3D *in vitro* cancer models for drug screening

A study of glucose metabolism and drug response in 2D and 3D culture models

by

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the requirements for the degree of
Philosophiae Doctor
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In loving memory of Steven Dee Hall,
the man who volunteered to be there as a father
offering unconditional love and support,
who lived an inspiring life imbued with kindness and hard work.

"What we do see depends mainly on what we look for.

...

In the same field the farmer will notice the crop,
the geologists the fossils,
botanists the flowers,
artists the colouring,
sportmen the cover for the game.
Though we may all look at the same things,
it does not at all follow that we should see them."
Sir John Lubbock,
The Beauties of Nature, 1892

Preface

This thesis is submitted in partial fulfillment of the requirements for the degree of *Philosophiae Doctor* at the University of Stavanger. The research presented here was conducted at the University of Stavanger, University of Bergen, and Western Norway University of Applied Sciences under the supervision of Associate Professor Hanne R. Hagland, Ph.D., Professor Kjetil Søreide, MD Ph.D., Professor Karl Johan Tronstad, Ph.D., and Gro Vatne Røsland, PhD. This work was funded by the University of Stavanger and a Plogen grant from Validé.

The thesis is a collection of three papers, presented in chronological order of writing. The papers are preceded by chapters that provide background information (Chapter 1), motivation (Chapter 2) and methodological considerations (Chapter 3), then finally discuss the results (Chapter 5), and conclude with a view to the future (Chapter 6).

Acknowledgements

Throughout my Ph.D. there are many people that have been supported and assisted me, big and small, and for that I am thankful.

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Finally, my friends and family. To my late stepfather and mother for raising me, loving me, and letting me be free to follow my interests whatever and wherever they may be, even if that meant leaving the country. To my father, an inspiration of dedication and courage, for always voicing your support and pride in me going ever further after my dreams. To my lady, Hayley, my first life partner who has been there since before science trumped art in my life plans. It has been uplifting to watch you forge your own incredible path in a completely different arena, but face the obstacles together.

And my star, Steve. With whom I have forged a path so completely unexpected and delightful. Thanks for tolerating all the weeknights and weekends I've spent quality time with cancer cells (or writing about them) instead of you. I can't imagine my life without you and now our own little entropy machine, Xavier. Special thanks to Xavi for being there to give me little kicks of motivation during the last sprints of Seahorse assays and drug treatments, but also once out in the world, forcing me to take things slower, for the good of finishing my thesis in a timely fashion or not :)

Tia R. Tidwell
Oslo, December 2021

Contents

Abbreviations

2D two-dimensional

3D three-dimensional

ATP adenosine triphosphate

CCCP carbonyl cyanide m-chlorophenyl hydrazone

CRC colorectal cancer

ECAR extracellular acidification rate

FCCP carbonyl cyanide-p-trifluoromethoxyphenylhydrazone

GST glycolysis stress test

MST mitochondrial stress test

OCR oxygen consumption rate

OXPHOS oxidative phosphorylation

PDAC pancreatic ductal adenocarcinoma

PPR proton production rate

Abstract

Current drug screening protocols use *in vitro* cancer cell panels grown in 2D to evaluate drug response and select the most promising candidates for further *in vivo* testing. Most drug candidates fail at this stage, not showing the same efficacy *in vivo* as seen *in vitro*. An improved first screening that is more translatable to the *in vivo* tumor situation could aid in reducing both time and cost of cancer drug development. 3D cell cultures are an emerging standard for *in vitro* cancer cell models, being more representative of *in vivo* tumour conditions. To overcome the translational challenges with 2D cell cultures, 3D systems better model the more complex cell-to-cell contact and nutrient levels present in a tumour, improving our understanding of cancer complexity. Furthermore, cancer cells exhibit altered metabolism, a phenomenon described a century ago by Otto Warburg, and possibly related to changes in nutrient access. However, there are few reports on how 3D cultures differ metabolically from 2D cultures, especially when grown in physiological glucose conditions. Along with this, metabolic drug targeting is considered an underutilized and poorly understood area of cancer therapy. Therefore, the aim of this work was to investigate the effect of culture conditions on response to metabolic drugs and study the metabolism of 3D spheroid cultures in detail. To achieve this, multiple cancer cell lines were studied in high and low glucose concentrations and in 2D and 3D cultures.

We found that glucose concentration is important at a basic level for growth properties of cell lines with different metabolic phenotypes and it affects sensitivity to metformin. Furthermore, metformin is able to shift metabolic phenotype away from OXPHOS dependency. There are significant differences in glucose metabolism of 3D cultures compared to 2D cultures, both related to glycolysis and oxidative phosphorylation. Spheroids have higher ATP-linked respiration in standard nutrient conditions and higher non-aerobic ATP production in the

absence of supplemented glucose. Multi-round treatment of spheroids is able to show more robust response than standard 2D drug screening, including resistance to therapy. Results from 2D cultures both over and underestimate drug response at different concentrations of 5-fluorouracil (5-FU). A higher maximum effect of 5-FU is seen in models with lower OCR/ECAR ratios, an indication of a more glycolytic metabolic phenotype.

In conclusion, both culture method and nutrient conditions are important consideration for *in vitro* cancer models. There is good reason to not maintain *in vitro* cultures in artificially high glucose conditions. It can have downstream affects on drug response and likely other important metrics. If possible, assays should also be implemented in 3D. If not in everyday assays, at least as a required increase in complexity to validate 2D results. Finally, metabolism even in the small scope presented here, is complex in terms of phenotypic variation. This shows the importance of metabolic screening *in vitro* to better understand the effects of these small changes and to model how a specific tumor may behave based on its complex metabolism.

List of Papers

Paper I

Alhourani, A., Tidwell, T.R., Bokil, A., Røsland, G.V., Tronstad, K.J., Søreide, K., Hagland, H.R. "Metformin treatment response is dependent on glucose growth conditions and metabolic phenotype in colorectal cancer cells". In: *Scientific Reports*. Vol. 11, (2021), DOI: 10.1038/s41598-021-89861-6.

Paper II

Tidwell, T.R., Røsland, G.V., Tronstad, K.J., Søreide, K., Hagland, H.R. "Metabolic flux analysis of 3D spheroids reveals significant differences in glucose metabolism from matched 2D cultures of colorectal cancer and pancreatic ductal adenocarcinoma cell lines". *Submitted for publication.*

Paper III

Tidwell, T.R., Røsland, G.V., Tronstad, K.J., Søreide, K., Hagland, H.R. "Enhanced drug screening of CRC and PDAC multicellular spheroids using long-term culture and multi-round exposure to 5-fluorouracil". *Submitted for publication.*

Paper IV

Tidwell, T.R., Søreide, K., Hagland, H.R. "Aging, Metabolism, and Cancer Development: from Peto's Paradox to the Warburg Effect". In: *Aging & Disease*. Vol. 8 No. 5 pp. 662–676, (2017), DOI: 10.14336/AD.2017.0713. [Review].

Introduction

In the early 1900s cancer was recognized as a disease formed from an aberration of our own cells, and not an infection from an external source [1]. Unfortunately, this is also why cancer is so difficult to treat, and what makes it challenging to find treatments that target only cancer cells and leave normal cells unharmed. As each cancer is as unique as its host, finding common denominators to other like cancers and common differentiators from normal cells is a challenge. The preferred first-line treatment is to surgically resect a tumor, but this is only possible if is localized, does not involve non-resectable structures such as major arteries or veins, and the patient is in good health. In other cases, however, chemotherapy or radiotherapy treatment is necessary and this is where eradicating cancer cells is balanced with harsh systemic side effects. Both are toxic to cancer cells, but unfortunately they are also toxic to normal, healthy cells. Historically, systemic side effects have been a necessary outcome in order to achieve cancer remission. Because of the harsh treatment to the entire body, many people are not healthy enough to tolerate the treatment or are completely debilitated by it. Attempts have been made to target these cytotoxic therapies as much as possible to cancer cells only. With radiation, this means very fine resolution and for chemotherapy, innovative drug delivery methods. The field has been trying to move beyond standard chemotherapy by developing more targeted molecular treatments to different cancers and also by getting the body to recognize and attack cancer itself via immunotherapy.

The number of new cancer drugs actually approved for use is startlingly low, though. The likelihood of success from phase I of testing is 9.6% overall and just 5.1% for oncology drugs [2]. Drug approval is a long and expensive process

so starting with the right candidates has double the benefit, both for improved treatment of cancer and better economic outcomes, both public and private. To improve the drugs entering the development process, drug targets must be based on acquired knowledge of cancer biology and be assessed using functional testing and performance measures that actually yield some predictive value for clinical success [3]. Additionally, there is opportunity to more effectively target already approved drugs for better clinical outcomes. In both cases, more relevant cell model systems should be used for better understanding and predictability of targeted drug responses. This thesis attempts to address these needs by focusing on metabolism as a drug target and response metric, but also studying metabolism and drug response in an enhanced *in vitro* cell model to yield better predictive validity. To explain this further, some background is given on cellular metabolism and its relevance in cancer (1.1). In the next section, the current state of modelling cancer *in vitro* and different treatment options (1.2) are described. The last section concludes with considerations for improved targeting and testing of cancer drugs given this context (1.3).

