our data indicating that menin can regulate miR-3156-5p (Figure 2), which in turn can regulate MORF4L2 expression (Figure 3) provides additional novel insights into the importance of miRNA regulation in NET development. A role for miRNAs in the regulation of menin expression, and in the development of MEN1-associated tumours has previously been reported. For example, in a Men1 knockout mouse model, loss of cell cycle control and pituitary tumourigenesis were associated with miR-15a, miR-16-1 and let-7a downregulation and cyclin D1 upregulation in pituitary adenomas compared to normal wild-type pituitaries (Lines, et al. 2019). Further in vitro functional studies in AtT20 mouse pituitary cell lines transfected with Men1 siRNA, confirmed that loss of menin expression resulted in decreased miR-15a expression (Lines, et al. 2019). In addition, overexpression of miR-17 has been shown to promote pancreatic beta cell proliferation by downregulating menin
expression in the MIN6 mouse insulinoma-derived pancreatic beta cell line (Lu, et al. 2015). Studies of human parathyroid adenomas have also demonstrated negative feedback between, MEN1 mRNA, menin and miR-24-1, whereby miR-24-1 silences menin expression post-transcriptionally, to mimic the second hit of Knudson’s model of tumourigenesis (Luzi, et al. 2016). Parathyroid adenomas from MEN1 patients with a heterozygous MEN1 mutation are also reported to have reduced MEN1 mRNA levels, lack menin expression and overexpress miR-24-1, despite the presence of one wild-type MEN1 allele (Luzi, et al. 2012). More recently, studies have reported additional miRNAs (such as miR-28, miR-4258, miR-1301 and miR-664) as potential mediators of MEN1 parathyroid tumourigenesis by similarly silencing MEN1 or other tumour suppressor genes, such as CCND1, RET, CDKN1B, RB1, VDR, PRDM2, CDKN2C and CDC73 (Grolmusz, et al. 2017; Luzi, et al. 2017). Thus, the interaction of miRNAs and menin may be crucial in regulating and monitoring NET development, and could provide novel biomarker panels for MEN1 patients. For example, a biomarker panel consisting of multiple dysregulated miRNAs with their corresponding target proteins (e.g. miR-3156-5p and MORF4L2) could provide blood biomarkers with high sensitivity and specificity. This approach could also be utilised to identify novel miRNA-protein target combinations that act as specific biomarkers for different tumours, including gender specific tumours, for example thymic carcinoids in males, and bronchial carcinoids in females, which will also help inform on the underlying biology.

In summary, our results, which reveal an inverse relationship between miR-3156-5p and MORF4L2 expression, may help to increase the reliability of non-invasive blood biomarkers for the diagnosis, progression and treatment outcomes of NETs in MEN1 patients.

**Declaration of interest**

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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Figure legends

**Figure 1. miR-3156-5p downregulation in serum of MEN1 patients.** MiR-3156-5p expression was reduced in the serum of 5 MEN1 patients, when compared to sex matched control (unaffected) relatives (A). N=3-4 technical replicates were undertaken for each patient and controls with n=5 biological replicates. A t-test was used to determine significance; ***p<0.005. Data on all 5 patients (P5-9, Table 1) was then assessed individually, with samples arranged by the number of MEN1-associated tumour manifestations, to determine if all showed statistically significant reductions in miR-3156-5p compared to controls. A patient (P10) who had undergone extensive treatment and was described as tumour free has also been included for comparison (B). N=3-4 technical replicates were undertaken for each patient and control. One-way ANOVA was undertaken to determine statistical significance; *p<0.05, ***p<0.0005.

**Figure 2. Expression of miR-3156-5p after menin knock-down.** BON-1 cells were treated with siRNA for MEN1, or control non-targeting (NT) siRNA. Untransfected (UT) cells were used as controls. Quantitative reverse transcription PCR (qRT-PCR) was used to evaluate MEN1 expression (A). Data is represented relative to UT cells. Studies were undertaken in n=4 biological replicates. Statistical significance comparing all treatments to each other were assessed by one-way ANOVA; *p<0.05. Menin protein, encoded by MEN1, was assessed by Western blot analyses, with calnexin (housekeeper) used as a control (B). A representative Western blot is shown. Protein expression from the Western blot was quantified using densitometry analysis from n=4 biological replicates (C). Data is represented relative to UT cells. Statistical significance comparing all treatments to each other was assessed by one-way ANOVA; *p<0.05. Mir-3156-5p expression after MEN1 knock-down was evaluated using qRT-PCR (D). Data is represented relative to UT cells. Studies were undertaken in n=4 biological replicates, and statistical significance comparing all treatments to each other assessed by one-way ANOVA; **p<0.005.
Figure 3. Regulation of MORF4L2 expression by miR-3156-5p. BON-1 cells were transfected with either miR-3156-5p mimic or inhibitors for 48 hours, and MORF4L2 expression assessed. Confirmation of miR-3156-5p mimic transfection was undertaken using qRT-PCR (A). Untransfected (UT) cells, and non-targeting (NT) scrambled RNA treated cells were used as controls. Experiments were performed in n=4 biological replicates. Data is represented relative to UT cells, with statistical significance comparing all transfections to each other assessed by one-way ANOVA; ***p<0.0001. To determine if inhibitor transfection altered miR-3156-5p expression qRT-PCR analysis was undertaken (B). UT and control miRNA inhibitor transfections were used as controls. Experiments were performed in n=4 biological replicates. Data is represented relative to UT cells, with statistical significance comparing all transfections to each other assessed by one-way ANOVA; ns = not significant. MORF4L2 expression after mimic treatment was assessed by Western blot analyses (C). A representative image from n=4 biological replicates is shown. GAPDH was used as a housekeeper. Western blot analyses were quantified using densitometry analysis (D) Data is represented relative to UT cells. Studies were undertaken in n=4 biological replicates, and statistical significance comparing all treatments to each other assessed by one-way ANOVA; **p<0.005. MORF4L2 expression after inhibitor treatment was also assessed by Western blot analyses (E). A representative image from n=4 biological replicates is shown. GAPDH was used as a housekeeper. The Western blots were quantified using densitometry analysis (F). Data is represented relative to UT cells. Studies were undertaken in n=4 biological replicates, and statistical significance comparing all treatments to each other assessed by one-way ANOVA; *p<0.05.

