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Eva Maher
Technological University of the Shannon, Midlands Midwest, Athlone, a00264315@student.ait.ie

Ava O'Meara
Technological University of the Shannon: Midlands, a00241207@student.ait.ie

Cathy Brougham
Technological University of the Shannon Athlone, cathy.brougham@tus.ie

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Regulation of miRNAs in obesity and diabetes

Eva Maher[1], Ava O'Meara-Cushen[2] and Cathy L. Brougham[1*]
[1] Department of Pharmaceutical Science and Biotechnology, Faculty of Science of Health, Technological University of the Shannon Midlands Midwest, Athlone, County Westmeath, Ireland

[2] Department of Nursing and Healthcare, Faculty of Science of Health, Technological University of the Shannon Midlands Midwest, Athlone, County Westmeath, Ireland

*Corresponding Author e-mail: cathy.brougham@tus.ie

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Abstract

Introduction: The prevalence of obesity and obesity-related secondary disorders has increased dramatically in recent years. Dysregulated microRNA (miRNA) expression has been linked to the development of obesity and its associated disorders. Adipose derived stem cells (ADSCs) are promising cell models of obesity, obesity-related inflammation and diabetes. Furthermore, miRNA expression profiles have emerged as promising targets for the novel treatment of diseases such as obesity. In order to enhance miRNA-based therapies, the miRNA expression profile of obesity and its related disorders must be monitored to further understand the underlying mechanisms of obesity and to identify novel targets.

Aim: The aim of this study was to identify the expression profile of six target miRNAs and three target cytokines. The expression of these targets was compared to control ADSCs, ADSCs differentiated into adipocytes and ADSCs from diabetic patients using a series of tissue culture, molecular biology, and biochemical techniques. This study compares these findings with available literature to identify miRNAs and cytokines that could be novel targets in obesity and diabetes-based therapeutic approaches.

Results: The expression of miR-A, miR-221, miR-222, miR-21, miR-146 and miR-133 were significantly dysregulated in obese and diabetic patients compared to controls. MiR-A, a novel miRNA, was identified in this study and was significantly increased in diabetes compared to control samples. Transforming Growth Factor-beta (TGF-β), Tumour Necrosis Factor-alpha (TNF-α) and Vascular Endothelial Growth Factor A (VEGFA) were differentially expressed across each of the cell cohorts investigated.

Conclusion: This study identified a novel miRNA and revealed that significant changes in miRNA and cytokine expression have the potential to identify obese patients from diabetic patients. The identification of dysregulated miRNAs and in particular novel dysregulated miRNAs, may lead to the development of miRNA-based personalised therapeutics for obesity and diabetes.

Keywords: ADSCs, obesity, diabetes, mRNA, cytokines.

1. Introduction

The hallmark characteristic of obesity is a chronic low-grade inflammatory state caused by the release of proinflammatory cytokines from fat tissue (Kim and Nam, 2020). Low-grade inflammation associated with obesity has been known to increase insulin resistance, and hence has emerged as a promising target to treat diabetes (Rohm *et al.*, 2022; Zatterale *et*

al., 2020). In addition, previous research has identified genes in obese and diabetic individuals that are associated with processes such as inflammation and adipocyte differentiation (Molina-Ayala *et al.*, 2022). Such genes associated with obesity and diabetes are regulated by miRNAs. MiRNAs are a class of small noncoding RNAs approximately 22-nucleotides in length that regulate gene expression by degrading their messenger RNAs (mRNAs) and/or inhibiting their translation at the posttranscriptional level (O'Brien *et al.*, 2018). Transcription of miRNAs is performed in the nuclease by polymerase II where the stem loop is removed from the DNA transcript to create pri-miRNA (Nguyen *et al.,* 2020). Drosha enzyme catalyses pri-miRNA to pre-miRNA and is transported to the cytoplasm where cleavage is carried out by the Dicer enzyme to form double-stranded RNA (dsRNA) (Yoshida *et al.,* 2021). Dicer cuts the dsRNA to isolate the miRNA strand of interest where it is inserted into the RNA induced silencing complex (RISC), a ribonucleoprotein complex, forming miRNA-induced silencing complex (miRISC). Thus, miRNAs can regulate gene expression by degradation or inhibition of the translation and stability of the target mRNAs (Oliveto *et al.,* 2017).

Cytokines are small proteins secreted by ADSCs which have a role in cell-cell communication and can induce the secretion of angiogenic and anti-inflammatory factors (Kany *et al.,* 2019; Zhang and An, 2007). The role of cytokines in immunoregulation and inflammation is clearly understood. It has been documented that polymorphisms and mutations of cytokine receptors and their signalling components, leads to dysregulated cytokine expression, which can contribute to autoimmune disorders such as diabetes (Banerjee and Saxena, 2014). Recent research has investigated the specific impact of miRNAs on the immune system and has shown that cytokine expression is targeted by miRNAs, which can act on upstream signals and functional outputs, which in turn can affect miRNA levels (Garavelli *et al.,* 2018). MiRNAs have recently been recognized as powerful regulators of numerous genes and pathways in the pathogenesis of inflammatory and autoimmune diseases. Several miRNAs have been investigated to be up- or down- regulated in relation to these diseases, and could potentially be used as tools for the prevention and treatment of such diseases. However, for miRNAs to be used for therapeutic management, the molecular mechanism, and pathways they target need to be fully elucidated (Tang *et al.,* 2021).

Stem cells are capable of self-renewal and differentiation into several cell types (Zakrzewski *et al.,* 2019). In particular, adult stem cells have gained popularity in recent years as they have less legal considerations related to their use compared to embryonic stem cells, and they can be isolated relatively easily from many types of adult tissue (Lo and Parham, 2009). Sources of these adult stem cells include the bone marrow, muscle, blood, and adipose tissue (Gurusamy *et al.,* 2018; Łos *et al.,* 2019). Mesenchymal stem cell (MSC) are a class of non-haematopoietic adult stem cell than can isolated from most tissues, such as from the stromal vascular fraction (SVF) of adipose tissue in the form of ADSCs (Pittenger *et al.,* 2019). ADSCs are commonly used in research as they can be isolated through non-invasive methods and in large quantities from adipose tissue, which is an abundant tissue in the human body (Si *et al.,* 2019).