1.1 Cellular Metabolism

At the cornerstone of cell biology, and therefore also cancer biology, is cell metabolism. All cells are driven by energy transfer reactions from catabolic (destructive) and anabolic (constructive) metabolism, for growth, cell division and every cellular process. “Normal” cell metabolism is a thus a large, complex, web of connected molecular pathways (Figure 1.1).

1.1.1 Glucose metabolism

Glucose metabolism forms the backbone of cellular metabolism as the major pathway for conversion of glucose to energy in the form of adenosine triphosphate (ATP), the primary energy currency of the cell. ATP is produced when adenosine diphosphate (ADP) is phosphorylated. When the bonds between these groups are hydrolyzed under physiological conditions, 30.66 kJ [4] are released making ATP the most important energy transfer unit in a cell. In normal healthy cells, glucose metabolism is variable and adjusts according to nutrient changes in the cellular environment. For example, nutrient gradients are formed in tissues with diffusion from vasculature, whereby some tissues need to be highly vascularized

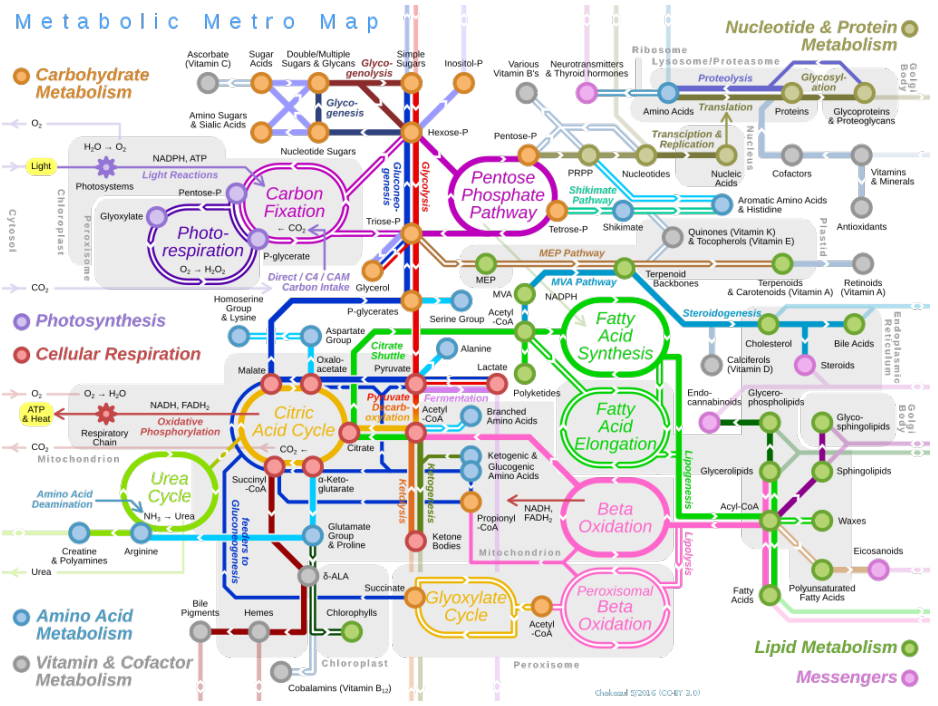

Figure 1.1: Metabolic Metro Map. Attribution: Chakazul. For interactive exploration of metabolic pathways, visit the full Metabolic Pathways Map.

to perform their regular metabolism; cells that outgrow the vascular system will eventually perish if cell metabolism does not compensate until more vessels are developed. Other biomolecules that can fuel ATP production include fatty acids (the other major contributor for energy conversion), amino acids, and lactate.

Glycolysis

In all cells, glucose is imported into the cell via glucose transport receptors (GLUTs) and catabolized through glycolysis (Figure 1.2). It is a multi-step process involving several enzymes, split into two phases. The first preparatory phase requires investment of 2 ATP molecules to oxidize glucose. The second pay-off phase generates 2 NADH molecules, 2 H^+, 2 H_2O molecules, and 4 ATP for a net gain of 2 ATP per glucose molecule. Glycolysis is able to produce ATP very quickly, despite the low output per glucose molecule. Additionally, many glycolytic intermediates support macromolecular synthesis essential for

cell proliferation, such as nucleotides, lipids, and some amino acids [5]. The end product of glycolysis is pyruvate and in hypoxic conditions, it is converted to lactate via lactate dehydrogenase (LDH) in the cytosol for export out of the cell. In normoxic conditions, it is transported into the mitochondria and converted to acetyl-CoA by pyruvate dehydrogenase (PDH).

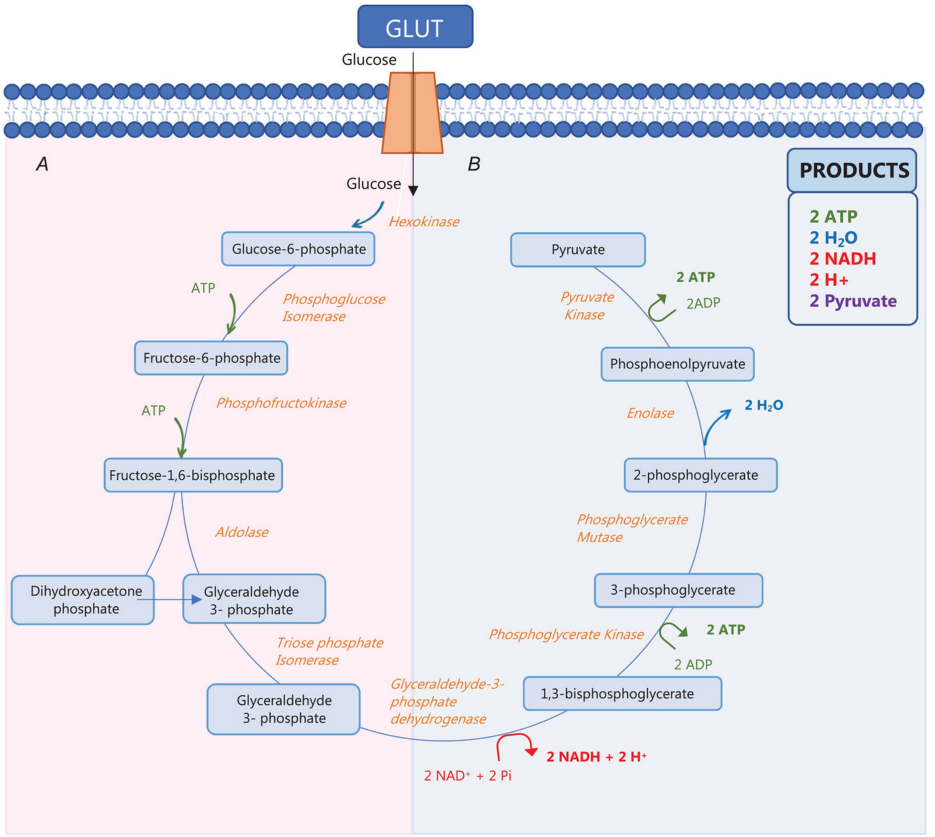

Figure 1.2: Overview of glucose metabolism through glycolysis. (A) Oxidation of glucose in the preparatory phase. (B) Production of NADH, H^+, H2O, and net 2 ATP in the pay-off phase [6]. Reproduced with permission from Wiley.

Oxidative phosphorylation

In the mitochondria, acetyl-CoA is subsequently decarboxylated through several intermediate steps in the tricarboxylic acid (TCA) cycle (Figure 1.3). The TCA cycle supplies essential cofactors for reactions in the electron transport chain

(ETC), such as NADH and $FADH_2$. This electron transport process in the mitochondria is known as oxidative phosphorylation (OXPHOS), in reference to the oxygen consumed and ATP produced from phosphorylation of ADP. The ETC is a supercomplex spanning the mitochondrial inner membrane and consists of five protein complexes. The movement of electrons and protons over the first four complexes results in a proton surplus in the mitochondrial intermembrane space. Proton shuttling occurs specifically at complexes I, III, and IV, while complex II transfers electrons to complex III via ubiquinone. Electrons are carried further from complex III to complex IV via cyctochrome c. At Complex IV, oxygen is reduced to water by electron transfer. Complex V, or ATP-synthase, combines ADP and a free phosphate to form ATP using the proton motive force driven by the proton surplus in intermembrane space. Consequently, the ability of the mitochondria to produce ATP is heavily reliant on its membrane integrity, being able to uphold a proton motive force to support the ATP synthase to produce ATP [7]. Assuming full coupling to ATP production, 32 ATP are produced from catabolism of one glucose molecule [4], from glycolysis to oxidative phosphorylation. Any disruption in the mitochondrial matrix allowing for proton backflux outside of ATP synthase restricts ATP production, but can still allow the running of the TCA cycle by re-cycling of NADH and $FADH_2$ through the ETC. Uncoupling proteins (UCPs) are one way for protons to re-enter the mitochondrial matrix uncoupled from ATP-production [8].

