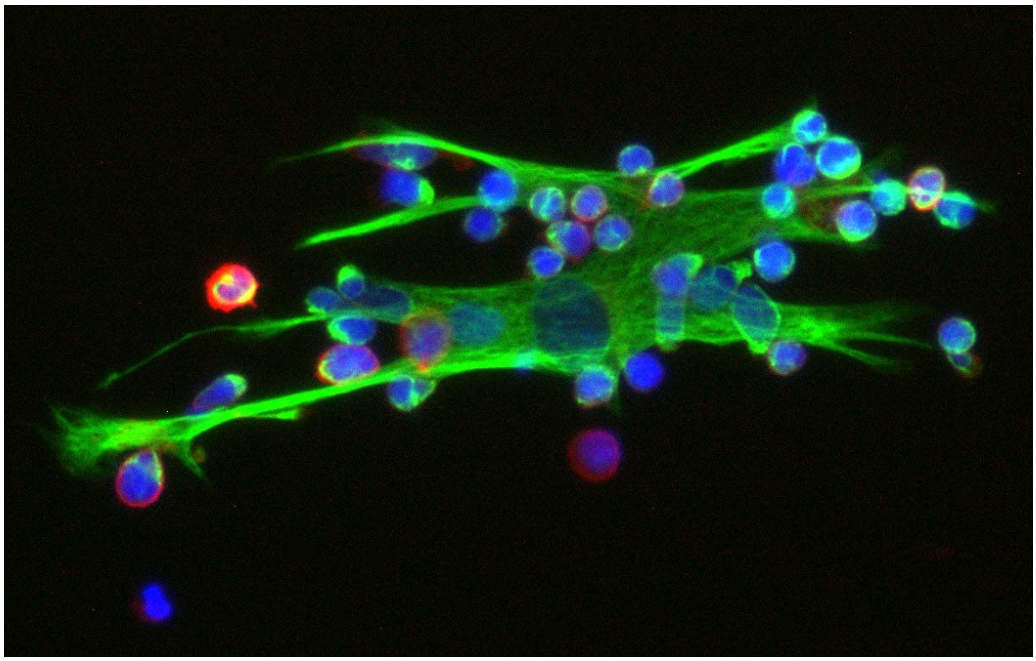
AALBORG UNIVERSITET
STUDENTERRAPPORT

DEVELOPMENT OF AN IN VITRO MODEL FOR ACUTE LYMPHOBLASTIC LEUKEMIA



Master thesis

Medicine with Industrial Specialization, biomedicine

School of Medicine and Health, Aalborg University

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Abbreviations

ALL	Acute lymphoblastic leukemia
ASC	Adipose-derived stem cells
AraC	Cytosine β-D-arabinofuranoside
α-SMA	Alpha smooth muscle actin
Ang-1	Angiopoietin 1
B-ALL	B-cell ALL
Bcl-2	B-cell lymphoma 2
CAF	Cancer associated fibroblast
CAR cells	CXCL12-abundant reticular cells
CM	Cytokine mix
CXCL12	C-X-C motif ligand 12
ECM	Extracellular matrix
FAP	Fibroblast activation protein
FGF	Fibroblast growth factor
FLT-3	Fms-like tyrosine kinase receptor 3
HC	Hydrocortisone
HIF-1	Hypoxia-inducible factor
hPL	Human platelet lysate
HS	Human serum
HSC	Hematopoietic stem cells
LepR	Leptin receptor
MSC	Mesenchymal stromal cells
ODD	Oxygen-dependent degradation domain
OPN	Osteopontin
PDGFR-α	Platelet-derived growth factor-receptor α
P-MSC	Primary human bone marrow derived MSC
SCF	Stem cells factor
SS	Stem Span II
T-ALL	T-cell ALL
TERT-MSC	Human EGFP-hTERT transduced MSC
TGF-β	Transforming growth factor-β
THPO	Thrombopoietin
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

English abstract

Background: Acute lymphoblastic leukemia (ALL) is a hematological cancer affecting the lymphoid cell line and is the most common type of cancer in children. Even though the survival has increased a lot in pediatric patients over the last decades, the prognosis for ALL in both older adults and infants remains poor why further research is still needed. ALL cells have, however, proven to be difficult to study, as they die rapidly in vitro. It has been suggested that the microenvironment of the bone marrow contains important components for supporting ALL cells, such as supportive stromal cells, cytokines, growth factors, ECM components, and hypoxia. The aim of this study is therefore to create an in vitro model for ALL with inspiration from the microenvironment in the bone marrow.

Methods: In this study, an ALL in vitro model consisting of ALL cells in co-culture with primary mesenchymal stromal cells (P-MSC) on a 1:1:1 collagen, laminin, and fibronectin coat was developed. The optimal medium for ALL cells was examined and the supportive effect of selected cytokines (Flt-3, IL-2, IL-3, Il-7, SCF) and the growth factor rich human platelet lysate (hPL) was investigated by flow cytometry with an apoptosis assay. Stromal cells, such as P-MSCs, human EGFP-hTERT transduced MSC (TERT-MSCs), and adipose-derived stem cells (ASCs) were investigated with ICC for the stem cells marker nestin in normoxia and hypoxia in both monoculture and ALL co-culture, as nestin positive cells has been linked to improved survival of ALL cells in co-culture. Survival and Cytosine β-D-arabinofuranoside (AraC) sensitivity of ALL cells in model 0.1 in both normoxia and hypoxia was investigated with sytox stain by automated microscopy. Statistical analysis of the result was performed in IMB SPSS Statistics version 25.

Results: The results obtained from the medium optimization experiment showed increased survival of ALL cells supplemented with cytokines and hPL ($p<0.05$). The ICC staining showed labeling of nestin in all stromal cell types, with the strongest nestin labeling in stromal cells in co-culture with ALL cells. The sytox stain of ALL cells in co-culture and model 0.1 showed improved survival compared to monoculture ($p<0.05$) in both normoxia and hypoxia ($p<0.05$).

Conclusion: Previous studies have suggested that multiple factors are important in the support of ALL cells in vitro, and this could also be concluded based on the results obtained in this study. Factors such as cytokines, hPL, ECM components, and hypoxia all seemed to improve the survival of ALL cells in vitro.

Dansk resumé

Baggrund: Akut lymfoblastisk leukæmi (ALL) er en hæmatologisk kræftsygdom, der påvirker lymfoide cellelinjen og er den mest almindelige kræftform hos børn. Selvom overlevelsen er steget meget hos de pædiatriske patienter i løbet af de sidste årtier, er prognosen for ALL hos både ældre voksne og spædbørn stadig dårlig, hvorfor der stadig er behov for yderligere forskning. ALL-celler har i midlertidig vist sig at være svære at studere, da de dør hurtigt in vitro. Det er blevet foreslået, at mikromiljøet i knoglemarven indeholder vigtige komponenter til at understøtte ALL-celler, såsom understøttende stromale celler, cytokiner, vækstfaktorer, ECM-komponenter og hypoxi. Formålet med dette studie er derfor at skabe en in vitro model for ALL med inspiration fra mikromiljøet i knoglemarven.

Metoder: I dette studie blev der udviklet en ALL in vitro model bestående af ALL-celler i co-kultur med primære mesenkymale stromale celler (P-MSC) på en 1:1:1 kollagen, laminin og fibronektin coat. Det optimale medium for ALL-celler blev undersøgt, og den understøttende virkning af udvalgte cytokiner (Flt-3, IL-2, IL-3, Il-7, SCF) og det vækstfaktorrige humane blodpladelysat (hPL) blev undersøgt ved flowcytometri med en apoptoseanalyse. Stromale celler, såsom P-MSC'er, humane EGFP-hTERT-transducerede MSC'er (TERT-MSC'er) og adipøst deriverede stamceller (ASC'er) blev undersøgt med ICC for stamcellemarkøren nestin i normoksi og hypoxi i både monokultur og ALL co-kultur, da nestin-positive celler er blevet forbundet med forbedret overlevelse af ALL celler i co-kultur. Overlevelse og Cytosin β-D-arabinofuranosid (AraC) følsomheden af ALL-celler i model 0.1 i både normoksi og hypoxi blev undersøgt med sytox farvning ved automatiseret mikroskopi. Statistisk analyse af resultatet blev udført i IMB SPSS Statistics version 25.

Resultater: Resultaterne opnået fra medium-optimeringseksperimentet viste øget overlevelse af ALL-celler suppleret med cytokiner og hPL ($p < 0,05$). ICC-farvningen viste farvning af nestin i alle stromale celletyper, med den stærkeste nestin-farvning i stromale celler i co-kultur med ALL-celler. Sytox-farvningen af ALL-celler i co-kultur og model 0.1 viste forbedret overlevelse sammenlignet med monokultur ($p < 0,05$) i både normoksi og hypoxi ($p < 0,05$).

Konklusion: Tidligere undersøgelser har antydet, at flere faktorer er vigtige for at støtte ALL-celler in vitro, og dette kunne også konkluderes baseret på resultaterne opnået i dette studie. Faktorer som cytokiner, hPL, ECM-komponenter og hypoxi syntes alle at forbedre overlevelsen af ALL-celler in vitro.

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1 Introduction

1.1 Acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is a hematological cancer affecting the lymphoid cell line. The cells undergo a malignant transformation that results in the uncontrolled proliferation of lymphoid progenitor cells that invade the bone marrow and blood. ALL can also migrate from the primary focus in the bone marrow and invade extramedullary sites such as the brain (Meyer & Hermiston, 2019; Terwilliger & Abdul-Hay, 2017).

ALL is the most common type of cancer in children, and pediatric patients account for around 80% of all cases. Unfortunately, while the 5-year overall survival for the pediatric patients has increased to approximately 90%, the prognosis for ALL in both older adults (>50 years) and infants (<1 year) remains poor, which is why further treatment improvements are needed (Bassan & Hoelzer, 2011; Lee & Cho, 2017).

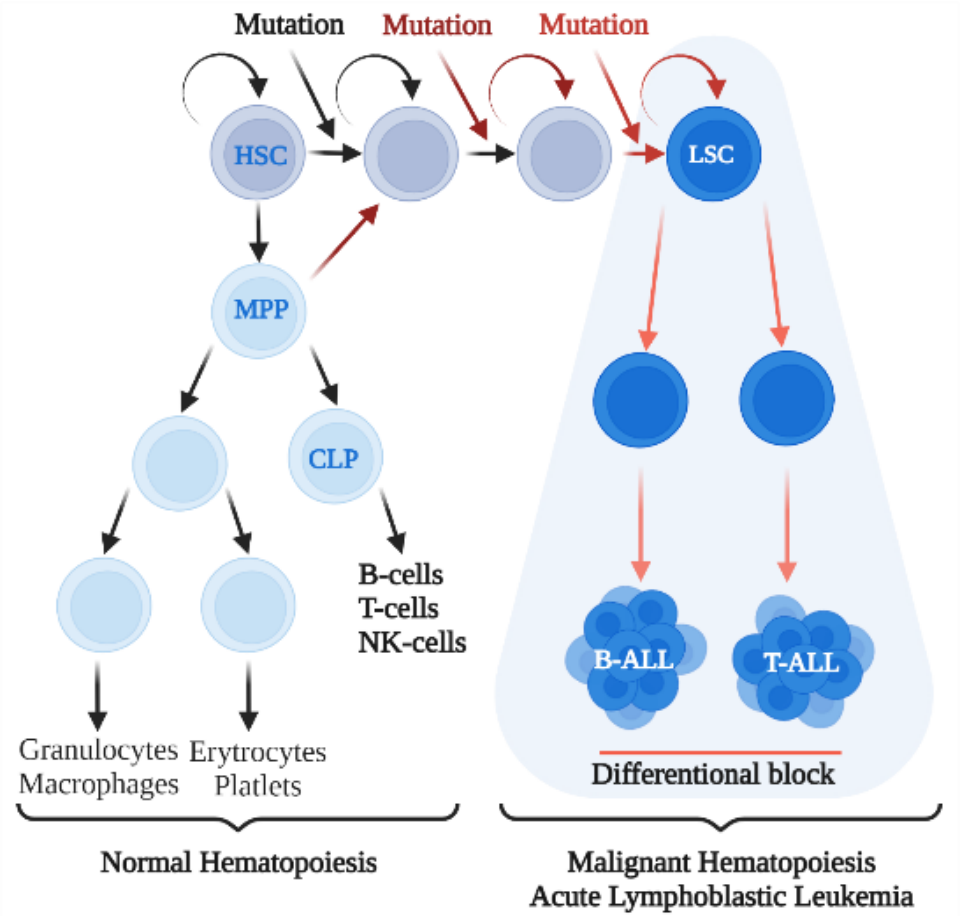
ALL can be divided into three major subtypes based on the affected lymphocyte type and the appearance of genetic or chromosomal changes: B-cell lymphoblastic leukemia (B-ALL) characterized by recurrent genetic abnormalities or not specified B-ALL and finally, the T-cell lymphoblastic leukemia (T-ALL). The B-ALL types are the most common and represent approximately 75% of the cases, while the T-ALL accounts for around 25%; a mixed phenotype also exists, but this type is very rare and seen in 3-5% of all cases. Different types of ALL are associated with different prognoses and may also require other treatment strategies (Sakurai et al., 2021; Terwilliger & Abdul-Hay, 2017; Weinberg & Arber, 2010)

Lymphocytes and other blood cells are derived from hematopoietic stem cells (HSC) through a complex and highly regulated series of events called hematopoiesis. Hematopoiesis starts during embryogenesis but changes drastically from neonates to children to adults. Preterm hematopoiesis occurs in multiple sites in the body including the preterm bone marrow, the fetal liver, and the extraembryonic yolk sac. After birth, the hematopoiesis gets restricted to bone marrow and the production of new red blood cells diminishes abruptly. With age, the haematopoietically active red marrow gets replaced with yellow marrow, and active hematopoietic sites get restricted to the sternum, ribs, skull, pelvis, and vertebrae. HSCs are crucial in providing cells that transport oxygen

to tissue throughout the body and cells that populate the immune system (Dixit & Luqman, 2016; Kawahara, 2007; Klamer & Voermans, 2014; Prabhakar et al., 2009).

In ALL, the hematopoietic function is disrupted and changed in a malignant manner (*Figure 1*). There is an accumulation of early lymphoblastic progenitor cells in the bone marrow due to lymphoid cells proliferating uncontrollably and a differentiation blocking at an immature state (Terwilliger & Abdul-Hay, 2017).

Figure 1, Malignant Hematopoiesis (Created with BioRender.com)

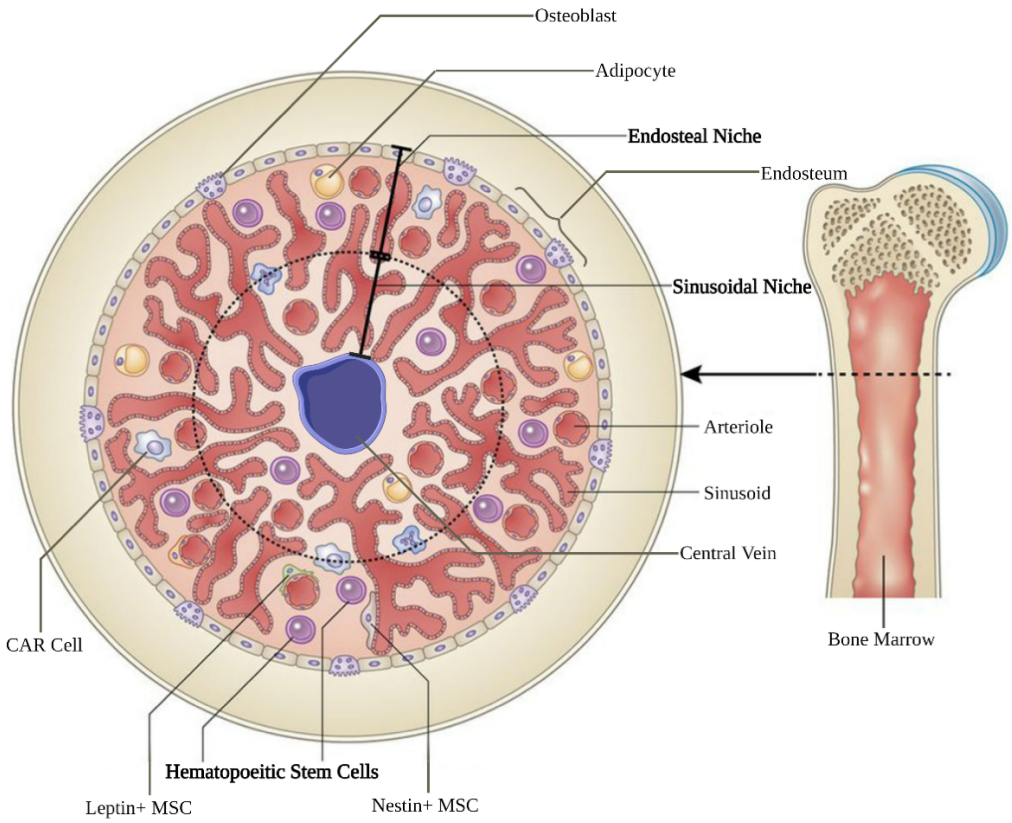


1.2 The bone marrow and its niches

The bone marrow is a complex tissue that influences the cellular fate of the HSCs. Histologically the bone marrow is divided into a central vascular compartment consisting of networks of sinusoids and arterioles that branch out into the surrounding stroma delimited by the endosteum. The non-hematopoietic part of the stroma comprises a variety of cells from the mesenchymal, endothelial,

and neural origin that is organized to provide structural cues that aid in creating a microenvironment that supports hematopoiesis employing proliferation, differentiation, and maturation of the HSC. The HSCs and their progenitor cells are neatly distributed throughout the bone marrow stroma and have been found to reside in niches, (*Figure 2*). Two niches have previously been described, a sinusoidal or central niche representing 90% of the bone marrow and an endosteal or peripheral niche representing 10% of the bone marrow (Chatterjee et al., 2021; Kopp et al., 2005; Travlos, 2006; Woods & Guezguez, 2021).

Figure 2, The Bone Marrow - The Endosteal Niche and The Sinusoidal Niche (Modified from (Mendelson & Frenette, 2014)



The endosteal niche is located at the interface between the mineralized bone, the endosteum, and the bone marrow. The majority of the HSCs found in this niche are quiescent and possess a high self-renewal potency. In both the endosteal- and the vascular niche, supportive cells like mesenchymal stromal cells (MSC) are found, but these cells have a high 'osteolineage' capacity in the endosteal niche. Osteocytes and osteoblasts are also found in the endosteal niche. The osteoblasts have been shown to provide important structural support for the HSCs by binding N-cadherin and cell-cell interactions mediated through Notch/Jagged-1. Both osteocytes, MSCs, and

their osteoprogenitor cells are known to secrete various factors that preserve the stemness and quiescence of the HSCs. These factors include osteopontin (OPN), C-X-C motif ligand 12 (CXCL12), thrombopoietin (THPO), stem cell factor (SCF), and angiopoietin-1 (Ang-1), and upon binding to their corresponding receptor on the HSC, the HSC becomes resistant to proliferation signals and stay in their dormant stage. Besides preventing the release of HSC, these factors also promote cell division that helps maintain the stem cell pool. HSC expresses calcium-sensing receptors and has been shown to home and retain in calcium-enriched regions. The endosteal niche is just that as trabecular bone is continually remodeled due to osteoclastic activity, which results in the release of calcium (Chatterjee et al., 2021; Klamer & Voermans, 2014; Sugiyama et al., 2006; Woods & Guezguez, 2021; Yamada et al., 2013).