ADSC-based therapies have advanced modern medicine to provide novel approaches in regenerative medicine, tissue reconstruction, inflammatory diseases treatments e.g., Crohn's disease, obesity and diabetes management (Mazini *et al.,* 2021; Qi *et al.,* 2021; Yang *et al.,* 2021; Yu *et al.,* 2019; Zhou *et al.,* 2020). Several factors, such as miRNAs,

have been linked to adipocyte differentiation and adipogenesis, and their dysregulation can influence the precipitation of obesity and diabetes (Heyn *et al.*, 2020; Pant *et al.*, 2021). Previous studies have investigated the relationship between miRNAs, adipogenesis and obesity, however the underlying mechanism of regulation needs to be fully elucidated (Zaiou *et al.*, 2018; Zhang *et al.*, 2019; Zhang *et al.*, 2021).

Inflammatory cytokines and adipokines secreted by adipose tissue have been implicated in chronic low-grade inflammation observed in obesity, with miRNAs playing a key role in the modulation of these signalling molecules (Lischka *et al.*, 2021). Animal derived cell lines (murine, porcine, and feline) have studied adipogenesis, with the use human ADSCs becoming increasing popular due to the advancement of ADSC models in recent years (Bahmad *et al.*, 2020). The exact effect of ADSCs in diseases such as obesity has not yet been definitively defined and there is a need for a complete understanding of the mechanisms and regulatory influences in adipogenesis, in order to advance potential ADSC-based treatments for obesity and its related disorders (Matsushita and Dzau, 2017). **Figure 1** depicts the link between miRNA expression in ADSCs, obesity and diabetes, with a suggested link to cancer.

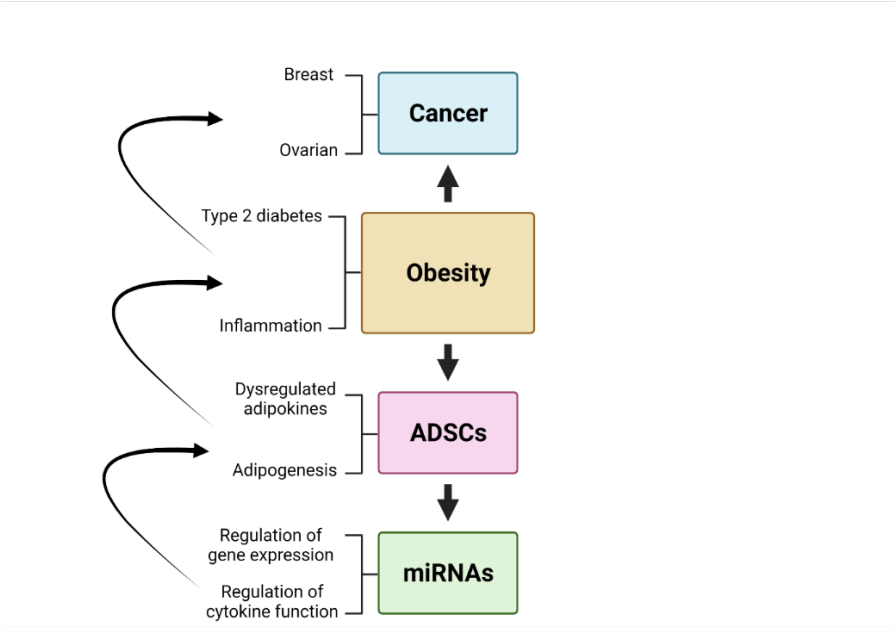

Figure 1: The link between miRNA dysregulation and obesity (Maher *et al.*, 2022)

The aim of this study was to examine miRNA and cytokine profiles in different cohorts of ADSCs, focusing on obesity and diabetes. This study seeks to determine if miRNAs are expressed and moreover dysregulated in obesity and diabetes, with the aim of contributing to future studies that identify potential novel therapeutic targets for the treatment of these diseases.

2. Materials and Methods

Cell Culture

Control ADSCs and ADSCs from patients with diabetes: With informed patient consent and ethical approval, primary human ADSCs (hADSCs) were isolated from adipose tissue from healthy volunteers and diabetic patients. hADSCs were digested using collagenase digestion. Isolated cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (SigmaAldrich) supplemented with 10% Foetal Bovine Serum (FBS) (SigmaAldrich), 1% Penicillin/Streptomycin (SigmaAldrich) and 1% L-Glutamine (SigmaAldrich). The cells were maintained at 37 °C in a 5% CO_2 incubator. hADSCs were cultured with fresh media for 48 hours. After 48 hours, the conditioned media (CM) was isolated and stored at -80 ° C for analysis by enzyme-linked immunosorbent assay (ELISA). The cell monolayer was harvested, and the miRNA content was extracted from the cells using the mirVana™ miRNA isolation kit (ThermoFisher). The isolated miRNA was stored at -80 ° C for quantification by quantitative real time polymerase chain reaction (RT-qPCR).

Adipocytes: Cultured hADSCs were maintained at 37 ° C and 5 % CO_2. Once the cells achieved confluency, after 2-3 days incubation, adipogenesis was induced by the addition of adipogenic induction medium (HG-DMEM, 10 % FBS, 1 % Penicillin/Streptomycin, 1µM Dexamethasone, 10µg/ml Insulin, 200µM Indomethacin and 500µM methyl-iso-butyl-xanthine). Cells were cultivated in the induction media for 3 days. During this time, control cells received normal hADSC growth medium. After a 3-day incubation, the induction media was aspirated to waste and adipogenic maintenance medium was applied to the cells for 1 day. This induction-maintenance media cycle was repeated three times. The ceiling culture method was then performed to isolate a pure culture of adipocytes, separating differentiated adipocytes and hADSC populations by exploiting the different buoyancy characteristics of these cells. This was performed by adding a suspension of differentiated hADSCs to a T25 flask filled to the top with adipogenic maintenance medium. The flask was incubated with the adherent surface facing upwards for 24 hours at 37 °C and 5% CO_2. The adipocytes floated to the surface of the media and adhered to the adherent surface of the flask. Any remaining undifferentiated cells remained on the bottom of the flask. The undifferentiated cells were removed. The adipocytes were harvested and maintained for further analysis.

