

**Towards unravelling the kinetics of an Acute Myeloid Leukaemia
model system under oxidative and starvation stress: A comparison
between two and three dimensional cultures**

Eirini G. Velliou^{1,*}, Susana Brito Dos Santos², Maria M. Papathanasiou^{2,3},
Maria Fuentes-Gari^{2,3}, Ruth Misener⁴, Nicki Panoskaltsis⁵,
Efstratios N. Pistikopoulos^{3,6}, Athanasios Mantalaris²

¹ *Department of Chemical & Process Engineering, Faculty of Engineering and Physical Sciences,
University of Surrey, Guildford, Surrey, GU2 7XH, UK*

² *Biological Systems Engineering Laboratory (BSEL), Department of Chemical Engineering, Imperial
Imperial College London, South Kensington Campus, London SW7 2AZ, UK*

³ *Centre for Process Systems Engineering (CPSE), Department of Chemical Engineering, Imperial
Imperial College London, South Kensington Campus, London SW7 2AZ, UK*

⁴ *Department of Computing, Imperial College London, South Kensington Campus, London
SW7 2AZ, UK*

⁵ *Department of Haematology, Imperial College London, Northwick Park & St. Mark's Campus,
London HA1 3UJ, UK*

⁶ *Artie McFerrin Department of Chemical Engineering, Texas A&M University, Jack E. Brown
Building, 3122 TAMU, College Station TX 77843-3122, USA*

**Corresponding author. Fax: +44-(0)-1483683010*

Tel: +44-(0)-1483686577

E-mail address: e.velliou@surrey.ac.uk (E. G. Velliou).

Running Title: AML kinetics in 2D and 3D under environmental stress

Abstract

A great challenge when conducting *ex vivo* studies of leukaemia is the construction of an appropriate experimental platform that would recapitulate the Bone Marrow (BM) environment. Such a 3D scaffold system has been previously developed in our group [1]. Additionally to the BM architectural characteristics parameters, such as oxygen and glucose concentration are crucial as their value could differ between patients as well as within the same patient at different stages of treatment, consequently affecting the resistance of leukaemia to chemotherapy.

The effect of oxidative and glucose stress –at levels close to human physiologic ones– on the proliferation and metabolic evolution of an AML model system (K-562 cell line) in conventional 2D cultures as well as in 3D scaffolds were studied.

We observed that the K-562 cell line can proliferate and remain alive for 2 weeks in medium with glucose close to physiological levels both in 20% and 5% O₂. We report interesting differences on the cellular response to the environmental, i.e., oxidative and/or nutritional, stress stimuli in 2D and 3D. Higher adaptation to oxidative stress under non-starving conditions is observed in the 3D system. The glucose level in the medium has more impact on the cellular proliferation in the 3D compared to the 2D system.

These differences can be of significant importance both when applying chemotherapy *in vitro* and secondly when constructing mathematical tools for optimisation of disease treatment.

Keywords

Acute Myeloid Leukaemia, starvation stress, oxidative stress, 3D scaffolds, K-562 cell line.

55

56 **Introduction**

57 Acute Myeloid Leukaemia (AML) is the most aggressive type of leukaemia. According
58 to Cancer Research UK, approximately 8000 cases of AML occur annually in the UK.
59 AML is a type of leukaemia which is characterized by an increased proliferation of
60 immature blasts in the myeloid lineage and, as a consequence, insufficient normal
61 blood cell production. These blasts have low proliferation capability. However, a
62 small population of cells with higher proliferation capacity and self renewal potential,
63 i.e., the Leukaemic Stem Cells (LSC)s, are the key component for the maintenance of
64 the disease [2]. AML is usually the result of somatic mutations in either a pluripotent
65 hematopoietic stem cell or a slightly differentiated progenitor cell and leads to a
66 deregulation and/or inhibition of normal haematopoiesis due to space restrictions as
67 well as inhibitory and clonal factors specific to the disease [3-6].
68 The most common treatment for AML is intensive chemotherapy, which aims at
69 eradicating the leukemic cell population. Current chemotherapy treatment protocols are
70 designed based on pre-clinical animal experiments and on empirical clinical trials as well
71 as the acquired experience of subspecialist physicians. The criteria- parameters for these
72 protocols consist of the patient bone marrow aspirate examination (blasts percentage,
73 immunophenotype, cytogenetics and molecular analysis) and physiological patient
74 characteristics (height, weight) for the normalization of the dose according to the body
75 surface area (BSA). It is well known that therapy based on the above can result in
76 several life-threatening complications as only few patient-specific factors are taken
77 into consideration in current protocols and the choice of treatment often depends on
78 the physician's experience. Furthermore, inter-patient and intra-leukaemia
79 heterogeneity significantly increase the complexity in determining optimum treatment

80 protocols. As a consequence, no widely accepted ‘optimal’ chemotherapy treatment
81 protocol exists, as the evolution of the cancer as well as the chemotherapy (drug)
82 metabolism is highly dependent on patient characteristics such as age, weight, height,
83 medical and family history as well as on a variety of unknown factors/parameters.
84 A first step towards understanding and further optimising chemotherapy for AML
85 treatment is to conduct *in vitro* studies to investigate these unknown parameters in an
86 appropriate experimental platform that could efficiently reproduce *ex vivo* the *in vivo*
87 environment [7]. Therefore, parameters derived from the *in vitro* platform could serve
88 as an input for the construction of appropriate *in silico* tools for the optimisation of
89 chemotherapy [8].

90 A variety of research in *ex vivo* studies of both normal and abnormal haematopoiesis
91 is conducted in two dimensions (2D), i.e., laboratory T-flasks or micro-plates (see as
92 examples [9-11]). Despite the fact that 2D cultures enable the understanding of
93 growth and differentiation of normal and abnormal Haematopoietic Stem Cells
94 (HSC), they require high amount of exogenous growth factors that differ from those
95 *in vivo*, therefore, creating an artefact in the culture and, consequently in the cell
96 kinetics. Moreover, they are unable to recapitulate the structure of the *in vivo* BM
97 microenvironment, especially the 3D niches that are protective for leukaemic cells as
98 well as micro-environmental aspects that may affect the cancer evolution and
99 chemotherapy resistance ([6], [12 -19]). Overall, efficient *in vitro* cultivation of
100 normal and abnormal HSC should reproduce as closely as possible the *in vivo*
101 conditions ([6], [17], [20], [21]).