The vascular niche relates to the area proximal to the central vein in the sinusoidal network. The sinusoidal endothelial cells have a dual function in this niche. First, they have a structural role that provides a permeable and tightly regulated barrier between the bone marrow and the blood. They provide chemical cues that contribute to a microenvironment where HSCs can self-renew, expand, and maintain their population. The endothelial cells, e.g. express E-selectins that promote HSC proliferation and CXCL12, which is crucial in the differentiation process of the HSC together with induction of the trans-endothelial migration of the HSCs to the circulation. Surrounding the sinusoidal compartment in the bone marrow, many different cell types are found, including adipocytes, pericytes, neurons, and MSCs. The adipocytes have shown to be of great importance for the bone marrow, as they secrete many nutrients together with factors and cytokines like CXCL12, SCF, IL-3, and IL-6, which are known to be involved in the maintenance of HSC. A cell type that has proven to be of great importance in both the endosteal and vascular niche is the MSCs (He et al., 2014; Kim et al., 2020; Klamer & Voermans, 2014; Kopp et al., 2005; Woods & Guezguez, 2021).

1.3 Mesenchymal stromal cells of the bone marrow

MSCs are colony-forming, plastic-adherent cells that have a trilineage differentiation potential. They have proven to have a huge impact on the HSCs despite their very small fraction in the bone marrow (<0.1% of adult bone marrow cells). MSCs reside in many different tissues besides the bone marrow and are described as a heterogeneous cell population known for their

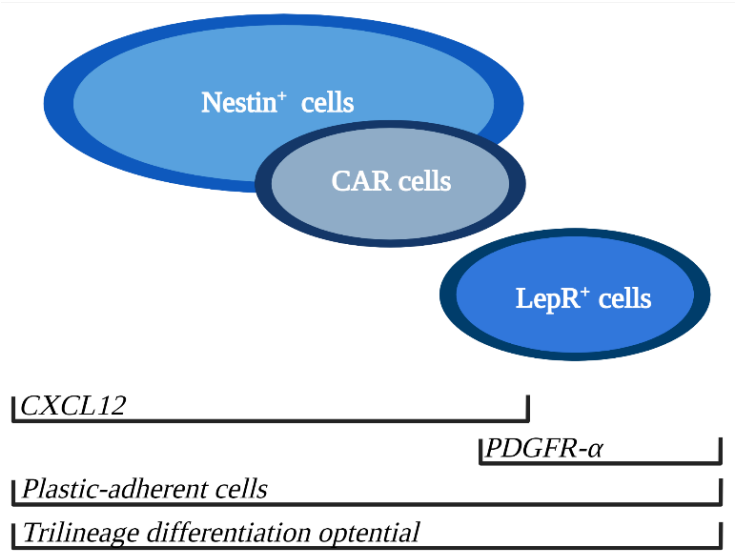
immunomodulatory functions and ability to produce nutrients (Bello et al., 2018; Horwitz et al., 2006; Woods & Guezguez, 2021).

The bone marrow MSCs are, as mentioned, a heterogeneous cell population, and different subpopulations have shown to possess different functions in the bone marrow (*Figure 3*). Previous studies have identified the subpopulation leptin receptor (LepR) positive MSC. These LepR+ MSC also possess the marker platelet-derived growth factor-receptor α (PDGFR-α) and have given rise to most adipocytes and osteoclasts in the bone marrow. In addition, the LepR+ MSC is a major source of the cytokines CXCL12 and SCF that are crucial for HSC (Matsuzaki et al., 2014; Woods & Guezguez, 2021).

The LepR+ MSC's secretion of the cytokine CXCL12 is similar to another stromal subpopulation in the bone marrow called the CXCL12-abundant reticular cells (CAR cells). The CAR cells have proven to be particularly important for the HSCs as they possess the CXCL12 that can bind to the receptor CXCR4 found on most cells, including HSCs. This CXCL12-CXCR4 signaling plays an important role in the maintenance of the HSC pool in the bone marrow as CXCR4 and CXCL12, upon binding, activate signaling pathways that are involved in hematopoiesis, cell migration, cell homing, and cell retention in the bone marrow (Bianchi & Mezzapelle, 2020; Sugiyama et al., 2006).

Another subpopulation of the bone marrow MSCs is the nestin-positive cells (nes+ MSC) that have shown to be more similar to earlier progenitor cells than other bone marrow MSC, e.g. due to their self-renewing properties besides their trilineage differentiation potential. The nes+ MSC also has supportive effects on HSCs regarding homing and maintenance. They have shown high expression levels of HSC regulatory genes, such as Cxcl12, IL-7, c-kit ligand, OPN, and vascular cell adhesion molecule-1 (VCAM-1). Nes+ MSCs have been localized near the vascular compartment in the bone marrow and also in co-localization with HSCs, where they are associated with cobblestone-formation of hematopoietic progenitor cells (Lindsay & Barnett, 2017; Méndez-Ferrer et al., 2010; Pinho et al., 2013; Xie et al., 2015).

Figure 3, Subpopulations of Mesenchymal Stromal Cells in the Bone Marrow (Created with BioRender.com)



As mentioned, the MSCs have an important exocrine function for the cells in the bone marrow, where they secret an array of different cytokines, some more important than others. The cytokines involved in the hematopoiesis can be hierarchically arranged, depending on how broadly the cytokines act. At the top of the hierarchy, cytokines like Fms-like tyrosine kinase receptor 3 ligands (Flt-3 ligand), SCF, IL-2, IL-3, and IL-7 are found, as they can act on both multipotent cells but also more lineage-specific cells (Robb, 2007).

Flt-3 ligand and SCF work by regulating hematopoietic growth together with maintenance of self-renewal and proliferation of the hematopoietic stem cells, and SCF has specifically proven to be required for the long-term preservation of HSCs in the bone marrow. In addition, IL-2 and IL-7 are both involved in the survival and proliferation of lymphocytes through the activation of STATA5, and IL-3 can stimulate growth across multiple hematopoietic lineages (Fry & Mackall, 2002; Giampaolo et al., 2017; Li & Wu, 2011; Robb, 2007; Woods & Guezguez, 2021).

Growth factors like vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and transforming growth factor-β (TGF-β) have also proven to be important in the maintenance and proliferation of HSCs (Allouche & Bikfalvi, 1995; Gerber et al., 2002; Vaidya & Kale, 2015).

1.4 Stiffness

Besides biochemical factors, the biophysical factors in the bone marrow have also proven to be of great importance for the HSCs, such as oxygen levels, extracellular matrix (ECM) presentation, stiffness, and topography. The topographic surface and the stiffness change across different regions in the bone marrow niche due to changes in the cellular composition. In the central region of the bone marrow, the vascular niche, cells like endothelial cells and adipocytes are found. Therefore, the relative stiffness is lower, around 3 kPa, compared to the proximal region of the bone marrow, the endosteal niche, where the relative stiffness is much higher, as cells like osteoclasts, osteoblasts and their progenitor cells are present. The relative stiffness is around 35-40 kPa (Bello et al., 2018).

1.5 Extracellular matrix components of the bone marrow

The cells in the bone marrow are arranged within an ECM scaffold, thus, the ECM provides structural support for the cells, but this is far from the only important mechanism of the ECM in the bone marrow niche. The ECM can influence associated cells in many ways with physical properties such as porosity, pore size, viscoelasticity, and stiffness which can be crucial for the cell's differentiation pathway. MSCs have, for instance, presented the ability to differentiate in a lineage-specific way based on the stiffness of the ECM. The spatial organization of the ECM is also important as this determines the presentation of cell adhesion epitopes that are available to the associated cells, and this dimensionality can be vital for the fate of stem cells. Finally, there is the biochemical complexity, which describes the specific composition of matrix proteins. This can be niche specific and play important roles in activating intracellular signaling pathways regulating the growth and differentiation of cells within the bone marrow. Some of the most important ECM-proteins in the bone marrow are collagens, fibronectin, and laminins (Akhmanova et al., 2015; Bello et al., 2018; Engler et al., 2006).

Collagen is the most abundant ECM protein in the bone marrow; it is made of chains of amino acids that have been bound together, forming a triple helix. Collagen is found in radiating supportive strands throughout the bone marrow stroma and in layers surrounding the blood vessels, the trabeculae, and the sinusoidal endothelial alongside the basal surface. Different collagens can be found in the ECM in the bone marrow, but the most abundant is collagen IV, which accounts for 60% of the total amount of collagen found in the bone marrow, the second in line is collagen I,

which accounts for 30%. Collagen seems to form a supportive framework where other ECM proteins can attach. One such protein is fibronectin, which has a collagen-binding domain. Fibronectin is the second most abundant ECM protein found in the bone marrow and is made up of two amino acid chains forming a heterodimer. Fibronectin is a cell adhesion protein that can bind to cell surface receptors, such as integrins, and is known to play an important role in hematopoiesis as fibronectin-binding integrins are found on the surface of hematopoietic stem cells. Fibronectin has been involved in cellular processes such as proliferation, differentiation, apoptosis inhibition, and cell attachment and migration (Hamilton & Campbell, 1991; Marinkovic et al., 2020; Wirth et al., 2020).

Both fibronectin and co-culture with stromal cells have proven to support the anchorage of hematopoietic stem cells. Since most of the cells found in the bone marrow niche can produce fibronectin, it has been hypothesized that the stromal cells ability to produce fibronectin provides a beneficial effect on the HSCs (Wirth et al., 2020).

The ECM protein laminin, like fibronectin, has also proven to be an important factor in the bone marrow niche regarding adhesion and differentiation of hematopoietic progenitor cells and can also interact with other ECM proteins like collagen. Laminin is a heterotrimeric ECM protein that consists of three chains, an α-chain, a β-chain, and a γ-chain. As different types of these three chains exist, there are many different isoforms of laminin, and not all of these are found in the bone marrow niche. For example, α2 laminin, α4 laminin, and α5 laminin are all known components of the ECM in the bone marrow and can bind to HSCs, partly to anchor the cells to the niche and partly to activate intracellular signaling pathways. However, it is important to note that not all laminin isoforms are present in the bone marrow niche. Even though other laminin isoforms might also be cell adhesive ECM molecules, the presence of non-relevant ECM can lead to the activation of non-beneficial patterns like the induction of apoptosis or even malignant transformation (Akhmanova et al., 2015; Sagar et al., 2006; Susek et al., 2018).

1.6 Hypoxia in the bone marrow

The bone marrow is considered a hypoxic environment, as the oxygen tension ranges from 1-6%. The small arteriolar diameter, low blood flow, and placement of the arterioles result in differences in the oxygen concentrations within the bone marrow, and two different microenvironmental

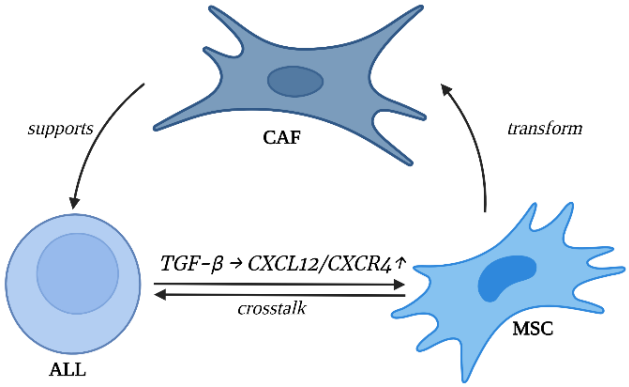
regions have been described as two separate niches as previously mentioned, the endosteal niche with the lowest oxygen levels and the vascular niche with the highest. The most important molecular response to hypoxia is governed by the transcription factor hypoxia-inducible factor (HIF)-1. HIF-1 is a heterodimeric transcription factor that comprises two subunits, HIF-1α, which is oxygen-sensitive, and HIF-1β, which is insensitive to oxygen. It is the post-translational modifications of the oxygen-dependent degradation domain (ODD) in the α-subunit that regulates the activity of HIF-1. In normoxia, HIF-1α is degraded by 26s proteasome as proline residues 402 and 564 in the ODD are hydroxylated, allowing the binding of ligase von Hippel-Lindau tumor suppressor protein. In hypoxic environments, HIF-1α dimerizes with HIF-1β and becomes the stable heterodimeric transcription factor that can activate the transcription of numerous genes involved in cell survival (Chatterjee et al., 2021; Eliasson & Jönsson, 2010; Petit et al., 2016).

1.7 Crosstalk between the bone marrow niche and leukemic cells

Upon the development of leukemia, the leukemic cells interact ('cross-talk') and change the microenvironment in the bone marrow by reprogramming stromal components and reshaping the microenvironment in favor of the survival and growth of the leukemic cells. It is widely believed that many malignancies are dependent on interactions with their local microenvironment, and this is also the case in ALL. Previous studies have shown that MSCs maintain leukemic cells in B-ALL and exert a chemo-protective effect on the ALL cells. Furthermore, bone marrow MSC has been shown to develop a specific phenotype when activated by leukemic cells and become cancer-associated fibroblasts (CAFs), (*Figure 4*). The CAFs express high levels of α-smooth muscle actin (α-SMA) and fibroblast activation protein (FAP), and interestingly these markers only remain in the presence of leukemic cells. In a study by Pan et al. 2020, they found that bone marrow MSC with the CAF-phenotype supported the proliferation of leukemic cells in B-ALL and promoted the colony-formation of the leukemic cells. The exact mechanism behind the transformation of MSC to CAFs remains unknown but Pan et al. (2020) have shown that the cytokine TGF-β plays an

important role in promoting this transformation through the CXCL12/CXCR4 pathway (Burt et al., 2019; Pan et al., 2020, 2021).

Figure 4, MSC transformation to the CAF phenotype (Created with BioRender.com)



HSCs normally express CXCR4 on their surface, but cancer cells in hematological malignancies have been shown to overexpress CXCR4, which is associated with progressive disease. In addition, the expression of CXCR4 and CXCL12 in the malignant bone marrow is partially dependent on the activation of HIF-1α by the hypoxic environment found in the bone marrow (Burger et al., 1999; Tsaouli et al., 2019).

1.8 Limitations of current acute lymphoblastic leukemia in vitro models

Because ALL cells arise from the hematopoietic lineage in the bone marrow and therefore show many similarities to hematopoietic cells, the HSC niche is of great interest and inspiration in developing an in vitro model for ALL. Much effort has been made previously to recreate a healthy bone marrow with the aim to expand HSCs in vitro, but even this has only proven to be a difficult task (Bello et al., 2018).

ALL cell line models have been made and used to study things like drug sensitivity in vitro. Still, even though the original driver mutations are present in the cell line, the molecular complexity is not the same. The cell lines have also been adapted to survive without the support from stromal cells, making it impossible to directly translate the data generated from these models. Culture conditions can also vary a lot from the primary cells because of the lack of complexity in the cell line models. Primary leukemic cells from ALL patients have previously been used in studies, but

they have proven to be very difficult to culture, as they rapidly undergo apoptosis ex vivo. In a study by Nijmeijer et al. 2008, they observed that extensive cell death occurred during the initial 24 hours of in vitro cell culture, making it difficult to study the cells and try out therapeutic interventions (Nijmeijer et al., 2009; Pal et al., 2016).

Like other cancer cells, ALL cells have been shown to overexpress the B-cell lymphoma 2 (Bcl-2) protein. Bcl-2 is a part of the BCL-2 superfamily that regulates the intrinsic apoptosis pathway, whereas Bcl-2 is known to suppress the onset of apoptosis (McBride et al., 2019).

It has been hypothesized that the overexpression of Bcl-2 contributes to the metastatic ability of cancer cells to migrate to and live at suboptimal sites in the body. A study by Campana et al. 1993 observed that the Bcl-2 expression in some ALL patients decreased when cultured in monoculture, which could explain why an extensive cell death occurs in the initial culture period (Campana et al., 1994).

As of now, the in vitro models available for primary ALL cultures are very simplified and rely on cytokine supplemented medium and/or co-cultures with bone marrow niche-cells, such as MSCs or osteoblasts. Some of the most commonly used co-culture systems consist of ALL cells in co-culture with primary bone marrow-derived MSCs from doners, and several studies (Campana et al., 1994; Manabe et al., 1992; Pal et al., 2016) have shown that these co-cultures can actually reduce the extensive amount of apoptosis that has been observed in the early culture phase of ALL cells. In the study by Pal et al., they also found that nes+ human MSCs were superior compared to murine M210B4 cells and human TERT-immortalized MSCs when it comes to the long-term culture of ALL cells (Pal et al., 2016).

Even though 2D cultures have been used for drug testing and pathophysiology studies they lack true representation of the in vivo environment, as the 3-dimensional (3D) structure has been neglected. In the bone marrow, the ECM is providing this structure and it has proven to be important not only for the 3D function it exerts but also for the interactions the ECM components can provide (Scielzo & Ghia, 2020).

1.9 Aim of study

This study aims to create an in vitro model for ALL; as previous studies have suggested, several factors are involved in the survival and maintenance of HSCs in the healthy bone marrow. These factors include support from a niche-specific ECM, co-existing MSC, a hypoxic environment, and certain cytokines. Therefore, this study aims to investigate if these normal niche factors can improve the survival of the ALL cells in vitro.

2 Materials and Methods

2.1 Study design

This study was performed in four parts, as illustrated in *Figure 5*.

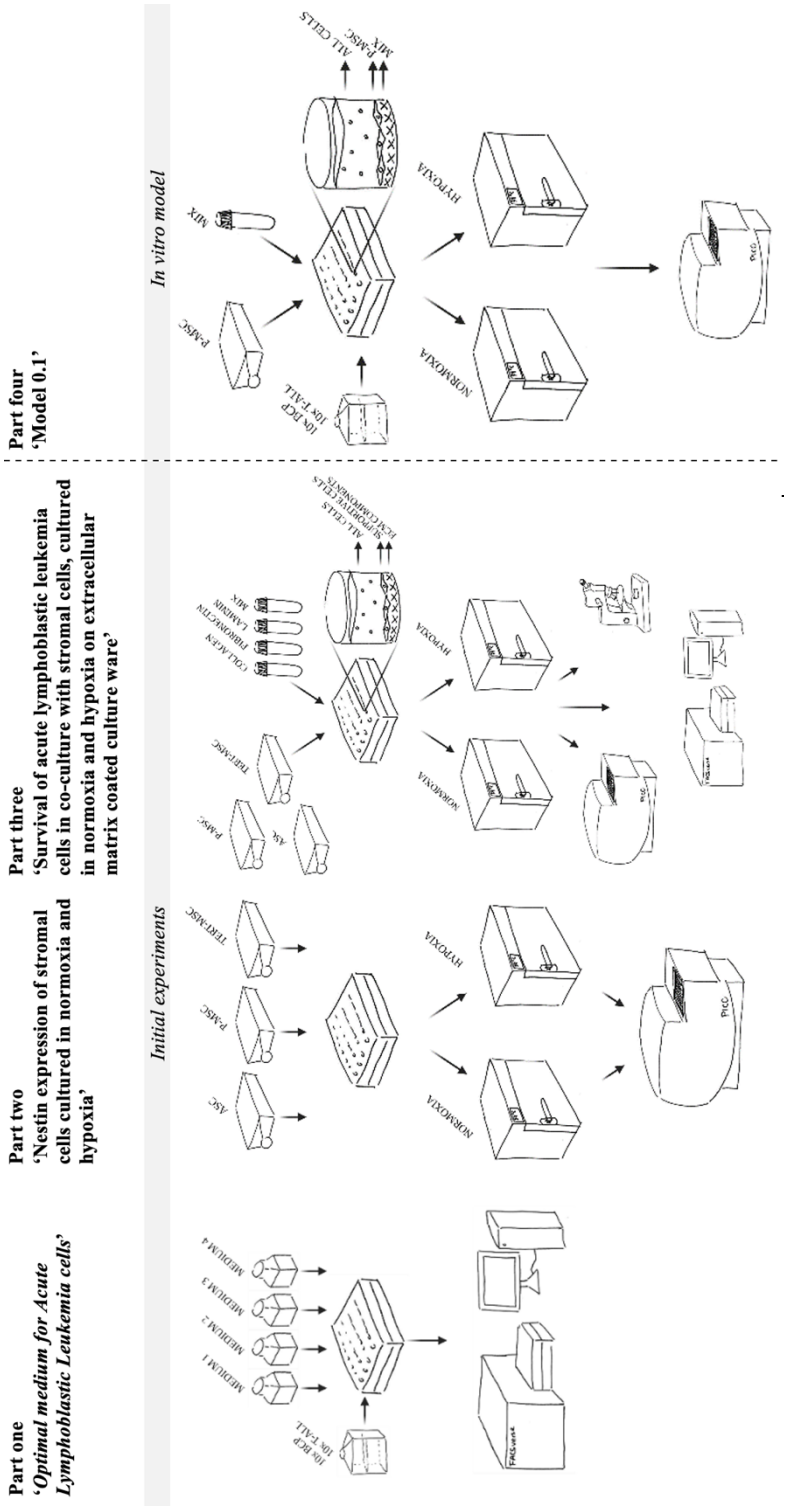


Figure 5, Study design

In the first part, the aim was to identify the most optimal medium for ensuring the viability of B-ALL and T-ALL cells cultured in various cell culture media. The survival was assessed using a flow cytometric assay.