Normal metabolic heterogeneity

Metabolic activity and fuel dependence varies in different cell types, from slower-growing cells to fast-growing or highly-active cells. Neurons are an example of slow-growing cells; they rely on oxidative phosphorylation to generate ATP as they have a low capacity for glycolysis and fatty acid oxidation [10]. In contrast, astrocytes have low OXPHOS, higher glycolytic rates, and an ability to oxidize fatty acids. They are known to shuttle lactate and ketone bodies to neurons as fuel [10]. Resting immune cells such as microglia [10] and lymphocytes [11] rely on OXPHOS. However, when these cells are activated, they switch to utilize more glycolysis. Slower-growing cells are the typical normal phenotype used for comparison with cancer phenotypes. Fast-growing and active normal cells shift metabolism from the mitochondria-centric OXPHOS to glycolysis for proliferation and biosynthesis. The same occurs in muscle cells during intense exercise at the

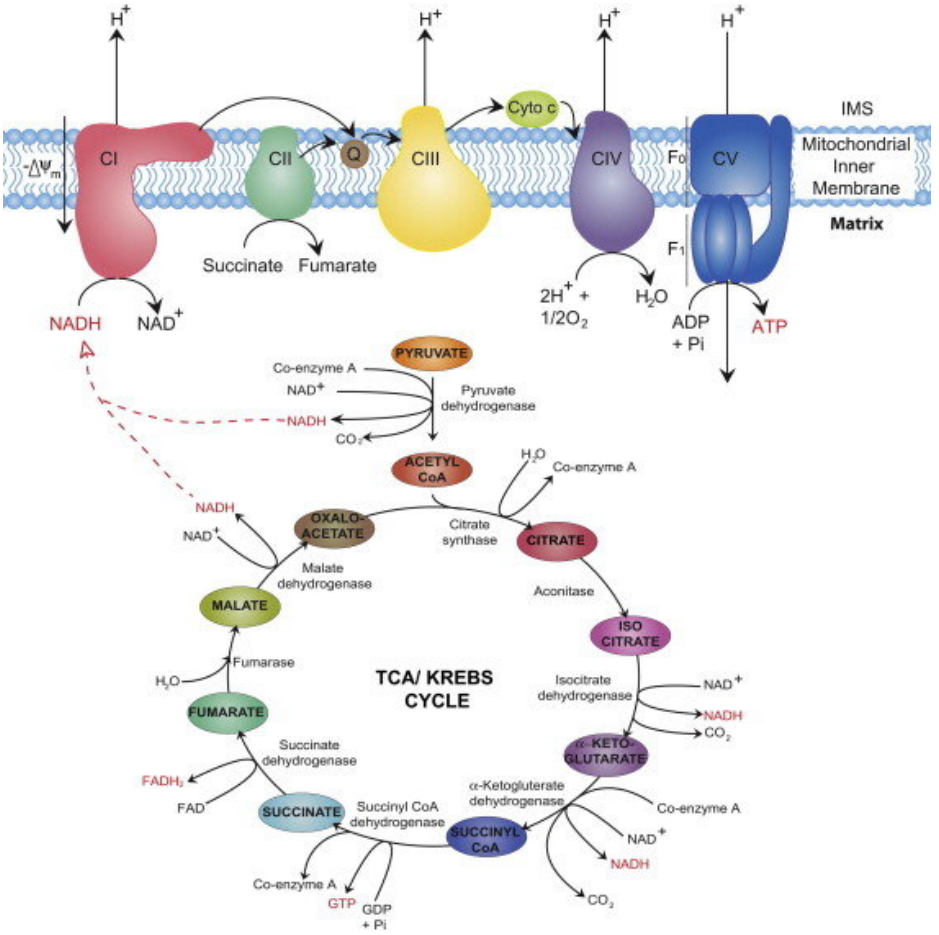

Figure 1.3: Bioenergetics of the electron transport chain and the TCA/Krebs cycle [9].

peak of maximal oxygen uptake; when cells need to maximize ATP production under limited oxygen conditions, they resort to anaerobic glycolysis, churning through available glucose and glycogen [12].

1.1.2 Cancer metabolism

As cells age, dysfunction accumulates in many essential cellular processes, such as mitophagy, proteostasis, and nutrient sensing, ultimately resulting in altered bioenergetics (reviewed in Paper IV). As autophagy is inhibited, these cells persist

despite their dysfunction and perhaps due to the dysfunction conferring survival in the surrounding degraded microenvironment [13]. Tissue remodeling [14], aging vasculature [15], and lifestyle can alter access to nutrients and oxygen, further selecting for those cells that thrive in such conditions. Eventually, the cells' dysfunction hits a tipping point whereby the cells are no longer reacting to regulatory checkpoints on growth and are no longer recognizable as normal. In contrast with normal cells, cancer cells do not require signalling from growth factors and adhesion for continued proliferation, key hallmarks of cancer [16]. Metabolic reprogramming to support cancer growth and its microenvironment is accepted as a hallmark of cancer as well [17].

From Warburg to present

It is almost 100 years since Otto Warburg first reported that cancer cell metabolism differs from normal cells through their use of aerobic glycolysis [19], now termed the Warburg effect. In hypoxic conditions, normal cells will utilize anaerobic glycolysis, but in the presence of oxygen a return to oxidative phosphorylation occurs. Warburg suggested this must be due to defective mitochondria and a resulting energy shortage or adaptation to oxygen deprivation. In 1956 [20], he reported on further experiments quantifying and supporting his former conclusions. He confirmed that this shift can occur through injury to respiration and oxygen deprivation. However, not all cancer cells express this exact Warburg phenotype. Even Warburg alludes to this in his mention of "sleeping cancer cells" and description of two cell lines from one clone with differing malignancy and levels of aerobic glycolysis. Metabolic heterogeneity in cancer can lead to a potential divergence in drug response and even detection and visualization of cancer cells using standard methods. Chemotherapy targets fast-growing cells, which typically exhibit Warburg (aerobic glycolysis) metabolism [5]. Meanwhile, cells that are slower-growing and depend upon OXPHOS are less targeted or destroyed by this treatment, leaving the resistant cells remaining, thus providing the potential for recurrence or metastasis [21]. Understanding the inherent differences between these two metabolic states can identify biomarkers to better stratify patient responses to current drugs and reveal other, more effective anti-cancer therapies.

Some arguments against typical Warburg metabolism are that it is an artifact of *in vitro* cell culture, driven by saturation with glucose in culture media [22, 23] and that this is not a phenotype found in tumors *in vivo* [24].

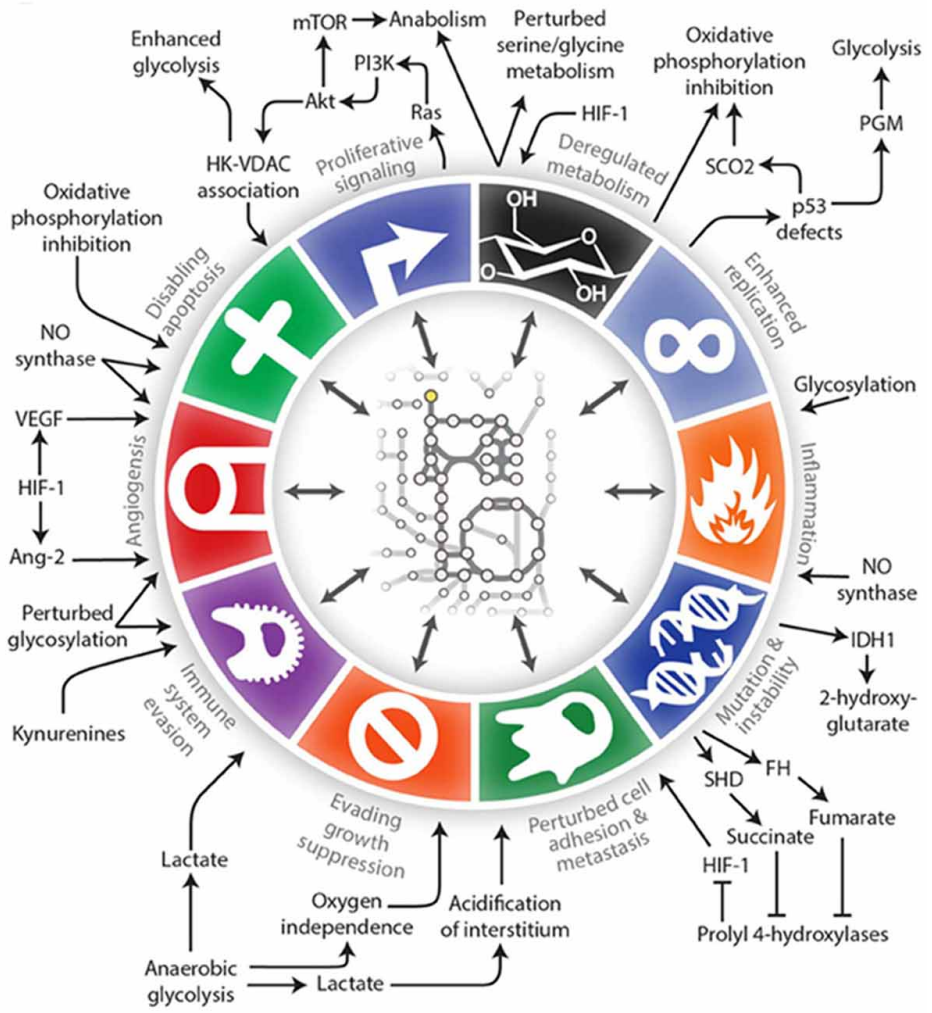

Figure 1.4: Relationship of cell metabolism with cancer hallmarks [18].