102 Lately, several researchers have focused on the development and further studying of
103 normal and leukaemic haematopoiesis in three dimensional (3D) culture systems. In
104 particular, these cultures recapitulate the architectural characteristics of the BM

micro-environment by using a variety of polymer-based biomaterials and/or the BM extracellular matrix by using specific proteins and/or the BM stromal support by co-culturing HSC with appropriate cellular systems [22].

For example, Tun *et al.* (2002) [23] observed an increased expansion and proliferation of murine BM cells in a 3D polyvinyl formal (PVF) porous scaffolding system compared to suspension cultures. da Silva *et al.* (2005) [24] observed expansion and maintenance of HSC from adult BM as well as umbilical cord blood (UC) in serum free medium in presence of human stromal layers. Mortera Blanco *et al.* (2010) [1] showed that AML cell lines can be efficiently expanded for up to 6 weeks in 3D polyurethane collagen coated scaffolds. Leisten *et al.* (2012) [25] proposed a three-dimensional collagen based gel system as an efficient *in vitro* bone marrow niche model in which Haematopoietic Progenitor Cells (HPC) proliferate and expand highly when co-cultured with mesenchymal stem cells. Alijtawi *et al.* (2014) [26] observed an increased resistance of the HL-60 and Kasumi leukaemic cell lines to drug induced apoptosis (cytarabine) when co-cultured with human bone marrow mesenchymal cells in 3D PGA/PLLA copolymer disks, compared to cells co-cultured in 2D.

Additionally to the actual 3D structural characteristics a variety of other environmental factors should be taken into consideration when studying the evolution of AML *in vitro*. For example alterations of parameters such as oxygen and glucose concentration on the one hand in the different body compartments, e.g., in the BM and the peripheral blood or the liver, and, on the other hand, between patients, i.e., individual cases of hypoglycemia, hyperglycemia, may lead to a different stress adaptation of the leukaemic population. The latter may consequently affect the cancer

growth and inactivation kinetics as well as the response to a chemotherapeutic drug *in vivo*.

A variety of research has revealed the strong relation between resistance, i.e., longer survival and increased proliferation of haematopoietic or leukaemic cells and resistance to chemotherapy, and (1) oxygen or (2) starvation stress *in vitro* and/or *in vivo*.

For example, Fecteau *et al.*, (2013) [27] observed an increased *in vitro* survival of cells from BM aspirates of patients with Chronic Lymphocytic Leukaemia (CLL) in 5% O₂ compared to 20% O₂. Wilkinson *et al.* (2012) [28], state that chronic oxidative stress may contribute to increased resistance of lymphoma patients to chemotherapy, due to alterations in the mitochondria. Zhou *et al.* (2010) [29], pointed the possible relation between the relapse of AML and increased oxidative stress *in vivo*. Lodi *et al.* (2011) [30] observed that hypoxia is a key factor that affects the metabolic activity, i.e., adaptation of phospholytic and glycolytic metabolism, and evolution of KG1a and K562 leukaemic cell lines. Herst *et al.* (2011) [31] has pointed a possible relation between the level of glycolytic metabolism of AML blasts and resistance to chemotherapy: analysis of 26 BM aspirates showed that AML cells with higher glucose consumption were more tolerant to *in vitro* apoptosis caused by chemotherapy.

In general, from all the above studies it is clear that a systematic monitoring of environmental factors such as oxygen and glucose concentration are essential, on the one hand for a better understanding of the (normal and abnormal) haematopoietic cellular proliferation and metabolic activity and on the other hand for more efficient *in vitro* mimicry of *in vivo* systems in which fluctuations of environmental factors naturally occur. However, there is very limited available literature on the impact of

such stress factors on the leukaemic evolution in 3D cultures and at stress levels close to physiological.

The aim of the current research is to study and compare the proliferation and metabolic evolution of an AML model system in 3D and 2D cultures for different oxygen and glucose conditions close to physiological (*in vivo*) levels. More specifically, the K-562 leukaemic cell line was cultivated in 3D polyurethane porous scaffolds (that mimic the 3D architecture of the BM and have been developed and optimised within our group) coated with collagen type I (to mimic the extracellular matrix present in the BM), as described in [1], as well as in 2D suspension cultures in 5% (hypoxia) as well as 20%, (normoxia) oxygen and three different glucose levels, i.e., 4.3 g/L (optimal level generally applied in laboratory growth media), 1.3 g/L (highest human physiological level *in vivo*) and 0.6 g/L (lowest human physiological level *in vivo*) for 2 weeks. This time span was selected as the total chemotherapy cycle for AML treatment lasts approximately 10 days (NHS-Trust, UK). Consequently, understanding the effect of environmental factors on a leukaemic population within that time frame would be appropriate for comparison and/or future combination with *in vitro* chemotherapy experiments. The cellular proliferation and metabolic evolution was monitored in order to enable the understanding of the cellular response and possible adaptation to the environmental stress stimuli.

Materials and Methods

Fabrication, coating and sterilization of 3 dimensional scaffolds

3D scaffolds were fabricated and coated as previously described by our group in [1]. For the *scaffold fabrication* 3 g of polyurethane (PU) (Noveon, Belgium) were added in 60 mL of Dioxan (99,8% pure, Sigma-Aldrich, UK). The foams were fabricated by

Thermally Induced Phase Separation (TIPS) of polymer solution followed by solvent sublimation as described in [32]. The PU solution was frozen at -186°C and maintained there for 2h. Removal of the solvent took place by freeze-drying in an ethylene glycol bath at -15°C for approximately 3 days. Prior to coating scaffolds were cut into 5x5x5 mm³ cubes. Scaffold porosity was approximately 90-95% and average pore size 100-250 µm.

After cutting, the *scaffolds were coated* with collagen type I from calf skin (Sigma Aldrich UK). The cubes were first dipped in ethanol (70% v/v) for 1 min and then transferred in phosphate buffered saline (PBS, Gibco, UK) for 10 min and then centrifuged at 2500 rpm for 10 min. Hereafter, the scaffolds were transferred in 62.5µg/mL of collagen type I solution –soluble in 0.1 M acetic acid and dissolved in deionised water- of pH 7 (re-adjusted with addition of 0.1 M NaOH) and centrifuged at 2000 rpm for 20 min. At the end scaffolds were washed once more with PBS at 1500 rpm for 10 min.

Prior to cell seeding *sterilisation* of the scaffolds took place by combinatory exposure to ethanol (70% v/v) and UV (230v, 50Hz, 0.14A, Kedro Laboratory Products UK). More specifically, the scaffolds were immersed in ethanol for 2 h and then washed twice with PBS –in which they were kept for 15 min for each washing step- before exposure for 8 min under UV. After sterilisation the scaffolds were added to the growth medium (see section 2.3) in a humidified incubator for 3 days at 37°C and 5 % CO₂ in order to be examined for possible contaminations.

