The Effect of Chemotherapy Treatment on Bone Marrow Mesenchymal Stromal Cell Adipocyte Differentiation

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Abstract

In an effort to understand the cause of late onset cardiac, metabolic, and musculoskeletal conditions in paediatric acute lymphoblastic leukaemia (ALL) survivors, the adipogenic differentiation of bone marrow (BM) mesenchymal stromal cells (MSCs) has been studied. There is a complex network of factors influencing adipogenesis, which to date is not completely understood. Hence, the overall aim is to better understand the cellular and molecular basis behind the development of these conditions in survivors. To this end, we asked whether treating BM MSCs *in vitro* with cancer drugs, Doxorubicin and Dexamethasone, will initiate a skewed differentiation towards adipogenesis. BM MSCs were analysed with respect to lipid accumulation, gene expression, and adipokine production. In general, our hypothesis was not confirmed. No lipid accumulations were detected in the cells. In analysis of gene expression of the adipogenic transcription factors PPARγ and C/EBPα, certain changes were seen; however, due to lack of biological replicates, no statistical analyses could be applied to the results. Lastly, the inflammation and adipogenesis associated cytokine IL-6 displayed a slight increase, whereas the cytokines IL-8 and TNF-α were undetectable.

Sammanfattning

I ett försök att förstå orsakerna bakom de kardio-, metaboliska- och muskuloskeletala sjukdomar hos barn som överlevt akut lymfatisk leukemi (ALL) har vi studerat den adipogena differentieringen hos mesenkymala stromaceller från bennärg (BM MSCs). Det komplexa nätverket av faktorer som påverkar adipogenes är hittills inte helt kartlagt. Därför är vårt övergripande mål att få en bättre förståelse för den cellulära och molekylära grunden bakom utvecklingen av dessa tillstånd hos ALL-överlevare. Vi undersökte om behandling av BM MSC *in vitro* med cancerläkemedel, Doxorubicin och Dexamethason, kan påverka differentieringen mot adipogenes. BM MSCs analyserades med avseende på lipidadkumulering, genuttryck och adipokinproduktion. Vår hypotes kunde inte bekräftas. Inga lipidadkumuleringar kunde detekteras i cellerna. Vid analys av genuttryck av de adipogena transkriptionsfaktorerna PPARγ och C/EBPα sågs vissa förändringar; men på grund av brist på biologiska replikat kunde inga statistiska analyser tillämpas på resultaten. Slutligen sågs en liten ökning i den inflammation- och adipogenes-associerade cytokinen IL-6, medan cytokinerna IL-8 och TNF-α inte gick att detektera alls.

Keywords

Human Bone Marrow Mesenchymal Stromal cells, Acute lymphoblastic leukaemia, Doxorubicin, Dexamethasone, adipogenesis, cytokines
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ALL</td>
<td>Acute Lymphoblastic Leukaemia</td>
</tr>
<tr>
<td>BM</td>
<td>Bone Marrow</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT enhancer binding protein</td>
</tr>
<tr>
<td>CHOP</td>
<td>CCAAT/enhancer-binding protein</td>
</tr>
<tr>
<td>CTCM</td>
<td>Complete tissue culture medium</td>
</tr>
<tr>
<td>Doxo</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>Dexa</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>KLF5</td>
<td>Kruppel-like factor 5</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stromal cell</td>
</tr>
<tr>
<td>PPARγ</td>
<td>peroxisome proliferator-activated receptor-γ</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>TZDs</td>
<td>Thiazolidinediones</td>
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1. Introduction

1.1 Acute-Lymphoblastic leukaemia and its treatment

Acute lymphoblastic leukaemia (ALL) is the most common type of blood cancer, and the most common paediatric malignancy worldwide. ALL is manifested by the expansion of immature B or T cells, caused by disturbances in hematopoietic progenitor cells (1,2). This rapid increase in B or T cells causes a decrease in oxygen-transporting red blood cells, resulting in anemia, and in addition thrombocytopenia and neutropenia (1,3,2). The progress of the disease is rapid and requires immediate treatment (4). To date, the main treatment of ALL is chemotherapy, which is performed repeatedly during a 2-year period with the aim of eliminating the malignant immature white blood cells and preventing the disease from relapse (5). The intensity of treatment for ALL depends on factors such as age, ALL phenotype, and white blood cell counts at diagnosis. Additionally, a cytogenetic test is performed, where the chromosomal examination aims to find structural abnormalities in the leukemic blast cells that add prognostic information. Taken together, all these factors pave the way for risk stratification of patients (3,6), dividing the patients into different risk categories; standard-, high and very-high, all of will receive chemotherapy during treatment.