Additionally, The "reverse Warburg-effect" is seen in primary and metastatic breast cancer, and potentially in prostate cancer and melanoma. This is whereby the tumor-associated stromal cells (fibroblasts, immune cells, adipocytes) have been transformed to be more glycolytic by the cancer cells, and secrete lactate that then fuels oxidative metabolism in cancer cells [25, 26]. That cancer cells use lactate as a fuel is replicated in an analysis of non-small cell lung carcinoma cells [27], but it is also important to note that the same study points to higher

levels of oxidation of glucose *in vivo*. However, when performing metabolomic analysis of tissue biopsies from pancreatic adenocarcinomas, Battini et al [28] found significant differences from normal samples, pointing to higher glycolysis in tumor cells. The reliance on glucose versus other fuels depends mainly on the local environment and available nutrients and oxygen, with the main takeaway being that a tumor is advantageously heterogeneous in its metabolism [29–31]. The space around tumors are found to be a lower pH than normal tissues, between 6.3 and 7.0 versus between 7.35 and 7.45, respectively. This is also typically explained by a metabolic shift to aerobic glycolysis [19] and thereby increased secretion of lactate in the tumor microenvironment (TME). However, it has been found that what contributes most to the acidity is secretion of CO_2, not lactate, via increased catalysis by carbonic anhydrase IX [32].

Metabolism and the hallmarks of cancer

Altered metabolism can be tied to all of the hallmarks of cancer [17, 18] (Figure 1.4). This makes metabolism a potential target for inhibiting many cancer processes, especially as most cancer cells exhibit divergent metabolism from normal. Increased glycolysis can be linked to immune system evasion [33, 34] and perturbed cell adhesion and metastasis [35, 36] via increased lactate secretion. Perturbed OXPHOS affects both apoptosis and growth suppression, in favor of cancer cell survival. Glycosylation changes can be linked to angiogenesis, immune system evasion, and inflammation. Not only responsible driving hallmarks, metabolic changes are driven by other hallmarks, such as genetic mutations and instability (p53, SDH, FH, IDH1, Myc) [37]. Key aberrations to metabolism in cancer have been defined into their own metabolic hallmarks [38] which can be helpful to classify cells and tumors accordingly.

1.1.3 Metabolic biomarkers

Given the importance of metabolic abnormalities in cancer, analysis and detection of metabolic phenotype is essential for clinical translation. This kind of insight is typically provided by biomarkers. According the the National Cancer Institute's Dictionary of Cancer Terms [39], a biomarker is, "a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease." Metabolism itself is not currently a standard clinical marker for prognosis or drug response. However, research suggests a link between

tumor metabolism and clinical outcomes [28]. Biomarkers of metabolism are therefore important for translating basic metabolic research into clinical practice. Potential metabolic biomarkers include high content clinical imaging, protein expression, and metabolic flux analysis.

High content imaging includes positron emission tomography (PET), combined with computed tomography (CT, combined as PET/CT), is already used for basic detection of cancer using 18F-fluorodeoxyglucose (FDG) to identify tissues consuming a high amount of glucose. The radioactive glucose tracer is used to visualize increased glucose uptake and overlayed with the detailed imaging of bones, tissues, and organs from CT. Normal tissues that have high uptake are the brain (high glucose consumption), kidneys (clearing of tracer), and bladder (clearing of tracer), and any sites with active inflammation or infection. Any other areas that have abundant FDG signal in the body are suspected cancerous and a biopsy is taken to confirm, if possible. As mentioned previously, not all cancers exhibit significantly increased glucose consumption so other indicators may can be useful for further characterization. Other molecules of interest include markers of proliferation (such as 11C-acetate, 11C-choline/18F-fluorocholine, and 18F-fluorothymidine), markers of hypoxia (18F-fluoromisonidazole and 18F-fluoroazomycin arabinoside) [40], and 13C-glycerate for tracing glycolysis specifically [41].

Tissue biopsies are already assessed histologically for some indicators, such as Ki67 (marker of proliferation), H&E (visualization of the extracellular matrix and nuclei to assess cell abnormalities), and other tumor specific molecular markers such as hormone receptors in breast, prostate, uterine, and ovarian cancers. There are many proteins that are involved in glucose metabolic flux, as shown in Figure 1.1, that could serve as metabolic biomarkers. Gene expression or sequencing also offer some insight into molecular differences between cells, but are indirect measures of functional units such as protein.

Specifically, essential proteins for metabolic function may serve as good biomarkers of metabolic phenotype (Figure 1.5). Glucose transport proteins play the most important role for glucose import into cells. The glucose transporter family consists of 14 members where glucose transporter 1 (GLUT1) is the best described and ubiquitously expressed [42]. Its increased expression is shown to be associated with cancer aggressiveness [43]. Cancer cells that heavily rely on glucose have altered metabolism to support the increased flux through glycolysis [44] where lactate is shuttled out of the cell via the monocarboxylate transporters

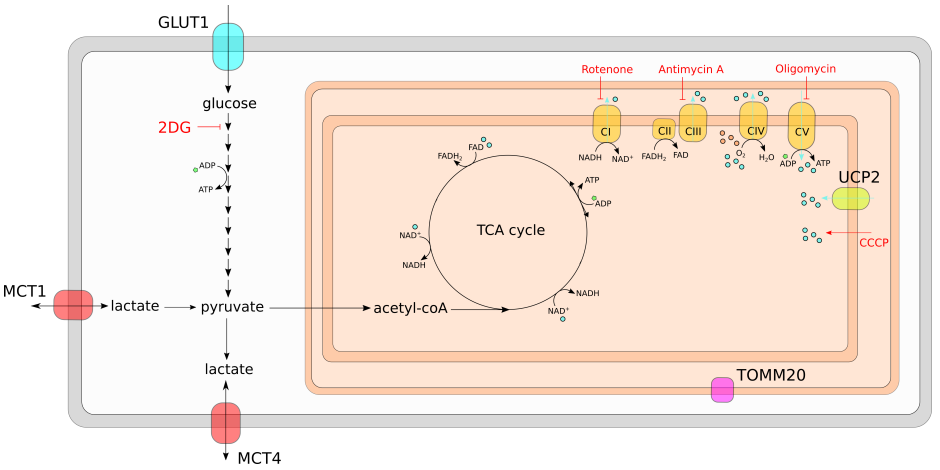

Figure 1.5: Simplified overview of glucose metabolism pathways with relevant proteins labelled. Compounds used in metabolic flux assays are in red. The lines show their respective targets in glycolysis and oxidative phosphorylation. Other details of glucose metabolism are shown for further reference. Blue circles represent protons. Orange circles represent electrons. Green pentagons represent inorganic phosphate (Pi).

[45]. Monocarboxylate transporter 1 and 4 (MCT1 and MCT4, respectively) are implicated in lactate import and export [46]. Their expression has been related to glycolytic dependency [47] and already shown potential as metabolic biomarkers [48]. Mitochondrial mass or volume in a cancer cell can give information regarding the dynamics of metabolic flexibility [49]. Translocase Of Outer Mitochondrial Membrane 20 (TOMM20) is a commonly used marker of mitochondria, as it is involved in recognition and translocation of cytosolically synthesized mitochondrial pre-proteins [50], thus crucial in mitochondrial biogenesis [51]. Furthermore, another mitochondrial marker and involved in stress responses, is the uncoupling protein 2 (UCP2), found ubiquitously expressed in mitochondria of many tissues, and associated with tumorigenesis in CRC [52] and pancreatic cancer [53]. Specific cancer metabolites such as 2-HG [54] may serve as metabolic markers as well. However, normal concentrations can vary widely so monitoring baselines and changes in metabolite levels on an individual basis is important. Analysis via magnetic resonance imaging, blood or urine sampling over time in the same patient is what can be the most useful to capture metabolic dynamics for the best prognostic value.

1.1.4 Metabolism-targeting drugs

The rationale for targeting metabolism in tumors is that in their altered state, optimized for survival in restrictive environment or for their specific dysfunction, they are less flexible and unable to adapt to different processes as well as a normal cell might. However, this remains a relatively small focus in cancer therapy. In 2017, Amoedo et al [55] identified 2358 studies in the clinical trials database (clinicaltrial.gov) from the search combination of "cancer" and "metabolism". As of August 2021, this had increased to 4770, but is still only 5.7% of the total registered cancer trials (84 322).