In part two we investigated the expression of nestin in MSCs cultured in normoxia and hypoxia, in monoculture, and in co-culture with ALL cells. The nestin expression was determined using immunocytochemistry.

In part three co-culture with stromal cells, ECM components, and hypoxia were examined, the optimal medium from part one was included, and ALL cell viability and proliferation were investigated.

In part four the optimal medium from part one was included together with appropriate components from part three to create 'model 0.1'. The viability and Cytosine β-D-arabinofuranoside (AraC) sensitivity of five ALL patients were assessed in model 0.1 through viability staining.

The following sections will describe the methodology and protocols for cell cultures used in the experiment and the four experimental parts.

The studies are approved by the Capital Regional Committee on Biomedical Research Ethics (H-2-2010-002) and the Danish Data Protection Agency (journal number 2012-58-0004). All patient samples were collected with informed consent at time points where samples are collected for clinical purposes. The patients did not undergo additional procedures to obtain samples for this project.

2.2 Cell culture of stromal cells and acute lymphoblastic leukemia cell

2.2.1 Cell culture

ALL cell cultures, human EGFP-hTERT transduced mesenchymal stromal cells (TERT-MSC) cultures, and primary mesenchymal stromal cell (P-MSC) (StemCell Technologies, cat.no: 70071) cultures were initiated from cryopreserved cells. In addition, adipose-derived stem cells (ASC) were a gift in culture from Cardiology Stem Cell Centre at Rigshospitalet.

ALL patient cells were obtained from the biobank at the Laboratory of Pediatric Oncology, Rigshospitalet. For this project, ALL cells collected at the time of diagnosis were used.

Mononuclear cells were isolated from the bone marrow and peripheral blood by using sucrose density gradient centrifugation (Lymphoprep, density 1.077 g/ml, Nycomed Pharma, Oslo, Norway).

Table 1, Stromal cells

Stromal cells type	Abbreviation	Manufacture	Passage	....
Primary mesenchymal stromal cells	P-MSC	StemCell technologies cat.no: 70071	P6	
Adipose-derived stem cells	ASC	Gift from Cardiology Stem Cell Centre at Rigshospitalet	P5	
Human EGFP-hTERT transduced mesenchymal stromal cells	TERT-MSC	Obtained from St. Jude's Children's Hospital.	-	

Table 2, ALL patients, BCP and T-ALL

Patients with Acute Lymphoblastic Leukemia

Patient number	country	Sex	Age	ALL subclassification	WBC	CNS status *	cytogenetic analysis	Risk group
1	Denmark	Female	33	B-precursor	13	CNS1		High Risk
2	Denmark	Male	2	B-precursor	51,1	CNS1		Intermediate
3	Denmark	Male	3	B-precursor	5,4	CNS1	t1221	Standard Risk
4	Denmark	Male	8	B-precursor	180,1	CNS1		High Risk
5	Denmark	Male	35	B-precursor	157	CNS1	mllor11q23	High Risk
6	Denmark	Female	5	B-precursor	47,8	CNS1	dic920	Intermediate Risk
7	Denmark	Male	4	B-precursor	6,3	CNS1	Hyperdiploid >/=50 chromosomes	Standard Risk
8	Denmark	Male	26	B-precursor	87,5	CNS1		Intermediate Risk
9	Denmark	Male	23	B-precursor	9,3	CNS1		Standard Risk
10	Denmark	Female	5	B-precursor	106	CNS 3, only cells in CSF		Intermediate Risk
11	Denmark	Female	5	T-cell	237,6	CNS1	dnalt085 (Yes <0.85)	High Risk
12	Denmark	Male	5	T-cell	275	CNS2		High Risk
13	Denmark	Male	10	T-cell	60,5	CNS3, only cells in CSF		High Risk
14	Denmark	Male	15	T-cell	317	CNS2		Intermediate Risk
15	Denmark	Male	10	T-cell	225,1	CNS1		High Risk
16	Denmark	Female	6	T-cell	122	CNS2		Intermediate Risk
17	Denmark	Male	20	T-cell	161	CNS1		High Risk

18	Denmark	Male	16	T-cell	19	CNS1		Intermediate Risk
19	Denmark	Male	6	T-cell	33	CNS1		Intermediate Risk
20	Denmark	Male	43	T-cell	42	CNS1	mllor11q23	High Risk

**CNS1: No blasts on cytospin, no other signs of CNS leukemia.*

CNS2: >0 and <5 cells per µl (= x106 per litre) CSF that on cytospin are regarded to represent leukemic blasts, with no other signs of CNS-leukemia.

CNS3: ≥ 5 cells per µl (= x106 per litre) CSF that on cytospin are regarded to represent leukemic blasts, or cranial nerve palsy, or an intracranial "leukemic" mass on MRI (if performed), or retinal (or other eye) involvement confirmed by MRI or a biopsy (not mandatory).

2.2.2 Culture initiation

ALL cells and TERT-MSCs were thawed similarly. Vials were retrieved from the liquid nitrogen tank and inspected for liquid nitrogen entrapment. The lid was loosened to release pressure and firmly closed before the vial was thawed in a 37 °C water bath. The vial content was diluted 1 to 10 in medium one (RPMI) and gently mixed before the content was centrifuged for five minutes (ALL cells at 129 x g, TERT-MCS at 290 x g). The supernatant was removed, and the pellet was resuspended in culture medium (ALL in medium two (SS, HS, CM), TERT-MSC in medium five). TERT-MSC were seeded in a T75 culture flask (Sigma Aldrich, cat.no: C7106) and incubated (37°C, 5% $C0_2$, 21 % 0_2) and the medium was changed every other day, and the cell culture was sub-cultured approximately once per week. The ALL cells were always used directly in an experiment.

P-MSC vials were also retrieved from the liquid nitrogen tank and thawed in a 37 °C water bath. The cell suspension was moved to a 50 ml conical tube upon thawing. The vial was rinsed with one ml of medium six (MesenCult) that was hereafter added dropwise to the cell suspension while gently swirling to mix the medium and cell suspension. The cell suspension was further diluted 1 to 20, by dropwise addition of medium six (MesenCult), still while gently swirling the conical tube. The cell suspension was centrifuged for 10 minutes at 300 x g before the supernatant was removed, and the pellet was carefully resuspended in medium six (MesenCult) before being seeded in a T75 culture flask. The P-MSC culture was incubated (37°C, 5% C02, 21 % 02), and the medium was changed every other day. The cell culture was approximately sub-cultured once every week.

Table 3, Media and supplements

Media

Medium	*Serum*	*Antibiotics*	*Other*

Medium one, (RPMI)			
RPMI 1640 + GlutaMAX (Gibco, cat.no:61870)	*10% Fetal Bovine Serum (FBS)(In Vitro A/S, cat.no:BI-04-007-1A)*	*1000 U/mL penicillin (1x) + 1000 mg/mL streptomycin (1x)(Sigma Aldrich, cat.no: P7539)*	
Medium two (SS, HS, CM)			
StemSpan™ SFEM II (StemCell Technologies, cat.no:09655)	*1% human serum (Sigma-Aldrich, cat.no:H5667-20ML)*	*1000 U/mL penicillin (1x) + 1000 mg/mL streptomycin (1x).*	*0,5 % cytomix [50 ng/ml of Interleukin-2 (IL-2) (Sigma-Aldrich, cat.no: H7041), 20 ng/ml of Interleukin 3 (IL-3) (Sigma-Aldrich, cat.no: H7166), 20 ng/ml of Interleukin 7 (IL-7) (Sigma-Aldrich, cat.no: SRP3266), 20 ng/mL of Flt3-ligand (Sigma-Aldrich, cat.no: H5416), 50 ng/mL of Stem cell factor (SCF) (Sigma-Aldrich, cat.no: H8416)]*
Medium three (SS, hPL)			
StemSpan™ SFEM II	*5% Stemulate® Xeno- and heparin-free pooled platelet lysate (hPL) (sextonbiotechnologies)*	*1000 U/mL penicillin (1x) + 1000 mg/mL streptomycin (1x).*	-
Medium four (SS, hPL, CM)			
StemSpan™ SFEM II	*5% hPL*	*1000 U/mL penicillin (1x) + 1000 mg/mL streptomycin (1x)*	*0,5 % cytomix [IL-2, IL-3, IL-7, FLT-3, SCF]*
Medium five (RPMI, HC)			
RPMI 1640 + GlutaMAX	*10% FBS*	*1000 U/mL penicillin (1x) + 1000 mg/mL streptomycin (1x).*	*$1*10^{-6}$ M hydrocortisone (Sigma-Aldrich, cat.no: H0888-1G)*
Medium six (MesenCult)			
MesenCult ™-ACF Plus Medium (StemCell Technologies, cat.no:05446)	-	*1000 U/mL penicillin (1x) + 1000 mg/mL streptomycin (1x)*	*1% L-Glutamine 200 mM (StemCell Technologies, cat.no:07100)* *0,2% MesenCult™-ACF Plus 500X Supplement (StemCell Technologies, cat.no:05447)*
Medium seven (α-MEM)			
A-MEM (Gibco, cat.no:22561021)	*5% hPL*	*1000 U/mL penicillin (1x) + 1000 mg/mL streptomycin (1x)*	-

2.2.3 Sub-cultivation and seeding of cell cultures

The P-MSC, TERT-MSC, and ASC cultures were sub-cultivated when approximately 80-90% confluency was reached after one week.

TERT-MSCs and ASCs cultured in T75 culture flasks were washed three times with Dulbecco's phosphate-buffered saline (D-PBS)(Gibco, cat.no: 14190144) before the addition of trypsin-EDTA 1x in D-PBS (Sigma Aldrich, cat.no: T3924). The flasks were then incubated for one-two minute and tapped on the side to detach the cells. Next, culture medium (ASC in medium one (RPMI), TERT-MSC in medium five (RPMI, HC)) was added to the flasks to inhibit the trypsin-EDTA, and the cells were then split. The TERT-MSC were split 1/5 and the ASC 1/2.

P-MSC cultured in T75 culture flasks were washed once with D-PBS before ACF Enzymatic Dissociations Solution (StemCell Technologies, cat.no: 05427) was added. The cells were incubated at 37 ℃ for six minutes before the flask was tapped on the side to detach the cells. When the cells had detached, an equal volume of ACF Enzyme Inhibition solution (StemCell Technologies, cat.no: 05428) was added, and the flask content was moved to a 50 ml centrifuge tube. The cell suspension was centrifuged for eight minutes with brakes at 300 x g. The supernatant was removed, the pellet was resuspended in medium six (MesenCult) and the cells split 1/2.

2.3 Optimization of culture medium for acute lymphoblastic leukemia cells and stromal cells

2.3.1 Medium for stromal cells

P-MSCs, TERT-MSCs, and ASCs were seeded in their respective culture medium in a 12-well culture plate (Sigma Aldrich, cat.no: M0812) at a density of 100.000 cells/well. The plate was incubated at 37℃ overnight to ensure adhesion to the culture ware before the cells were photographed under the microscope (ZEISS Axio Vert.A1) on day zero. Medium one (RPMI), medium five (RPMI, HC), medium six (MesenCult), and medium seven (α-MEM) were prepared and heated up to 37 ℃ in a bead bath. According to the plate layout found in *Appendix, A.1*, the wells were washed three times with D-PBS before the four different culture media were added to

the plate. The cells were cultured for five days, the culture medium was changed once after three days, and the cells were photographed on days zero, two, and five.

2.3.2 Optimal medium for acute lymphoblastic leukemia cells

Medium one (RPMI) and un-supplemented StemSpan™ SFEM II (StemCell Technologies, cat.no: 09655) with 1000 U/mL penicillin (1x) + 1000 mg/mL streptomycin (1x) was prepared and heated up to 37°C in a bead bath.

Four different medium supplements were prepared up-concentrated to ensure the desired concentrations when mixed with cell suspension. Supplement 1: RPMI 1640 + GlutaMAX with 10% FBS and 1000 U/mL penicillin (1x) + 1000 mg/mL streptomycin (1x), supplement 2: StemSpan™ SFEM II with 1% human serum, 1000 U/mL penicillin (1x) + 1000 mg/mL streptomycin (1x) and 0,5 % cytomix (50 ng/mL IL-2, 20 ng/mL IL-3, 20 ng/mL IL-7, 20 ng/mL Flt3-ligand and 50 ng/mL SCF), supplement 3: StemSpan™ SFEM II with 5% hPL and 1000 U/mL penicillin (1x) + 1000 mg/mL streptomycin (1x), and supplement 4: StemSpan™ SFEM II with 5% hPL, cytomix, and 1000 U/mL penicillin (1x) + 1000 mg/mL streptomycin (1x). Medium one (RPMI), medium two (SS, HS, CM), medium three (SS, hPL), and medium four (SS, hPL, CM), respectively.

The different media were loaded in a 96-well suspension plate with U-bottom (SigmaAldrich, cat.no: M9436) according to the plate layout found in *Appendix A.2* and incubated at 37°C. At the same time, ALL cells from patients were thawed. ALL cells were thawed, as described in *2.2.2*, and counted before the cell suspension was divided into two parts, A and B. The two parts were centrifuged for five minutes at 800RPM. The supernatant was removed, A was resuspended in medium one (RPMI), and B was resuspended in un-supplemented StemSpan™ SFEM with 1000 U/mL penicillin (1x) + 1000 mg/mL streptomycin (1x). ALL cells were seeded in the plate with 50.000 cells/well. Cells from A were seeded in supplement 1 and in the positive control, and cells from B were seeded in supplements 2, 3, and 4. The plate was then incubated at 37 °C for five days.

2.3.2.1 AnnexinV and Live/Dead green assay

On day five, the plate was centrifuged for five minutes at 290 x g and flipped to discard the supernatant before binding buffer (Hepes 10 mM, NaCl 140 mM, $CaCl_2$ 2,5 mM, BSA 0,1 % in

LCMS H_2O, pH 7,4) was added. Ethanol was added to the positive controls to a final concentration of 30%. The plate was centrifuged and flipped again before staining solution with AnnexinV V450 (BD Biosciences, cat.no: 560506) 1:500 and Live/Dead green stain (Invitrogen, cat.no: L23101) 1:1000 in binding buffer was added to each well and left to stain for 30 minutes at room temperature in the dark. After staining, the plate was centrifuged and flipped, and binding buffer was added before the plate was run on the BD FACSVerse™ instrument. First, cells were gated from debris on a forward scatter- area (FSC-A) versus side scatter- area (SSC-A) dot plot. Then singlets were gated in a FSC- height (FSC-H) versus FSC-A dot plot. Then all AnnexinV positive cells and live/dead green positive cells were gated to identify unstained live cells. Gating example can be found in *Appendix A.8*.

2.3.3 BAX channel blocker treatment in acute lymphoblastic leukemia cells

Medium one (RPMI) and medium two (SS, HS, CM) were prepared and heated up to 37°C in a bead bath, while the BAX channel blocker (Sigma Aldrich, cat.no: 196805) was diluted in DMSO (Sigma Aldrich, cat.no: D2650) to reach a stock concentration of 21.4 mM. Five concentrations of BAX channel blocker working solution were prepared in medium two (SS, HS, CM): 3.3 µM, 1.1 µM, 0.3 µM, 0.1 µM, and 0.03 µM. According to the plate layout found in *Appendix A.3,* these were loaded in a 96-well plate with U-bottom along with negative control, controls with DMSO, and positive control. The plate was incubated at 37°C while the ALL cells were thawed. ALL cells were thawed, as described in *2.2.2*, counted, and seeded in a density of 50.000 cells/well before the plate was incubated at 37°C for 24 hours.

After 24 hours, AnnexinV and Live/Dead green assay were performed, as described in *2.3.2.1*.

2.4 Nestin expression of stromal cells

Stromal cells were passaged as described in *2.2.3* and seeded in two 96-well culture plates, F-bottom (Sigma Aldrich, cat.no: M0812). 950 cells/well of C-MSCs, P-MSCs, and ASCs were seeded in accordance with the plate layout found in *Appendix A.4* in their respective culture medium. The cells were incubated for 48 hours at 37°C till a 50% confluency was reached.

2.4.1 Initiation of co-culture

On day three, co-cultures were initiated. The wells containing stromal cells were washed three times with D-PBS. ALL cells were thawed as described in *2.2.2*, except that the ALL cells were resuspended in medium four (SS, hPL, CM), Table 3. The ALL cells were seeded in a density of 50.000 ALL cells/well following the plate layout found in *Appendix A.4*.

The plates were incubated for five days, one plate in normoxia (37°C, 5% C02, 21 % 02) and the other in hypoxia (37°C, 5% C02, 3.2 % 02).

2.4.2 Immunocytochemistry

On day eight, the plates were centrifuged for five minutes at 290 x g, the supernatant was removed, and the wells were fixed in 4% paraformaldehyde (Sigma Aldrich, cat.no: 158127) for 10 minutes at room temperature. The wells were then washed three times with D-PBS before permeabilizing in 0.3% Triton X-100 (Sigma Aldrich, cat.no: T8787) for five minutes at room temperature. The wells were washed three times with D-PBS and blocking buffer (5% FBS in D-PBS) was then added for one hour while the primary antibody solutions were prepared. Three antibody solutions were made, one with anti-Nestin and anti-Fibronectin, the second with anti-Nestin and anti-Vimentin, and the third with anti-CD45 and anti-Vimentin. All solutions were made in blocking buffer in concentrations according to *Table 4*. After one hour, the blocking buffer was aspirated. The primary antibody solutions were added following the plate layout found in *Appendix A.4*. The plates were left overnight at 4 °C.

Table 4, Primary antibodies

Primary antibodies

Antibody	*Class*	*Clone*	*Host*	*Species reactivity*	*Stock concentration*	*Dilution*	*Positive control*	*Company*	*Cat. no*
Anti-CD45	Mono-clonal	MEM-28	Mouse	Human	100 µg at 1 mg/ml	1:10	Human peripheral blood mononuclear cells.	Abcam	AB8216
Anti-Fibronectin	Mono-clonal	F1	Rabbit	Human	100 µl at 0.054 mg/ml	1:300	HepG2 and human	Abcam	Ab32419

							mesenchymal stem cells		
Anti-Ki67	Mono-clonal	B56	Mouse	Human	100 µg at 1.015 - 1.122 mg/ml	1:50	HeLa cells	Abcam	ab279653
Anti-Nestin	Mono-clonal	10C2	Mouse	Human	1 mg/mL	1:200	WI-38 (ATCC# CCL75) or U251 cell lines	Sigma Aldrich	MAB5326
Anti-Vimentin	Mono-clonal	EPR3776	Rabbit	Human	100 µl at 0.232 - 0.268 mg/ml	1:1000	HeLa, human adenocarcinoma, and wild-type HAP1 cells	Abcam	ab92547

After 24 hours of incubation time with primary antibodies, the primary antibody solutions were aspirated, and the wells were washed three times with D-PBS. The secondary antibody solutions were prepared with goat anti-Mouse secondary antibody labeled with Alexa Fluor™ 555 and goat anti-Rabbit secondary antibody labeled with Alexa Fluor™ 488 in 1:400 in blocking buffer, *Table 5*. The secondary antibody solution was added to the wells and incubated at room temperature in the dark for one hour. After incubation, the secondary antibody solution was aspirated, and the wells were washed once with D-PBS. A Hoechst staining solution was made with Hoechst diluted in blocking buffer at a concentration of 5 µg/ml, *Table 6,* and was added to the well to stain for five minutes. The staining solution was hereafter aspirated, and the wells were washed three times with D-PBS. The wells were photographed on the ZEISS Axio Vert.A1 fluorescent microscope.