Inoculum preparation

The human leukaemic cell line K-562 (human erythromyelo-blastoid leukaemic cell line, ATCC®UK, CCL-243) which was originally derived from a patient with a blast

crisis of chronic myeloid leukaemia, i.e., therefore resembling acute myeloid leukaemia, was chosen as an AML model system. It has been previously reported by our group that this cell line can successfully expand in three dimensional polyurethane (PU) collagen coated scaffolds [1]. After thawing, as recommended by the cell line supplier, the cells were expanded by cultivation on standard polystyrene non-pyrogenic tissue culture flasks (Fisher Scientific) and in a humidified incubator at 37°C in an atmosphere of 5% CO₂. The cultivation medium was IMDM medium (Invitrogen Ltd, UK), supplemented with 10% foetal bovine serum (FBS, heat inactivated, Invitrogen Ltd), and 1% Penicillin/Streptomycin (Invitrogen Ltd). Feeding and appropriate re-suspension/sub-culturing of the cells in fresh medium took place every 48 hours, as indicated by the supplier, until a cell number, sufficient for the experimental purposes was reached.

Cell culturing conditions

During the experiments cells were cultured in DMEM, no glucose medium (Invitrogen Ltd, UK) supplemented with 10% foetal bovine serum (FBS, heat inactivated, Invitrogen Ltd), and 1% Penicillin/Streptomycin (Invitrogen Ltd) and either 4.3 g/L (optimal glucose concentration as suggested by the cell line supplier and, therefore, considered as control, [CTR]), 1.3 g/L (highest concentration in the human blood, resembling hyperglycemia, [HIGH]) or 0.6 g/L (lowest concentration in the human blood, resembling hypoglycemia [LOW]) of glucose.

For culturing the cells in suspension, i.e., 2D, 100 µL of cell suspension (5 x 10⁵ cells/well) were added in a high throughput micro-tetri plate system (24 well micro-plate), that contained 1.5 mL of medium exhausted at 37°C and 5% CO₂ and in either normoxia (20% O₂) or hypoxia (5% O₂) with appropriate glucose concentration,

i.e., [LOW] or [HIGH] or [CTR]. The plates were placed in either a hypoxic or a normoxic incubator. Medium was changed approximately every 2-3 days and the volume was re-adjusted when needed –depending on the cell counts- in order to maintain the cell density suggested by the supplier, i.e., between 2×10^5 - 2×10^6 cells per mL for a time period of 2 weeks.

For *culturing the cells in the scaffolds*, i.e., 3D, 100 μ L of cell suspension (6×10^5 cells/well in order to have approximately the same initial inoculum level as the 2D system (5×10^5), since we have observed that the seeding efficiency of the K-562 cell lines was approximately 70-80% -see also [1]) were seeded onto the sterilized collagen scaffolds, placed in 24-well tissue culture plates and incubated for 15 min at 37°C, 5% CO₂ and either 5 or 20% O₂ (depending on the oxygen condition of the experiment) in order to allow the cells to settle into the scaffolds. Hereafter, 1.5 mL of appropriate medium –as described above for the 2D cultures- was added in each well. The plates were placed in either a hypoxic or a normoxic incubator and medium was changed approximately every 2-3 days for 2 weeks.

Determination of growth in two dimensions

Determination of growth took place almost approximately on a daily basis for all the conditions under study. For growth determination in the 2D system the whole medium of 3 different wells was removed (triplicate) and a cell count was performed by using the haemocytometer.

Determination of growth in three dimensions

Quantitative assessment of the cell proliferation in the 3D scaffolds took place by means of changes in the number of metabolically active cells using the tetrazolium

compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTS, Promega, CellTiter96[®] AQueous Solution Cell Proliferation Assay)[33]. MTS together with fresh medium was added and incubated with the scaffolds seeded with cells over 3h at 37°C, 5% CO₂ and either 5 or 20% O₂ (depending on the oxygen condition of the experiment). Absorbance was measured at 490 nm. In order to correlate absorbance with actual cell numbers a calibration curve was previously constructed correlating the cell number in known (enumerated with the haemocytometer) 2D suspension with the absorbance at 490 nm after applying the MTS assay (see Figure A.1). This calibration curve was used for the calculation of cell numbers, enabling the comparison with the 2D system.

Analysis of metabolites

Analysis of nutrients and metabolites took place with use of the Nova Bioprofiler 400 (Nova Biomedical, UK). 1.5 ml of (waste) medium was pipetted and transferred in an eppendorf tube. Centrifugation took place at 2000 rpm for 5 min for removal of cells that could be present in the medium. Supernatants were stored at -20°C prior to analysis. Samples for nutrient and metabolite analysis were collected approximately on a daily basis for all the conditions under study, and from two different wells/condition in order to account for possible variability between different wells. Concentration of nutrients in the growth medium (free of cells) can be found on Table A.1, for all the conditions under study. The Nova Bioprofiler can detect glucose at level down to 0.1mM. For glucose levels lower than that, a glucose assay kit (ab65333, abcam, UK) was used, which is able to determine glucose levels lower than 1 mM.

278 *Scanning Electron Microscopy (SEM) of the 3D scaffolds*

279 3D scaffolds for all conditions under study were examined by SEM (JEOL JSM-
280 840A, JEOL Ltd., Welwyn Garden City, U.K) in order to observe possible space
281 limitations within the 3D matrix. Scaffolds were collected for examination at different
282 time periods, i.e., days 1-7-14. After collection, incubation of the scaffolds took place
283 in 5 ug/mL of fibronectin for 2 h -to ensure the maximum attachment and
284 maintenance of cells on the scaffold since a series of washing steps is introduced next-
285 , followed by fixation in 3% gluteraldehyde in Sorenson's buffer for 1 h and washing
286 with Sorenson's Buffer for 5 min. Post-fixation with 1% Osmium Tetraoxide in
287 Sorenson's buffer took place for 1 h. Afterwards a first serial dehydration process of
288 the scaffolds took place with washing in EtOH/Sorenson's buffer solutions as follows:
289 50% EtOH/50% Sorenson's Buffer, 70% EtOH / 30% Sorenson's Buffer, 90%
290 EtOH/10% Sorenson's Buffer, 95% EtOH/5% Sorenson's Buffer. The scaffolds
291 remained in each combination of solutions for 15 minutes. Thereafter, the scaffolds
292 were immersed in 100% EtOH for 15 minutes for two successive washings. A second
293 serial dehydration process of the scaffolds took place with washing in EtOH/HMDS
294 solutions as follows: 0% HMDS/50% EtOH, 90% HMDS/10% EtOH. The scaffolds
295 remained in each combination of solutions for 15 minutes. Hereafter, the scaffolds
296 were immersed in 100% HMDS for 15 minutes for two successive washings. After
297 the two serial dehydration procedures, the scaffolds were left to air dry overnight in an
298 aseptic environment. The specimens were sectioned and then sputter-coated with gold
299 in argon atmosphere for 2 min prior examination by SEM at an acceleration voltage of
300 20 kV.