There are many anti-cancer agents being investigated for their ability to target different aspects of cellular metabolism, from glycolytic and TCA cycle enzymes to transport proteins to mitochondrial complexes; an exhaustive list can be found elsewhere [56, 57]. The two drugs used in the studies here, metformin and 5-fluorouracil, both target metabolism. Metformin gained attention as a possible anti-cancer drug in 2005 due to its correlation with reduced colorectal cancer incidence in diabetic patients prescribed the drug [58]. Other studies have also shown improved survival in patients prescribed metformin compared to diabetic patients on other drugs [59]. There are also studies showing no cancer-related effects of metformin [60]. However, it continues to be of interest due to its low cost and lack of major side effects. Unfortunately, it has not been as successful in clinical trials as hoped. 5-fluorouracil (5-FU), an essential chemotherapy agent, inhibits thymidylate synthase, which is responsible for production of dTMP (one of the four base nucleotides in DNA) from dUMP, thereby inhibiting DNA replication. Through its action within nucleotide metabolism, it is categorized as a metabolism-targeting drug but its relationship to glucose and energy metabolism is less understood.

1.2 Modelling cancer *in vitro*

The first steps to identify and test drugs that have anti-cancer activity largely take place in 2D cell cultures. This is then tested in small animals such as mice and rats and typically in non-human primates. Assuming efficacious effects are still observed at this stage, it then goes into humans for a Phase I trial. However, 2D culture does not mimic *in vivo* human results [61] despite being human cells. Cells in 2D culture are not exposed to physiological substrate levels and gradients.

They are grown with a flat morphology that provides no cell-cell interaction, extracellular matrix (ECM), and do not mimic the tumor microenvironment (TME) (Figure 1.6A). In a 2D model, approximately 50% of the cell surface is exposed to nutrients, an environment rarely achieved *in vivo* (Figure 1.6B). The basic cell culture media used to supplement the cultures are not physiologically relevant. Normal glucose concentration in blood plasma after fasting is 3.9-5.6 mM, with diabetes diagnosed in those with fasting blood glucose over 7 mM [62], while standard culture medium contains 25 mM glucose. The renal threshold for glucose in the blood is 10 mM [63], with glucose beyond this excreted in urine. However, glucose concentration may reach 10 mM post-meal in a diabetic individual. This concentration can be relevant for diabetic *in vitro* models, but not for normal disease modeling, and certainly no physiological relevance exists for glucose levels as high as 25 mM. The effect of these morphological and environmental differences has a significant effect on the cancer cell behavior [64], which may mask real responses to treatments *in vitro*.

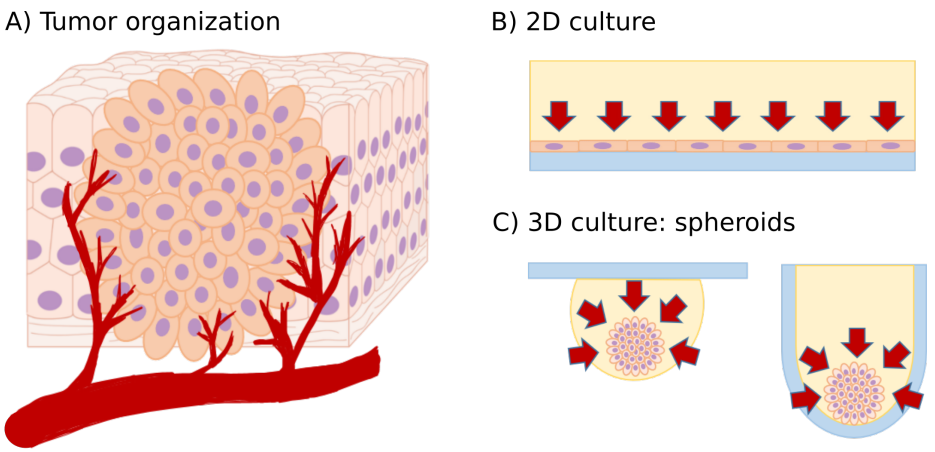

Figure 1.6: Illustrated representation of A) tumor organization *in vivo*, B) 2D culture of cells *in vitro*, and C) one example of 3D culture of cells *in vitro* in spheroids. Red arrows denote direct exposure to nutrients and drugs and how this differs between 2D and 3D culture.

1.2.1 3D culture models

In contrast with 2D cultures, where cells are cultured side-by-side, 3D culture comprises cells on top of one another as well, adding a third dimension (Figure 1.6C). Pre-clinical tests in small animals are commonly the first complex 3D model of the cancer in the development process. However, it has been shown that in addition to causing undue harm to the animals, the results are neither reliable or reproducible, and difficult to translate to humans [65]. Animals used in testing are normally homogenous, immuno-compromised, represent specific exclusive phenotypes, and have divergent responses from humans in many ways [66]. 3D *in vitro* culture offers the ability to model the major characteristics of the *in vivo* tumor environment namely the ECM, multicellular organization, and the possibility co-culture with fibroblasts and immune cells. For this reason, 3D cultures are recognized as the way forward for enhanced screening in cancer research. Because of the more complex structure, gradients are formed from the surface of the culture to the core (Figure 1.7); most notably of oxygen and nutrients, thus driving changes to energy dynamics of these regions.

Different options exist for culture of 3D models. At the most basic level is 2.5D, which is just 2D cell growth on top of a matrix [67]. Although providing a bottom layer of ECM for cell attachment, it lacks the same 360° environment for both cell-cell and cell-matrix contact. As well reviewed by Weiswald et al [68], there are many ways to refer to 3D cultures in literature, but the terminology is inconsistent. The first mention of 3D cell culture was by Sutherland [69], as "multicell spheroids". While these cultures were sourced directly from tumor-derived cells, the term "spheroids" now refers more to culture of established cell lines. This is in contrast to freshly derived cells from tissue which is referred to generally as an organoid [70]. It is also important to note the difference from cell aggregates, that are not organized in 3D, but are just a collection of detached cells, loosely associated, which should not be described as a spheroid or organoid.

3D cultures can include scaffold-free cultures, cultures in a scaffolding matrix, and the most complex models, microfluidic chip devices. The matrices and scaffolds used for 3D culture come in a wide variety from synthetic polymers [71] to biological ECM from animal [72–74] and plant sources [75, 76]. Although not the focus of this thesis, 3D cultures also offer the ability to study biophysical forces present in the 3D TME, such as between cells, cells to matrix [77], interstitial pressure, and oxygenation [78].

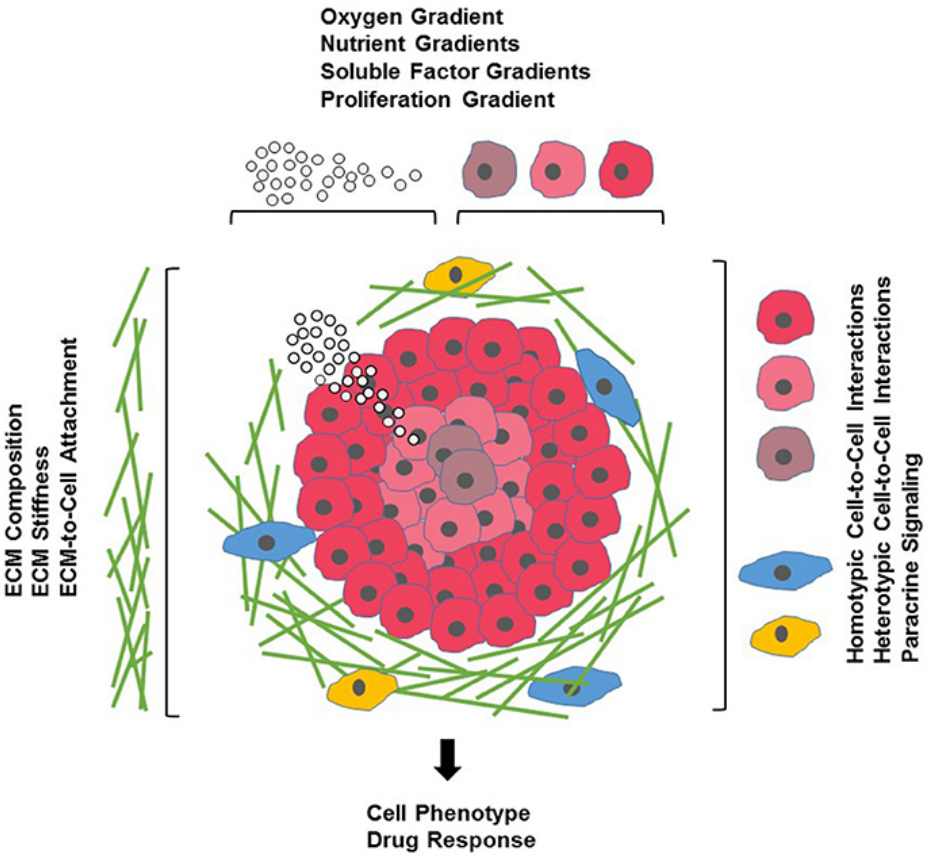

Figure 1.7: 3D culture microenvironment [64].

1.3 Improving cancer targeting and testing

The potential for 3D culture to enhance drug targeting and improved testing in cancer lies early in the drug development process. The current drug development process in the USA, regulated by the FDA, is comprised of many steps and can take 10-15 years from start to finish [2]. The first step encompasses research and development (lead generation) and pre-clinical testing (lead optimization) can take from 1-3 years, and perhaps even more if basic research into targets and compounds is included before this. During this time, short-term animal testing may begin in order to identify the translation potential of findings, safety, dosage, and efficacy. The next step involving clinical trials in humans is done in three

phases which together can take 2-10 years to complete. Long-term animal testing can take place concurrently. After clinical testing is done, the FDA approval process for a New Drug Application (NDA) takes up to a year.