Quantitative reverse transcription PCR (*qRT-PCR)*

RNA was isolated from ADSCs using the mirVana™ miRNA isolation kit (ThermoFisher) as per manufacturers guidelines. Following organic extraction and subsequent precipitation, the isolated RNA was stored at -20°C for complimentary DNA (cDNA) synthesis. To convert mRNA to cDNA, a master mix containing dNTP nucleotides, reverse transcription buffer, nuclease free water, RNase inhibitor, MultiScribe reverse transcriptase, stem loop primers and isolated miRNA was prepared. Samples were placed in the Applied Biosystems (ABI) Gene Amp PCR system 9700 to reverse transcribe mRNA sample to cDNA. The samples were then heated at 16 °C for 30 minutes (mins) and 42 °C for 30 mins, followed by 5 mins at 85 °C. The cDNA was stored at -20 °C for qRT-PCR. For quantification by qRT-PCR, 9.3µl of ABI Fast MasterMix to each cDNA sample and the samples were cycled on the ABI 7500 Fast Real-Time PCR Systems to achieve raw cycle threshold (C_T) values for each target miRNA.

ELISA

A sandwich ELISA was used for analysis of the cytokine secretion from ADSCs (R&D Systems). The antigen was bound between the capture antibody and detecting antibody, where they each detect a different epitope of the antigen. A 96-well plate was coated with the cytokine antibody. The plate was left overnight at 4 °C to allow the antibody to adhere to the plate. 100µl of the controls and samples were added to the appropriate wells. 100µl of the appropriate enzyme conjugate was added to each

well. The plate was incubated in the dark at room temperature for one hour. The plate was washed with 300µl deionized water. The wash step was repeated six times, ensuring no overflow of wash solution mixing in the wells. 100µl of the 3,3', 5,5-tetramethylbenzidine (TMB) horseradish peroxidase (HRP) substrate solution was added to each well. The plate was incubated in the dark at room temperature for 30 mins. 100µl of stop solution was added to the wells. The absorbance was measured using a spectrometer at 450 nm within 15 mins of the addition of the stop solution.

Data Analysis

MiRNA Analysis: Raw C_T values for each miRNA were determined by qRT-PCR. The raw C_T values were processed to generate $\log_{10}$ values for each target miRNA. Boxplots were generated using Minitab 21.1.0 to represent the $\log_{10}$ miRNA expression in each cell cohort (ADSCs, ADSC adipocytes and ADSC diabetes). Statistical differences in miRNA expression between the cell cohorts were denoted by p-values obtained from a series of 2 sample t-tests and Analysis of Variance (ANOVA). Statistical significance was established at the alpha value of 0.05 or lower.

Cytokine Analysis: The absorbance data was processed to determine the concentration of each cytokine in each cell cohort (ADSC, ADSC adipocytes and ADSC diabetes). Cytokine concentrations were analysed using Minitab 21.1.0. Statistical significance between cytokine concentrations in each cell cohort were determined by a series of 2 sample t-tests and ANOVA to generate p-values. Statistical significance was established at the alpha value of 0.05 or lower.

3. Results

MiRNA expression analysis

Several miRNAs were investigated in ADSCs, ADSC adipocytes and diabetic ADSC cohorts. To establish an endogenous control for this particular patient cohort, three potential endogenous controls, miR-16, miR-497 and U6, were assessed. MiR-497 was the most stably and highly expressed endogenous miRNA investigated across all samples, as shown in **Figure 2**. Therefore, miR-497 was utilised as the most suitable endogenous control when investigating the expression of the target miRNAs in this patient cohort.

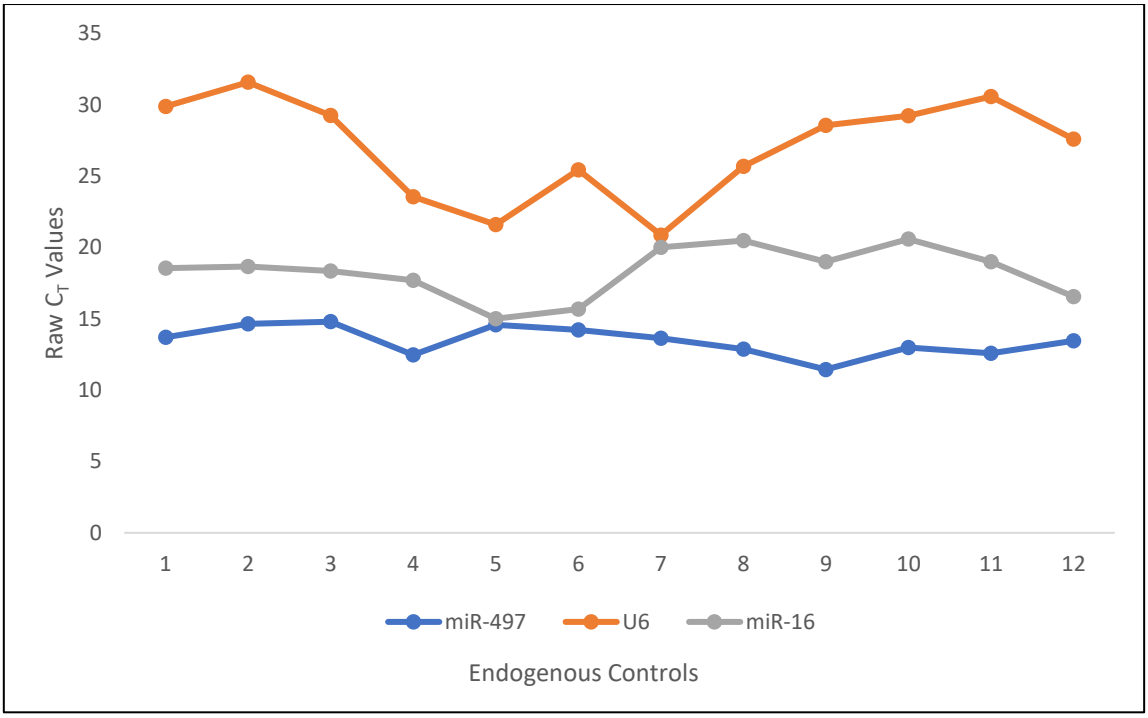

Figure 2: Raw C_T values of endogenous controls, miR-497, U6 and miR-16, using qRT-PCR.