Doxorubicin (Doxo) is an anthracycline widely used against many types of cancer, including ALL (6). Doxo is administered intravenously and acts by inhibiting cell proliferation by intercalating base pairs in the DNA helix, thus inhibiting DNA replication. Doxorubicin further inhibits topoisomerase 2, an enzyme playing a key role in DNA replication. (5) Another key component that is administered in all paediatric ALL patients belongs to corticosteroid class of drugs, among which Dexamethasone (Dexa) is the most common. Dexe is orally administered and highly impacts the white blood cells by, for instance, inducing G1-phase cell-cycle arrest and cell apoptosis (7).

1.2 Bone marrow mesenchymal stromal cells

1.2.1 BM MSCs and their differentiation mechanisms

Mesenchymal stromal cell (MSC) are multipotent cells with the potential to differentiate into a large variety of cell types including adipocytes (fat tissue), osteoblasts (bone) or chondrocytes (cartilage) (2,3). As they possess the ability to self-renew they serve as very attractive therapeutic components in the field of regenerative medicine (8). Furthermore, in the Bone marrow (BM) subsets of MSCs, the cells provide survival factors for immune memory cells playing an important role for maintaining long lived immunity of the host (9). Human BM MSCs are characterized by their positive expression of the surface molecules including CD73, CD105 and HLA-class I molecules together with negative expression of CD34 and CD45 (10) The fate of MSCs is determined by various microenvironmental factors in the stem cell niche, including growth factors, mechanical and physical stimulation, cell-cell interactions and attachment, and cell density (11). Upon stimuli from these factors, cell signaling pathways are activated which further advances the progression of differentiation. The differentiation of MSCs is divided into two major phases: the determination phase, in which the MSCs commit to lineage-specific progenitors, and the termination phase, in which the MSCs mature from these progenitors into the specific cell type.
1.2.2 Adipogenesis

During the determination phase of adipogenesis, multipotent MSCs lose their ability to differentiate into other mesenchymal lineages, and commits to the adipocyte lineage by forming preadipocytes, whom still are morphologically indistinguishable from their fibroblastic precursors. During the termination phase, the preadipocytes are matured into spherical adipocytes with the ability to synthesize and transport lipids, secrete adipocyte-specific proteins, and insulin sensitivity machinery, which is crucial for substrate metabolism.

The differentiation mechanisms of MSCs into adipocytes and osteoblasts are competitively balanced; the mechanisms that promote adipogenesis actively suppress mechanisms promoting osteogenesis, and vice versa. This balance is maintained though cross talk between the complex signaling pathways derived from a number of proteins and transcriptional regulators (12). Deviations from this balance are for instance manifested in age related bone loss and osteoporosis. Both conditions are caused by loss of osteoblasts, in which osteoporosis patients have been found to have an excessive amount of BM adipocytes compared to healthy controls with a maintained balance between osteoblasts and adipocytes (13). For adipogenesis in particular, the inducing factors ultimately directly or indirectly activates the two key adipogenesis associated transcription factors, peroxisome proliferator-activated receptor-γ (PPARγ), and CCAAT enhancer binding protein-α (C/EBPα) (14,11).

1.2.2.1 PPARγ

PPARγ is often referred to as the master regulator of adipogenesis, and a dramatic increase of PPARγ expression is the characterization of adipogenesis in BM MSCs. PPARγ belongs to the nuclear hormone receptor gene superfamily of ligand-activated transcription factors, and exists in two isoforms, PPARγ1 and PPARγ2. Both transcription factors are associated to adipogenesis, but PPARγ1 is expressed in other cell and tissue types, and only in low levels in adipose tissue, while PPARγ2 is exclusively expressed in high levels in adipose tissue. The role of each PPARγ isoforms remain unclear, but to date, no factor is found that can induce adipogenesis in the absence of PPARγ,
and ectopically expressed PPARγ can induce adipogenesis in other cell types than adipocytes (12). Studies conducted in mice have further shown that (12) disrupting expression of PPARγ leads to a decreased number of adipocytes, as well as decreased functioning of the adipocytes, ultimately leading to metabolic defects (15,16). The increased expression of PPARγ acts as a molecular switch and either directly activates or induces the expression of multiple genes that are responsible for the adipocyte phenotype, including fatty acid synthesis, Glut4, acetyl CoA carboxylase, adipocyte-selective fatty acid binding protein, and the insulin receptor. (12) In addition to an increased expression of PPARγ, ligand-dependent activation of PPARγ has been found to induce adipogenesis through lineage-commitment. Exogenous ligands activating PPARγ include derivatives of long-chain polyunsaturated acids and thiazolidinediones (TZDs), synthetic agonists utilized clinically for their insulin sensitizing actions (12).