When looking at the total cost of developing a single successful new molecular entity (NME), it requires on average 24 candidates in the beginning of the pipeline and a total of US$1.778 billion (15.2 billion NOK) (Figure 1.8) [2]. This bottleneck in the development process has been referred to as "the productivity crisis" [79] and has been described as Moore's law in reverse, or Eroom's law [80]. While Moore's law describes the exponential growth in efficiency in a technology ("the number of transistors that can be placed at a reasonable cost onto an integrated circuit") and many R&D processes have experienced this, drug approval has had the opposite trend. Whereby the number of new drugs per billion US dollars spent in R&D has halved every 9 years since 1950 [81]. If this bottleneck can be reduced, with just half the number of candidates entering the process due to better pre-screening methods and thus increased success rates, it could reduce costs to US$889 million (7.6 billion NOK). Data since 2010 do suggest that drug approval is on an upswing in the past decade [80], however this is not specific to oncology and it remains to be seen if the trend will continue.

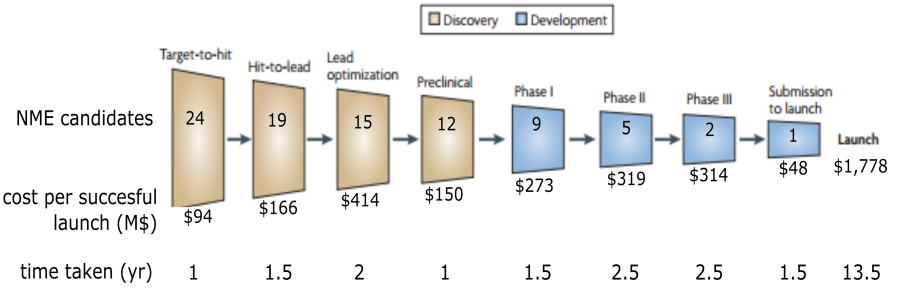

Figure 1.8: R&D costs to discover and develop a single new molecular entity (NME). Cost per successful launch is capitalized cost. Prior to Target-to-hit, there is target identification and target validation where many potential candidates are also screened. Figure reproduced and adapted from Paul *et al* [2] with permission from Springer Nature.

1.3.1 Drug discovery

In the 1990s, in response to the productivity issues in R&D, there was a shift to target-based high-throughput screening (HTS) [81], but this did not coincide with an increase in drug development efficiency. When discussing HTS there is a range of what this includes. In compound screening, this could mean testing a collection of thousands (10^5) of drugs using highly-parallelized, automated, and miniaturized 2D assays in 384-, 1536-, or 3456-well microtiter plates [82]. However, when talking about high-content phenotypic assays, with more complex analysis and sample handling, HTS peaks at the level of 96-well or perhaps 384-well plates. For 3D culture formats, a reasonable goal is this magnitude of reproducible HTS. This is a consideration in the development of 3D culture assay systems. A microfluidic or lab-on-a-chip system, while possibly more biologically relevant, may only serve as a basic research tool and not for drug development if there is no option for higher-throughput parallelization. The right balance of complexity and resource cost has to be found to have a quality *in vitro* model to improve the drug development process.

Target-based and Phenotypic Drug Discovery

The ideal cancer drug would target all cancer cells and leave normal cells unharmed. However cancer biology is complex and targeting treatment in groups of like cancers is more feasible. Knowing which groups are most sensitive to a potential treatment based on certain markers is how we achieve personalized medicine. In recent history, this has been undertaken via target-based drug discovery (TDD) [83], focusing on common gene mutations and important signalling pathway proteins (Figure 1.9). Many targets identified in this manner come from promising early research, but have resulted in lackluster outcomes later in validation testing. Working on simplified, homogeneous model systems can amplify small positive effects and blind to inhibiting factors and complexities. There are many new drugs are targeted to gene mutations [84], while only 10% of cancers have been tied to familial gene mutations. For this reason, a more foundational and phenotypic approach to target cancer is more promising. Phenotypic drug discovery (PDD) is in contrast with TDD, but is how most drugs were discovered before the current technological and molecular science revolution. PDD is supported by the ability to monitor behaviors in a culture of primary cells from patients and 3D cultures. However, a combination of biomarkers and TDD while monitoring the

real effect of such targets with PDD results in mechanism-informed phenotypic drug discovery (MIPDD), a powerful tool in the search for clinically relevant therapies [83] (Figure 1.9).

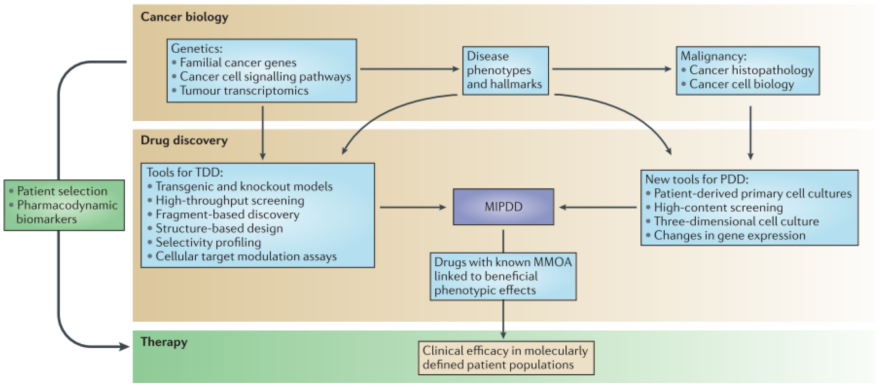

Figure 1.9: Personalized medicine and target identification in drug discovery [83]. Reprinted with permission from Springer Nature.

Mechanism-informed phenotypic drug discovery

Many robust options exit that can yield insight into functional phenotypes of cancer cells for MIPDD, specifically metabolic phenotypes. Analyzing metabolic flux is one way to do this. Metabolic flux can be described as the movement of molecules through metabolic pathways. The balance of different metabolites in the process, their rates of turnover, and what pathways are used are characteristics of flux and help define the cell's metabolic phenotype. Different methods exist that can indirectly assess this flow of molecules. Stable isotopes (13C, 18F) can be used to label substrates like glucose to trace how the labelled carbon is integrated into final measured metabolites. The positioning of the labelled carbon gives some insight into the pathway taken to get to a specific endpoint. It requires heavy data analysis and modelling to extract metabolic flux maps from the raw data. Metabolomics, assessing pools of metabolites in a cell population, gives some data on the ratio of molecules and thus some insight into favored pathways or substrates. Live measurement of oxygen consumption and proton excretion is another way to assess metabolic flux. Monitoring these metrics while injecting different reagents that affect metabolic pathways is the principle of the

Seahorse Analyzer instrument. By combining reagents in specific orders, insight is gained into the cell's metabolic phenotype with regard to mitochondrial function, glycolytic capacity, and also utilization of other substrates. However, to achieve the most relevant insight into function, the cells should be in more relevant culture conditions such as 3D culture.

Aims of Study

The aim of this thesis was to investigate metabolic phenotypes of cell line models, the potential biomarkers thereof, and how this could relate to drug response in cancer. To achieve a more physiological culture environment for both metabolism and drug response experiments, physiological glucose levels and 3D cancer cell line models were used.

2.1 Objectives

- Study the effect of glucose concentration and metabolic phenotype on response to mitochondria-targeting drug metformin in colorectal cancer cell line models
- Analyse the metabolic flux of models in a Seahorse metabolic analyzer to compare differences between 2D and 3D cell line models of colorectal cancer and pancreatic ductal adenocarcinoma
- Measure the response of 3D culture models of colorectal cancer and pancreatic ductal adenocarcinoma to chemotherapy drug, 5-fluorouracil

Material and Methodological Considerations

3.1 Culturing cancer cells *in vitro*

A major goal in cancer research is to understand cancer biology at a deeper level and test relationships between markers and drug response. However, only so much information can be gleaned from endpoint testing of tumor biopsies, and animal models have many disadvantages. Because of this, *in vitro* cell culture is how fundamental cancer research is performed. Here we are working with *in vitro* culture models using established cell lines. Colorectal cancer has been the focus of our group due to the high incidence rate and direct metabolic relevance of the gastrointestinal cancer type. Pancreatic cancer is also of high relevance due to its high mortality rates, association with metastatic colorectal cancer, and low representation in metabolic research [55]. In the first paper, two cell lines were used, SW948 and SW1116, that were already under study for their metabolic characteristics in the Hagland lab. As the focus in later papers were on 3D spheroid models, SW1116 was not included due to the suboptimal culturing and 3D characteristics (discussed more below). HCT116 was chosen as an alternate CRC model due to its interesting metabolic phenotype and well-documented 3D qualities. The PDAC cell lines (Panc1 and MIA-Pa-Ca-2) were also chosen to be of differential metabolic phenotypes and having recorded spheroid forming properties.