301
302 *Statistical analysis*

The experiments were conducted in duplicate (N=2) and cell proliferation was determined in triplicate (n=3) and bioprofiler in duplicate (n=2) for each experiment. Analysis of variance was performed with a level of significance of $p < 0.05$ in order to evaluate whether there was a statistical difference between different conditions.

Results and Discussion

Cellular Proliferation

In general, the cellular proliferation was higher in normoxia (20% O₂) compared to hypoxia (5% O₂) for both the 3D and 2D system (Figure 1) apart from the [CTR], i.e., 4.3 g/L glucose, in the 3D system at which no significant ($p < 0.05$) difference in the cell proliferation was observed for the two different oxygen conditions (Figure 1c, 1d). The latter highlights that –under non starving conditions, i.e., 4.3 g/L glucose which is normally used in most laboratory media- probably there is a higher oxidative stress response and further stress adaptation of the culture in the 3D system, leading to an increased resistance to hypoxia and consequent cell proliferation which is similar to the one in normoxia. The fact that the same trend in proliferation is not observed under conditions of nutrient limitation, i.e., 1.3 g/L and 0.6 g/L, (Figure 1c, 1d) further indicates that the observed trend for optimal nutritional conditions in hypoxia is not due to features of the 3D culture system (for example increased cell to cell interaction due to space limitations) but it is actually most probably resulting as a cellular response to the environmental stress stimuli. In contrast, in the 2D system oxidative stress seems to act as an inhibitor to cell growth leading to slower proliferation compared to normoxia (Figure 1a, 1b).

In normoxia, i.e., 20 % O₂, for both the 2D and the 3D system no effect ($p>0.05$) of the glucose concentration on the cellular proliferation is observed until day 9 (Figure 1a, 1c) after which significant differences ($p<0.05$) can be seen for different glucose feeding levels. More specifically, in the 2D culture system significantly lower ($p<0.05$) cell numbers are observed for the [LOW] level of glucose, i.e., 0.6 g/L, compared to [HIGH], i.e., 1.3 g/L, and [CTR], i.e., 4.3 g/L, from day 9 up to day 12. For this time period no significant differences were observed in the cell numbers between [CTR] and [HIGH] and only at the end of the culturing period, i.e., day 14, [CTR] level leads to significantly higher ($p<0.05$) cell numbers compared to the lower glucose levels (Figure 1a). A similar trend can be observed for the 3D system, i.e., from day 9 on [LOW] level of glucose results in significantly lower cell proliferation compared to [CTR] and [HIGH]. Moreover, no significant difference on the proliferation between the two latter conditions is observed (Figure 1c).

In contrast, in hypoxia, i.e., 5% O₂, for both the 2D and 3D systems, there is a higher impact of the glucose level on the cell proliferation (Figure 1b, 1d). More specifically, for the 2D culture, the effect of glucose on the K-562 growth becomes significant ($p<0.05$) from day 9 on. From that time point, lower glucose, i.e., [LOW] leads to lower proliferation and the highest proliferation is observed for the [CTR] (Figure 1b). The difference in the cell number for [CTR], [HIGH] and [LOW] is more evident compared to normoxia (Figure 1a, 1b). In the scaffolds the impact of the level of glucose on K-562 expansion, becomes significant ($p<0.05$) between the [CTR] and the two lower glucose concentrations ([HIGH], [LOW]) on day 2 and among all three glucose levels from day 4 on. Especially the lowest glucose level, i.e., [LOW], seems to be limiting for growth in the 3D scaffolds in 5 % O₂ as the cell expansion

throughout 2 weeks is very low (Figure 1d). As an overall, under oxidative stress the impact of the glucose level on the cellular proliferation is higher in the 3D system. To our knowledge, there is very limited literature available especially in 3D scaffold systems studying the proliferation of (normal or abnormal) haematopoietic cells under oxygen and glucose concentrations close to physiological levels. However, the trends of several of our findings are in agreement with the limited literature. For example, lower growth of K-562 for lower glucose levels and in hypoxic compared to normoxic conditions was also observed by Guintoli *et al.* (2011) [34] in a 2D culture system. More specifically, K-562 cells proliferated lower in hypoxia (0.1% O₂) compared to normoxia (20% O₂) and low glucose level in the growth medium (1.1 g/L) lead to lower proliferation for both oxygen conditions compared to higher glucose (4.3 g/L). A reversed effect of the impact of oxygen on cell survival and proliferation, compared to our findings, was observed by Fecteau *et al.* (2013) [27] as well as dos Santos *et al.* (2010) [35] but for mesenchymal stem cells derived from the BM of patients with Chronic Lymphocytic Leukaemia (CLL) and normal human BM mesenchymal cells, respectively, in 2D culture: higher proliferation was observed in hypoxic in comparison to normoxic conditions.

Metabolic evolution

Next to the cell growth, the metabolic activity of the cells for all the conditions under study was monitored and compared for all different environmental conditions ([LOW], [HIGH], [CTR] glucose and 5 & 20% O₂) and systems (2D vs 3D), as, potential differences in the metabolism among different conditions or culturing systems could affect the AML progression and secondarily the cancer drug-response (Herst *et al.*, 2011).

The metabolic production of individual cells (Figures 2 & 3) as well as the yields of metabolites such as Lactate (Lac) and Glutamate (Glu) over their basic nutrients, i.e., Glucose (Gluc) and Glutamine (Gln) respectively, were calculated for all environmental conditions and culturing systems, for each feeding cycle (Tables 1 & 2).