1.2.2.3 C/EBPα

C/EBPs, belonging to the basic-leucine zipper class of transcription factors, have six isoforms: C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, and transcription factor homologous to CCAAT/enhancer-binding protein (CHOP). The C/EBPα, C/EBPβ, and C/EBPγ isomers are involved in promoting adipogenesis, while C/EBPδ and CHOP rather have an inhibitory effect on adipogenesis by inactivating C/EBPβ (12,17). In BM MSCs, adipogenesis induces a rapid increase in C/EBPβ, C/EBPγ, which consecutively induces the major adipogenic transcription factors C/EBPα and PPARγ. C/EBPα directly activates PPARγ and other adipogenic genes, and upon induction of PPARγ, the two genes stimulate each others expression, maintaining high levels throughout the life of the cell.

![Figure 2](image_url)

**Figure 2. A schematic overview of the different factors inducing adipogenesis.** The different factors involved in induction of adipogenesis, including insulin, corticosteroids, TZDs, pCREB, C/EBPβ, C/EBPγ, C/EBPα, PPARγ, SREBP1c, KLF5, and RXRa (the last three not included in the scope of this article). Adapted from Cell. Mol. Life Sci. 66 (2009) 236 – 253.

Under *in vitro* MSC differentiation conditions, stimulation from other supportive cell types (for instance in the form of cytokines triggering differentiation) is usually absent, why certain induction factors are supplemented to the *in vitro* cultures. Some chemical factors have been proven to induce adipogenesis and osteogenesis during *in vitro* cultivation of MSCs, through regulation of key transcription factors. For adipogenesis, commonly added supplements include
isobutylmethylxanthine, indomethacin, Dexa, and insulin. Dexa acts by activating expression of the C/EBPα precursor C/EBPb by binding intracellular corticosteroid receptors (14). Altogether, the supplements induce phosphorylation of cAMP response element binding protein (CREB) which in turn upregulates expression of C/EBPα and Kruppel-like factor 5 (KLF5), the latter inducing several adipogenic genes, predominantly PPARγ. These two transcription factors are expressed throughout the entire life span of the adipocyte, regulating a group of genes that promote the adipocyte phenotype (11,18).

1.3 Long-term effects of ALL treatment and its connection to chemotherapy

The long-term survival in paediatric ALL has improved remarkably during the last decades; however, recovering from ALL often comes with complications. A recent large population study from a cohort of childhood ALL survivors (19) showed a 7-fold increased risk of developing cardiac, metabolic, and musculoskeletal conditions. Although it is known that certain components in chemotherapy are toxic to the body, the exact cell- and molecular mechanisms of the long-term ALL-treatment effects are yet unknown. Several drugs used against ALL are genotoxic and may impact cancer survivor’s health by inducing changes in gene expression (2). An excessive production of adipocytes or dysregulation in adipogenesis is a known cause of cardiac and metabolic conditions. Furthermore, inflammatory cytokines such as IL-6 and IL-8 (also referred to adipokines) which can be released by fat cells have been connected to the pathophysiology of metabolic diseases (20). In connection to a study on BM MSC’s effect on memory B-cells (2) the group in which I performed my thesis discovered that that BM MSCs exposed to Doxo had an increased tendency to differentiate into adipocytes, by means of positive lipid staining using Oil Red O (unpublished data). In other studies, Dexametaphone has been shown to induce adipogenesis in BM MSCs, and inhibit maturation of osteoblasts, and is thereby responsible for a widespread problem of bone deficiencies in ALL survivors (21). This is contrary to the fact that Dexamethasone is used as a supplement for osteogenesis induction, as previously mentioned. In order to better understand the development of the late-onset conditions following ALL treatment, further studies at cell- and molecule level are required.

1.4 Aim and hypothesis

The overall aim is to better understand the cellular and molecular basis behind the late development of metabolic conditions in survivors. The specific aim for this project is to study if and how Doxo, with or without Dexamethasone, can influence BM MSCs adipocyte differentiation in terms of lipid accumulation, gene expression, and adipokine production.

The working hypothesis of the project is that treating MSCs in vitro with cancer drugs will initiate a skewed BM MSC differentiation towards adipocytes.

2. Materials and Methods

2.1 In vitro culture and drug exposure of the cell line HS27A and primary BM derived MSCs

In the initial experimental phase of this project, the human BM MSCs cell line HS27A was used in an optimization study, conducted in order to establish suitable conditions for later cultivation and drug exposure of the primary BM MSCs. In short, the cells were thawed and expanded in cell culture flasks, as described in detail in section 2.2. For Doxo exposure, the cells were seeded in 6-well plates with starting cell numbers of 25x10⁴, 50x10⁴, and 100x10⁴ per well and Doxo concentrations of 0.5
µg/ml and 1.0 µg/ml. Following 2h of exposure cells were washed with phosphate-buffered saline (PBS, Gibco), then provided fresh complete tissue culture medium (CTCM, consisting of RPMI 1640, 10% Fetal Calf Serum, 1% L-glutamine, 1% Pen-Strep) followed by rest in incubator (37°C with 5% CO2) overnight, and exposed to Doxo for 2h more hours. The cells were washed and provided with fresh CTCM followed by rest in incubator (37°C with 5% CO2) for 3, 7, 10 and 14 days. At these time points cells were followed morphologically by means of cell density and viability.