Beyond the cell lines used, the media is the next most important variable *in vitro*. Here, we used standard DMEM supplemented with high (25 mM) and

low or physiological glucose (5 mM) in Paper I as a comparison of the effect. After this first study, the others were carried out only in physiological glucose. The importance of nutrient supplementation is well researched, but perhaps is not as widely known as it should be, especially outside the metabolism and stem cell fields. In stem cell cultures, growth factors and nutrients are known to activate and differentiate cells [85]. With the propensity of cancer cells to also exhibit stemness properties [86], it is surprising it is also not a more substantial consideration in cancer research. Glucose as a supplement is the obvious focus for our research, but other supplements are also important to consider *in vitro* such as uric acid [87] and several micronutrients [88, 89]. The media surrounding the cells can only be optimized so much, however. There is a certain point where no matter how optimized the liquid formulation is, the morphology and micro-environment of the cells in 2D are very different from the *in vivo* TME. This is why we began working with 3D culture models.

3.1.1 3D Culture

3D culture is a very general term that covers many methodological techniques. There were two main methods used in this thesis, culture in hanging drops and low-attachment round-bottom plates. There are many commercial solutions available for spheroid culture, and they can be useful for specific applications, but basic lower-cost methods are equally easy to work with as more expensive options. The reason these two methods were chosen was to start with simple methods that produce reproducible single spheroids [90]. Before introducing more variables such as embedding in a matrix or co-culture, basic characteristics had to be assessed in these simple scaffold-free cultures which are easier to work with and analyse. Low cost was another requirement, both to reduce our own costs, but also because price and complexity are major perceived barriers to entry in to 3D culture, in addition to reproducibility [91]. The cost to run basic assays in 3D should not be much more than 2D if more labs are going to implement them. This is another consideration: being able to achieve the same high-throughput level that is achieved in 2D. There are perhaps some better options for more high-throughput formation of spheroids for some destructive assays, but the methods here fit the level of high-throughput limitations in other areas, such as microscopy (Paper III) and also Seahorse assays (Paper II). The decision to use hanging drops and round-bottom plates depended on the analysis needed and the

best method upstream of these. The microscopic analysis of the spheroids could have been improved in a 384-well format but this increases the difficulty of media exchange in such low-volumes and best fits the use of a liquid handling system. This could be a next step. Other methods to produce high numbers of spheroids at once would be ideal for the flow cytometry analysis such as micro-well mesh [92] or hydrogel droplet formation of spheroids [93].

The need to use cell lines with documented spheroid-formation presents a major limitation in this type of work. When working with suspension cultures only, some cell lines may not form spheroids (for example, SW1116). Embedding in a matrix can help, as we experimented with collagen I matrices, but even then formation is not guaranteed. This may force analysis to only take place on certain cell models and bias results. More research is needed on the difference between cell lines and what affects the ability to form spheroids. This could be an important characteristic to consider in itself and what it may represent in terms of cancer aggressiveness and potential drug response. The contribution of epithelial-to-mesenchymal transition (EMT) properties are an interesting avenue to investigate for this [94]. Media supplements that may be essential for spheroid-formation should be considered as well, such as methylcellulose and collagen [95].

Some preliminary work was done with more complex, microfluidic systems. However, with time taken to initialize the set-ups and throughput being an issue, the results did not reach a publishable stage. The CellDirector3D from Gradientech is a microfluidic device with a single chamber large enough to accommodate spheroids and also offer continuous fluid flow, as well as gradient formation across the culture area. It was investigated for studying the effect of nutrient and drug gradients on spheroid(s) growth and cell migration in a collagen matrix. While we did author an application note [96], further development was not able to be done to take this project further. AIMBiotech 3D cell culture chips offer the opportunity to introduce fluid flow and form gradients in a 3D channel. They were used for 3D culture of colorectal cell lines in collagen matrices with fibroblast co-culture to investigate the effect of directional fluid flow and cancer-associated fibroblasts on cancer cell growth and migration. A master student project was dedicated to this study [97], but was not developed further. The dimensions of these chips do not allow for culture of spheroids of relevant sizes. Finally, the Ibidi μ-slide spheroid perfusion slide is designed for spheroids cultured in parallel, with fluid flow through the channel possible from volume

differentials in opposing media ports. This was tested for graphene nanosheet uptake [98] in spheroids, but also holds interesting potential for cancer co-culture and metastasis modeling.

3.1.2 Drug treatment and long term culture

When planning the experiments to test response of spheroids to 5-fluorouracil, biological replicates had to be balanced with technical considerations of longer-term culture and throughput limitations of microscopy. The cell lines were treated in tumor groups, with CRC together and PDAC together, and two plates of each cell line were prepared, resulting in 20 replicates at once for each experiment. This was the same in 2D as well, and as the imaging of (8) 96-well plates takes some time, only transmitted light images were taken at regular intervals. The more time-consuming volume stacks of viability using fluorescent dyes were limited to after treatment rounds. In total, the spheroids were cultured for over 3 weeks. Between treatments, media was exchanged every other day to avoid glucose starvation, but as shown in Paper II, over the 4-day treatment period, glucose was not entirely exhausted in the cultures, only dropping to 1.1 mM. Also, at these low glucose levels, lactate remained within a normal range [99].

3.2 Metabolic flux

Seahorse was used for metabolic flux analysis because it probes the live, dynamic metabolic state of the cell. The metabolic flexibility to adapt to acute changes in the environment is an interesting phenotypic characteristic we wanted to incorporate. This is still a relatively new system from 2006, performing some of the same measurements of other systems such as the Oxygraph-2K [100], the Clark electrode [101], and other fluorescent or luminescent reporter systems [102]. The Oxygraph is the most accurate for mitochondrial analysis as it is completely closed system, with very high sensitivity and resolution. The main benefit of Seahorse over the Oxygraph is that it enables assay of adherent cell culture in 2D or 3D, therefore requiring less processing and potentially more relevant results. It is designed to form a microassay chamber within the well when the probe lowers, decreasing the assay volume to 7 ul and able to largely minimize oxygen exchange during the short measure time. The potential oxygen leak is taken into consideration in any data analysis where this makes a difference, such as ATP

production. Other benefits include higher throughput testing and simultaneous measurement of pH and oxygen. As the semi-closed chamber is formed, this does not require a completely closed system. Injections are integrated into the assay cartridge and it automatically calibrates. The disadvantages are perhaps not exclusive to the Seahorse system and are detailed below to some degree. Other options for metabolic flux analysis are isotope tracing and metabolomics, but these are more indirect measurements and do not allow for the same acute functional assays as Seahorse and other respirometers.

3.2.1 Technique: 2D v 3D

Seahorse has good standard operating protocols for measurement in 2D, but common practices in 3D measurement are lacking. More experience and data for this application area was one of the outcomes of this thesis. One of the major hurdles in 3D analysis is working with cell lines that form well-structured spheroids. Since the spheroids have to be transferred to the assay plates, a successful assay depends on effective transfer. Here the choice was made to grow spheroids in hanging drops because of the relative ease of transfer from these to the plates in a multichannel format. It is much easier to control the pipetting and track if a spheroid is in the pipet from the plate lid than from the wells of a round-bottom plate. After transfer, placement is very important. The spheroid is ideally placed in the center of the well for the best detection of signal by the sensor. This can be the most time-consuming part of the process and introduces significant variation in incubation time before the assay is run. For the studies described in Paper II, a pipetting guide was designed, 3D printed, and used for this purpose [103].

The seahorse plate is designed to have beneficial flow through the well to promote exchange of molecules in the media and also to prevent movement of the spheroid. However, coating the plate with CellTak is still recommended by Seahorse to make spheroids more stable. Because of the design of the plate, the complete removal of the high-pH CellTak solution is difficult, which can cause some effect on the assay pH as well as introduce bubbles. This is a hindrance for normalization as well. Avoiding CellTak may be recommended for these reasons.

3.2.2 Assay design and chemical considerations: 2D v 3D

In 3D Seahorse assays, higher cycle numbers are used after some reagent injections to ensure enough time for diffusion into the spheroids. Basal readings are more unstable at first, perhaps due to settling of the spheroid in the well after initial agitation. Oligomycin was the most difficult to infiltrate the spheroid, and this is experienced by others as well [104]. However, response to CCCP was immediate and did not change over several readings. CCCP is recommended by Agilent to be titrated over a range to find the maximum respiration achievable. This was done for both 2D and 3D, so this could be why there are clear reactions to the concentration used. This is not currently a suggestion for oligomycin, but given the results, it should be a consideration. Even with a higher concentration, they may not show a rapid reaction, primarily due to the limitation of the method of action of oligomycin. This response to oligomycin could be an interesting characteristic of spheroid metabolism, specifically ATP-synthase function.

3.2.3 Data analysis and normalization

Agilent and Seahorse have produced many standard assays and analysis workflows for metabolic analysis, but consistency in data analysis and presentation is still a problem in the field. Setting aside the limitations of Seahorse macros for custom assay designs, there are no recommendations or workflows for assessing data quality. Some published data from Seahorse is still not normalized, making it very difficult to compare between studies. When normalized, the method tends to vary. The two main ways to normalize are by protein content and cell number. Both of these have their limitations and values are not comparable between the two. This is why the Seahorse data here (Papers I and II) are presented normalized to the basal reading. This loses resolution at the absolute level, but as the purpose here was to compare metabolic phenotypes, it enables comparison of cells that vary in absolute metabolic activity. Finally, the overinterpretation of Seahorse data should be avoided. While it has the ability to yield important information on the dynamic metabolism of cells, it is not a representation of physiological environment and some parameters are ambiguous [105].