The yield of lactate over glucose is, almost for all conditions under study, relatively higher than 2, indicating that the anaerobic metabolic pathway is followed (Tables 1 & 2). The fact that in some cases this yield is much higher than 2, indicated that lactate is produced not only by glycolysis, but also from amino acids present in the FBS or the medium. With respect to lactate synthesis per cell, apart from the [CTR] level of glucose in normoxia at which, the lactate production was the same for 2D and 3D (Figure 2a) for all other conditions significantly higher ($p < 0.05$) lactate was produced in 2D compared to 3D, as cellular proliferation is much higher in 2D (Figure 2b, 2c, 2d, 2e, 2f). For all glucose concentrations under study, as expected, higher lactate and, consequently, relatively higher yield of lactate over glucose was gained in hypoxia, confirming that the cells follow anaerobic cell metabolism (Figure 2). Nevertheless, significant amounts of lactate were also produced in normoxia as well, possibly as a result of the Warburg effect. The latter is suggested to be a characteristic of highly proliferative and/or cancerous cells that -due to alterations or damage at the mitochondria- independently of the presence of oxygen, do not enter the TCA cycle, therefore, following the anaerobic pathway ([35], [36-38]). The K-562 cell line is cancerous, highly proliferative, and therefore, most likely following partly the anaerobic pathway under normoxic conditions both in the 2D and the 3D system (Figure 2). Additional to the Warburg effect another possible phenomenon that might occur as a result of the very high proliferation rate is the inability of the cells to

efficiently produce at all times all the enzymes that are essential for the TCA cycle, therefore, the cells shift their metabolism towards the anaerobic pathway.

The yield of glutamine production over glutamate is significantly indifferent ($p < 0.05$) for all conditions under study (Tables 1 & 2). However, it is rather difficult to draw conclusions relying on this yield as, during one feeding cycle, glutamate may be re-consumed, reacting with ammonia in order to release cellular energy, i.e., ATP [39].

With respect to the glutamate (Glu) production per individual cell, for [CTR] glucose for almost all days no significant ($p < 0.05$) difference between 2D and 3D cultures can be seen in neither hypoxia nor normoxia (Figure 3a, 3b). This was most likely due to the fact that glucose was present in excess and the cells did not prioritise the glutamine (-glutamate) pathway as a glucose supplement in cellular metabolism [40].

In contrast, under glucose limitations, i.e., [LOW] & [HIGH] glucose, significantly higher ($p < 0.05$) glutamate production per cell took place in the 2D compared to the 3D system for most days (Figure 3c, 3d, 3e, 3f), most likely, due to glucose limitations in the growth medium: Glutamine acts supplementary to glucose metabolism for macromolecule production and cellular energy demands ([40], [41]).

Therefore, due to the higher proliferation in 2D, higher glutamate production took place in order to meet the cellular demands for energy. In general, higher glutamate synthesis took place in hypoxia, compared to normoxia both for the 2D and the 3D system (Figure 3), not surprisingly, as several studies have shown that under hypoxia a switch from oxidative metabolism (alteration of acetyl-CoA to citrate ratio) to reductive glutamine metabolism (increase in α -ketoglutarate to citrate) takes place [42]. This switch leads consequently to an enhanced glutamate production.

Note that, especially on days 7-8 the metabolic production per cell –both for glutamate and lactate- increased extremely in the 2D system (Figures 2 & 3) due to

the fact that the cells were transferred on day 7 from a 24 to a 6-well tissue culture plate (from approx. 1.5 mL to approx. 10 mL of medium). This transfer took place as significantly higher volume of medium (not appropriate for the 24-well plates) was requested in the 2D culture during feeding in order to maintain the cell concentration levels at an acceptable range, according to the supplier's instructions (see also Materials & Methods). The cells were exposed at a higher volume of metabolites and on that day and for approximately 1 feeding cycle, fewer cells/well were present, i.e., cells were given more space. We observed (by running experiments both in 24 and 6 well plates and for different medium volume combinations) that the increased metabolic activity on that day was not singly due to the space or the increase on the medium volume but due to combination of the two (data not shown).

At this point it has to be mentioned that the possibility of having nutrient and/or oxygen mass diffusion limitations within the scaffolds was examined computationally. A partial differential equation (PDE) model based on Fickian diffusion was constructed and simulated in gPROMS ModelBuilder®. The model considers the diffusion of oxygen & nutrients over time in three dimensions within the highly porous scaffold, including oxygen consumption by the cells 20-50% higher than the maximum consumption reported by Ma et al. (2012) [43] for leukocytes. The latter has been chosen as a representative average oxygen consumption rate for the K-562 leukaemic blasts as according to Ma et al. (2012) [43], the proliferation rate of leukaemic cells is approximately 20-50% higher than that of leukocytes, therefore, we assumed oxygen consumption 20-50% higher than the maximum reported for leukocytes. According to simulation results, even at the end of the culturing period (in which approximately 4 million cells (i.e. maximum cell level observed) were present in the scaffold (Figure 1c, 1d) steady state both for oxygen and glucose was reached at

the centre as well as at the bottom of the scaffold within a few seconds (computational data not shown). Moreover, SEM images indicate that no space limitations were observed in the highly porous 3D scaffolds, not even for the condition at which maximal cell density was reached, i.e., on day 14 for 4.3g/L glucose in the medium (Figure 4).

Therefore, it is fairly assumed that the kinetic and/or metabolic differences –both between different stress conditions in the 3D as well as between 2D and 3D- are not due to (1) diffusional limitations of nutrients in the scaffolds that could lead to areas of no oxygen and/or no glucose, neither due to (2) space limitations in the 3D matrix, but are due to the fact that cells experience differently, from a biological point of view, the environmental stress in the two different culturing systems.

Conclusion

From our findings it can be concluded that the K-562 cell line can proliferate and be maintained for 2 weeks in medium with glucose close to *in vivo* physiological levels (much lower than generally applied in laboratory systems) both in normoxia (20% O₂) and hypoxia (5% O₂). Generally, the cells proliferate more and are metabolically more active in suspension, i.e., 2D, compared to the PU collagen coated 3D scaffolds. Higher glutamate production was observed in hypoxia for all glucose conditions under study. Lactate is also produced in normoxia, pointing that the anaerobic metabolic pathway is followed, possibly as a result of the Warburg effect. It is remarkable that in, widely used, conventional 2D culture systems glucose fluctuations do not significantly affect the leukaemia evolution. In contrast, in the 3D BM mimicry glucose has a much higher impact on the cell proliferation, especially under low oxygen level (which recapitulates more realistically the *in vivo* bone marrow

environment in which the conditions are hypoxic). Additionally, under optimal glucose levels the cells evolve almost identically in 3D for normoxia and hypoxia but evolve completely differently in 2D for different oxygen levels. We, therefore, speculate that in the 3D system the cells most probably perceive differently the low oxygen level as their proliferation is not altered in comparison to the one in normoxia. This hypothesis seems to be supported by the observation that in the 2D system the proliferation is significantly lower in hypoxia compared to normoxia. Moreover, the fact that in our highly porous 3D scaffolding system no diffusion limitations occur, points out that the different behaviour in the 3D system is purely biological. It is evident that the 2D system is an artificial system that boosts proliferation in an unrealistic way, therefore, failing to capture the actual cell stress responses to realistic environmental stress factors.