2.2 In vitro culture and drug exposure of primary BM derived MSCs

Following above optimizations on HS27A cells, primary BM MSCs (purchased from TCC-LGC Standards, ref: PCS-500-012) (2) from healthy donors (n=2) were included in the experiments. BM MSCs were thawed and expanded in cell culture flasks with complete tissue culture medium (CTCM, consisting of RPMI 1640, 10% Fetal Calf Serum, 1% L-glutamine, 1% Pen-Strep). The CTCM was changed every 3-4 days until the adherent HS27A cells reached almost 100% confluence. The cells were detached by incubation with 0.25% Trypsin-EDTA (37°C) for a maximum of 10 minutes, and upon cell detachment the enzyme activity was blocked by addition of an equivalent volume of CTCM. The cells were centrifuged and counted by trypan blue staining and re-seeded at 30-40% confluency in new flasks. A total of 6x10³ cells in 2 ml CTCM were transferred into each well of six-well culture plates and kept until cell confluence reached above 80% (approximately after 2 days). After removal of CTCM, BM MSCs were incubated for 2 h with 0.5 µg/mL Doxo with or without 0.5 nM Dexa. BM MSCs in CTCM only served as negative control. Next, BMMSCs were washed twice with PBS to remove Doxo and Dexa, followed by addition of fresh CTCM. After overnight rest, the same drug exposure was repeated, but this time with 3 h exposure. After 2 days of recovery (detailed in figure 3) BM MSCs were analyzed in downstream assays with respect to their morphology, phenotype, lipid accumulation, mRNA expression, and cytokine production described in the following sections. All analyses were completed before the BM MSCs reached five passages. In addition, aliquots from all conditions were processed for chromatin modification studies for follow-up studies.

![Figure 3. Flow chart of the experiment.](image)

A schematic flow chart of the experiment. After initial expansion of the cells, the cells were seeded in 6 well plates in CTCM (control) and with Doxo, with or without Dexa in two cycles of 2 hours and 3 hours, respectively. After recovery, the cells were analyzed in downstream assays.

2.2 Primary BM MSC morphology and phenotype analysis by microscopy and flow cytometric analysis

The morphology and phenotype of the BM MSCs were studied in order to detect any alterations of the cells. Following recovery, the MSCs were observed in a microscope (Nikon Eclipse TS100, Nikon Instruments) to monitor visible alterations in their morphology. Images were acquired at 10x, 20x, and 40x magnification (figure 4) using software LAS V4.4 (Leica) with a Leica DFC420C camera (Leica Microsystems).
Further, the MSCs were phenotyped by means of expression of surface molecules to confirm that the cells were intact after storage in liquid nitrogen and drug exposure. The MSCs were phenotyped using fluorochrome-tagged antibodies specific for CD34, CD45, CD73, HLA-ABC, and CD105, and a live/dead cell probe (referred to as dead cell marker (DCM) that discriminates between live and dead cells. The MSCs were washed two times with PBS (10 min, 400g, room temperature (RT)) and incubated with DCM (10 min, 4°C, in dark). The cells were washed with FACS buffer (PBS, 2mM EDTA, 0.1% BSA) (5 min, 400g, 4°C) and the supernatant discarded. Next, the cells were incubated in a cocktail of the listed (table 3, Tables and figures) antibodies in FACS buffer (10 min, 4°C, in dark) and washed with FACS buffer as in the previous step. The cells were fixed with 4% paraformaldehyde (PFA, 5 min, 4°C, in dark). Last, the cells were washed and resuspended in FACS buffer. Phenotype data was acquired with Novocye flow cytometer, and the data was analyzed with FlowJo software.

2.3 Lipid staining

For detection of lipid accumulations in the BM MSCs after drug exposure, cells were washed with PBS, then fixed by covering the cell monolayer in 4% PFA (20-30 min, RT). The cells were washed with distilled water and then covered with 60% isopropanol for 1 minute in RT. After aspiration of the isopropanol, Oil Red O solution (0.5% in isopropanol, mixed to a working stock of 6:4 ratio with distilled water, homogenized for 10 minutes, then filtered with a 0.2 µm syringe filter) was added to cover the cell monolayer and incubated for 10-15 minutes in RT. The cell monolayer was washed with distilled water five times. After drying the plate, PBS was added to the wells and the cells were observed in microscope.

2.4 Gene expression upon RNA extraction, cDNA synthesis, primer pair evaluation and qPCR

2.4.1 Cell collection

The gene expression of the MSCs were quantified to detect alterations in the expression of the genes of interest following Doxo exposure. One 6-well plate of BM MSCs was harvested following incubation with 0.25% Trypsin-EDTA (37°C) until the cells detached from its surface, as previously described.

