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Cover Page Footnote

I am grateful to the Technological University of the Shannon for the opportunity to study within their fantastic facilities, to extend my knowledge in science, amongst Ava O'Meara and Cathy Brougham, whose expertise were invaluable in formulating the research questions and methodology as well as providing never-ending support and guidance throughout my studies within the lab and externally.

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The Interaction of Adipose Derived Stem Cells and Breast Cancer

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Abstract

Introduction: Mesenchymal stem cells are adult stem cells capable of self-renewal and multilineage differentiation (Schweizer *et al.,* 2015). Adipose derived stem cells have been used in breast reconstruction following surgical intervention in breast cancer patients. MicroRNAs(miRNA) have been linked to gene regulation essential in oncogenic, and tumour suppression as well as cell signalling pathways in BC.

Aim: To research the hypothesis of ADSCs and their therapeutic properties in BC patients.

Methods: Proliferation assays were carried out to demonstrate how ADSC conditioned media influenced BC cell lines MDA-MB-231, SKBR3, and T47D. The expression of six miRNAs (miR-21, miR-133, miR-222, miR-146, miR-221, and miR-A) and three cytokines (TGF-β, RANTES, TNF- α) was determined using a variety of functional assays. Statistical analysis was performed using Minitab 20.1.0.

Findings: Upregulation of miRNA expression all miR-21 co-culture samples, miR-222 T47D co-culture, and both miR-146, and miR-221's SKBR3 BC co-culture cell line. All co-culture cells within miR-133 expression displayed downregulation of high significance, and co-culture cells lines MDA-MB-231 and SKBR3 expressing miR-222. Down-regulation was observed in all cytokine samples ($p<0.001$) of BC cell co-cultures, apart from RANTES concentration within SKBR3 ($p<0.05$). This research demonstrated how ADSCs have properties as a double-edged sword by providing insight upon ADSCs influence on BC malignancy properties. Which in future could be a target for novel treatment therapies, thus giving patients a better prognosis and survival rate by providing a personalised molecular approach of treatment.

Keywords: ADSCs, Breast Cancer, microenvironment, exosomes, miRNA

1. Introduction

Breast Cancer (BC) is the most diagnosed form of cancer for women worldwide. With over 3,600 women diagnosed annually in Ireland alone, this disease proceeds to be the primary cause of death in women worldwide (Alam *et al.,* 2022). The most common treatment option for BC patients is surgical intervention, although often successful, these methods mutilate the breast tissue and frequently require fat engraftment (Schweizer *et al.,* 2015; Scioli *et al.,* 2019). Mesenchymal stem cells (MSCs) are multipotent adult stem cells which are capable of self-renewal and multilineage differentiation *in vitro*. Sources of MSC include bone marrow, adipose tissue, umbilical cord blood and tissue, or amniotic fluid, and potentially, although less frequent, dental pulp and peripheral blood. Each source can differentiate into various lineages, as shown in **Figure 1**. Bone marrow stem cells (BMSCs) are the gold standard source of stem cells, but more recently adipose derived stem cells (ADSCs), isolated from white adipose tissue (WAT) are shown to have many benefits. ADSCs extraction is much less invasive procedure, provides

a much higher yield of stem cells by 1000-fold, requiring less time, consumables, and expense when compared to high risk, invasive bone marrow extraction (Berebichez-Fridman & Montero-Olvera, 2018; Wang *et al.*, 2020).