The differences that we report on the K-562 proliferation under different oxygen and glucose levels as well as between the 2D and 3D cultures can be of significant importance when applying chemotherapy *in vitro*. Most chemotherapy agents generally applied for the treatment of patients with AML are targeting highly proliferative cells, i.e., anthracyclines such as DNR which are targeting cells of the G1 phase of the cell cycle (Cancer Research UK). Therefore, under oxidative or glucose stress especially in a 3D microenvironment, cells proliferating slower may be less susceptible to chemotherapeutics. Future work should focus on unravelling the mechanisms of interaction of the cells with the 3D microenvironment in order to shed more light on the observed differences in proliferation. Moreover, studying the combined effect of environmental stresses of level close to that observed *in vivo* in tandem with chemotherapy in the 3D system would be of high importance for deriving realistic cell kinetics and for moving towards chemotherapy optimisation.

Acknowledgements

This work is supported by ERC-BioBlood (no. 340719), ERC-Mobile Project (no. 226462), by the EU 7th Framework Programme [MULTIMOD Project FP7/2007-2013, no 238013] and by the Richard Thomas Leukaemia Research Fund. R.M. is further thankful for a Royal Academy of Engineering Research Fellowship.

References

1. **Mortera-Blanco, T., Mantalaris, A., Bismarck, A., and Panoskaltsis, N.:** Development of a three-dimensional biomimicry of human acute myeloid leukemia ex vivo. *Biomaterials*, **31**, 2243-2251 (2010).
2. **Bonnet, D. and Dick, J.:** Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Med.*, **3(7)**, 730–737 (1997).
3. **Grech, G., Avellino, R., and Schembri- Wismayer, P.:** Molecular mechanisms in haematological malignancies. *Malta Med. J.*, 21(3), 6-11 (2009).
4. **Lowenberg, B., Downing, J., and Burnett, A.:** Acute Myeloid Leukemia. *N. Engl. J. Med.*, **341(14)**, 1051-1062 (1999).

- 523 **5. Lichtman, M.A., and Williams, J.W.:** Hematology in the aged pp 501-540 In:
524 Beutler, E., Lichtman, M.A., Coller, B.S., Kipps, T., Seligsohn, U. (Eds) Williams
525 Hematology (6th edition), McGraw-Hill, USA (2001).
526
- 527 **6. Panoskaltsis, N., Reid, C.D.L., and Knight, S.C.:** Quantification and cytokine
528 production of circulating lymphoid and myeloid cells in acute myelogenous leukemia
529 (AML). *Leukemia*, **17**, 716-730 (2003).
530
- 531 **7. Mayani, H., Flores-Figueroa, E., and Chavez-Gonzalez, A.:** In vitro biology of
532 human myeloid leukaemia. *Leuk. Res.*, **33**, 624-637 (2009).
533
- 534 **8. Velliou, E., Fuentes-Garí, M., Misener, R., Pefani, E., Rende, M., Panoskaltsis,**
535 **N., Pistikopoulos, E. N., and Mantalaris, A.:** A framework for the design, modeling
536 and optimization of biomedical systems. *Comp. Aid. Chem. Eng.*, **34**, 225-236
537 (2014).
538
- 539 **9. Collins, P.C., Miller, W.M., and Papoutsakis, E.T.:** Stirred culture of peripheral
540 and cord blood hematopoietic cells offers advantages over traditional static systems
541 for clinically relevant applications. *Biotechnol. Bioeng.*, **59(5)**, 534-543 (1998).
542
- 543 **10. Koller, M.R., Emerson, S.G., and Palsson, B.O.:** Large-scale expansion of
544 human stem and progenitor cells from bone marrow mononuclear cells in continuous
545 perfusion cultures. *Blood*, **82(2)**, 378-384 (1993).
546
- 547 **11. Giarratana MC, Kobari L, Lapillonon H, Chalmers D, Kiger L, Cynober T,**

et al.: *Ex vivo* generation of fully mature human red blood cells from hematopoietic stem cells, *Nat. Biotechnol.*, **23**, 69–74 (2005).

12. **Colmone, A., Amorim, M., Pontier, A.L., Wang, S., Jablonski, E., and Sipkins, D.A.:** Leukemic cells create bone marrow niches that disrupt the behaviour of normal hematopoietic progenitor cells. *Science*, **19(322)**, 1861-1865 (2008).

13. **Engelhardt, M., Douville, J., Behringer, D., Jahne, A., Smith, A., Henschler, A., and Lange, W.:** Hematopoietic recovery of *ex vivo* perfusion culture expanded bone marrow and unexpanded peripheral blood progenitors after myeloblastic chemotherapy, *Bone Marrow Transplant.*, **27(3)**, 249-259 (2001).

14. **Kirito, K., Fox, N., and Kaushansky, K.:** Thrombopoietin stimulates Hoxb4 expression: an explanation for the favourable effects of TPO on hematopoietic stem cells. *Blood*, **102(9)**, 3172-3178 (2003).

15. **Levac, K., Karabu, F., and Bhatia, M.:** Identification of growth factor conditions that reduce *ex vivo* cord blood progenitor expansion but not alter human repopulating function *in vivo*. *Haematologica*, **90(2)**, 166-172 (2005).

16. **Maltman, D.J., and Przyborski, A.:** Developments in three-dimensional cell culture technology aimed at improving the accuracy of *in vitro* analyses. *Biochem. Soc. Trans.*, **38(4)**, 1072-1075 (2010).