2.4.2 RNA extraction

The total RNA was extracted using RNeasy Micro Kit (Qiagen) according to published methods (22). In short, the cells were lysed using lysis buffer and the cell lysate homogenized by pipetting. The lysate was then added to the appended membrane spin columns to which the RNA binds under optimal binding conditions provided by addition of ethanol. The RNA was eluted and quantified using Nanodrop at a wavelength of 260 nm.

2.4.3 cDNA synthesis

cDNA was synthesized using SuperScript™ III First-Strand Synthesis SuperMix for RT-qPCR (Invitrogen). In short, approximately 500 ng RNA for each condition was used for synthesis through addition of reverse transcriptase and dNTPs and insertion into SimpliAmp™ Thermal Cycler (25°C
for 10 min, 50°C for 30 min, 85°C for 5 min, kept on ice and added E. coli RNase H, 37°C for 20 min). The cDNA was stored in -20°C until use.

2.4.4 Primer pair evaluation

Primer pairs binding to each gene of interest (GOI), two different pairs for PPARγ and one for C/EBPα, were designed. In addition, evaluation of two different housekeeping genes, transferrin receptor (TFRC), and Hypoxanthine guanine phosphoribosyl transferase (HPRT), were conducted. Quantification of housekeeping genes in qRT-PCR, used as an endogenous control, was later used for normalization of gene of interest expression (see calculations) in each of the conditions. The primers were altogether evaluated to identify the most suitable options.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer sequence (5’-3’)</th>
<th>Reverse primer sequence (5’-3’)</th>
</tr>
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<tbody>
<tr>
<td>TFRC</td>
<td>TCTGGATCCAGCTGTTGCCTTCAACTC</td>
<td></td>
</tr>
<tr>
<td>HPRT</td>
<td>CGTCTTGCTGAGATGTGATG</td>
<td>TTTAGCCCCCTTGAGCAC</td>
</tr>
<tr>
<td>PPARg1</td>
<td>GCCCAAGTTGAGTTTGTGCTG</td>
<td>TCAATGGGCTTCACATTAGC</td>
</tr>
<tr>
<td>PPARg2</td>
<td>AGATCCAGTGTTCAGATTA</td>
<td>GGAGATGCGAGCTCCACTTT</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>TGTATACCCCTGTTGGAGA</td>
<td>TCAATACCGTCCCTCTG</td>
</tr>
</tbody>
</table>

2.4.5 Gene expression quantification

For gene expression quantification, 2 µg cDNA, mixed with optimized forward and reverse primers for the genes of interest and the reference genes, and Power SYBR™ Green (Applied Biosystems™), was added to a 96 well plate and analyzed in a qRT-PCR assay (Power SYBR™ Green) with respect to the PPARγ gene and C/EBPα gene, accordingly: (95°C for 10 min, 95°C for 15 sec, 60°C for 1 min) repeated 40 times, 95°C for 1 min, 55°C for 1 min, and establishing melt curve from 55°C to 95°C with a 0.5°C increment for 10 sec). The Ct values obtained from the qRT-PCR analysis were used to calculate the normalized expression of each GOI (see equation 1-3).

Step 1: Normalisation to housekeeping gene (HKG): \[ΔCt = Ct(HKG) − Ct(GOI)\]

Step 2: Normalisation to calibrator: \[ΔΔCt = ΔCt(\text{treated sample}) − ΔCt(\text{untreated sample})\]

Step 3: Exponential expression transforms: \[\text{Fold change} = 2^{−ΔΔCt}\]

Equation 1-3. Equations used for calculation of the normalized expression of the genes of interest.

2.5 Cytokine quantification by ELISA

The supernatants from the well plates were collected and cellular residues were removed by centrifugation (4 min, 600xg, 4°C) The supernatants were stored at -80°C until analysis. The supernatants were thawed for analysis and the cytokines IL6, IL-8, and TNF-α quantified using an ELISA assay. An ELISA plate (Corning™ 96-well, High Binding, Flat-Bottom, Half Area
Microplate) was prepared according to protocol (Mabtech Human IL-6, IL-8, and TNF-α ELISA Basic kit (ALP)) using capture monoclonal antibody (mAb) 13A5 (0.5 μg/mL in PBS) and biotinylated detection mAb 39C3 (1 μg/mL) for IL-6 assay, capture mAb MT8H6 (2 μg/mL) and biotinylated detection mAb 26E5 (0.1 μg/mL) for IL-8 assay, and capture mAb MT1P14 (2 μg/mL) and biotinylated detection mAb MT859 (0.5 μg/ml) for TNF-α assay. The supernatants were added to the plate in duplicates along with standard curves and the plates were subsequently analysed in an ELISA reader (Thermo Scientific Multiskan FC) in wavelength 405 nm.