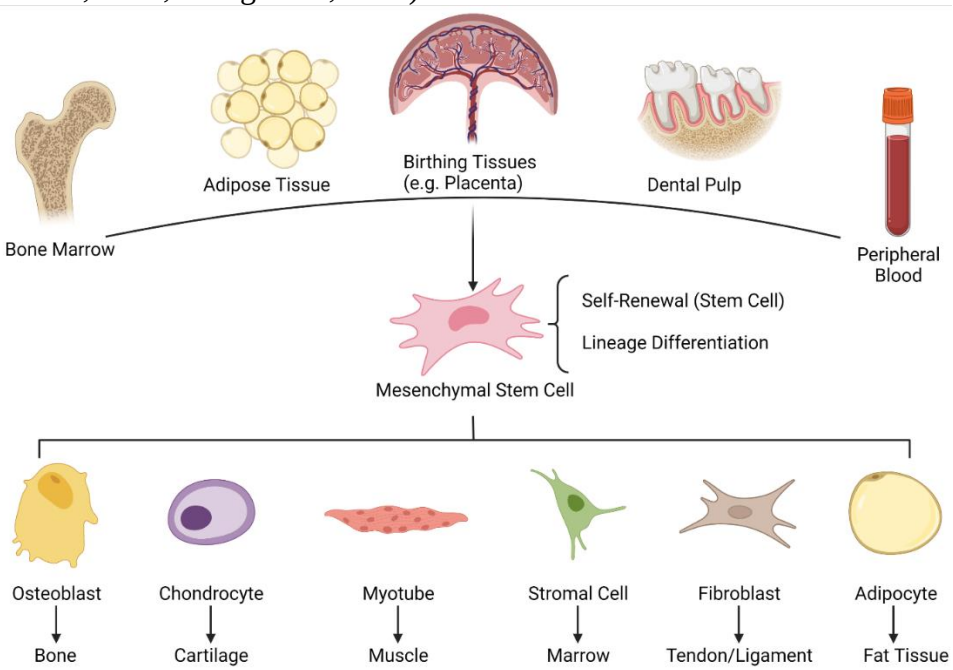

Figure 1: *MSC sources and differentiation schematic (Cunningham et al. (2022).*

Legislation and Ethics of Stem Cell Research

Challenges within stem cell research include both the scientific challenges, as well as ethical and regulatory confronts. Research on adult stem cells is legal and currently being conducted in several locations in Ireland, even though there is no specific legislation toward its research in Ireland (European Commission, 2013). The legal situation regarding embryonic stem cell research is less well defined, which has urged the drive to resource alternative stem cell sources with less ethical implications. ADSCs have emerged as a promising alternative stem cell source and have served as useful models in researching various biological interactions, like microRNA (miRNA) dysregulation in several diseases and disorders, e.g., obesity, diabetes, and breast cancer (Abente *et al*., 2016; S. Li *et al.*, 2020). The concern of using ADSCs is their potential to influence the progression of the BC tumour microenvironment at the site of surgical intervention in BC patients, who are undergoing breast reconstruction via adipose tissue (Zocchi *et al.*, 2019). Research of reliable scientific sources debates ethical concerns of whether ADSC application is beneficiary or destructive (Si *et al.* 2019).

This research will contribute to the question of how ADSCs interact with BC through miRNA mediated communication.

ADSCs: The double-edged sword in BC therapeutics

ADSC-based therapies exert their regenerative and therapeutic properties by releasing exosomes, secreted by MSCs into their extracellular matrix (e.g. conditioned media (CM)) (Singh *et al.*, 2021). Cell-free therapies have been developed that exploit the therapeutic action of ADSC-exosomes via ADSC-CM (Cai *et al.*, 2020). Exosomes aid the transfer of molecules like cytokines, genes and miRNAs from one cell to another (Maqsood *et al.*, 2020). Evidence has suggested that these extracellular vesicles can alter gene expression and function in recipient

cells, particularly when the extracellular vesicle is acting as a carrier of RNA from one cell to another (O'Brien *et al.*, 2020).

ADSC-based therapies in the treatment of diseases like BC has been described as a double edged sword encompassing both therapeutic and potential cancer enhancing effects (Lee and Hong, 2017). ADSCs secrete many growth factors in vitro including TGF-β, which participate in increased collagen and extracellular matrix deposition necessary for tissue repair (Li *et al*., 2020). Schweizer *et al.* (2015) observed the benefit of ADSC incorporation in fat engraftment by the induction of endothelial cell proliferation, retaining volume and shape of the fat engraftment whilst reducing scarring effects upon the tissue (Scioli *et al.*, 2019).