17. **Panoskaltsis, N., Mantalaris, A., and Wu, D.J.H.:** Engineering a Mimicry of

- Bone Marrow Tissue *Ex vivo*, *J. Biosci. Bioeng.*, **100**, 28-35 (2005).
18. **Rabinowitz, J., Petros, W., Stuart, A., Peter, W.:** Characterization of endogenous cytokine concentrations after high-dose chemotherapy with autologous bone marrow support. *Blood*, **81(9)**, 2452-2459 (1993).
19. **Vunjak-Novakovic, G., and Scadden, D.T.:** Biomimetic platforms for human stem cell research. *Cell Stem Cell*, **8**: 252-261 (2011).
20. **Cabrita, G.J.M., Ferreira, B.S., da Silva, C.L., Gonçalves, R., Almeida-Porada, G. and Cabral, J. M. S.:** Hematopoietic stem cells: from the bone to the bioreactor. *Trends Biotechnol.*, **21(5)**, 233-240 (2003).
21. **Rodriguez, C.A., Fernandes, T.G., Diogo, M.M., da Silva, C.L., and Cabral, J.M.:** Stem cell cultivation in bioreactors. *Biotechnol. Adv.*, **29(6)**, 815-829 (2011).
22. **Lee-Thedieck, C., and Spatz, J.P.:** Artificial niches: biomimetic materials for haematopoietic stem cell culture, *Macromol. Rapid Commun.*, **33**, 1432-1438 (2012).
23. **Tun, T., Miyoshi, H., Aung, T., Takahashi, S., Shimizu, S., Kuroha, T., Yamamoto M., and Ohshima, N.:** Effect of growth factors on *ex vivo* bone marrow cell expansion using three-dimensional matrix support. *Artif. Organs*, **26(4)**: 333-339 (2002).

24. **Da Silva, C.L., Gonçalves, R., Crapnell, K.B., Cabral, J.M.S., Zanjani, E.D., and Almeida-Porada, G.:** A human stromal-based serum-free culture system supports the *ex vivo* expansion/maintenance of bone marrow and cord blood haematopoietic stem/progenitor cell. *Exp. Hematol.*, **33**(7), 828-835 (2007).
25. **Leisten, L., Kramann, R., Ventura Ferreira, M.S., Bovi, M., Neuss, S., Ziegler, P., Wagner, W., Knuchel, R., and Schneider, R.K.:** 3D- co-culture of hematopoietic stem and progenitor cells and mesenchymal stem cells in collagen scaffolds as a model of the hematopoietic niche. *Biomaterials*, **22**, 1736-1747 (2012).
26. **Alijtawi, O.S., Li, D., Xiao, Y., Zhang, D., Ramachandran, K., Stehno-Bittel, L., Van Veldhuizen, P., Lin, T.L., Kambhampati, S. and Garimella, R.:** A novel three-dimensional stromal-based model for *in vitro* chemotherapy sensitivity testing of leukemia cells. *Leuk. Lymphoma*, **55**, 378-391 (2014).
27. **Fecteau, J.-F., Messmer, D., Zhang, S., Cui, B., Chen, L., and Kipps, T.J.:** Impact of oxygen concentration on growth of mesenchymal stromal cells from the marrow of patients with chronic lymphocytic leukemia, *Blood*, **121**, 971-974 (2013).
28. **Wilkinson, S.T., Tome, M.E., and Briehl, M.M.:** Mitochondrial Adaptations to Oxidative Stress Confer Resistance to Apoptosis in Lymphoma Cells, *Int. J. Mol. Sci.*, **13**, 10212-10228 (2012).
29. **Zhou, F.-L., Zhang, W.-G., Wei, Y.-C., Meng, S., Bai, G.-G., Bai- Wang, B.- Y., Yang, H.-Y. , Tian, W., Meng, X., Zhang, H., and Chen, S.-P.:** Involvement of

Oxidative Stress in the Relapse of Acute Myeloid Leukemia, *J. Biolog. Chem.*, **285**, 15010-15015 (2010).

30. **Lodi, A., Tiziani, S., Khanim, F.L., Drayson, M.T., Gunther, U.L., Bunce, C.M., and Viant, M.R.:** Hypoxia Triggers Major Metabolic Changes in AML Cells without Altering Indomethacin-Induced TCA Cycle Deregulation, *ACS Chem. Biol.*, **6**, 169-175 (2011).

31. **Herst, P.M., Howman, R.A., Neeson, P.J., Berridge, M.V., and Ritchie D.S.:** The level of glycolytic metabolism in acute myeloid leukemia blasts at diagnosis is prognostic for clinical outcome, *J. Leukoc. Biol.*, **89**, 51-55 (2011).

32. **Safinia, L., Mantalaris, A., and Bismarck A.:** Towards a methodology for the effective surface modification of porous polymer scaffolds. *Biomaterials*, **26**:7537-7547 (2005).

33. **Yang, J., Shi, G., Bei, J., Wang, S., Cao, Y., Shang, Q., Yang, G., and Wang, W.:** Fabrication and surface modification of macroporous poly(L-lactic acid) and poly(L-lactic-co-glycolic acid) (70/30) cell scaffolds for human skin fibroblast cell culture. *J. Biomed. Mater. Res.*, **62**: 438-446 (2002).

34. **Giuntoli, S., Tantarli, M., Gesualdo, F.D., Barbetti, V., Rovida, E., and Sbarba, P.D.:** Glucose availability in hypoxia regulates the selection of chronic myeloid leukemia progenitor subsets with different resistance to imatinib-mesylate, *Haematologica*, **96(2)**, 204-212 (2011).

35. **Dos Santos, F., Andrare, P.Z., Boura, J.S., Abecasis, M.M., da Silva, C.L., and Cabral, J.M.S.:** Ex vivo expansion of human mesenchymal stem cells: A more effective cell proliferation kinetics and metabolism under hypoxia. *J. Cell. Physiol.*, **223(1)**, 27-35 (2010).
36. **Vander Heiden, M.G., Cantley, L.C., and Thompson, C.B.:** Understanding the Warburg Effect: The metabolic requirements of cell proliferation. *Science*, **324**, 1029-1033 (2009).
37. **Vander Heiden, M.G., Locasale, J.W., Swanson, K.D., Sharfi, H., Heffron, G.J., Amador-Noguez, D., Christofk, H.R., G. Wagner, Rabinowitz J.D., Asara, J.M., and Cantley L.C.:** Evidence of an alternative glycolytic pathway in rapidly proliferating cells. *Science*, **329**, 1492-1499 (2010).
38. **Warburg, O.:** On the origin of cancer cells. *Science*, **123**, 309-314 (1956).
39. **Barnes L.M., Bentley C.M. and Dickson A.J.:** Advances in animal cell recombinant protein production: GS-NS0 expression system. *Cytotechnology*, **32**, 109-123 (2000).
40. **DeBerardinis, R.J., and Cheng, T.:** Q's next: the diverse function of glutamine in metabolism, cell biology and cancer. *Oncogene*, **29(3)**, 313-324 (2010).
41. **Wise, D.R., and Thompson, C.B.:** Glutamine addiction: a new therapeutic target. *Trends Biochem. Sci.*, **35**, 427-433 (2010).