Increased expression in target miRNA expression

MiRNA expression in ADSCs, ADSCs differentiated into adipocytes and ADSCs isolated from diabetic individuals are represented in **Figure 3**. MiR-A expression across ADSC cohorts was significantly dysregulated (ANOVA, $p<0.01$, **Figure 3(A)**). In particular, the expression of miR-A was significantly upregulated in ADSCs from diabetic patients compared to ADSC adipocytes (t-test $p<0.001$, **Figure 3 (A)**). There was no significant difference in miR-A expression when control ADSCs were compared with adipocytes or diabetic ADSCs (t-test $p>0.05$, **Figure 3 (A)**). This may be attributed to the broad range of miR-A expression in the ADSC controls, with some donors exhibiting increased miR-A expression and others displaying decreased miR-A expression.

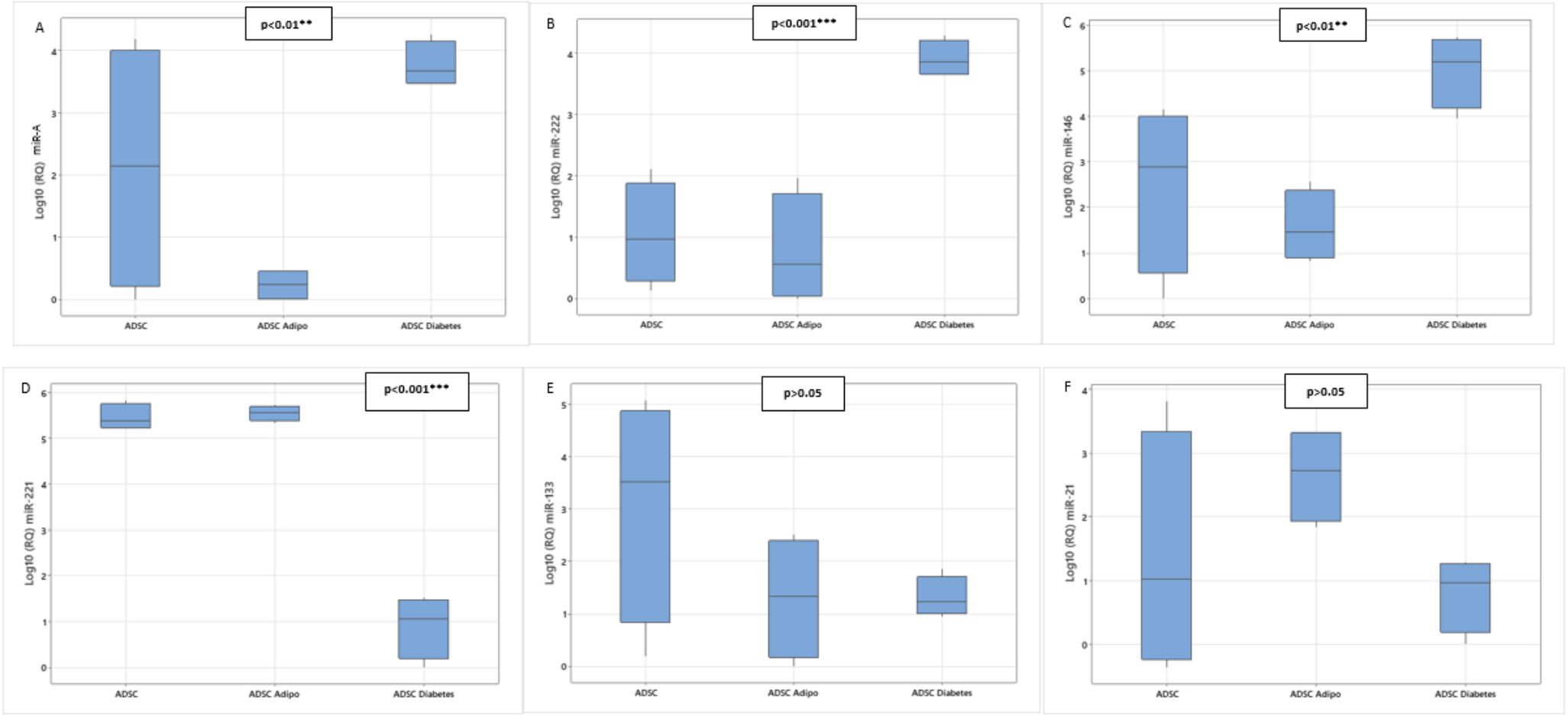

Figure 3 (A-F): MiRNA expression in ADSCs, adipocytes and diabetic ADSCs.

Figure 3 (B) represents miR-222 expression in ADSCs, adipocytes derived from ADSCs and diabetic ADSCs. MiR-222 has been implicated in obesity-related diabetes development, and treatments that target miR-222 could alleviate the adverse effects on insulin signalling associated with its dysregulation. In this study, miR-222 was expressed at significantly different levels in each ADSC culture investigated (ANOVA $p<0.001$). MiR-222 was significantly upregulated in diabetic patient ADSCs compared to control ADSCs and ADSC differentiated adipocytes (t-test $p<0.01$). There was no significant alteration in miR-222 expression across the control ADSC and ADSC adipocyte cohort, which is like miR-221 (t-test $p>0.05$, **Figure 3 (B)**).