Table 5, Secondary antibodies

Secondary antibodies

Antibody	*Class*	*Host/ isotype*	*Species reactivity*	*Conjugate*	*Stock concentration*	*Dilution*	*Excitation/ Emission wavelength*	*Company*	*Cat. no*
IgG (H+L)	Polyclonal	Goat / IgG	Mouse	Alexa Fluor™ 555	2 mg/mL	1:400	Red: 590/617 nm	Thermo Fisher	A-21422
IgG (H+L)	Polyclonal	Goat / IgG	Rabbit	Alexa Fluor™ 488	2 mg/mL	1:400	Green: 495/519 nm	Thermo Fisher	A-11008

Table 6, Stains

Stains

Stain	*Concentration*	*Dilution*	*Company*	*Cat. No*
Hoechst 33342	5 mg/ml	1:1000	Sigma Aldrich	B2261
SYTOX™ GREEN	5 mM	1:50.000	Thermo Fisher	S7020
SYTOX™ ORANGE	5 mM	1:50.000	Thermo Fisher	S11368

2.5 Survival of acute lymphoblastic cells in monoculture and co-culture with stromal cells, cultured in normoxia and hypoxia on extracellular matrix coated culture ware

Laminin (Sigma Aldrich, cat.no: L6274), collagen (Sigma Aldrich, cat.no: 234149), and fibronectin (Gibco, cat.no: PHE0023) were thawed slowly at 4 ℃ for a couple of hours before use. The ECM coats were made one at a time and used for coating immediately after dilution. The collagen stock was diluted in 10 mM acetic acid to reach a working dilution of 0.5 mg/ml to match the stock concentration of fibronectin and laminin. Four coating solutions were made, one with laminin, the second with fibronectin, the third with collagen, and the fourth with a 1:1:1 mix of all the components. All ECM components were diluted 1:100 in Hanks' Balanced Salt solution (HBSS) (Sigma Aldrich, cat.no: H6648), and a minimal volume was used to coat six 96-well culture plates, F-bottom following the plate layout found in *Appendix A.5*. The coated plates were incubated at 37 ℃ for two hours before receiving three HBSS washes. Stromal cells were passaged as described in *2.2.3* and 950 cells/well of TERT-MSCs, P-MSCs, and ASCs were seeded in their respective culture medium following the plate layout found in *Appendix A.5*. The cells were incubated for 48 hours at 37℃ until 50% confluent.

On the third day, ALL cells were thawed as described in *2.2.2*, and co-cultures were initiated as described in *2.4.1* and seeded according to the plate layout found in *Appendix A.5*.

The plates were incubated for five days, with three plates in normoxia (37℃, 5% C02, 21 % 02) and three in hypoxia (37℃, 5% C02, 3.2 % 02).

2.5.1 Assessment of the acute lymphoblastic cells in monoculture and co-culture with stromal cells, cultured in normoxia and hypoxia on extracellular matrix coated culture ware

On day eight, the six plates were assessed in one of three assessment methods: 1) Annexin-V & Live/Dead Green apoptosis assay (flow cytometry), 2) Immunocytochemistry for Nestin, fibronectin, etc, and 3) Sytox dead stain (microscopy).
Plates A, B, and C were incubated in normoxia, and plates D, E, and F were incubated in hypoxia.

2.5.1.1 Assessment one (Annexin-V & Live/Dead green)

Plate A and D were washed three times with D-PBS before TrypLE™ Express Enzyme (1X) (Gibco, cat.no: 12605028) was added for 10 minutes. The plates were gently bumped on the side to loosen the cells before the TrypLE Express was diluted with medium one (RPMI). The unattached cells in suspension were moved to the same position in a U-bottom 96 well plate for the AnnexinV and Live/Dead staining. The staining was performed as described in *2.3.2.1*.

2.5.1.2 Assessment two (Immunocytochemistry)

Plate B and E were fixed and permeabilized as described in *2.4.2*. The primary antibody staining solution was made with anti-Ki-67 and anti-Vimentin in blocking buffer in concentrations according to *Table 4*. The antibody solutions were added to the plate. On the following day, the plates were stained with a secondary antibody solution and assessed on the ImageXpress Pico.

2.5.1.2.1 Cell scoring assay

On the ImageXpress Pico, a cell scoring assay was performed, where ALL cells were manually identified based on size and nuclear intensity before an algorithm was used to analyze dead (sytox) or proliferating (Ki-67) ALL cells. In co-culture systems stromal cells were excluded by adjustment of the nuclear intensity and size, so only ALL cells were detected and analyzed.

2.5.1.3 Assessment three (Sytox dead staining)

A SYTOX orange staining solution and a Hoechst staining solution were made in HBSS in concentrations according to *Table 6*. Plate C and F were centrifuged at 290 x g for five minutes and washed once with HBSS. Ethanol was added to the positive control well to reach a concentration of 30%. The plates were centrifuged again before the HBSS were aspirated, and the sytox staining solution was added to stain for six minutes in the dark at room temperature. After the sytox staining, the wells were washed once before being stained with the Hoechst staining solution for five minutes in the dark at room temperature. After the second staining, the plates were washed three times before it was photographed on the ImageXpress Pico and assessed as described in *2.5.1.3.1*.

2.6 Test of the survival of acute lymphoblastic leukemia cells in model 0.1

A 1:1:1 laminin, collagen, and fibronectin in HBSS coating solutions were made in concentrations described in *2.5*, and a minimal volume was used to coat four 96-well culture plates with F-bottom, following the plate layout found in *Appendix A.6*. The coated plates were incubated at 37 ℃ for two hours, then washed three times with HBSS before P-MSCs were passaged as described in *2.2.3* and seeded in a density of 950 cells/well according to the plate layout found in *Appendix A.6.* The cells were then incubated for 48 hours at 37℃ till 50% confluent. On the third day, ALL patients' cells were thawed as described in *2.2.2*, and co-cultures were initiated as described in *2.4.1*, and seeded in the plates, following the plate layout found in *Appendix A.6*.

The plates were incubated for five days, with plates A and B in normoxia (37℃, 5% C02, 21 % 02) and plates C and D in hypoxia (37℃, 5% C02, 3.2 % 02).

Plate A and C were stained with sytox, as described in *2.5.1.3*, but with SYTOX green instead of SYTOX orange, and assessed on ImageXpress Pico as described in *2.5.1.3.1*.

2.7 Cytosine β-D-arabinofuranoside sensitivity test of acute lymphoblastic leukemia cells in model 0.1

Plate B and D were used to test the chemosensitivity of Cytosine β-D-arabinofuranoside (AraC) (Sigma-Aldrich, cat.no: C1768) on ALL cells in co-culture with P-MSCs seeded on a coat made of a 1:1:1 mix of laminin, collagen, and fibronectin in either hypoxia or normoxia for 24 hours.

Cytosine β-D-arabinofuranoside was diluted in medium four from a 200 µM stock to a working dilution of 0.0625 µM. The plates were centrifuged for five minutes. The supernatant was removed. AraC working solution was loaded into the plate according to the plate layout in *Appendix A.7*. Plate B was incubated in normoxia and plate D in hypoxia for 24 hours.

After incubation, the plates were stained with sytox, as described in *2.5.1.3*, but with SYTOX green instead of SYTOX orange, and assessed on ImageXpress Pico as described in *2.5.1.3.1*.

2.8 Statistical analysis

The data generated in this report have been represented with standard deviations unless otherwise stated. The results have been tested for normal distribution using the Shapiro-Wilk test of normality. Normally distributed data were analyzed using repeated measures analysis of variance (ANOVA), and non-normally distributed data were assessed using the non-parametric Kruskal-Wallis test. Data from *2.3.3, 2.6,* and *2.7* proved to be normally distributed ($p<0.05$). Data generated from *2.3.2* proved to be non-normally distributed. Statistical analysis was performed using IBM Statistics SPSS 25 and the level of significance was set to 95%. Results from *2.3, 2.4,* and ICC were analyzed through qualitative assessment.

3 Results

3.1 Qualitative assessment of medium for stromal cells

Stromal cells were cultured in different media types to determine which medium would generate similar growth rates, as it was of interest to find culture media that would allow the cells to reach the same level of confluency within the same culture period.

Images of TERT-MSCs show that all four media types can support the growth of TERT-MSCs, and an increase in cells can be observed from day zero to day two to day five in all media types (*Figure 6*). TERT-MSC seems to grow the fastest in medium seven (α-MEM), second fastest in medium six (MesenCult), and slower in medium one and medium five (RPMI, HC).

Figure 6, Human EGFP-hTERT transduced mesenchymal stromal cells cultured in medium one, medium five, medium six and medium seven for five days. 4x.

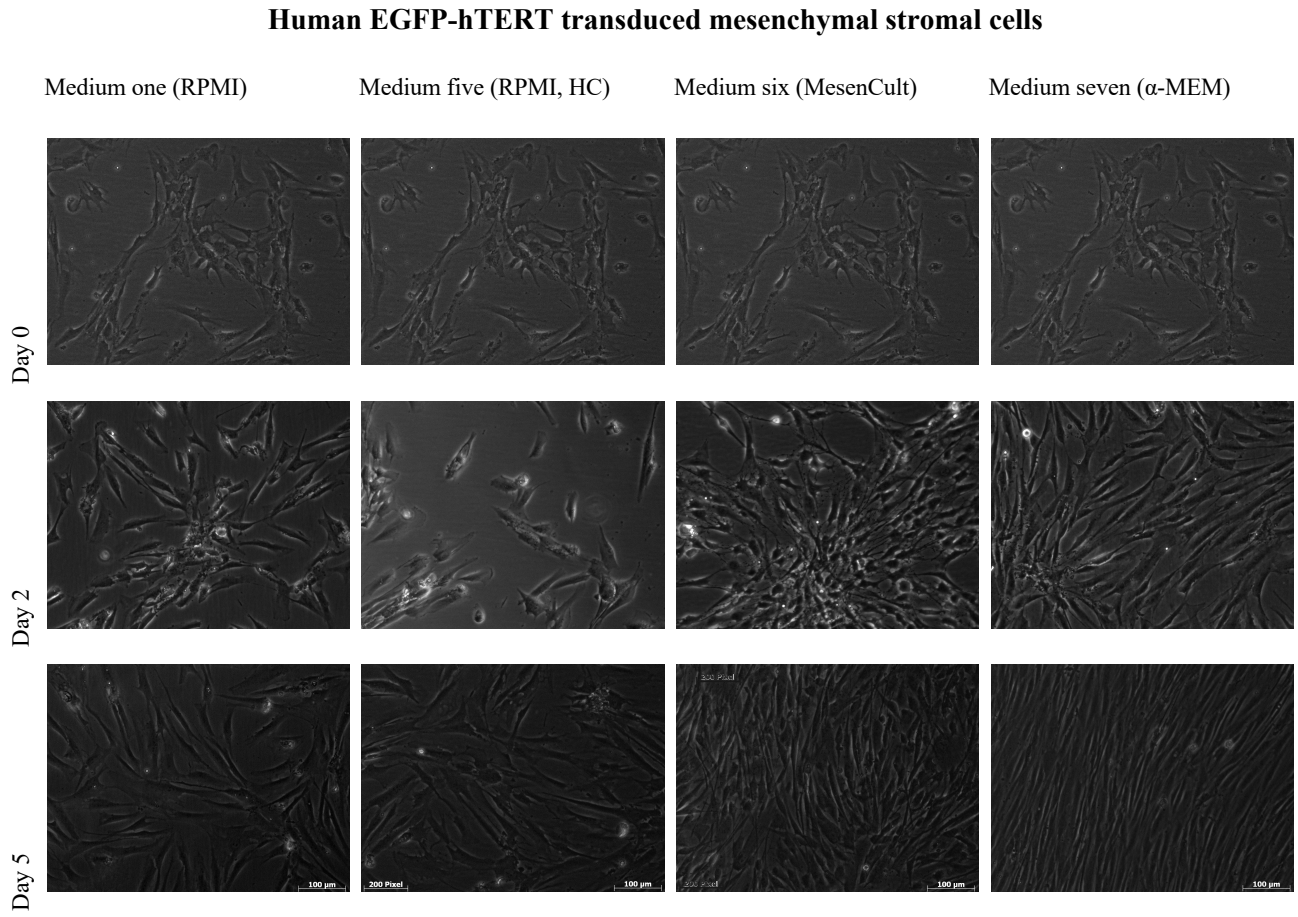
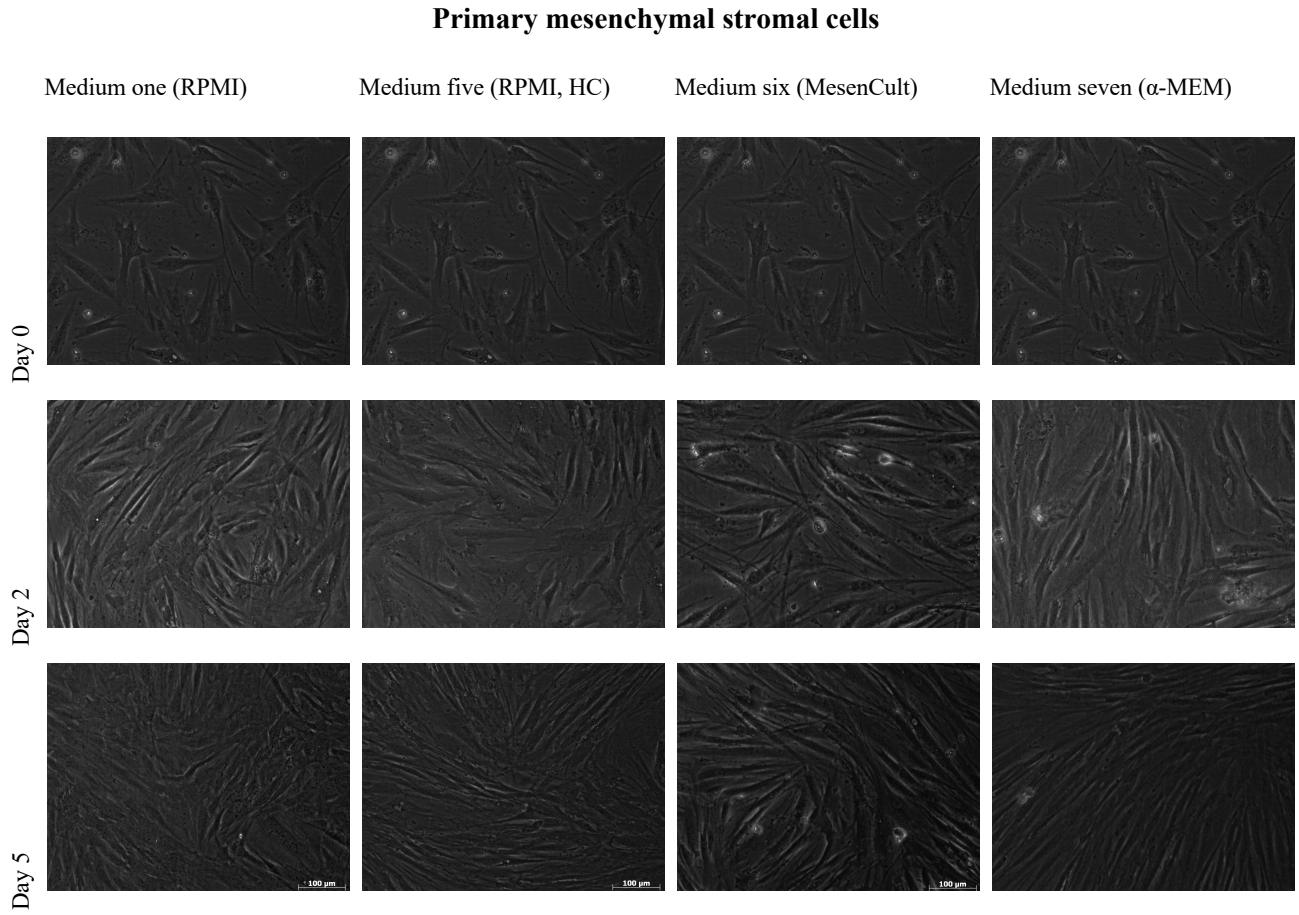
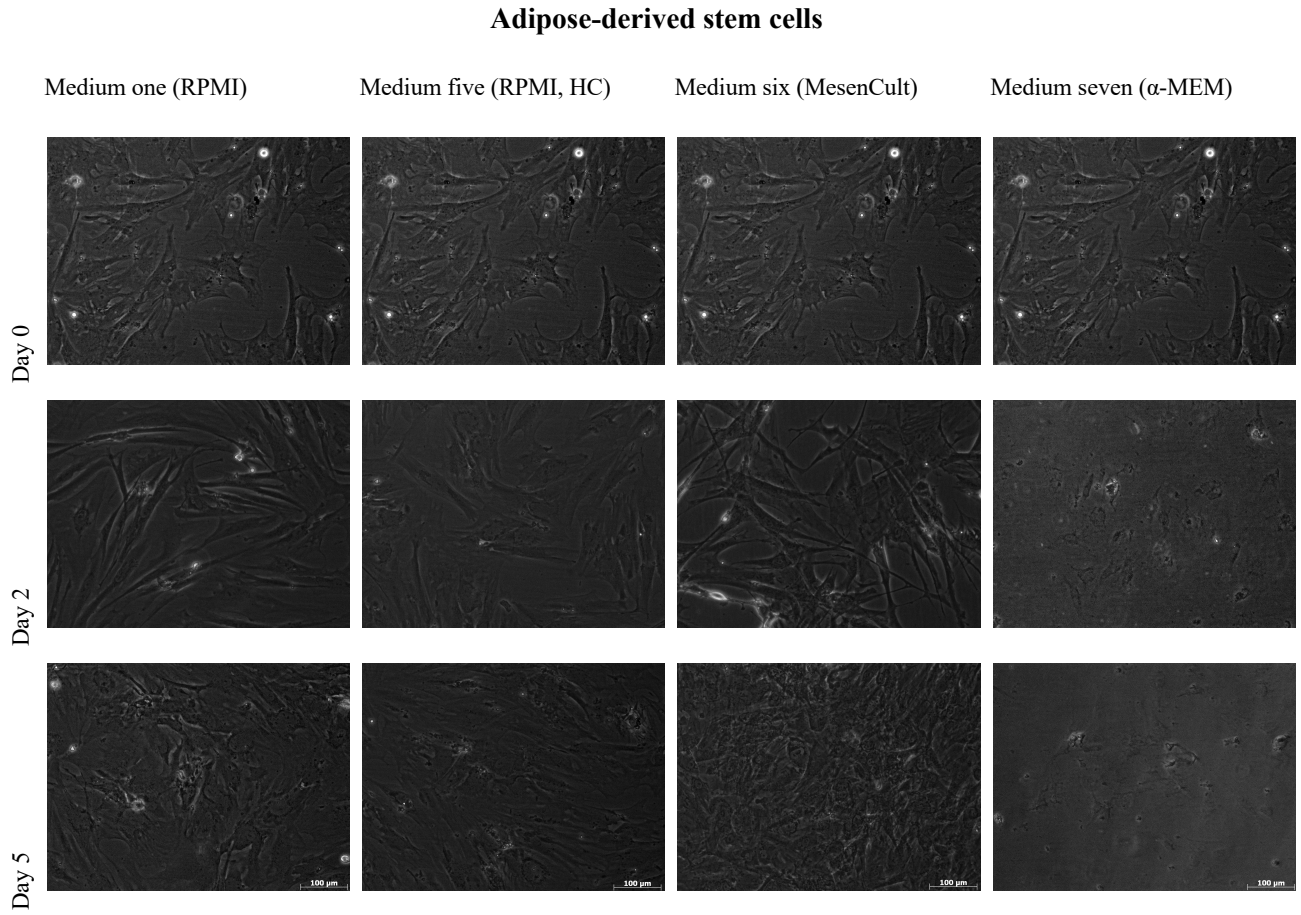


Figure 7, Primary mesenchymal stromal cells cultured in medium one, medium five, medium six and medium seven for five days. 4x.