2.5.2 Statistics

As analyses are made on material from human donors, biological replicates were a limiting factor and the data was not considered normally distributed. Therefore, a matched non-parametric Friedman’s one-way ANOVA was used for analysis of cytokine quantification. P-values <0.05 were considered statistically significant.

3. Results

3.1 Cell morphology and phenotype of primary BM MSCs

The images were analyzed with respect to the general morphology of the BM MSCs in order to monitor any visible alterations arising from response to the cell-cytotoxic drugs. No obvious morphological changes were detected when comparing the different conditions in either of the two BM MSC donors (figure 4A).

FACS analysis was conducted for phenotyping of the MSCs, using antibodies positively and negatively expressed on the BMMSCs cell surface. The analysis confirmed that the BM MSCs had maintained their phenotype through freezing, and through drug exposure (figure 4B). Doxo intracellular content in non-exposed and drug-exposed BM MSCs was measured in the phycoerythrin bandpass filter but revealed no differences between the conditions (figure 4C).

3.2 Accumulation of fat in BM MSCs

The BM MSCs were stained using Oil Red O lipid staining solution to detect lipids, as a correlate of adipogenesis. After the conditions used in this study, no obvious red staining was detected in either of the conditions CTCM (control), Doxo, or Doxo+Dexa (figure 5) These findings were common for both donors.
Figure 4. Results from analysis of BM MSCs morphology and phenotype. A) Morphological studies of the MSCs, before treatment and in the three different conditions (untreated, Doxo, Doxo+Dexa) at 10x magnification. The morphology of the two donors were similar. B) Phenotype of BM MSCs reveal intact cells without C) residual Doxo content. The black curve corresponds to BM MSCs in CTCM (control), the red curve corresponds to BM MSCs in Doxo and the green curve to Doxo+Dexa. The mean fluorescence intensity in the control samples is similar to both of the treated samples.

3.3 Gene expression

The hypothesis of alteration in gene expression was evaluated by quantifying the gene of interest expressions on mRNA level using qRT PCR analysis. Pre-studies was conducted in order to find suitable primer pairs and reference gene. Later, the results from these pre-studies were incorporated into the analyses of the primary studies.
3.3.3 Refer

3.3.2 Primer pair evaluation PPARγ

For the PPARγ gene, two different primer pairs (referred to as primer pair 1 and 2) were evaluated in order to find the primers with highest binding specificity. A qRT-PCR assay was conducted with cDNA synthesized from mRNA from HS27A cell line cultivated in the three different conditions (CTCM, Doxo, Doxo+Dexa). The melt curves from the analysis (figure 8, Tables and figures) were used to evaluate the primer pairs. The two peaks in the melt curve of primer pair 1 (figure 8, Tables and figures) demonstrates that two products were formed in the qRT-PCR reaction, which indicates that the primer pair binds to genetic material beyond the gene of interest. Hence, primer pair 2 was selected for further analyses.

3.3.3 Reference gene evaluation

In order to identify a suitable reference gene for the qRT-PCR analyses, two reference genes, TFRC and HPRT, was used in a qRT-PCR assay with cDNA synthesized from mRNA from HS27A cell line cultivated in the three different conditions (CTCM, Doxo, Doxo+Dexa). The melt curve (figure 8, Tables and figures) showed unspecific binding of the HPRT gene, which indicates that the primer pair binds to genetic material beyond the gene of interest. Hence, the TFRC gene was selected for further analyses.

Figure 5. Lipid staining of the BM MSCs. The lipid staining of BM MSCs in CTCM (control), Doxo, and Doxo+Dexa in magnification 40x. The results were similar for both donors.

3.3.1 RNA extraction

The total RNA was extracted from the cell lysate. RNA was quantified using Nanodrop (wavelength 260 nm) according to table 2.

Table 2. The amounts of extracted RNA [ng/μL] from each donor and condition.

<table>
<thead>
<tr>
<th>Donor</th>
<th>CTCM (control) [ng/μL]</th>
<th>Doxo [ng/μL]</th>
<th>Doxo + Dexa [ng/μL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>253,6</td>
<td>135,7</td>
<td>143,9</td>
</tr>
<tr>
<td>2</td>
<td>47,5</td>
<td>59,4</td>
<td>69,9</td>
</tr>
</tbody>
</table>
3.3.4 Gene of interest quantification

The expression of the genes of interest C/EBPα and PPARγ were quantified with respect to the reference gene TFRC. qRT-PCR analysis of the C/EBPα in donor 1 (figure 6) showed similar expression values for CTCM and the Doxo sample. However, more than a 100% increase in the Doxo+Dexa treated sample were identified. For donor 2, C/EBPα expression for CTCM and Doxo were similar (figure 6), and a 40% decrease in Doxo+Dexa. Quantification of the PPARγ expression (figure 6) of donor 1 BM MSCs showed similar expression in CTCM and Doxo. In contrast, Doxo + Dexa displayed a 100% decrease in fold change. The PPARγ expression pattern of donor 2 (figure 6) displayed a similar expression in CTCM, Doxo, and Doxo+Dexa. However, any overall differences in the PPARγ expression between Doxo-treated cells and Doxo+Dexa-treated cells in both donor 1 and donor 2 were not detectable.