Figure 3 (C) represents miR-146 expression in each cell cohort (ANOVA $p<0.01$). MiR-146 is involved in the regulation of obesity-related inflammation, with its dysregulation leading to increased inflammation which can lead to the development of obesity-related disorders. MiR-146 was expressed at a significantly higher level in ADSCs from a diabetic individual than in ADSC differentiated adipocytes (t-test $p<0.01$, **Figure 3 (C)**). There was no statistically significant difference in miR-146 expression in ADSCs compared to both adipocytes and ADSCs from a diabetic patient (t-test $p > 0.05$). This may be attributed to the inconsistency in miR-146 expression in donor ADSCs shown by a broad range of expression.

Decreased target miRNA expression

MiR-221 was significantly differentially expressed across the 3 cohorts (ANOVA $p<0.001$, **Figure 3(D)**). MiR-221 expression was significantly decreased in diabetic ADSCs when compared to control ADSCs, and adipocytes (t-test $p<0.01$). MiR-221 was expressed at a significantly lower level in ADSCs from a diabetic patient (t-test $p<0.01$). There was no significant difference in miR-221 expression in adipocytes compared to control ADSCs (t-test $p>0.05$).

No significant changes in target miRNA expression

MiR-133 expression did not change significantly across donor ADSCs, ADSC differentiated adipocytes or ADSCs isolated from a diabetic individual (ANOVA $p>0.05$, **Figure 3 (E)**). MiR-133 was expressed at varying levels across the donor ADSC cohort, with some donors displaying increased miR-133 expression and others displaying decreased miR-133 expression. The same broad range of miR-133 expression can be observed in the adipocyte and diabetic ADSC cohorts, but not to the same extent as the donor ADSCs.

In addition, miR-21 was not significantly expressed across the three ADSC cohorts (ANOVA $p>0.05$, **Figure 3(F)**). MiR-21 was significantly downregulated in diabetic ADSCs compared to ADSC differentiated adipocytes (t-test $p<0.05$). MiR-21 was not consistently expressed in donor ADSCs, with some donors showing increased expression and some donors displaying decreased miR-21 expression. This may have contributed to the lack of a significant difference in miR-21 between donor ADSCs and the obesity and diabetes models (t-test $p>0.05$)

Cytokine Secretion Analysis

The cytokine secretion profile of each cell sample (ADSCs, ADSC differentiated adipocytes and ADSC diabetes) were analysed to investigate if different subsets of ADSCs secrete distinct levels of TNF-α, TGF-β and VEGFA. The cytokine secretion profiles of each ADSC cohort, ADSCs, ADSC

differentiated adipocytes and ADSC diabetes, were analysed to investigate if different subsets of ADSCs secrete distinct levels of TNF-α, TGF-β and VEGFA (**Figure 4**).

Donor ADSCs expressed the highest level of TNF-α and the lowest level of VEGFA out of each of the three cell populations. Adipocytes, derived from differentiated donor ADSCs, exhibited a different profile to donor ADSCs and diabetic ADSCs, as this group displayed the highest level of TGF-β secretion. TNF-α and TGF-β were expressed the lowest in diabetic ADSCs compared to the other two cohorts, with VEGFA displaying the highest expression of all cytokines investigated.

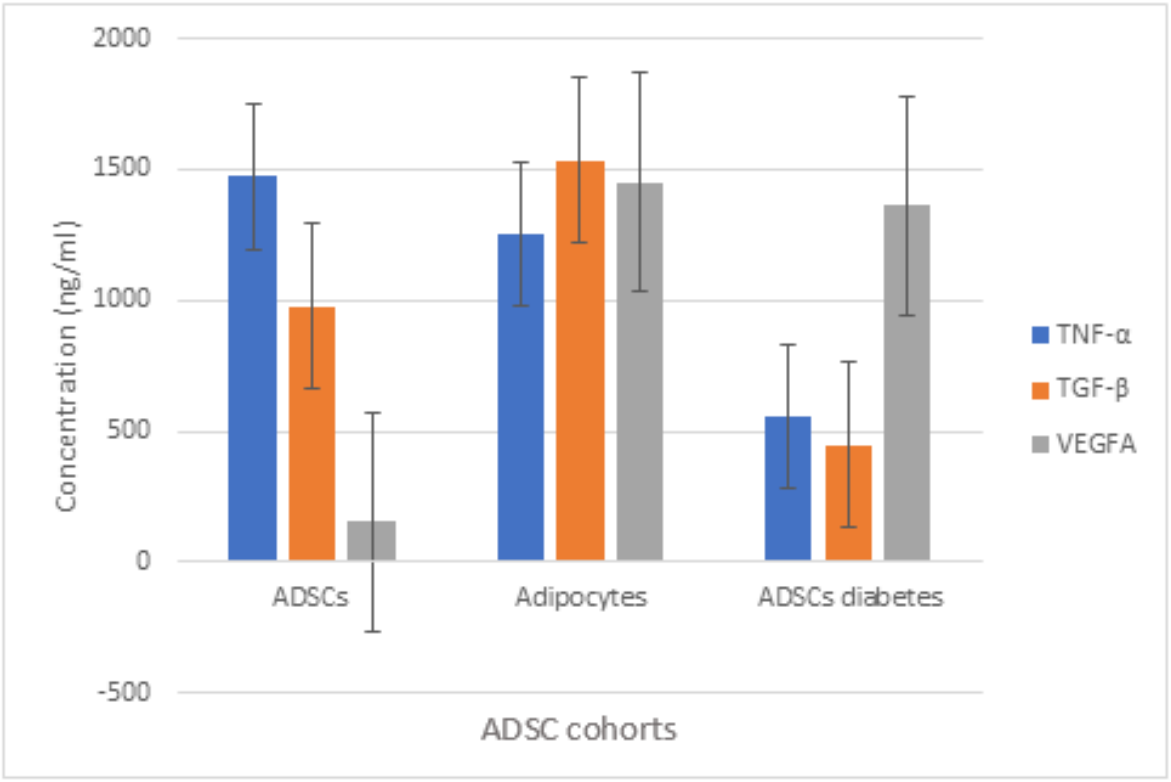

Figure 4: Target cytokine secretion profile (TNF-α, TGF-β and VEGFA) in ADSCs, adipocytes and diabetic ADSCs. Error bars represent standard error of the mean.