On the other hand, research has presented that the interaction between ADSCs and the BC tumour microenvironment can promote cancer progression and invasiveness, by stimulating ADSCs to undergo epithelial to mesenchymal transition (EMT) (Wu *et al*., 2019). Therefore, the use of ADSCs for tissue reconstruction in BC may potentially promote cancer metastasis, where residual cancer cells at the site of reconstruction may be stimulated by the presence of ADSCs to proliferate, or in contrast, transplanted ADSCs may be stimulated to become cancer cells (Scioli *et al*., 2019). ADSC-free therapies in BC treatment have been observed to supress BC migration and proliferation *in vitro*, with some authors observing the contrary. S. Li *et al*. (2020) identified a novel antitumour ADSC subtype, that when loaded with an antitumour miRNA was shown to be a promising therapeutic approach in BC treatment. In contrast, Wu *et al*. (2019) reported that non-contact co-culture of human ADSCs and the BC cell line MCF7 increased MCF7 migration and invasion potential by altering the expression of certain molecules involved in EMT. The interaction of ADSCs and the BC microenvironment, cancer initiation, and stem cell-initiated tumour growth is important in advancing ADSC-based BC therapies, and must be clarified (Fang *et al.*, 2021; Schweizer *et al.*, 2015). The hypothesis of this study is to establish miRNAs as key mediators in ADSC-BC interaction.

ADSC signaling via miRNAs

While the understanding of the interaction between ADSCs and the BC microenvironment is constantly developing as new research emerges, authors have suggested that an important factor in the communication mechanism of the BC microenvironment could be due to the presence of miRNAs (Qattan, 2020). MiRNAs regulate cancer cell proliferation, apoptosis, differentiation, and metastasis, and in cases with aberrant expression, and oncogenic dysregulation can implicate cellular signalling, thus stimulating cancer (Kaboli *et al*., 2015). MiRNAs have been shown to regulate cytokines following their transcription (Lischka *et al.*, 2021). Authors have highlighted that some miRNAs may play a role in the regulation of the pro-inflammatory cytokine activity in cancer-related inflammation, however, cytokines 1L-1β, TNF-α and IFN-γ have been demonstrated to control miR-21, miR-34a and miR-146a expression levels (Chakraborty *et al.*, 2020). Exosomal miRNAs have been demonstrated to act as oncogenic and tumour suppressor molecules in breast cancer (Wong, Abu Jalboush and Lo, 2020). Indirectly co-culturing breast cancer cells with ADSC-CM is an important step in understanding the interaction between ADSC and BC in terms of current therapies (i.e. tissue reconstruction), and also in terms of novel ADSC-based therapies where developing the understanding of how ADSC-CM (containing exosomes, miRNAs and cytokines) interacts with BC, potentially leading to the identification of novel drug delivery mechanisms or therapeutic targets to treat this prevalent disease (Hong *et al.*, 2019; Asgarpour *et al.*, 2020; Liu *et al.*, 2022).

This research will investigate the influence of ADSCs upon the BC tumour microenvironment with the aim of identifying novel targets for miRNA-based therapies.

2. Materials and Methods

BC Cell Line Selection

To understand the interaction between ADSCs and BC, three ADSC-BC cell line co-cultures were studied; ADSC-MDA-MB-231, ADSC-T47D and ADSC-SKBR3. These BC cell lines were chosen due to their suitability as BC cell models and due to their characteristics, as this study aimed to investigate the interaction of ADSC and BC as broadly as possible to account for varying degrees of cancerous properties (e.g., aggressiveness, invasiveness, and proliferation). To be inclusive of this, it was desired to include a basal, triple negative subtype (MDA-MB-231), a luminal, $HER2^+$ subtype (SKBR3) and a luminal A, HER^- subtype (T47D).

Table 1: BC Cell Lines considered.

BCC Line	Classification	ER	PR	$HER2^+$	In culture
MDA-MB-231	Basal B, Claudin low	-	-	-	Stellate
T47D	Luminal A	+	±	-	Mass
SKBR3	Luminal, $Her2^+$	-	-	+	Grape-like

(Schweizer *et al.* 2015; Lee *et al.,* 2017)

ADSC isolation

Human adipose tissue was obtained from healthy volunteers with informed patient consent and ethical approval. ADSCs were isolated via collagenase digestion. The isolated ADSCs were then cultured at 37°C and 5% CO_2 for 2-3 days with supplemented high glucose Dulbecco's Modified Eagle Media (DMEM) (10% FBS, 1% Penicillin/Streptomycin and 1% L-Glutamine). Upon confluency, cells were passaged and cultured with fresh supplemented media for a further 48 hours. After 48 hours, the ADSC-CM was collected and stored at -80°C for use in the ADSC-breast cancer cell co-cultures.