In the images of P-MSCs cultured in the four media types, it is seen that the growth is supported by medium one (RPMI), five (RPMI, HC), six (MesenCult), and seven (α-MEM), and the P-MSCs seem to reach 90% confluency on day two in all media types (*Figure 7*). A further increase in cells can be observed on day five, where the cells seem to be slightly over-confluent.

Figure 8, Adipose-derived stem cells cultured in medium one, medium five, medium six and medium seven for five days. 4x.



Images of ASCs in medium one (RPMI), medium five (RPMI, HC), and medium six (MesenCult) all show that the media can support the growth of ASCs, and medium one (RPMI) and medium six (MesenCult) seem to increase from day zero to day two, and further at day five (*Figure 8*). The growth of the ASCs does not seem to increase on day two in medium five (RPMI, HC), but an increase in cells can be observed on day five. Medium seven (α-MEM) do not show the ability to support ASCs, as a decrease in cells can be observed from day zero to day two and five.

3.2 Optimal medium for acute lymphoblastic leukemia cells

The quantitative analysis of the AnnexinV and LiveDead stain illustrated in *Figure 9*, show the number of live ALL cells cultured in four media types for five days. The number of live ALL cells is significantly higher in medium two (SS, HS, CM), medium three (SS, hPL), and medium four (SS, hPL, CM) compared to medium one (RPMI) ($p<0.05$).

Figure 9, Bar chart illustrating the number of live ALL cells in medium one (RPMI), medium two (SS, HS, CM), medium three (SS, hPL), and medium four (SS, hPL, CM) after five days in culture. AnnexinV and Live/dead green assay. ★ indicates a significant difference ($p<0.05$).

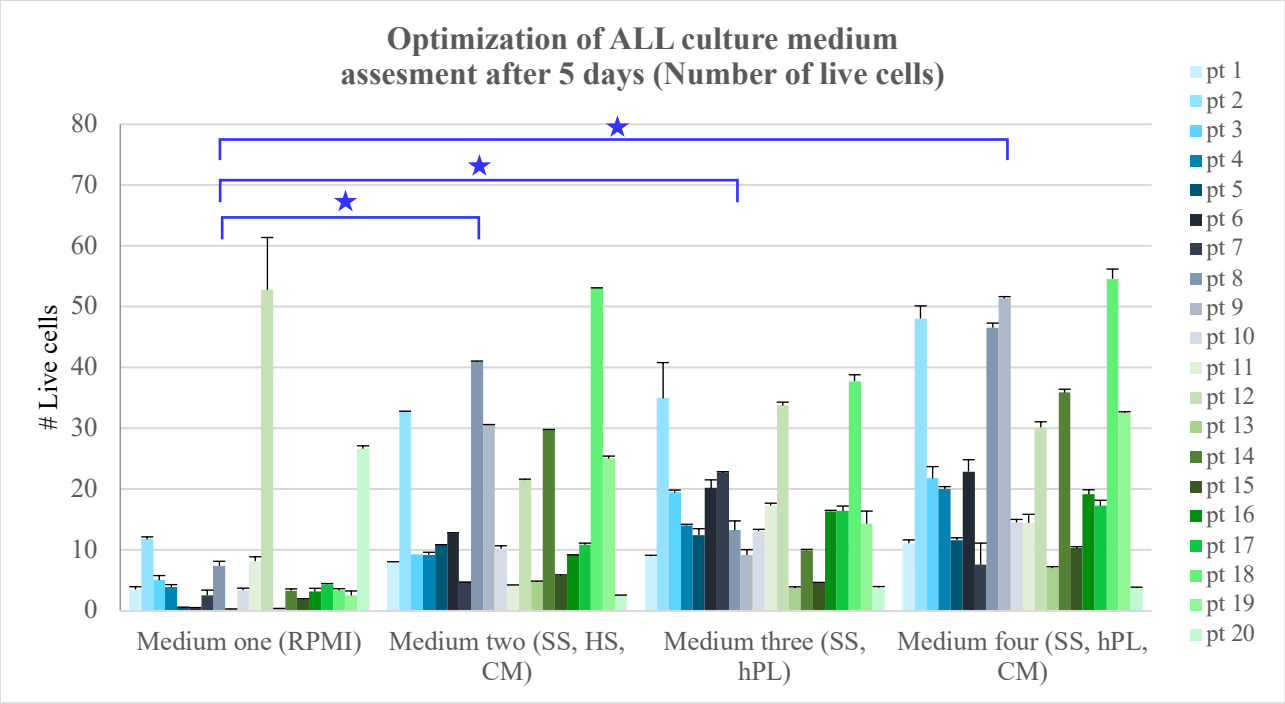
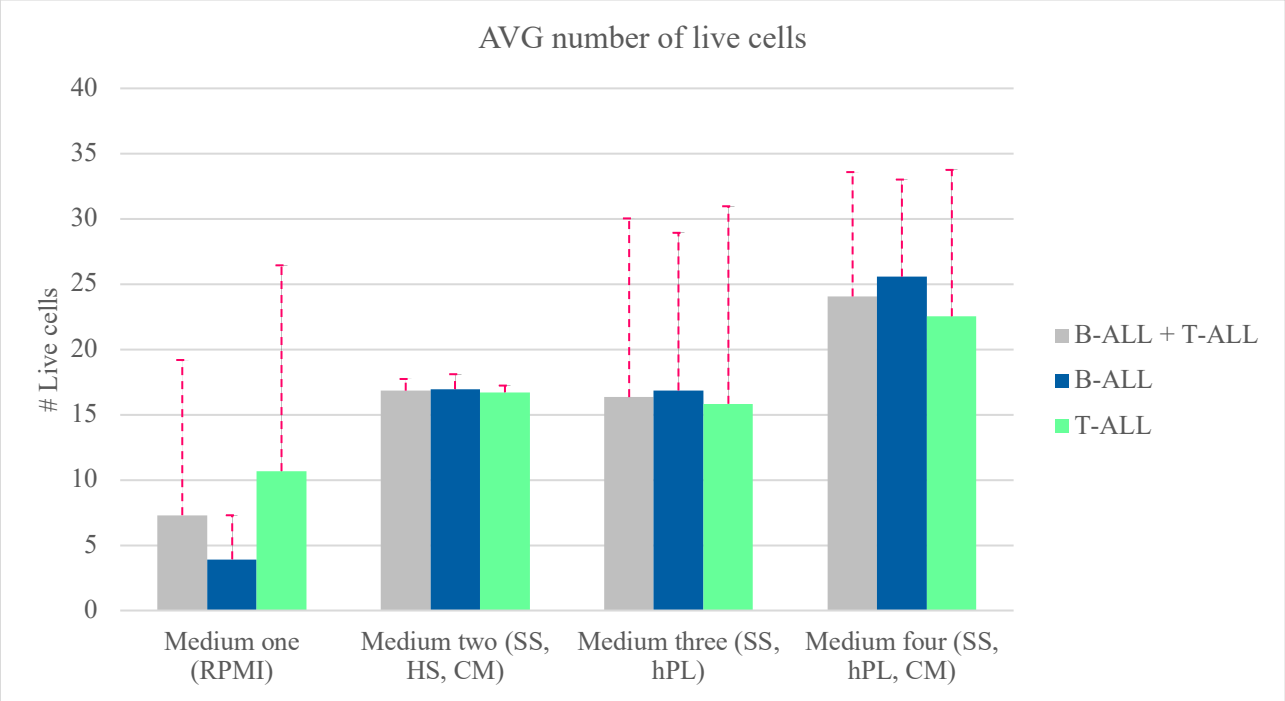


Figure 10, Average number of live cells. GREY: all patients pooled, BLUE: B-ALL patients pooled, GREEN: T-ALL patients pooled. Pink dashed standard deviations represent deviations between patients. AnnexinV and Live/dead green assay.

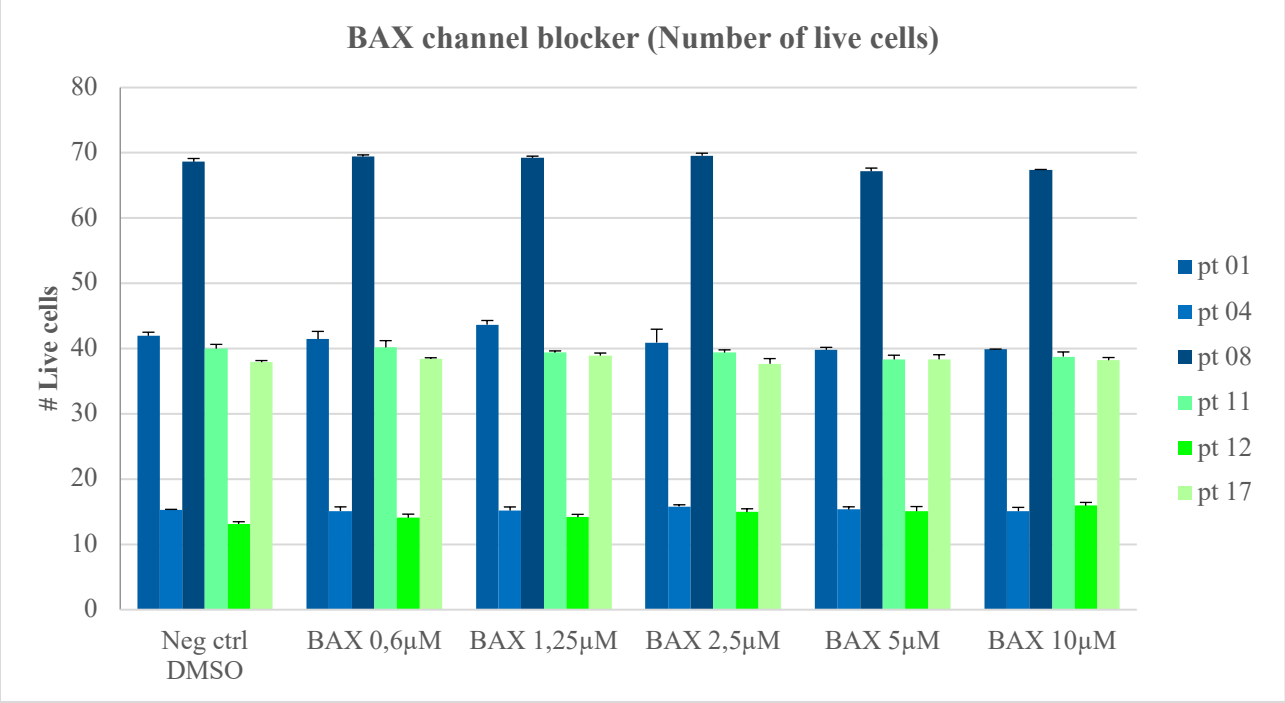


To illustrate the survival tendency across all patients and across ALL-types, patients have been pooled to provide a general overview. This show a small increase in ALL cell survival between medium four compared to medium two and medium three for both all patients pooled together and for B- and T-ALL patients separately (*Figure 10*). However, this difference did not reach a statistical difference ($p>0.05$) due to the large individual variation within the groups as illustrated in *Figure 10* in the pink dashed standard deviations.

3.3 BAX channel blocker treatment in acute lymphoblastic leukemia cells

ALL cells-death has been associated with BAX-mediated apoptosis why ALL cells were treated with a downstream BAX channel blocker. Patients with different cell survival were selected for the experiment, and the quantitative analysis of the AnnexinV and LiveDead stain from the dose response experiments is illustrated in *Figure 11*.

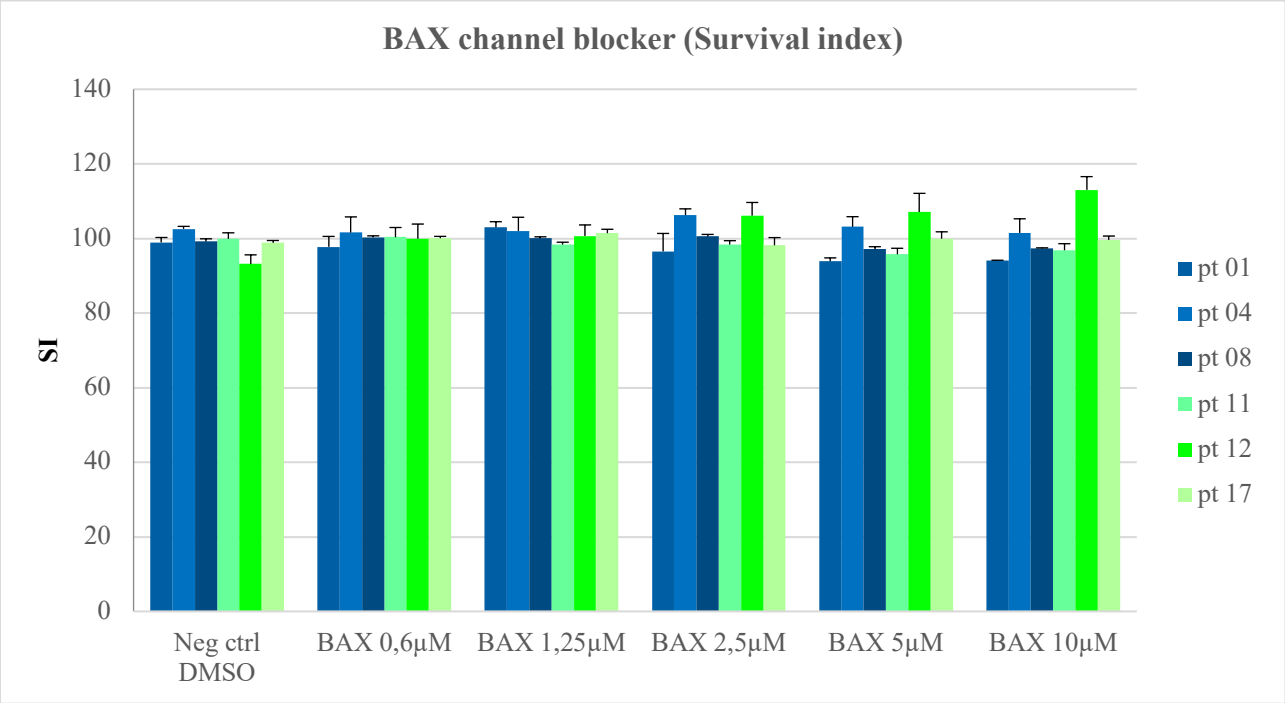
Figure 11, Bar chart illustrating the number of live ALL cells treated with 0.6µM, 1.25µM, 2.5µM, 5µM, or 10µM BAX channel blocker for one hour, and assessed after 24 hours. AnnexinV and Live/dead green assay.



No statistical difference can be found in the survival of ALL cells treated with 0.6µM, 1.25µM, 2.5µM, 5µM, or 10µM of BAX channel blocker for one hour compared to the negative control.

This is also illustrated in the survival index plotted in (*Figure 12)*, where all samples have been normalized to the negative control.

Figure 12, Bar chart illustrating the number of live ALL cells treated with 0.6µM, 1.25µM, 2.5µM, 5µM, or 10µM BAX channel blocker for one hour, and assessed after 24 hours normalized to negative controls. AnnexinV and Live/dead green assay.