TNF-α was significantly decreased across each of the cohorts investigated (ANOVA $p<0.001$). Donor ADSCs exhibited the highest level of TNF-α secretion. When compared, adipocytes secreted TNF-α at a significantly lower level than donor ADSCs (t-test $p<0.01$, **Figure 5 (A)**). ADSCs from diabetic patients displayed the lowest level of TNF-α expression when compared to donor ADSCs and adipocytes (t-test $p< 0.001$)

In **Figure 5(B)**, TGF-β was expressed at the highest level in adipocytes derived from differentiated ADSCs compared to donor ADSCs (ANOVA $p<0.001$, **Figure 5 (B)**). ADSCs from diabetic patients expressed TGF-β at a significantly lower level than donor ADSCs (t-test $p<0.001$) and ADSC differentiated adipocytes (t-test $p<0.001$).

Similarly, VEGFA was expressed at a significantly higher level in ADSC differentiated adipocytes than observed in donor ADSCs (ANOVA $p<0.001$, **Figure 5 (C)**). Furthermore, ADSCs isolated from diabetic patients expressed VEGFA significantly more than donor ADSCs (t-test $p<0.001$). ADSC differentiated adipocytes expressed VEGFA to a greater extent than diabetic ADSCs (t-test $p<0.05$).

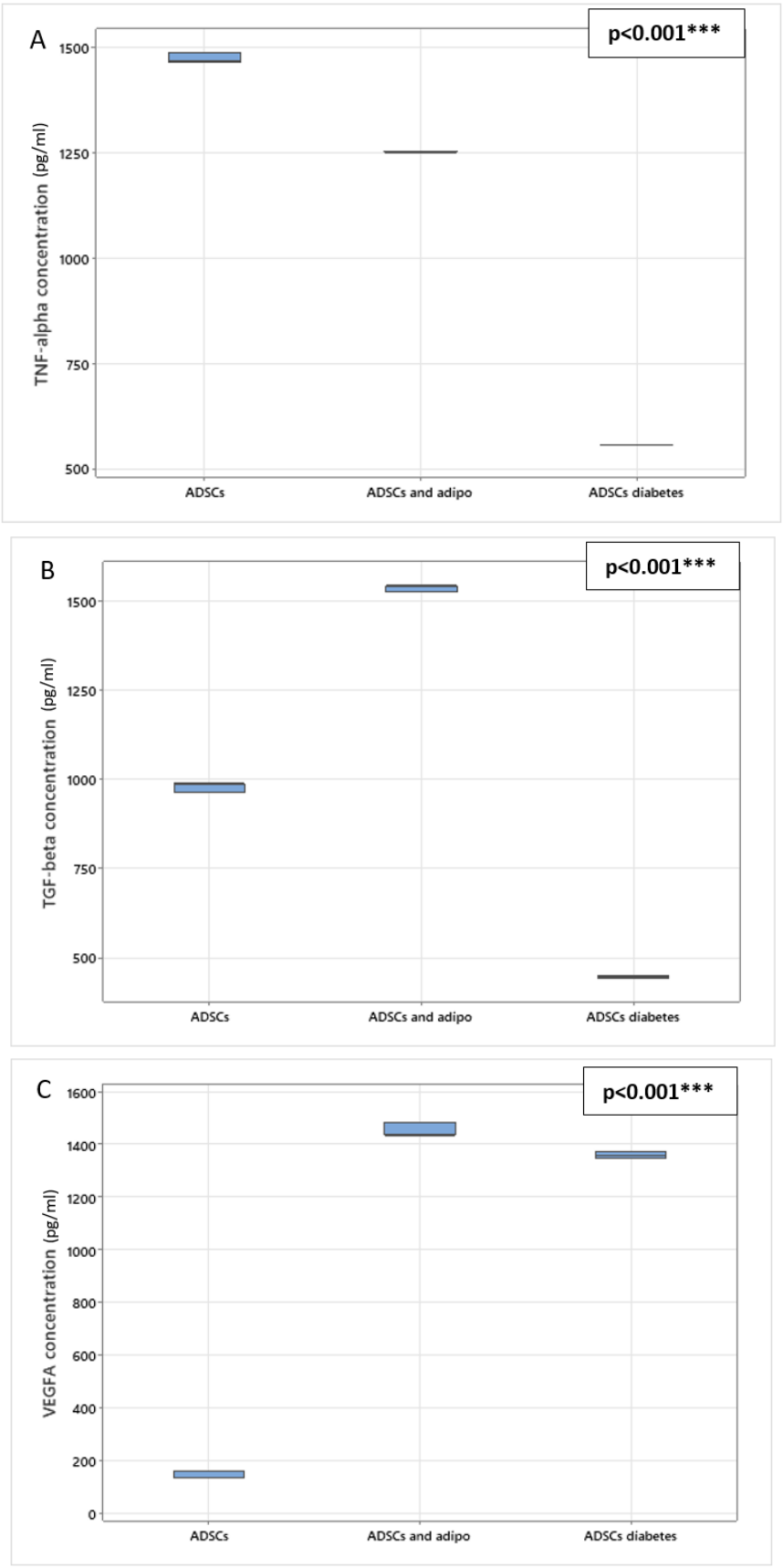

Figure 5 (A-C): Cytokine expression in ADSCs, adipocytes and diabetic ADSCs.