Indirect ADSC-BC co-culture

Breast cancer cell lines, MDA-MB-231, SKBR3 and T47D cells were seeded at 8 x 10^4 cells/well in separate 96 well plates and incubated for 24 hours. Following this 24-hour incubation, the BC cell lines were incubated with ADSC-CM for 24, 48, 72 hours and 7 days. After these incubation periods, culture media was collected and stored at -80°C for cytokine secretion analysis. The adhered cells were harvested and pelleted for miRNA isolation and expression analysis.

Proliferation Analysis

The effect of co-culture with ADSC-CM on MDA-MB-231, SKBR3, and T47D proliferation compared to BC cell lines cultured alone over a 24-hour period was determined by MTT assay. Absorbance readings at 540nmwere obtained and processed to establish the percentage proliferation.

Enzyme Linked Immunosorbent Assay (ELISA)

After 24, 48, 72 hours and 7-day incubation with ADSC-CM, ADSC-BC culture media was collected to quantify TGF-β, RANTES and TNF-α secretion by ELISA (ThermoFisher) as per manufacturer's guidelines.

RNA isolation and RT-qPCR analysis

MiRNA was extracted from control ADSCs and each ADSC-BC coculture using the mirVana™ miRNA Isolation Kit (ThermoFisher) as per the manufacturer's instructions. Isolated miRNA was converted to complementary DNA (cDNA) using Reverse transcription polymerase chain reaction (RT-PCR). The cDNA templates for each control (endogenous and negative) and each sample were primed with TaqMan primers (ThermoFisher), exposed to target probes (miR-21, miR-133, miR-222, miR146, miR-221 and miR-883), quantified by RT-qPCR and reported as raw Cycle Threshold (CT) values proportional to target miRNA expression levels. These TaqMan primers are within the current Sanger database to deliver comprehensive analysis with up-to-date annotation.

Data Analysis

The raw C_T data obtained from RT-qPCR (miRNA analysis) and the absorbance data from ELISA (cytokine analysis) were processed using Excel. The RT-qPCR data was interpreted and processed into Log10 values representing the relative expression of the target miRNAs. The absorbance values for the ELISA plates were processed in Excel to achieve the cytokine concentrations (ng/ml). The Log10 values (relative miRNA expression levels) and cytokine concentrations (ng/ml) were statistically analysed using Minitab 21.1.0 to determine if there were statistically significant differences in target miRNA expression across the control and test cohorts. In Minitab, boxplots were generated to graphically represent target miRNA expression across the ADSC control and each ADSC-BC cell co-culture (ADSC-MDA-MB-231, ADSC-SKBR3s and ADSC-T47Ds). Boxplots were also created to depict cytokine secretion levels in the control and ADSC-BC cell line cocultures. Two sample t-tests were executed to obtain p-values which were indicative of the level of significance in target miRNA expression (miR-21, miR-133, miR-222, miR-146, miR-221 and miR-883) across control ADSC and ADSC-BC cell line cocultures (ADSC-MDA-MB-231, ADSC-SKBR3 and ADSC-T47D). The levels of significances were no significance ($p > 0.05$), significant ($p< 0.05$*) and highly significant ($p< 0.01$** and $p< 0.001$***).

3. Results

BC Cell Line Selection

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4. Discussion

This study in an extension upon previous undergraduate research, concentrating on ADSCs effect on the BC tumour microenvironment. The findings in this paper demonstrate the molecular individuality of the disease between different subtypes of BC; basal (MDA-MB-231) and luminal A, HER^- subtype (T47D), and luminal, $HER2^+$ (SKBR3) (Scioli *et al.,* 2019). This study provides valuable insight toward the significance ADSC CM has upon the interaction with both BC malignancy, and tumour suppressive properties in the direction of the investigation of potential targeted therapeutic approaches in conjunction with gold standard cancer therapies (Scioli *et al*., 2019).

This study explored whether ADSCs are safe within the BC microenvironment. Recent clinical trials have shown that there is no significant difference in breast cancer recurrence rates with the use of ADSC in therapeutics, yet a pattern is demonstrated throughout literature that ADSCs contain both stimulatory and inhibitory effects as a double-edged sword (Scioli *et al.,* 2019). The proliferative activity of three BC cell lines was stimulated in the presence of ADSC CM compared to when these BC cells were cultured alone, with the greatest proliferation observed in the MDA-MB-231 cell lines. Many complex external factors within cell communities can influence this, therefore, miRNA expression and cytokine pathways were analysed to understand the underlying mechanism (Kumari & Gupta, 2021).