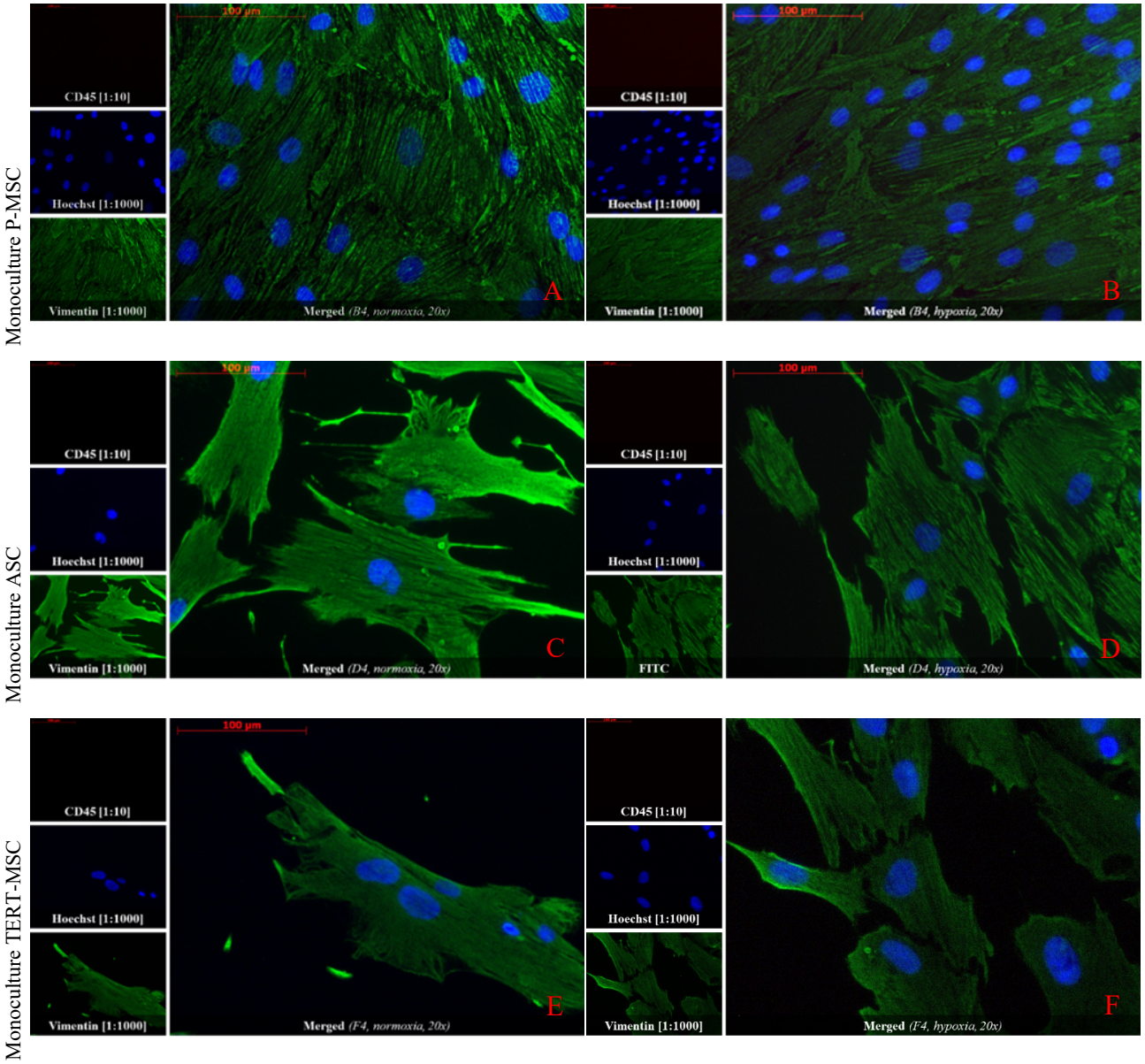


3.4 Nestin expression of stromal cells

3.4.1 Nestin expression of stromal cells in monoculture, in normoxia and hypoxia

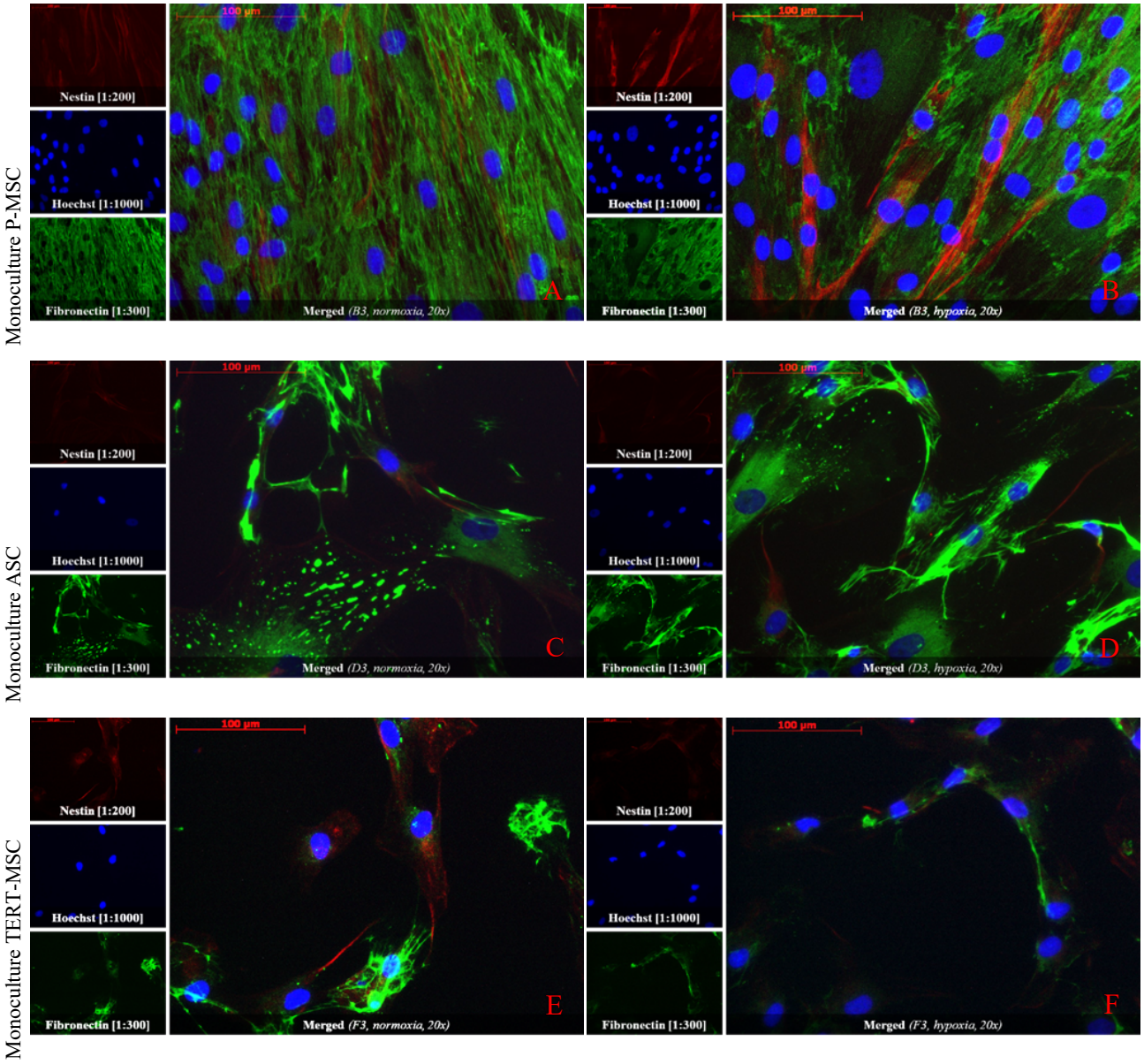
The ICC staining of monocultures with the P-MSCs, ASCs, and TERT-MSC cultured for five days shows strong labeling of vimentin (*Figure 13*) and fibronectin (*Figure 14*) in all stromal cells together with no labeling of CD45 (*Figure 13*). No difference is shown in the labeling of vimentin and fibronectin in stromal cells in normoxia compared to the stromal cells in hypoxia.

Figure 13, Monocultures 20x. A: P-MSC CD45 [1:10]+Vimentin [1:1000] normoxia. B: P-MSC CD45 [1:10] + Vimentin [1:1000] hypoxia, C: ASC CD45 [1:10] + Vimentin [1:1000] normoxia. D: ASC CD45 [1:10] + Vimentin [1:1000] hypoxia. E: TERT-MSC CD45 [1:10] + Vimentin [1:1000] normoxia. F: TERT-MSC CD45 [1:10] + Vimentin [1:1000] hypoxia.



The staining also shows medium-weak labeling of nestin in all the stromal monocultures (*Figure 14*) with no difference in the labeling of nestin in ASC and TERT-MSC cultures in normoxia compared to hypoxia (*Figure 14*, C-F). The ICC staining shows slightly stronger labeling of nestin in the P-MSCs cultures in hypoxia (*Figure 14*, B) compared to normoxia (*Figure 14*, A).

Figure 14, Monocultures 20x. A: P-MSC Nestin [1:200]+Fibronectin [1:300] normoxia. B: P-MSC Nestin [1:200]+Fibronectin [1:300] hypoxia, C: ASC Nestin [1:200]+Fibronectin [1:300] normoxia. D: ASC Nestin [1:200]+Fibronectin [1:300] hypoxia. E: TERT-MSC Nestin [1:200]+Fibronectin [1:300] normoxia. F: TERT-MSC Nestin [1:200]+Fibronectin [1:300] hypoxia.

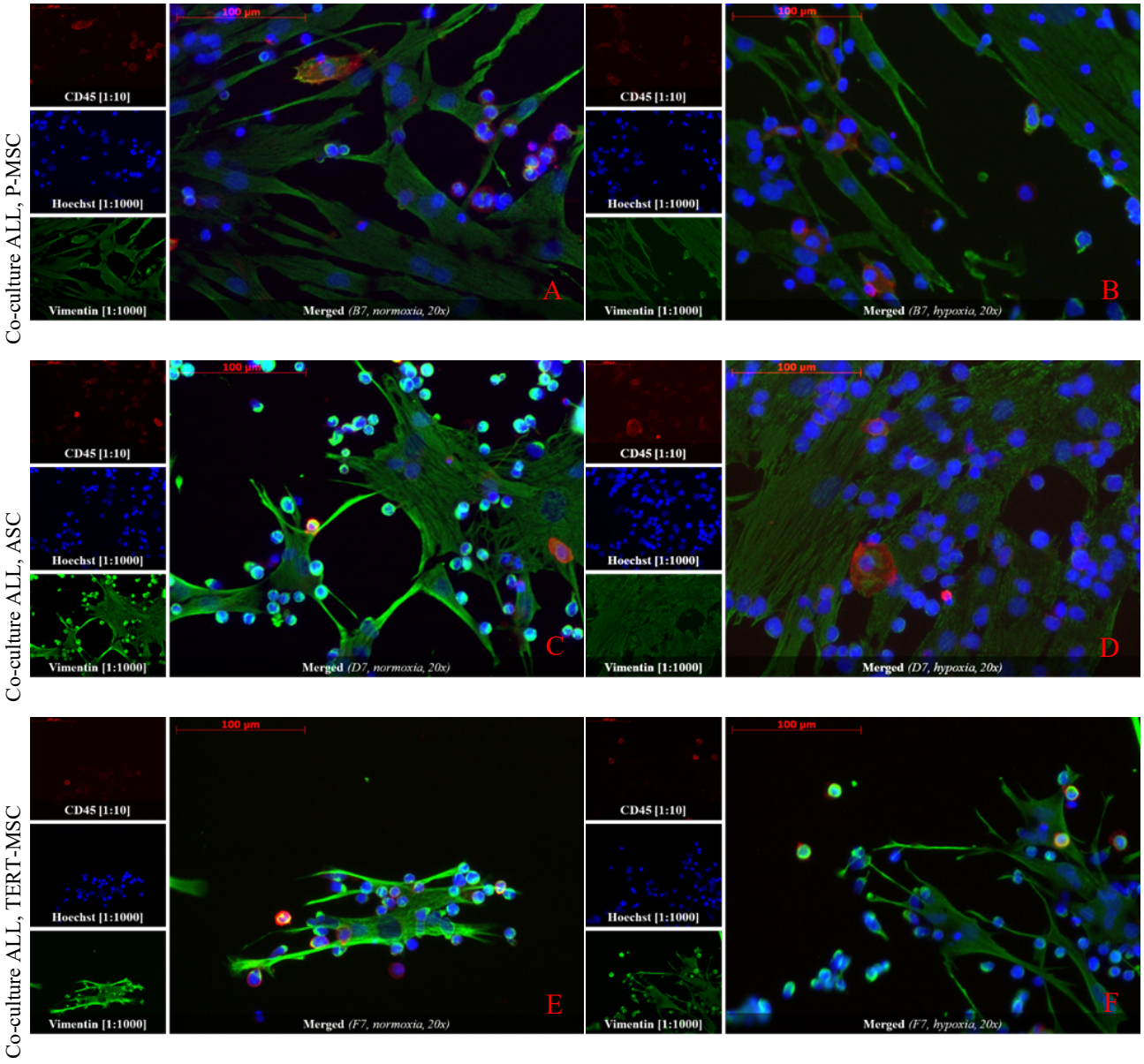


3.4.2 Nestin expression of stromal cells in co-culture in normoxia and hypoxia

ICC staining after five days of co-culture with P-MSCs and ALL cells, co-cultures with ASCs and ALL cells, and co-cultures with TERT-MSCs and ALL cells show strong labeling of vimentin (*Figure 15*) and fibronectin (*Figure 16*, *Figure 17*) throughout the stromal cells, and no labeling of CD45 (*Figure 15*). The ALL cells also show strong labeling of vimentin (*Figure 15*) and fibronectin

(*Figure 16*, *Figure 17*) together with CD45 labeling primarily restricted to the surface of the ALL cells (*Figure 15*).

Figure 15, Co-culture 20x. A: ALL + P-MSC CD45 [1:10]+Vimentin [1:1000] normoxia. B: ALL + P-MSC CD45 [1:10] + Vimentin [1:1000] hypoxia, C: ALL + ASC CD45 [1:10] + Vimentin [1:1000] normoxia. D: ALL + ASC CD45 [1:10] + Vimentin [1:1000] hypoxia. E: ALL + TERT-MSC CD45 [1:10] + Vimentin [1:1000] normoxia. F: ALL + TERT-MSC CD45 [1:10] + Vimentin [1:1000] hypoxia.



The staining shows labeling of nestin in both P-MSCs *(Figure 16*, A-B) and TERT-MSCs (*Figure 16*, E-F) and slightly weaker labeling of nestin in the ASCs (*Figure 16,* C-D). No difference in

nestin labeling can be observed in the P-MSCs, ASC, or TERT-MSC cultures in normoxia compared to cultures in hypoxia (*Figure 16, Figure 17*).

Figure 16, Co-culture 20x. A: ALL + P-MSC Nestin [1:200]+Fibronectin [1:300] normoxia. B: ALL + P-MSC Nestin [1:200]+Fibronectin [1:300] hypoxia, C: ALL + ALL + ASC Nestin [1:200]+Fibronectin [1:300] normoxia. D: ALL + ASC Nestin [1:200]+Fibronectin [1:300] hypoxia. E: ALL + ALL + TERT-MSC Nestin [1:200]+Fibronectin [1:300] normoxia. F: ALL + TERT-MSC Nestin [1:200]+Fibronectin [1:300] hypoxia.

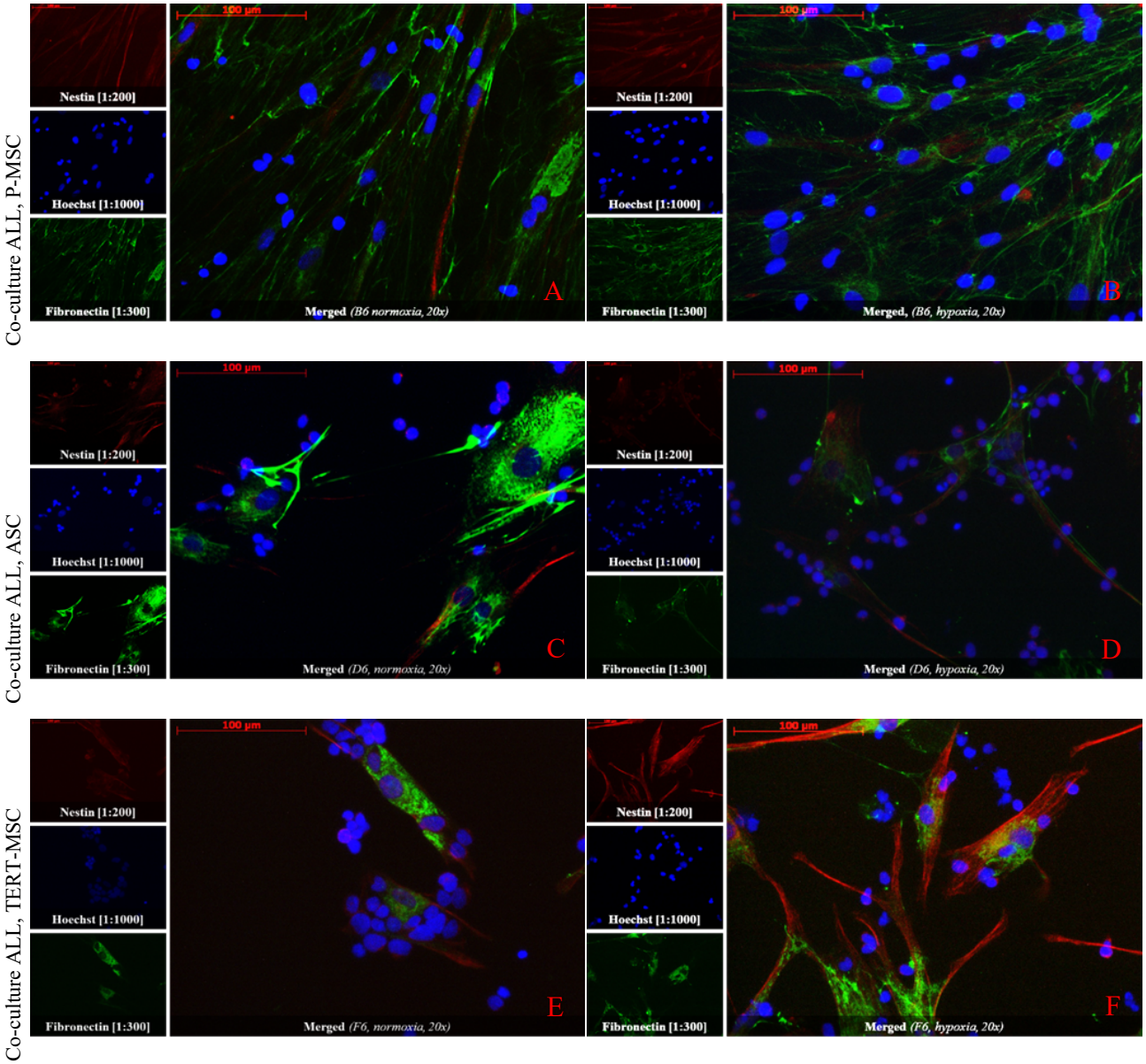
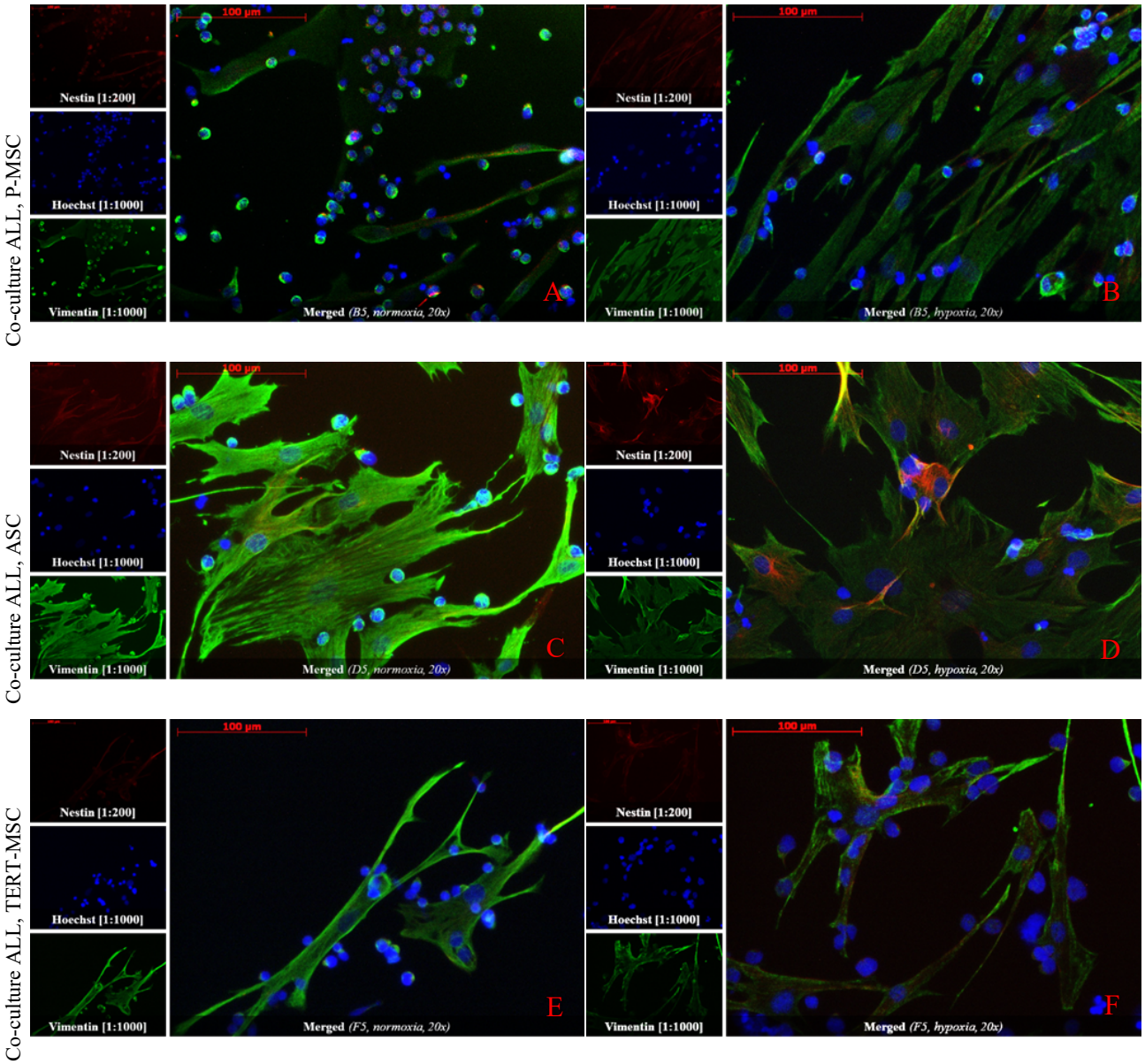


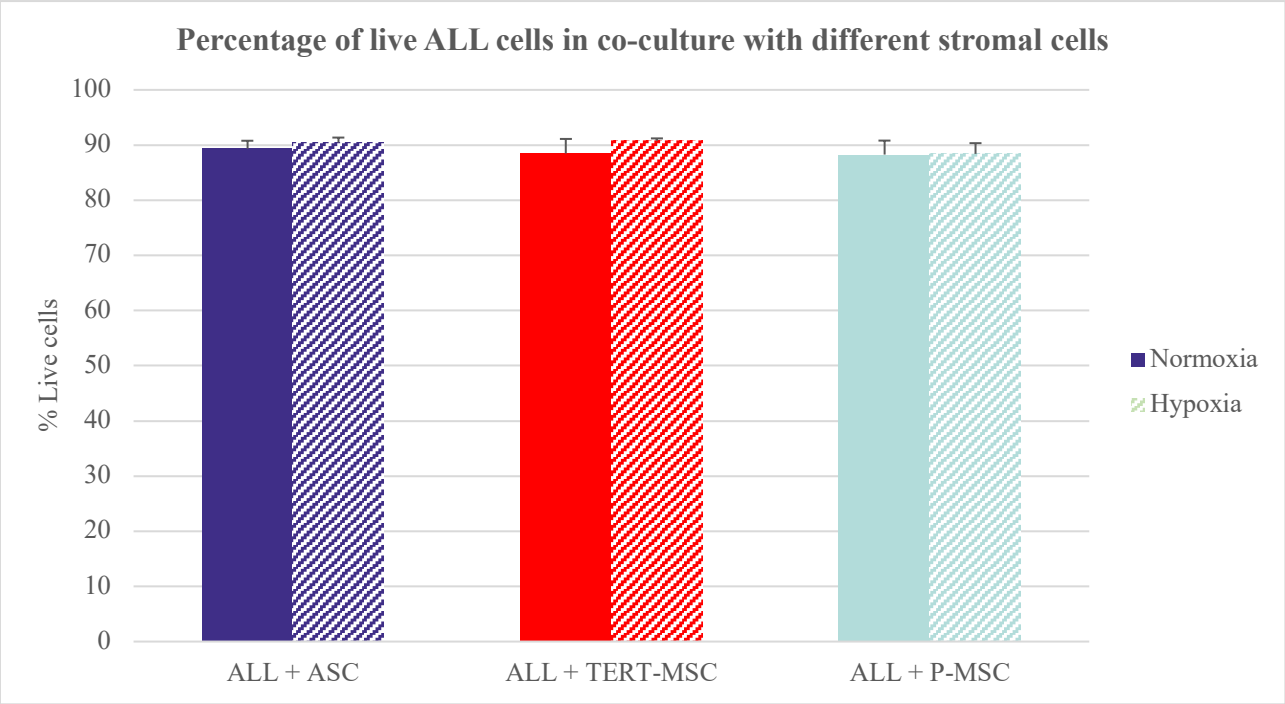
Figure 17, Co-culture 20x. A: ALL + P-MSC Nestin [1:200]+Vimentin [1:1000] normoxia. B: ALL + P-MSC Nestin [1:200]+Vimentin [1:1000] hypoxia, C: ALL + ALL + ASC Nestin [1:200]+Vimentin [1:1000] normoxia. D: ALL + ASC Nestin [1:200]+Vimentin [1:1000] hypoxia. E: ALL + ALL + TERT-MSC Nestin [1:200]+Vimentin [1:1000] normoxia. F: ALL + TERT-MSC Nestin [1:200]+Vimentin [1:1000] hypoxia.