![Gene expression quantification](image)

**Figure 6.** Gene expression quantification. The gene expression levels of the genes of interest C/EBPα (left graph) and PPARγ (right graph) in donor 1 and donor 2.

3.7 Cytokine production

The production of the three cytokines IL-6, IL-8, and TNF-α was quantified in the three conditions in order to detect alterations. The performance of the ELISA was validated by means of standard curves (figure 9, Tables and figures). Upon evaluating the concentrations of IL-6, IL-8 and TNF-α, only the first adipokine was measurable in the BM MSC cultures (figure 7). There were no significant changes in the amount of IL-6 after exposure to the cell-cytotoxic drugs.

![IL-6 quantification](image)

**Figure 7.** Quantification of cytokine IL-6. The cytokine production was analysed using an ELISA assay.
4. Discussion

The aim of this project was to study if and how cancer treatment, in particular the commonly used anthracycline Doxorubicin, can influence BM MSCs adipocyte differentiation, and hence cause metabolic conditions in cancer survivors. The study was conducted through treating BM MSCs in vitro and analyzing the BM MSCs differentiation in terms of lipid accumulation, gene expression, and adipokine production.

Microscopical analysis and phenotyping of the BM MSCs confirmed that the cells were intact throughout freezing and treatment, and any behaviour seen from the cells during downstream analysis should correspond to their normal behaviour. Measuring the residual Doxo content in the cells from each condition revealed no differences between the control samples and the treated samples. The lack of residual Doxo either indicates that the cells have metabolised the Doxo and survived, or that the Doxo were not taken in by the cells.

Several studies have reported that cytotoxic drugs in general impair viability in BM MSCs (23) (24). In conjunction with these findings, previous studies in the group showed that BM MSCs exposed to Doxo in vitro tend to grow slightly wider and rounder than their typical elongated fibroblast morphology (2). Cell swelling can be a result of cellular stress upon cytotoxic drug exposure, and additionally, it can be a result from adipocyte formation. However, such morphological changes by means of cell swelling could not be detected from studying the BM MSCs in microscope.

Lipid accumulations within the cells are one sign of differentiation skewed towards adipogenesis. After the 2-day recovery, no staining was detected in the cells. The absence of lipids indicates that there was no skewed differentiation; however, there is a possibility that a longer recovery time, and thereby a longer time for the MSCs to properly display their adipocyte characteristics, had given more visible staining. In a similar study, BM MSCs in adipogenic induction media required 21 days for formation of intracellular lipid droplets (25). Since the MSCs, especially in the negative control, rapidly overgrows in the 6 well plates, there were no possibility to let the cells recover for a longer period. An alternative culture strategy to halt the negative control cells may be a way forward in these settings.

Overall, no statistical analyses can be applied to the results from the qPCR analyses, due to limited number of biological replicates. Although the lack of statistical power limits our conclusions, the data shows patterns in conjunction with previous studies made (14,18). The expression of C/EBPα was similar in the control and the Doxo samples for both donors. However, the Doxo+Dexa sample displayed an increased expression of C/EBPα in donor 1, while donor 2 displayed a slight decreased expression. The expression of C/EBPα in BM MSCs is preceded by other C/EBP isomers, and time can yet again be a limiting factor; if the cells had been recovering for a longer period of time, an alteration in expression of C/EBPα might have been detected. If the other C/EBP isomers were included in the quantification, this might have given an indication on whether adipogenesis were actually induced in the cells. As regards to PPARγ, the expression only differed in the Doxo+Dexa sample of donor 2, in which the expression decreased by 100%. Interestingly, the same donor displayed the biggest increase in C/EBPα expression. Whether or not these results coincide is difficult to deduce, but since the two genes generally are co-dependent, the two gene expression might have affected one another. Interestingly, it is the Doxo+Dexa samples that deviates the most in gene
expression. As previously stated, Dexa is involved in induction of both C/EBPα and PPARγ, and
contradictorily also has the potential of inducing osteogenesis, which through the competitive balance
with adipogenesis inhibits adipocyte formation. The complex nature of the impact of Dexa on
adipogenesis might be an explanation for the differences in gene expression.