674 42. Fedt, S.M., Bell, L.M., Keibler, M.A., Olenchock, B.A., Mayers, J.R.,
675 Wasylenko, T.M., Vokes, N.I., Guarente, L., Vander Heiden, M.G., and
676 Stephanopoulos, G.: Reductive glutamine metabolism is a function of a-
677 ketoglutarate to citrate ratio in cells, *Nat. Commun.*, **4**, 2236 (2013).

678
679 43. Ma, C.Y.J., Panoskaltis, N., Kumar R., Xu, X.Y., and Mantalaris A.:
680 Simulation of ex vivo bone marrow culture: Application to chronic myeloid
681 leukaemia growth model. *Biochem. Eng. J.*, **61**, 66-77 (2012).

Tables:

Table 1: Yields of metabolites in the 2D cultures, i.e., Lac, Glu, over their nutrients consumption, i.e., Gluc, Gln, respectively (and their standard deviations) for the days at which medium exchange took place.

Initial Glucose level in medium	DAY	NORMOXIA		HYPOXIA	
		Y _{Lac/Gluc}	Y _{Gln/Glu}	Y _{Lac/Gluc}	Y _{Gln/Glu}
LOW (0.6 g/L)	2	2.46±0.51	0.21±0.14	4.07±0.78	0.16±0.12
	4	2.61±0.62	0.38±0.19	2.68± 0.57	0.22±0.15
	7	1.61±0.45	0.62±0.25	3.83± 0.42	0.19±0.20
	9	3.91±0.85	0.45±0.18	3.43±0.34	0.17±0.08
	11	3.15±0.62	0.32±0.15	2.69±0.47	0.19±0.12
	14	2.88±0.75	0.35±0.12	3.19±0.51	0.25±0.14
HIGH (1.3 g/L)	2	2.12±0.42	0.21±0.11	2.78±0.52	0.18±0.13
	4	2.63±0.57	0.38±0.17	3.20±0.64	0.26±0.21
	7	1.84±0.50	0.43±0.14	3.78±0.52	0.20±0.14
	9	1.55±0.32	0.18±0.16	3.11±0.43	0.26±0.19
	11	2.69±0.46	0.30±0.20	2.73±0.76	0.57±0.15
	14	2.98±0.79	0.31±0.18	3.01±0.61	0.69±0.17
CTR (4.3 g/L)	2	1.89±0.55	0.35±0.24	2.17±0.42	0.28±0.13
	4	0.97±0.69	0.12±0.28	1.43±0.64	0.17±0.21
	7	0.69±0.34	0.49±0.17	4.35±0.92	2.21±2.14
	9	1.99±0.51	0.32±0.19	1.29±0.21	1.39±0.71
	11	2.38±0.68	0.17±0.14	2.37±0.57	0.17±0.18
	14	1.16±0.47	0.21±0.13	2.12±0.41	0.18±0.14

Table 2: Yields of metabolites in the 3D cultures, i.e., Lac, Glu, over their nutrients consumption, i.e., Gluc, Gln, respectively (and their standard deviations) for the days at which medium exchange took place.

Initial Glucose level in medium	DAY	NORMOXIA		HYPOXIA	
		Y _{Lac/Gluc}	Y _{Gln/Glu}	Y _{Lac/Gluc}	Y _{Gln/Glu}
LOW (0.6 g/L)	2	3.63±0.85	0.25±0.08	3.25±0.58	0.24±0.14
	4	1.59±0.72	0.20±0.12	2.41±0.45	0.17±0.09
	7	2.03±0.34	0.31±0.15	1.39±0.67	0.19±0.12
	9	1.83±0.21	0.39±0.21	1.82±0.36	0.20±0.15
	11	2.32±0.15	0.41±0.11	2.08±0.40	0.08±0.14
	14	1.99±0.31	0.25±0.13	2.23±0.17	0.16±0.02
HIGH (1.3 g/L)	2	2.46±0.45	0.51±0.16	3.47±0.42	0.23±0.12
	4	2.19±0.14	0.47±0.13	2.45±0.37	0.23±0.17
	7	2.56±0.43	0.34±0.21	1.96±0.22	0.21±0.11
	9	2.17±0.35	0.40±0.26	2.58±0.56	0.28±0.19
	11	2.76±0.67	0.34±0.17	2.29±0.47	0.15±0.14
	14	2.32±0.17	0.33±0.19	3.05±0.29	0.25±0.18
CTR (4.3 g/L)	2	0.81±0.45	0.54±0.27	1.32±0.25	0.18±0.14
	4	2.03±0.72	0.36±0.15	1.80±0.37	0.13±0.16
	7	1.64±0.61	0.43±0.23	1.63±0.35	0.19±0.12
	9	2.22±0.31	0.39±0.13	2.18±0.77	0.18±0.17
	11	2.47±0.26	0.43±0.15	2.84±0.85	0.15±0.15
	14	3.52±0.29	0.38±0.18	3.54±0.62	0.15±0.11

Figure Legends

Figure 1: K-562 growth in the 2D (a), (b) and the 3D (c), (d) system, at different oxygen levels: (a), (c) 20% O₂ and (b), (d) 5% O₂. Different colours represent different glucose levels: (closed squares) [CTR], (closed diamonds) [HIGH], (closed circles) [LOW].

Figure 2: Evolution of Lactate, [Lac] for all the environmental conditions under study. Different colours represent the two different culturing systems: (closed bright-yellow squares) 2D cultures, (closed dark-purple squares) 3D scaffolds. Left column is normoxia and right column hypoxia.

Figure 3: Evolution of Glutamate, [Glu] for all the environmental conditions under study. Different colours represent the two different culturing systems: (closed bright-yellow squares) 2D cultures, (closed dark-purple squares) 3D scaffolds. Left column is normoxia and right column hypoxia.

Figure 4: SEM images for [CTR] glucose in normoxia (the condition at which the highest growth rate is observed) on day 14 for (a) x430 and (b) x950 magnification.

Figure A.1: MTS standard curve of the K-562 cell line for 2D culture.

Appendix A

This Appendix consists of Table A.1 & Figure A.1

A.1 Concentrations of nutrients in different growth media as detected by the NOVA bioprofiler

Table A.1: DMEM synthesis

Components (mM)	[CTR]	[HIGH]	[LOW]
Glutamine	2.89 ± 0.01	2.89 ± 0.01	2.89 ± 0.01
Glucose	23.80 ± 0.12	7.20 ± 0.03	3.30 ± 0.02
Glutamate	0	0	0
Lactate	0	0	0

A.2 Calibration curve of MTS vs live K-562 cells (2D system)

Figure A.1: MTS standard curve of the K-562 cell line for 2D culture.