3.5 Survival of acute lymphoblastic cells in co-culture with stromal cells in normoxia and hypoxia

The quantitative analysis of data derived from the sytox-stain is illustrated in *Figure 18*, where the survival of B-ALL cells from patient 02 is co-cultured with ASCs, TERT-MSCs, or P-MSC, in either normoxia or hypoxia for five days.

Figure 18, Percentage of live B-ALL cells from patient 02 in co-culture with ACS, TERT-MSC or P-MSC in normoxia (solid bars) or hypoxia (striped bars). Cell scoring assay on ImageXpress PICO, with sytox stain.

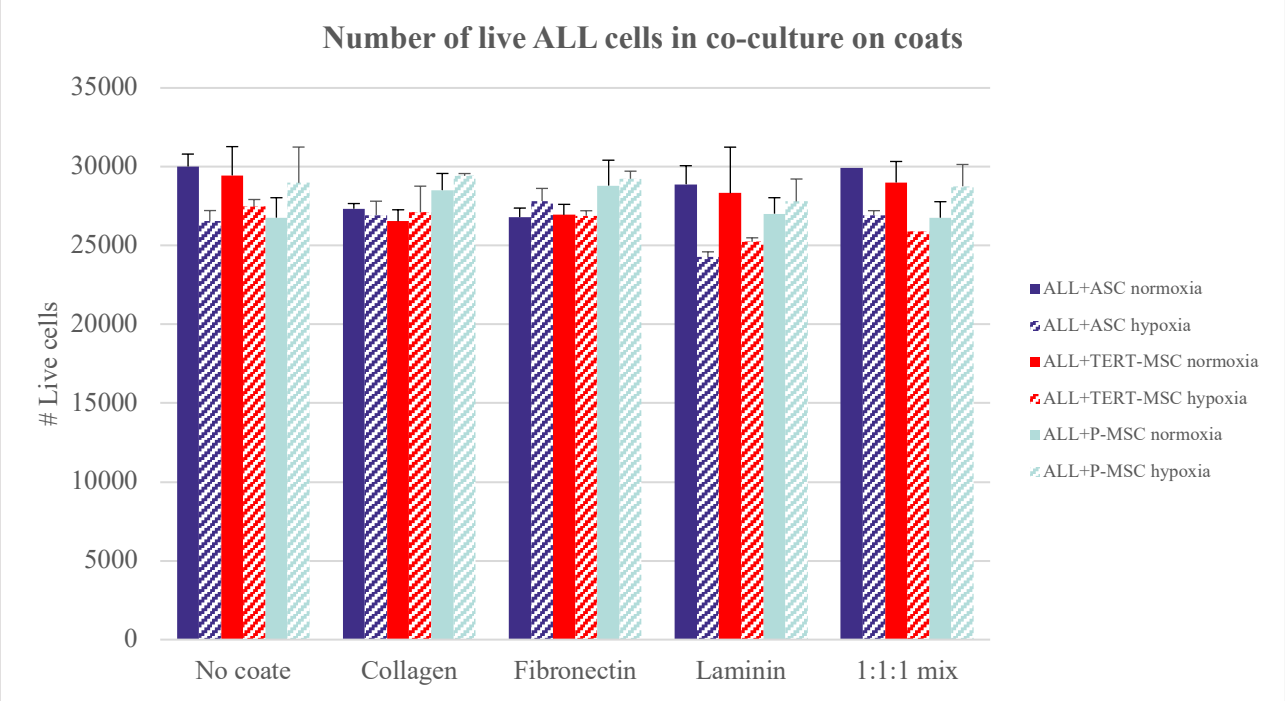


When comparing the survival of ALL cells in co-culture with ASCs, TERT-MSC, or P-MSCs cultured in five days no difference is found, and the same applies when comparing the survival of ALL cells in co-culture in normoxia to hypoxia (a table with the number of live cells can be found in the *Appendix B.1*).

3.6 Survival of acute lymphoblastic cells in monoculture and co-culture with stromal cells, cultured in normoxia and hypoxia on extracellular matrix coated culture ware

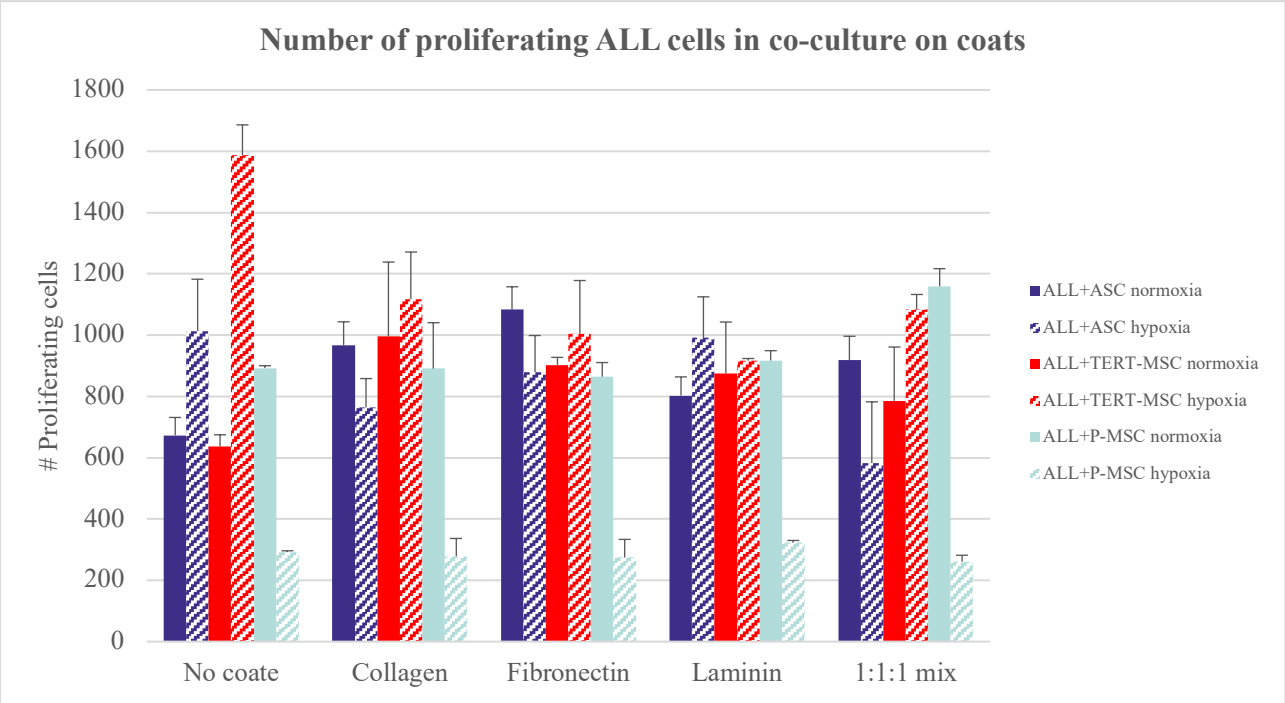
The quantitative analysis of the data derived from the sytox-stain of ALL cells in co-culture with ASC, TERT-MSC, or P-MSC in either normoxia or hypoxia on collagen coat, fibronectin coat, laminin coat, or 1:1:1 collagen-fibronectin-laminin coat cultures in five days is illustrated in *Figure 19,* where the percentage survival of B-ALL cells from patient 02 is shown.

Figure 19, Number of live B-ALL cells from patient 02 in co-culture with ACS, TERT-MSC or P-MSC in normoxia (solid bars) or hypoxia(striped bars) on no coat, collagen coat, fibronectin coat, laminin coat, or 1:1:1 collagen-fibronectin-laminin coat. Cell scoring assay on ImageXpress PICO, with sytox stain.



When comparing the survival of B-ALL cells from patient 02 in co-culture with ASCs, TERT-MSC, or P-MSCs on different coats after five days of culture, no difference is found, as illustrated in the solid bars in *Figure 19*. The same applies to the same conditions cultured in hypoxia, as illustrated in the striped bars in *Figure 19*, where, again, no difference is found.

Figure 20, Number of proliferation B-ALL cells from patient 02 in co-culture with ACS, TERT-MSC or P-MSC in normoxia (solid bars) or hypoxia(striped bars) on no coat, collagen coat, fibronectin coat, laminin coat, or 1:1:1 collagen-fibronectin-laminin coat. Cell scoring assay on ImageXpress PICO, with sytox stain.

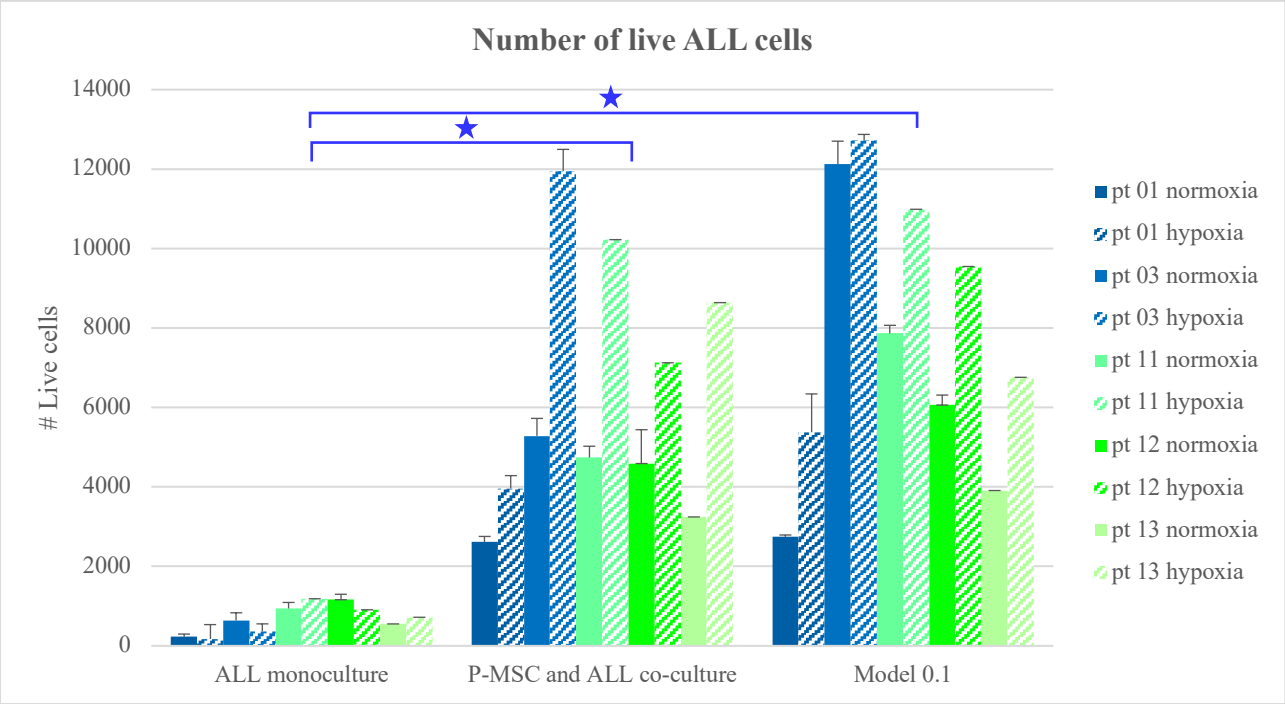


When comparing the number of proliferating B-ALL cells from patient 02 in co-culture with ASCs, TERT-MSC, or P-MSCs on different coats after five days of culture, no difference in proliferation is found between coats in ASCs and P-MSCs co-cultures, *Figure 20*. However, ALL cells seem to proliferate more in TERT-MSC co-culture on no coat in hypoxia compared to the other conditions. ALL cells in co-culture with P-MSC seem to proliferate less in hypoxia.

3.7 Test of the survival of acute lymphoblastic leukemia cells in model 0.1

Model 0.1 was created as a co-culture system with P-MSC on an ECM coat of 1:1:1 mixed fibronectin, laminin, and collagen. The P-MSCs were chosen, as they appeared to have slightly stronger labeling of nestin compared to the other stromal cells in monoculture. The 1:1:1 mixed fibronectin, laminin, and collagen coat were chosen, as none of the ECM components had shown a negative effect on the ALL cells, and because the literature had suggested that all components would be beneficial for supporting ALL cells. The quantitative analysis of the data derived from the sytox-stain of ALL cells in monoculture, co-culture with P-MSCs, or in model 0.1 in either normoxia or hypoxia is illustrated in *Figure 21*, where the percentage survival of ALL cells is shown.

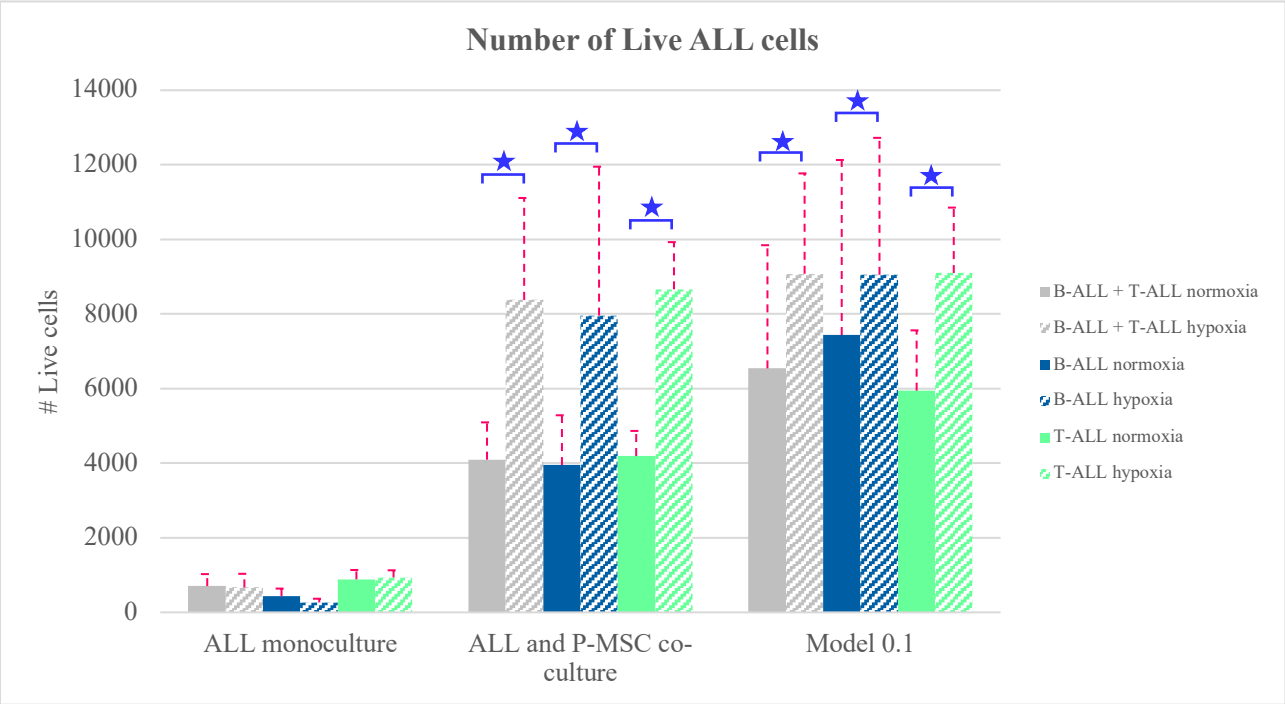
Figure 21, Number of live ALL cells monoculture, co-culture with P-MSC, and in model 0.1 in normoxia or hypoxia. ★ indicates a significant difference (p<0.05). Cell scoring assay on ImageXpress PICO, with sytox stain.



The bar chart in *Figure 21* shows that ALL cells have significantly higher survival in co-culture with P-MSCs and in model 0.1 after five days compared to the ALL monoculture ($p<0.05$).
A significantly higher survival is also found in hypoxia compared to normoxia and is illustrated in *Figure 22*, to remain overview ($p<0.05$).

To illustrate the survival tendency of ALL cells in monoculture, co-culture with P-MSC, and model 0.1 across all patients and across ALL-types, patients have been pooled to provide a general overview (*Figure 22*). An increase in live ALL cells from ALL in co-culture with P-MSC compared to model 0.1 is shown in *Figure 22*. This tendency is however not significant ($p>0.05$).

Figure 22, Average number of live ALL cells. GREY: all patients pooled, BLUE: B-ALL patients pooled, GREEN: T-ALL patients pooled. Solid bars: normoxia, stribed bars: hypoxia. Pink dashed standard deviations represent deviations between patients. ★ indicates a significant difference (p<0.05). Cell scoring assay on ImageXpress PICO, with sytox stain.