4. Discussion

This study explored miRNA regulation in different cohorts of ADSCs (control ADSCs, adipocytes derived from ADSC differentiation and ADSCs isolated from diabetic patients). There is a clear link between the dysregulation of miRNAs and its effect on the status and function of adipose tissue leading to obesity, as shown in **Figure 1.** qRT-PCR was used to quantify miRNA expression. To establish an appropriate endogenous control, various endogenous miRNAs including miR-16, miR-497 and U6 were assessed. MiR-497 was the most stably and highly expressed endogenous miRNA that was investigated, as shown in **Figure 2**. Consequently, miR-497 was determined as the endogenous control when investigating target miRNA expression in this study. U6 is the most used endogenous control for miRNA expression analysis, however miR-497 and miR-16 have been used previously in other studies (Duan *et al.*, 2018; Lou *et al.*, 2015). Therefore, it is possible that miR-497 may be the most suitable endogenous control for miRNA expression analysis in ADSCs and ADSC-derived subtypes. The target miRNAs investigated in this research were miR-A, miR-222, miR-146, miR-221, and miR-133. In addition, cytokine expression analysis was quantified by ELISA for target cytokines; TNF-α, TGF-β and VEGFA.

This research identified a novel miRNA, miR-A, that may be implicated in obesity and diabetes pathogenesis. There is little to no literature currently available that investigated the role of miR-A in disease, but these findings may allude to its potential role in the onset of diabetes, but it has not been identified in obesity and diabetes (Xiao *et al.*, 2019). MiR-A was upregulated to a higher level in diabetes compared to obesity (**Figure 3 (A)**). From this study, it can be concluded that miR-A dysregulation occurs prominently in diabetes, potentially highlighting a novel target in diabetes treatment and management. MiR-A exhibited a broad range of expression in the control ADSC cohort, which may have contributed to the lack of significant expression between the control and test cohorts. Further studies will be conducted to fully establish the role of this miRNA in obesity and diabetes, as a potential novel marker of disease.

MiR-222 was significantly higher in the diabetic cohort compared to control ADSCs and ADSC differentiated adipocytes (t-test $p<0.01$, **Figure 3 (B)**). However, previous literature observed significant upregulation of miR-222 in both diabetes and obesity (Ortega *et al.*, 2013; Sadeghzadeh *et al.*, 2020). There was no significant difference in miR-222 expression in the donor ADSC and ADSC adipocyte cohorts as some donors displayed increased miR-222 expression and some donors exhibited downregulated miR-222 expression (t-test $p>0.05$, **Figure 3 (B)**). This high variation in miRNA expression between ADSC donors may stem from ADSC donor variation which has been noted previously (Mori *et al.*, 2019).

MiR-146 expression is involved in the regulation of obesity-related inflammation, with its dysregulation leading to increased inflammation which can lead to the development of obesity-related disorders (Roos *et al.*, 2021). In this study, miR-146 expression was significantly increased in the diabetic model compared to the obesity model (t-test $p<0.01$, **Figure 3 (C)**). It must be noted that a broad range of miR-146 expression in control ADSCs was observed. Furthermore, this research found that miR-146 was upregulated in ADSCs isolated from diabetic individuals, undermining studies reporting its downregulation in both obesity and diabetes (Alipoor *et al.*, 2017; Barutta *et al.*, 2021; Lenin *et al.*, 2015; Runtsch *et al.*, 2019). Clarification of the miR-146 expression profile in obesity and diabetes will determine whether these findings compliment or contradict past studies on this miRNA. Identifying instances of dysregulated miR-146 expression can lead to miR-146 targeted therapies for obesity-related secondary disorders where obesity-associated inflammation is a key mediator.

In contrast, miR-221 expression was significantly lower in diabetes patients compared to the adipogenesis model of obesity (t-test $p<0.01$, **Figure 3 (D)**). This result contradicted previous literature findings where miR-221 expression was upregulated in both obesity and diabetic patients (Liu *et al.*,

2018; Yamaguchi *et al.,* 2022). MiR-221 was expressed at a comparable level in donor ADSCs and ADSC differentiated adipocytes (t-test p>0.05, **Figure 3 (D)).** This difference in miR-221 expression may be attributed to the disease model used, as this study investigated miR-221 expression in different ADSC cohorts, and this may also be attributed to donor variation (Brandao *et al.,* 2022). Furthermore, previous studies investigated circulatory miR-221 in diabetic serum and murine obesity models, whereas this study investigated miRNA expression in human ADSC tissue samples. Limited literature highlights the miR-221 expression profile in primary human obesity and diabetes cell models (Meerson *et al.,* 2013). This study elucidated that miR-221 expression is significantly lower in ADSCs isolated from diabetic patients, compared to donor ADSCs (control) and adipocytes derived from donor ADSCs (representing obesity). Dysregulated miR-221 has been linked to obesity-induced insulin resistance (Meerson *et al.,* 2013), with this study highlighting miR-221 as a potential target for the treatment of obesity-related diabetes. However, further studies should elucidate the miR-221 expression profile in human donor ADSCs.

In contrast to the other miRNAs targeted, there was no significant change in miR-133 expression across each of the ADSC cohorts (ANOVA p>0.05, **Figure 3 (E)**). Previous literature has shown that miR-133 expression has been dysregulated in obese and diabetic patients (Al-kafaji *et al.,* 2021; Oliveira *et al.,* 2020). This contrast in findings may be due to this study being performed in *in vitro* ADSC models, compared to miR-133 expression studies *in vivo* (Chen *et al.,* 2017). Future studies should investigate miR-133 dysregulation in obesity further to advance its potential as a therapeutic target.

MiR-21 expression was significantly lower than in diabetic ADSCs compared to donor ADSCs and adipocytes (t-test p<0.05, **Figure 3 (F)**). This downregulatory profile may indicate that the dysregulation of this miRNA may be implicated in diabetes development. These findings mirrored previous research on miR-21 expression in diabetes (Mahzad Mostahfezian *et al,* 2019), however, did not replicate miR-21 downregulation in obesity (Ghorbani *et al.,* 2018). Further studies are required to clarify miR-21 involvement in obesity and diabetes. This study highlights that miR-21 is expressed at a significantly lower level in diabetes compared to control and obesity ADSC models, potentially highlighting miR-21 as a novel target for diabetes prevention, management, and treatment. MiR-21 was not differentially expressed in the obesity ADSC model compared to donor ADSCs, as miR-21 was not consistently expressed across all donors highlighted by the broad range of expression observed (ANOVA p>0.05, **Figure 3 (F)**). Future studies are required to categorise the effect of miR-21 dysregulation in human ADSCs, so as miR-21 targeted therapies for obesity treatment can be advanced.