The cytokine quantification using ELISA gave varying results. The quantification of the fat and
inflammation associated cytokine IL-6 indicated an increased production in both donors, exposed to
both Doxo alone and to Doxo and Dexa in combination. This tendency of increased IL-6 production
has been shown earlier (2) and corresponds to a normal behaviour of the cells. In contrast, the
cytokines IL-8 and TNF-α was not detected in any of the conditions. The cytokine IL-8 (also known
as CXCL8) is released from BM MSCs during inflammation and similar environmental stresses, and
can promote cancer cell growth and metastasis. In healthy cells and tissues, IL-8 has been reported
both to be expressed at a basal level, and at an undetectable level (26,27). The expression is induced
from stimulation from other cytokines, for instance IL-1, IL-6, and TNF-α, and from stresses such as
inflammation and hypoxia (26). In addition, Dexa has been reported to inhibit the expression of IL-8
(2). Taken together, there are several explanations to why IL-8 is undetected in this experiment. Lack
of stress induced by the exposure to cytostatic drugs, lack of induction from other cytokines such as
TNF-α, have certainly affected the results. The effect of Dexa can not be confirmed, since IL-8 also
was absent in the cells treated only with Doxo. Neither the pro-inflammatory cytokine TNF-α was
detected in any of the conditions, which can be explained with the same causes as for IL-8.

The lack of significant differences in gene expression levels in the BM MSCs might have genetic
causes, and in future studies, a ChIP sequence analysis can rule out that alterations in the chromatin
levels of PPAR and C/EBP have influenced the expression. Studies have reported that 10-15% of
acute myeloid leukaemia (AML) patients harbour mutations in the C/EBP gene. These mutations lead
to expression of truncated isoforms of C/EBPα, for instance C/EBPα p30, that is able to regulate
transcriptional process by recruiting chromatin-modifying complexes. In addition, previous studies
have found that epigenetic downregulations of the PPAR gene are found in colon cancer patients.
Thus, studying the epigenetic modifications, i.e. alterations to the chromatin structure that alters gene
expression, of C/EBP and PPAR in BM MSCs could yield important insights into whether
chemotherapy can have long-lasting impact on the epigenetics of stem cells (28).

Overall, combination therapy makes it difficult to determine the effects of the effects of the individual
agents. Dexa can induce adipogenesis as well as inhibit osteogenesis, and this fact can have skewed
the results in this study (11). Given the insights on the intricate competitive balance between
adipogenesis and osteogenesis in BM MSCs differentiation, one possibility of proceeding with this
study is to expand analyses to include osteogenic factors, for instance in gene expression
quantification. The conditions for cultivation is crucial in the differentiation program, and could have
been optimized. For instance, previous studies have reported that incubating BM MSCs in
three-dimensional vessels, mimicking biological conditions, improve BM MSCs cultivation.

Our hypothesis could not be confirmed. Previous studies have seen cellular and molecular alterations
following exposure to both Doxo and Dexa. There are several explanations to why these results could
not be replicated in this experiment, in particular regarding the experimental setup, mentioned earlier.
The biggest limitation in this study is the low number of biological replicates. No conclusions can be
drawn from results yielded from two donors only; in order to draw conclusions from the results, a
bigger set of biological replicates.
5. Future perspectives

The survival rate of paediatric ALL patients has remarkably improved during the last decades, yet ALL survivors have a high risk of developing late onset complication following recovery. It is of greatest importance that these survivors are given good prerequisites for having a future free from further suffering. A lot of research is conducted in the field of cancer but more is required. The complex molecular basis behind adipogenesis is well studied but yet not completely understood. Were this project to proceed, increasing the number of donors would be crucial for retrieving significant results. In addition, there are many factors within the experimental setup that could be expanded or improved. Given the complex interactions between the transcription factors and the genetic expression, further transcription factors and genes could be studied. Improvements to certain cultivation conditions can be made, such as overcoming the dilemma of giving the cells enough recovery time after exposure without overgrowing them.

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Lastly, I would like to send love to the entire floor 9 at Bioclinicum. Thank you for always being helpful and supportive when I’ve been confused in the lab.
References


Tables and figures

Table 3. List of monoclonal antibodies used for flow cytometry.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Antibody</th>
<th>Fluorochrome</th>
<th>Dilution factor</th>
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Figure 8. Primer pair melt curves of the HPRT housekeeping gene in qPCR analysis.
Figure 9. Primer pair melt curves of the TFRC housekeeping gene in qPCR analysis.

Figure 10. Melt curves of the PPARγ primer pair 1 in qPCR analysis.
Figure 11. Melt curves of the PPARγ primer pair 2 in qPCR analysis.

Figure 9. Standard curves for the ELISA analyses of the cytokine IL-6.
Figure 10. Standard curves for the ELISA analyses of the cytokine IL-8.

Figure 11. Standard curves for the ELISA analyses of the cytokine TNF-α.
Table 4. Coefficient of determination (R2) for the ELISA standard curves for each of the cytokines IL-6, IL-8, and TNFα.

<table>
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<tr>
<th>Cytokine</th>
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