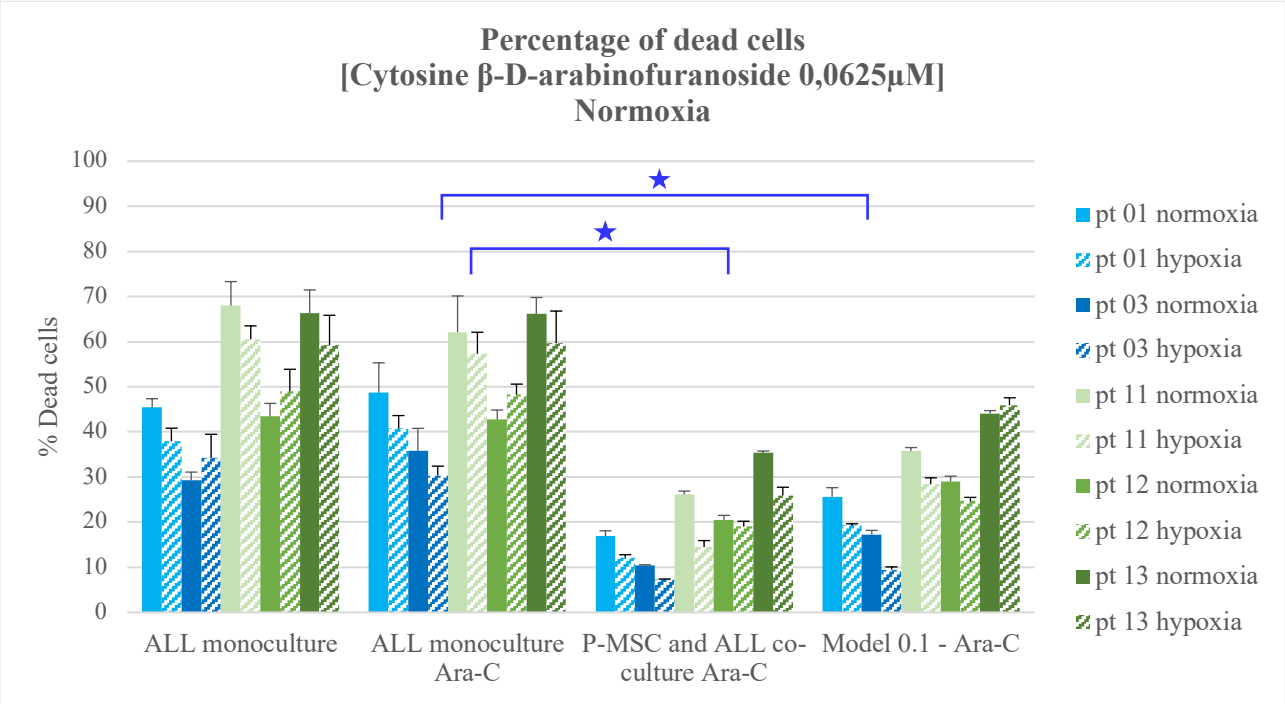


3.8 Cytosine β-D-arabinofuranoside sensitivity test of acute lymphoblastic leukemia cells in model 0.1

ALL cells in monoculture, co-culture with P-MSCs, and in model 0.1 were treated with AraC at a concentration of 0.0625 µM. The concentration based on unpublished studies made at the Laboratory of Pediatric Oncology, Rigshospitalet.

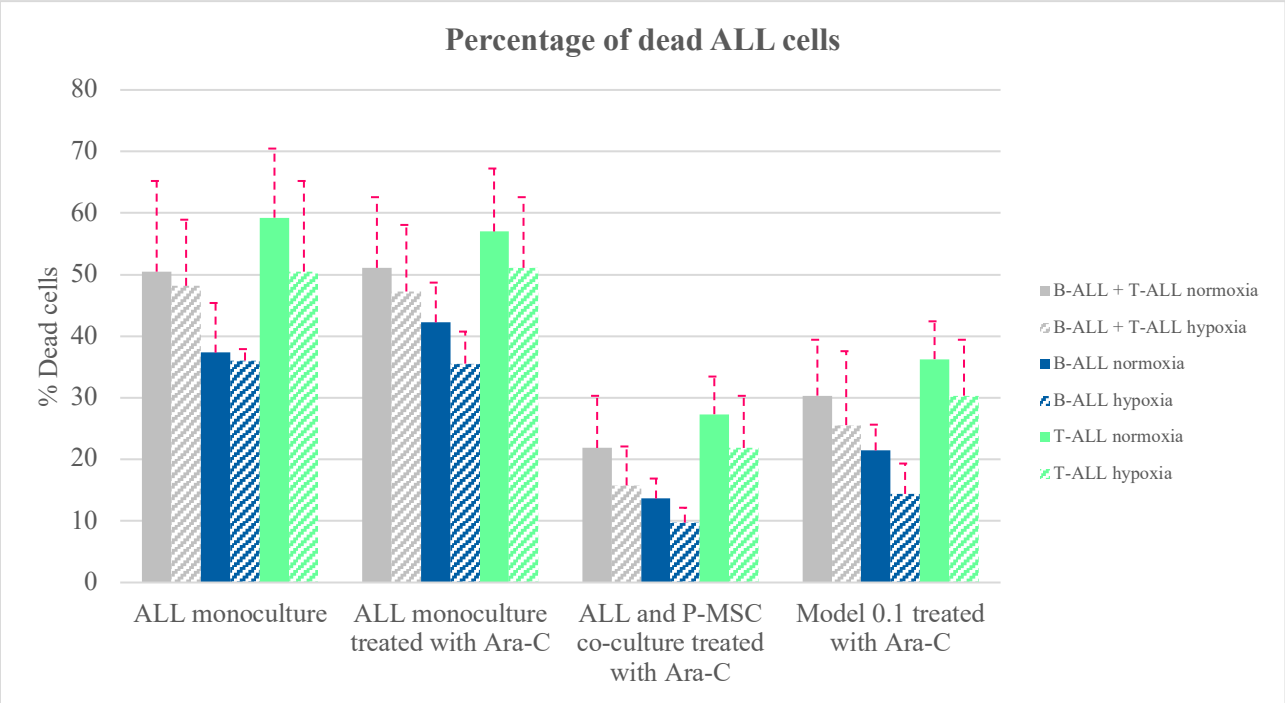
The quantitative analysis of the data derived from the sytox-stain of AraC treated ALL cells in monoculture, co-culture with P-MSCs, or in model 0.1 in either normoxia or hypoxia is illustrated in *Figure 23*, where the percentage of dead ALL is shown.

Figure 23, Percentage of dead ALL cells in monoculture, co-culture with P-MSC, and in model 0.1 in normoxia and hypoxia treated with 0.0625 µM Cytosine β-D-arabinofuranoside. Cell scoring assay on ImageXpress PICO, with sytox stain. ★ indicates a significant difference (p<0.05).



A statistical difference is shown in the number of ALL cells after treatment with 0.0625 µM AraC between ALL monoculture and P-MSC and ALL co-culture and between ALL monoculture and model 0.1 ($p<0.05$), as illustrated in *Figure 23*.

Figure 24, Percentage of dead ALL cells in monoculture, co-culture with P-MSC, and in model 0.1 in normoxia and hypoxia treated with 0.0625 µM AraC. GREY: all patients pooled, BLUE: B-ALL patients pooled, GREEN: T-ALL patients pooled. Pink dashed standard deviations represent deviations between patients. Cell scoring assay on ImageXpress PICO, with sytox stain.



For both pooled B- and T-ALL patients, together with their separate groups illustrated in *Figure 24* a general tendency of better survival in hypoxia compared to normoxia is shown, this difference is however not significant ($p>0.05$).

4 Discussion

This study aimed to create an in vitro model for ALL. As previously described, ALL cells have proven difficult to culture (Nijmeijer et al., 2009; Pal et al., 2016). Thus, different approaches to improve the survival of ALL cells in vitro were attempted. In the following sections, factors that might influence the survival of ALL cells are presented and discussed to investigate their validity and relevance for further study.

4.1 BAX channel blocker

ALL cells have been shown in previous studies to undergo extensive apoptosis in the initial culture period in monoculture (Nijmeijer et al., 2009). Therefore, it was interesting to investigate whether this could be avoided. In a study by Campana et al., a downregulation of the anti-apoptotic protein Bcl-2 was observed in some ALL patients when cultured in monoculture (Campana et al., 1994). In another study by Fuente et al., they found that an elevated Bcl-2/BAX ratio correlated with increased survival of B-Cell chronic lymphoblastic leukemia (B-CLL) cells, while a low ratio correlated with the opposite (Fuente et al., 1999). Thus, these findings might suggest that a decrease in Bcl-2 could result in increased BAX-mediated apoptosis. Therefore, in this study, it was interesting to investigate if the BAX channel blocker could prevent extensive apoptosis in the initial culture period (Peña-Blanco & García-Sáez, 2018). Both Campana et al. and Fuente et al. noted that the expression of Bcl-2 varied across patients, indicating heterogeneity among patients regarding Bcl-2 expression (Campana et al., 1994; Fuente et al., 1999), and a difference in the response was expected. In this study, however, B-ALL and T-ALL patients with different survival were investigated and treated for one hour with 0.6µM - 10µM of BAX channel blocker; thus, no statistical difference could be found in the survival of ALL cells at the different concentrations or compared to controls. Since the treatment did not appear to have any effect on the ALL cells in this study, it could be of interest to investigate whether the cell death is mediated through BAX since a BAX channel blocker would not be expected to prevent apoptosis otherwise.

4.2 Cytokines and growth factors

As described in the introduction, several soluble factors, including FLT-3, IL-2, IL-3, Il-7, SCF, FGF, VEGF, and TGF-β, are essential for the growth, support, and proliferation of HSCs (Allouche

& Bikfalvi, 1995; Gerber et al., 2002; Robb, 2007; Vaidya & Kale, 2015). Since ALL cells have shown to be compatible with HSC, it was theorized that the same soluble factors would indeed be beneficial in supporting ALL cells in vitro. The beneficial effect of cytokine has been confirmed previously by Bruserod et al., among others, that found an increased proliferation of ALL cells cultured with IL-7, IL-3, and especially FLT-3 ligand in vitro (Bruserud et al., 2003).

In this study, a medium optimization experiment was made as an initial step in the development of the in vitro model, where the effects of a cytokine cocktail of FLT-3, IL-2, IL-3, Il-7, and SCF and the growth factor-rich human platelet lysate (hPL) (Guiotto et al., 2020) was investigated and compared. In previous studies, the serum-free medium Stem Span II has been shown to improve the survival of ALL-cell (Bruserud et al., 2003; Pal et al., 2016), why it was included in this experiment. Three culture media, all made from Stem Span II supplemented with either the cytokine cocktail, 5% hPL, or a combined medium with both the cytokine cocktail and 5% hPL was compared to the standard medium RPMI with 10% FBS. In this study, all three Stem Span II media types significantly improved the survival of ALL cells when compared to the standard RPMI medium.

Even though no statistical difference was found between the three Stem Span II media types, a general tendency across patients showed that the combined Stem Span II medium containing the cytokine cocktail and 5% hPL had improved survival compared to the two other Stem Span II media.

This study also demonstrated an important discovery, the major difference in survival between patients. Although previous studies have also discovered this heterogeneous nature of ALL cells (Haegert et al., 1975), where B-ALL patients and T-ALL patients were pooled separately, no significant difference was found. However, the tendency favouring the combined Stem Span II medium containing the cytokine cocktail and 5% hPL was still present for both pooled B-ALL and T-ALL patients. The finding in this study contributes to the evidence that soluble factors like cytokines and growth factors have a beneficial effect on the survival of ALL cells, where the combined Stem Span II medium with cytokine cocktail and 5% hPL was used in the development of the in vitro model.

Even though the survival of ALL cells was significantly improved, it is still important to note that the survival was very different from patient to patient, and the development of an in vitro model advocates for a culture system that can embrace this heterogeneity or be patient-specific.

4.3 Supportive stromal cells

An attempt to improve the survival of ALL cells with inspiration from their natural microenvironment has been made with the introduction of stromal cells in co-culture systems, which have previously shown great results, and have therefore been a widely used method for sustaining ALL cells in vitro (Pal et al., 2016).

P-MSCs and immortalized MSC cell lines are the most common types of stromal cells used in co-culture systems with ALL cells. In a study by Burt et al., they found that both the human MSC cell line HS27a and P-MSCs could transform to the CAF-phenotype, which has been known to support ALL cells (Burt et al., 2019; Pal et al., 2016).

Another cell type that is gaining more interest is ASCs, which have proven to be compatible with P-MSCs but are easier to obtain (Kingham et al., 2007). In addition, previous studies have shown that ASCs are beneficial supportive cells for HSC (Zhang et al., 2020), where it was hypothesized that they could also be a candidate for co-culture systems with ALL cells.

In this study, ASCs, TERT-MSCs and P-MSCs were tested to support the survival of ALL cells in co-culture. Based on the results, they all seemed to possess this ability, with no difference in the survival of ALL cells between them.

Previously it has been implied that nes+ stromal cells possess an advantage in supporting HSC in co-culture (Pal et al., 2016). It was hypostasized in this study that ALL cells might also benefit from co-culturing with nes+ stromal cells. Previous studies have found that P-MSC, ASC (Foudah et al., 2014) together with TERT-MSC (Nikaido et al., 2013) express nestin in monoculture, and this was confirmed in this study. The P-MSC seemed to express slightly higher nestin levels in hypoxia than P-MSC in normoxia and compared to the other stromal cell types. In a study by Wong et al., they also investigated nestin expression in P-MSCs in hypoxia. They found that hypoxia increased nestin levels in P-MSCs through activation of HIF-1α (Wong et al., 2014), which could explain the slight increase in nestin expression found in this study.

As described in the introduction, leukemic cells can communicate and alter other cells in their tumor microenvironment in favour of their survival. A well-known outcome of this is CAFs. In a study by Burt et al., they found that CAFs in ALL had a two to eight-fold increase in the transcription of nestin. In this study, where stromal cells were co-cultured with ALL cells, P-MSCs, ASCs, and TERT-MSCs, all seemed to have a higher nestin expression, which could indicate that

the stromal cells in co-culture could have changed phenotype to CAF to support the ALL cells. Further investigation of stromal cells for CAF-markers such as α-SMA and FAP would need to be done to prove whether this transformation was happening (Pan et al., 2020).

4.4. Vimentin expression in ALL cells

Co-cultures were also investigated for vimentin expression, an intermediate filament expressed in various cell types, including mesenchymal cells and lymphocytes. Previous studies have suggested that vimentin is involved in multiple functions in lymphocytes, including apoptosis. (Su et al., 2019; Wu et al., 2018). In a study by Su et al. where apoptosis was investigated in the ALL-cell line, Jurkat found a significantly enhanced level of apoptosis in cells lacking vimentin compared to controls and cells overexpressing vimentin (Su et al., 2019). In this study, ALL cells showed intense vimentin labelling in co-culture with stromal cells, which could suggest protection from apoptosis. ALL protection from apoptosis through co-culture has already been described in this study. Still, it could be of interest to investigate if vimentin expression changes in ALL cells when cultured in co-culture and if it might be an anti-apoptosis marker. Therefore, it would be of great interest to investigate the vimentin expression of ALL cells in monoculture.

4.5 Oxygen tension

The bone marrow is naturally a hypoxic environment, and studies have suggested that this factor is a key element in the survival of leukemic cells, both in the culturing of ALL cells and the protection from chemotherapy (Deynoux et al., 2016; Petit et al., 2016). The beneficial effect of hypoxia is, as previously described, assigned to the HIF-1α, and in a study by Petit et al., they found overexpression of HIF-1α in ALL cells (Petit et al., 2016). HIF-1α is known to activate transcription of several genes, here among genes involved in cell survival, but as previously described, this activation requires a hypoxia environment. In this study, where ALL cells were cultured in co-culture, ALL cells had a significantly improved survival in hypoxia compared to normoxia.

4.6 Extracellular matrix components

As written in the introduction, the ECM is important for providing both structural and biochemical support for its associated cells. ECM components such as collagen, fibronectin, and laminin can bind cells through their integrin-binding domain and activate intercellular signalling pathways within these cells (Pan et al., 2021; P. Zhang et al., 2019).

An example of this is found in a study by Fuente et al., that found a correlation between elevated levels of Bcl-2 and prevented the onset of apoptosis in B-cell lymphoblastic leukemia (B-CLL) attached to fibronectin through integrin binding. In this study, different ECM components such as collagen, fibronectin, and laminin were used to coat flasks for ALL co-cultures with ASCs, P-MSCs, or TERT-MSCs, and even though the general survival was high, no difference was seen in the survival between co-cultures on collagen, fibronectin, laminin, 1:1:1 mixed coats or no coating. This finding could indicate that the ECM components did not have a beneficial effect on that particular patient used in the experiment, knowing that ALL patients have previously been shown to act very differently from one another. But it could also be that the beneficial effect from the supportive cells might have 'overruled' the effects of the ECM components. Leukemic cells can aberrantly express cell adhesion molecules on their surface and remodel the bone marrow microenvironment, including stromal cells and the ECM, to express more binding partners for the cell adhesion molecules on the leukemic cells (Kim et al., 2020). A study by Burt et al. clearly states that the cell-cell interaction between ALL cells and stromal cells is crucial for protection from oxidative stress associated with ALL cell death in vitro (Burt et al., 2019).

In a study by Scielzo et al., ECM was also highlighted as an essential component in developing improved in vitro models for leukemia, as it provides the 3D structure of tissue the conventional 2D models lack (Scielzo & Ghia, 2020). The introduction of 3-dimensionality was attempted in the final experiment in this study, comparing the survival of ALL cells from five patients in either monoculture, co-culture, or in model 0.1. The survival of ALL cells was significantly improved in co-culture and model 0.1 compared to monoculture, which was expected due to the introduction of stromal cells. Across all patients, however, slightly improved survival was also observed in model 0.1 compared to the co-culture. This slight improvement could be due to creating a more complex microenvironment facilitated by the ECM components. As described in the study by Scielzo et al., the physiological structure of the ECM provides for ECM-cell interactions and a scaffold for cell-cell interactions, which has proven to be important for the survival of leukemic cells (Scielzo &

Ghia, 2020). Thus, this could be the reason for the slight improvement found in model 0.1 in this study.

4.7 Cytosine β-D-arabinofuranoside sensitivity

MSCs provide crucial support for ALL cells that significantly improve their survival in vitro, but they have also been shown to exert a chemoprotective effect on ALL cells (Burt et al., 2019; Pal et al., 2016). In a study by Burt et al., they found that besides improvements in survival, MSCs also exert a chemoprotective effect on ALL cells from reactive oxygen species (ROS)-inducing chemotherapy. In monoculture of ALL cells, chemotherapeutic agents like AraC promote ROS, which results in ROS-induced apoptosis of the cells. Still, as demonstrated by Burt et al., AraC can also promote the transformation of MSCs to the CAF phenotype, which wields a chemoprotective effect on the ALL cells through mitochondrial transfer via nanotubes. In this study AraC mediated cells, death occurred at a significantly lower level in the co-culture and model 0.1 compared to the monocultures, which could imply a chemoprotective effect in the co-cultures and model 0.1, which could be due to the CAF transformation of the MSCs (Burt et al., 2019).

4.8 Assessment methods

In this study, ALL cells in co-culture were stained with SYTOX Green which stains nucleic acid in dead cells, as it cannot cross intact membranes (Lebaron et al., 1998). This viability assessment is very rapid but also very simple, where other assessment methods like flow cytometry were attempted. In this study, ALL cells in co-culture were found to adhere to the stromal cells after five days in co-culture. This phenomenon has previously been observed with HSCs in co-culture with nes+ MSC in a study by Méndez-Ferrer et al. However, the adhesion of ALL cells to the stromal compartment makes it impossible to assess the ALL cells by flow cytometry, as cells are required in suspension. In this study, an attempt was made to detach the ALL cells from the stromal cells with TrypLE, atlas only a very few cells were detected. Concurrently, with more complex culture systems development, assessment methods must also be re-evaluated to obtain the maximal level of information.

5 Conclusion

This study aimed to develop an in vitro model for ALL. Based on the experiments made in this study, it can be concluded that multiple factors are required for the survival of ALL cells in vitro.

Model 0.1 comprising of ALL cells in co-culture with P-MSCs on a 1:1:1 collagen, laminin, and fibronectin coat created in this study, generally, appears promising as cytokines, growth factors, stromal cells, and hypoxia all significantly improved the survival of ALL cells in vitro. The addition of ECM components also seemed to enhance the survival of ALL cells, where it is suggested that culture systems with more resemblance to the natural microenvironment are more efficient for propagating ALL cells in vitro. Even though ALL cells in model 0.1 had significantly higher survival than monocultures, the survival is still not at a desirable level, and further improvement of the model is needed.

To imitate the 3D structure of the bone marrow niches in the further development of the in vitro model, we intend to test the effect of two scaffolds, a gelatin scaffold with hydroxyapatite nanoparticles with encapsulated MSCs, and a β-tricalcium phosphate scaffold seeded with MSCs.

Characterization of the surviving ALL cells in model 0.1 would also be of great interest, to investigate how closely the in vitro ALL population resembles the in vivo population.

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