Cycle threshold (C_t) of target genes allowed for the selection of the endogenous control, miR-16, a miRNA with the least irregularity and the greatest stability for this cohort. The results found in this study provide information upon the ADSC-CM interaction with three BC subtypes in comparison to normal miRNA expression in ADSCs.

MiR-21 is a commonly studied oncogene in many human malignancies (Fu *et al.,* 2011). Previous studies have shown that its overexpression correlates with BC proliferation, progression and metastasis, deteriorating patient prognosis as it targets the inhibition of cell cycle arrest, thus making for a much more severe BC phenotype (Javanmardi *et al.,* 2017; Najjary *et al.,* 2020). MiR-21 expression contributes toward angiogenesis and vascularization of endothelial cells in tissue reconstruction, a commonly discussed feature of ADSCs double-edged sword (An *et al.,* 2019; Schweizer *et al.,* 2015). This research showed a significant

increase in miR-21 expression with co-cultured MDA-MB-231, SKBR3, and T47D, supporting documentation of oncogenic risk of ADSC CM use in BC patients.

MiR-133 has been identified as a significant tumour suppressor in BC. Its overexpression correlates with the suppress of proliferation and colony formation, as well as increased apoptosis of BC cells (Zhang *et al.*, 2020.), Moreover, miR-133 has previously been linked to BC patient survival rates, and a reduction in BC metastasis (Hesari *et al*., 2019) Consistently throughout literature, the down regulation and low expression of miR-133 is associated with BC cell proliferation. This study demonstrated a downregulated response miRNA expression, specifically in progesterone regulated T47D subtypes, as progesterone induces miR-133 expression from endothelial and epithelial cells (Pan *et al.,* 2017).

MiR-146 has been observed to stimulate cancer development, and was shown to promote proliferation and invasion of BC (Chen *et al.*, 2020; Liu *et al.*, 2020). The results in these findings present a significant increase in miR-146 in the SKBR3-ADSC co-culture and no significant change in the other BC subtypes. This disputes the literature's evidence of significant upregulation of miR-146 in MDA-MB-231 subtype, decreases expression of epidermal growth factor receptor (EGFR), to inhibit invasion and migration *in vitro* (Hurst *et al.,* 2009). Reasoning for this discrepancy may be due to ADSC donor variation, use of endogenous control, varying miRNA isolation methods or normalisation of miRNA expression profiles which has previously been observed in the literature (Pamedytyte *et al*., 2020).

Previous research has documented that both miR-221, and miR-222 are homologous miRNAs whose overexpression is associated with many malignant cancer types (Fu *et al.* 2011). MiR-221 may stimulate BC progression and metastasis (Liu, Wang, and Sun, 2019; S. Li *et al.,* 2020), whereas miR-222 may induce drug resistance in BC cells and potentially enhance BC malignancy (Shen *et al.*, 2017; Amini *et al.,* 2018). Both miRNAs have motility properties associated in metastatic cancer types, demonstrating a link between ADSC's part in EMT via paracrine signalling (Wang *et al.* 2020). This study found miR-221 expression significantly upregulated in SKBR3 co-culture, indicating proliferation and metastatic properties. Whereas, miR-222 expression in luminal A, HER^- subtype T47D-ADSC co culture was of no significance. This was refuted in the other BC subtype co-cultures MDA-MB-231 and SKBR3, where expression of miR-222 was significantly high, indicating the suppression of oncogenic properties. These results demonstrate the importance of individuality of disease subtypes and the influence even homologous miRNAs have on the specifics of the disease.

MiR-883 has not yet been documented in literature demonstrating a pattern of interaction with BC and ADSC CM. No co-culture samples had notable significance in its expression. The co-culture of T47D and ADSC CM had a similar downregulated expression to ADSC CM, opposite to the upregulated cellular response of MDA-MB-231, and SKBR3 co-culture samples.