In addition, the cytokine profiles of each cohort ADSCs, ADSC differentiated adipocytes and ADSC diabetes, were analysed to investigate if different ADSC cohorts secrete distinct levels of TNF-α, TGF-β and VEGFA **(Figure 4)**. The concentration of TNF-α, TGF-β and VEGFA (pg/ml) secreted by each of the cell cohorts is represented in **Figure 4**. Donor ADSCs expressed the highest level of TNF-α and the lowest level of VEGFA out of each of the three cell populations. Adipocytes exhibited the highest level of TGF-β secretion. TNF-α and TGF-β were expressed the lowest in diabetic ADSCs compared to the other 2 cohorts, with VEGFA being the highest secreted cytokine investigated in this cohort. TNF-α was significantly downregulated in diabetes (t-test p<0.001) and obesity (t-test p<0.01) when compared to control ADSCs (**Figure 5 (A)**). ADSCs isolated from diabetes expressed TNF-α to a significantly lower extent than obesity (p<0.001, **Figure 5 (A)**). The downregulatory presentation TNF-α secretion in obesity and diabetes observed in this study opposed previous reports that highlighted increased secretion of this cytokine in both obesity and diabetes (Aquilano *et al.,* 2019;

Lenin *et al.*, 2015). This may be attributed to age related alterations in ADSCs and donor variation (Li *et al.*, 2021). TNF-α may serve as a useful target in preventing obesity-related diabetes, as its dysregulation in obesity has been shown to promote insulin resistance (Nieto-Vazquez *et al.*, 2008).

TGF-β secretion was significantly lower in the diabetic ADSCs cultures compared to donor ADSCs (t-test $p<0.001$, **Figure 5 (B)**) and ADSC differentiated adipocytes (t-test $p<0.001$, **Figure 5(B)**). However, in contrast to TNF-α, increased TGF-β secretion levels in ADSC differentiated adipocytes aligned with that of prior studies (t-test $p<0.001$, **Figure 5 (B)**) (Chang *et al.*, 2016; Yadav *et al.*, 2011). TGF-β dysregulation has been linked to dysfunctional adipogenesis and obesity-related inflammation, highlighting that its reduction may alleviate these adverse effects observed in obesity that can lead to the development of obesity-associated disorders (Woo *et al.*, 2021). VEGFA displayed high expression in obesity and diabetes compared to control ADSCs (t-test $p<0.001$, **Figure 5 (C)**). ADSC differentiated adipocytes displayed higher levels of VEGFA expression than ADSCs isolated from diabetic individuals (t-test $p<0.05$, **Figure 5 (C)**). Increased VEGFA in obesity and diabetes has been reported previously (Herold and Kalucka, 2021; Liu *et al.*, 2018; Zafar *et al.*, 2018). Identifying VEGFA dysregulation in obesity and diabetes may be useful in the development of targeted therapies, as VEGFA is an angiogenic cytokine that has been implicated in the adipogenesis (Herold and Kalucka, 2021).

Where the data in this study contrasts prior findings, the conflict between the conclusions drawn may be attributed to the natural variation that occurs amongst donor samples (Mohamed-Ahmed *et al.*, 2018). Future studies should consider donor variation during experimental design and donor recruitment. Another point to note is the variation in miRNA isolation methods, endogenous controls used and normalisation of data which may contribute to the discrepancy in the literature (Drobna *et al*., 2018; Tan et al., 2023). Therefore, there is an urgent need to standardise miRNA expression analysis studies in order to identify novel therapeutic strategies for disease prevention and treatment (Condrat *et al*., 2020; Lee *et al*., 2020; Tan et al., 2023).

Nevertheless, this data highlights significant variations in miRNA and cytokine expression in obesity and diabetes, potentially leading to novel therapies that target these signalling molecules. This study has shown a clear linkage between dysregulated miRNA and cytokine expression in obesity and diabetes. MiRNAs and cytokines and work in conjunction with each other to activate signalling pathways responsible for adipogenesis and obesity-related inflammation, highlighting their potential benefit in novel therapeutic approaches. Further studies are required to investigate the correlation of specific miRNA expression on cytokine expression pertinent to obesity and diabetes.

5. Conclusion

These findings elucidated that miRNA dysregulation is a prominent phenotypic characteristic of obesity and diabetes. The most significant finding in this study is the identification of a novel miRNA, miR-A, with the potential to distinguish obesity and diabetic patients from healthy patients. This is the first evidence of this miRNA in obesity and diabetes to the best of the authors knowledge. Furthermore, significant differences in miRNA expression were observed between the obesity and diabetes models for other miRNAs. MiR-221 and miR-21 were significantly downregulated in ADSCs isolated from diabetic individuals. Similarly, miR-222, miR-A and miR-146 were significantly increased in ADSCs isolated from diabetic patients. No significant changes in miR-133 was observed across each ADSC population investigated. TNF-α, TGF-β and VEGFA were differentially expressed across donor ADSCs, adipocytes derived from donor ADSCs and ADSCs isolated from a diabetic individual. This study successfully established that miRNA dysregulation is characteristic of obesity and diabetes. This study has identified a novel miRNA (miR-A) that may be a potential target for the treatment of

diabetes. Further studies will focus on validating this finding and elucidating the involvement of this miRNA in obesity and diabetes. With authors recommending further investigation into miRNA involvement in diabetes, the findings of this study regarding miR-A may offer some novel insight. This data may lead to novel therapies for obesity and diabetes treatment by targeting these deregulated miRNAs. Identifying novel targets is a step toward more personalised, miRNA-based therapeutic approaches. Future studies should replicate the findings of this study and include compound testing that repair this dysregulated miRNA profile. Future research should further investigate the involvement of miR-A in obesity and obesity associated disorders, as the findings of this study has provided a link to its dysregulation in diabetes.

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