Figure 4. Functional analysis after dysregulation of miR-3156-5p in BON-1 cells. BON-1 cells were transfected with either miR-3156-5p mimic or inhibitors for 48 hours, untransfected (UT) cells, and non-targeting (NT) scrambled RNA treated cells, or control inhibitor treated cells were used as controls. All experiments were performed in n=4 biological replicates. Cell viability was assessed 5
days after transfection using Cell Titer blue assay. Data is shown relative to UT cells. Statistical analysis using one-way ANOVA indicted no significant difference (A). Apoptosis was assessed using Caspase 3/7 Glo assay 5 days post transfection. Data is shown relative to UT cells. Statistical analysis using one-way ANOVA indicted no significant difference (B). Cell migration was assessed 5 days post transfection using wound healing assays. Data is shown relative to UT cells. Statistical analysis using one-way ANOVA indicted no significant difference (C).

**Figure 5. Mir-3156-5p regulates MORF4L2 in HEPG2 cells.** HEPG2 cells were transfected with miR-3156-5p mimic for 48 hours. Untransfected (UT) cells, and non-targeting (NT) scrambled RNA treated cells were used as controls. Experiments were performed in n=4 biological replicates. Confirmation of miR-3156-5p mimic transfection was undertaken using qRT-PCR. Data is represented relative to UT cells. Statistical analysis was performed using one-way ANOVA, **p<0.005 (A).** MORF4L2 expression after mimic treatment was assessed by Western blot analyses. A representative image from n=4 biological replicates is shown. Calnexin was used as a housekeeper (B). Western blot analyses were quantified using densitometry. Statistical analysis was performed using one-way ANOVA, **p<0.005 (D).** After 5 days of transfection the effect of miR-3156-5p over expression was assessed in cell viability (E), apoptosis (F) and migration assays (G). No significant difference was observed in any of the three assays.
Figure 1
Figure 2

A

B

C

D

* UT

NT

MEN1

menin

calnexin

Relative MEN1 expression

Relative menin expression

Relative miR-3156-5p expression

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Figure 3
Figure 4
Figure 5
Table 1. Patients analysed for serum miRNA changes.

<table>
<thead>
<tr>
<th>MEN1 Patient</th>
<th>Gender</th>
<th>MEN1 mutation</th>
<th>~Age at time of blood sampling</th>
<th>Tumours present</th>
<th>Matched Relative Non-MEN1 Control</th>
<th>Gender</th>
<th>Relationship to patient</th>
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<tr>
<td>Test</td>
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<td></td>
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<td>1</td>
<td>Male</td>
<td>10BP insertion (exon 2) 63-66:fs51aaX</td>
<td>55</td>
<td>Gastrinoma, Parathyroid adenoma</td>
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<td>Brother</td>
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<tr>
<td>2</td>
<td>Male</td>
<td>GCT to CCT (exon 3) Ala160Pro</td>
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<td>3</td>
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<td>4 BP:CAGT (exon 3) 210/211:fs11aaX</td>
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<td>Sister</td>
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<td>7</td>
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<td>9</td>
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<td>10BP ins CCAGCCCCAGC (exon 2) 63-66:fs51aaX</td>
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<td>Surgical patient</td>
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<td>Glu 388 Stop</td>
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<td>Parathyroidectomy, partial pancreatectomy and gastrectomy</td>
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<td>Male</td>
<td>Brother</td>
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**Table 2.** Dysregulated miRNAs in 4 test MEN1 patients compared to unaffected relatives.

Data is sorted by fold change, and is represented as an average of the fold change occurring in the 4 MEN1 patients. LogCPM – log2 of counts per million reads.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Fold Change</th>
<th>LogCPM</th>
<th>P Value</th>
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<tbody>
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<tr>
<td>hsa-miR-125a-3p</td>
<td>4.38</td>
<td>5.78</td>
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<td>hsa-miR-582-3p</td>
<td>4.06</td>
<td>6.52</td>
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<tr>
<td>hsa-miR-654-5p</td>
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<td>hsa-miR-215</td>
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<td>hsa-miR-9-5p</td>
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Table 3. Predicted gene targets of miR-3156-5p according to miRDB (http://mirdb.org). In total there were 353 predicted targets, the top 25 of which are shown, ranked by their assigned target score.

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<thead>
<tr>
<th>Target Rank</th>
<th>Target Score</th>
<th>Gene Symbol</th>
<th>Gene Description</th>
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<td>divergent protein kinase domain 2A</td>
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<td>96</td>
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<td>MORF4L2</td>
<td>mortality factor 4 like 2</td>
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</table>
Supplementary Figure 1. Expression of candidate miRNAs in a validation cohort of 5 MEN1 patients. Quantitative reverse transcription-PCR (qRT-PCR) was undertaken on the serum of an additional 5 MEN1 patients, with data shown relative to that of a sex matched unaffected relative. The two most highly upregulated miRNAs in the profiling experiment (miR-125a-3p and miR-582-3p) are indicated in red, and the second most highly downregulated miRNA (miR-3168) is indicated in green. A significant alteration in expression in these miRNAs was not observed. All experiments were undertaken with n=4 technical replicates.