Furthermore, this study provided insight into ADSC-CM cytokine secretions within co-cultured BC cell lines, and their influence upon migratory factors and effects. Early studies highlight TGF-β tumour suppression properties in BC, and recent studies conclude that TGF-β dysregulation can promote cancer development via proliferation, apoptosis, differentiation, EMT, and migration (Zhang *et al*., 2020). The TGF-β signalling pathway crosstalk's with other signalling pathways to regulate cellular functions (Syed 2016).

Each co-cultured BC subtypes in this study displayed a significant downregulated response in TGF-β cytokine expression, particularly the luminal A HER^- subtype, T47D, complimenting the preliminary hypothesis that the ADSC-CM have the potential to supress this pathway in cancer

metastasis (Wahdan-Alaswad *et al.,* 2016). These results support previous research findings in with T47D cell line is not sensitive to TGF-β (Syed 2016; Xu *et al.,* 2018).

RANTES is a major chemoattractant chemokine secreted by BC cells which contributes to tumour progression by monocyte migration, increasing BC aggression. ADSC-CM have been documented to have a negative response to this as they promote cell motility and infiltration of leukocytes to tumours by secreting RANTES (Gallo *et al.,* 2018). This study demonstrated significant up-regulation of the SKBR3 cell line and RANTES chemokine expression, a prevalent risk in BC metastasis (Yaal-Hahoshen *et al.,* 2006).

TNF-α is a highly expressed inflammatory cytokine protagonist in BC tumours, leading to increased BC growth and metastasis. Literature has demonstrated contradictory qualities as it has tumour promoting factors in progression and tumorigenesis, whereas local administration of high doses of TNF-α has robust antiangiogenic and antitumoral effects, another example of the double-edged sword (Horssen *et al.,* 2006; Wu *et al.,* 2019). TNF-α involvement in EMT and BC progression also is a contributor toward acquired drug resistance. This study shows that co-culture of selected BC subtypes with ADSC CM enhanced the secretion of anti-inflammatory effects, particularly within T47D-ADSC-CM, suppressing carcinogenic properties. These results and previous literature provide the hypothesis of using TNF-α toward cancer suppression therapy (Cruceriu *et al.,* 2020).

Limitations which affected this study primarily revolved around the ongoing COVID19 pandemic, because of this increased pressure was implemented upon sourcing of materials and their viability due to extension on deliveries. This is turn had a cumulative effect of time constraints within the laboratory, as well as not having access to equipment within the research centre thus increasing the workload over a shortened period.

The novelness of this research also acted as a weakness, as throughout the literature ADSC implementation in BC is discussed as a double-edged sword, contrasting in a definite hypothesis of beneficiary or destructive effects on BC patients.

5. Conclusions

The focus of this study involved ADSC-CM interaction with the BC tumour microenvironment by concentrating upon the impact of changes in cellular proliferation, gene expression, and cellular communication by protein expression within co-cultured samples reflective of each BC subtype. Results demonstrated that ADSC-CM significantly affected multiple malignant features of BC cells *in vitro*, such as miRNA gene expression and proliferation. Luminal A, HER^- subtype T47D co-culture demonstrated an overall pattern on tumour suppression throughout miRNA and cytokine expression, despite increased proliferation. Whereas there was a large correlation between miRNAs, cytokines involved in EMT and carcinogenic properties providing an increased oncogenic risk in BC patients.

Additionally, this research provided a crucial insight into the importance of rigorously screening patients for premalignant or residual lesions prior to process of lipografting, with additional stem cell-augmentation, or isolated ADSC CM in the breast or adjacent tissues, to avoid a potential risk increase of disease.

Further investigation toward utilising miRNAs and their interaction with the BC microenvironment as a target for novel treatment therapies may potentially facilitate the gap in research for this prevalent disease, giving patients a better prognosis and survival rate by providing a personalised molecular approach of treatment. Reflecting upon this study as previous limitations are now less pronounced, this project would benefit from expanding BC

cell line classifications to include primary as well as commercial lines, thus increasing variety to correlate results. The introduction of compound testing to discover its effect upon miRNA regulator targeted therapies could also enhance suppressive factors of BC in ADSC implementation.

6. Acknowledgements

I am grateful to the Technological University of the Shannon for the opportunity to study within their fantastic facilities, to extend my knowledge in science, amongst Ava O'Meara and Cathy Brougham, whose expertise were invaluable in formulating the research questions and methodology as well as providing never-ending support and guidance throughout my studies within the lab and externally.

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