3.2.4 Terminology

Something that is not discussed enough is the terminology used around some of the assays. Specifically "maximum respiration" after FCCP/CCCP. This has been an issue for discussing our own data analysis during the peer-review process and it deserves more discussion. We find that some cancer cells do not experience measurable spare capacity in certain conditions and this could be due to the inability to overcome ATP-synthase inhibition by oligomycin [106] or due to only simulated ATP demand [107]. This is something not addressed in most Seahorse publications, but important to note. It may be worthwhile to also run an assay without oligomycin (both for titration and functional assay) to establish maximum oxygen consumption without ATP-synthase inhibition.

3.3 Metabolic biomarkers

Standard clinical biomarkers are typically assessed from liquid biopsies and also tumor biopsies. Liquid biopsies typically include blood and urine samples and analysis of these are quite robust but general. They are good candidates for analysis of metabolites via metabolomics. Tumor biopsies are used to study the standard markers and more specific molecular markers using basic cell stains and immunohistochemistry. Since this project is being carried out *in vitro* on cell culture, these standard clinical methods are not used and one objective of this thesis was to investigate new markers.

Metabolomics and gene expression are good for finding marker candidates with significant changes, but are costly. Protein expression was preferred due to the direct measurement of the functional properties of the cell. Gene expression can give insight into demands of the cell, but there can be some disconnect in gene expression and protein expression [108] due to complex epigenetic control processes. There are many steps and controls in the process from DNA code to gene expression (mRNA) and ultimately proteins.

Here flow cytometry was used in an attempt to quantify protein expression on single cells, with capability of resolving any heterogeneity in protein expression. Immunohistochemistry is an ideal method to compare to other studies though, due to less variables in the process, being a common method in research and the clinic, and offering spatial view of differential expression. The drawbacks are that it is not a high-throughput method and is very time and resource intensive. For these

reasons, flow cytometry was chosen. As mentioned above, spheroid production for this method was a limiting step. Implementing a much higher-throughput method of spheroid production could improve the data by yielding many more cells for analysis. The proteins analyzed were chosen based on their function in glucose metabolism, reporting on key transport processes in the cell (Figure 1.5).

Summary of Papers

4.1 Paper I

Metformin treatment response is dependent on glucose growth conditions and metabolic phenotype in colorectal cancer cells

Background

Cancer cells exhibit altered metabolism, a phenomenon described a century ago by Otto Warburg. However, metabolic drug targeting is considered an underutilized and poorly understood area of cancer therapy. Metformin, a metabolic drug commonly used to treat type 2 diabetes, has been associated with lower cancer incidence, although studies are inconclusive concerning effectiveness of the drug in treatment or cancer prevention. The aim of this study was to determine how glucose concentration influence cancer cells' response to metformin, highlighting why metformin studies are inconsistent. We used two colorectal cancer cell lines with different growth rates and clinically achievable metformin concentrations.

Results

We found that fast growing SW948 are more glycolytic in terms of metabolism, while the slower growing SW1116 are reliant on mitochondrial respiration. Both cell lines show inhibitory growth after metformin treatment under physiological glucose conditions, but not in high glucose conditions. Furthermore, SW1116 converges with SW948 at a more glycolytic phenotype after metformin treatment. This metabolic shift is supported by changed GLUT1 expression.

Conclusions

Thus, cells having different metabolic phenotypes, show a clear differential response to metformin treatment based on glucose concentration. This demonstrates the importance of growth conditions for experiments or clinical studies involving metabolic drugs such as metformin.

4.2 Paper II

Metabolic flux analysis of 3D spheroids reveals significant differences in glucose metabolism from matched 2D cultures of colorectal cancer and pancreatic ductal adenocarcinoma cell lines

Background

3D cell cultures are the emerging standard for *in vitro* cancer cell models, being more representative of *in vivo* tumour conditions. To overcome the translational challenges with 2D cell cultures, 3D systems better model more complex cell-to-cell contact and nutrient levels present in a tumour, improving our understanding of cancer complexity. However, there are few reports on how 3D cultures differ metabolically from 2D cultures, especially when grown in physiological glucose conditions (5 mmol/L). Well-described cell lines from colorectal cancer (HCT116 and SW948) and pancreatic ductal adenocarcinoma (Panc-1 and MIA-Pa-Ca-2) were used to investigate metabolism in 3D spheroid models. The metabolic variation under normal glucose conditions were investigated between 2D and 3D cultures by metabolic flux analysis and expression of key metabolic proteins.

Results

We found significant differences in glucose metabolism of 3D cultures compared to 2D cultures, both related to glycolysis and oxidative phosphorylation. Spheroids have higher ATP-linked respiration in standard nutrient conditions and higher non-aerobic ATP production in the absence of supplemented glucose. In addition, ATP-linked respiration is significantly negatively correlated with OCR/ECAR ($p=0.0096$). Mitochondrial transport protein, TOMM20, expression decreases in all spheroid models compared to 2D, and monocarboxylate transporter (MCT) expression increases in spheroids in 3 of the 4 cell models.

Conclusions

Studies in cancer cell metabolism should consider that 2D metabolic analysis does not replicate spheroid metabolism. This study presents a comparison of how metabolic flux, metabolic biomarkers, and nutrient utilization differ in corresponding 2D and 3D grown cancer cell lines of colorectal and pancreatic origin. The results emphasize the need to use more complex 3D cell models for investigation into nutrient utilization and metabolic flux for a better understanding of tumour metabolism and possible therapeutic targets.

4.3 Paper III

Increased response of CRC and PDAC multicellular spheroids using long-term culture and multi-round exposure to 5-fluorouracil

Background

The current drug screening protocols use 2D-grown cancer cell panels *in vitro* to evaluate drug responses and select the most promising candidates for further *in vivo* testing. Most drug candidates fail at this stage, not showing the same efficacy *in vivo* as seen *in vitro*. An improved first screening that is more translatable to the *in vivo* tumor situation could aid in reducing both time and cost of cancer drug development. Here we address some of the shortcomings in the current drug screening protocol. We show how treatment with 5-fluorouracil (5-FU) in 2D and mathed 3D culture models of colorectal (CRC) and pancreatic adenocarcinomas (PDAC) give very different responses with regard to growth inhibition. Growth and viability are assessed in spheroids over long-term multi-round treatment and results are discussed with regard to the metabolic phenotypes of the models.

Results

The multi-round 3D screening is able to show more robust response than standard 2D drug screening, including resistance to therapy. Results from 2D cultures both over and underestimate drug response at different concentrations of 5-FU. In 3D, only by the end of the second round of treatment do CRC models reach 50% inhibition at clinically achievable concentrations. The PDAC models are not strongly inhibited at clinical doses even after two rounds, with MIA-Paca-2 demonstrating regrowth after all but the highest dose. High content viability

metrics point to even lower response in the resistant PDAC models. Higher maximum effect of 5-FU is seen in models with lower OCR/ECAR ratios, an indication of a more glycolytic metabolic phenotype.

Conclusions

This study reveals the limitations of testing drugs in 2D cancer models and even short exposure in 3D models. Longer exposure and multi-round treatment is a viable and effective way to assess drug response. This is useful for evaluating sensitivities to drugs already widely in use, and screening those in the discovery pipeline. Lastly, identifying tumors with chemoresistance related to oxidative metabolism has high potential for targeting by metabolic drugs to increase chemosensitivity.

Discussion

5.1 Cell metabolism is complex yet important to characterize

Cell metabolism is dynamic and offers integral information about a cancer. The fact that cancer does exhibit altered metabolism compared to normal cells is an early insight by Otto Warburg that predates even a universal acceptance of the origin of cancer [19]. It is considered a cancer hallmark and is one of the major explanations in epigenetic or extragenetic drivers of cancer (Paper IV). However, it is not as simple as first theorized by Warburg. Cancer metabolic phenotypes exist on a sliding scale of dependence on glycolysis and oxidative phosphorylation, and this is just with respect to glucose metabolism. The importance of other metabolic pathways altered in cancer cannot be understated and there is much research being done on the metabolism of fatty acids [109] and amino acids [110] in cancer.

In Paper I, the differences between the two studied cell lines are very clear, despite displaying similar levels of OXPHOS, the faster-growing cell line (SW948) was significantly more glycolytic than SW1116. Upon analysis of even more cell lines as in Paper II, clear categorization into either glycolytic and OXPHOS are difficult. HCT116 is the most glycolytic when comparing based on OCR/ECAR ratio, and MIA-Pa-Ca-2 the most oxidative. However, if also considering spare capacity for glycolysis and respiration, measures of metabolic flexibilty under acute stress, and inclusion of other metabolic analysis methods, phenotypes become quite complicated [18]. This is why it is important to perform metabolic analysis of each sample for a clear picture of individual cell models' phenotypes. Having informative and quantitative metrics to report these metabolic differences

is needed so comparisons between studies can be done [111]. This is attempted in Paper II, but a concerted standardized approach by the field at large would be big step in the right direction. Metabolism and more specifically cancer metabolism is certainly a niche field, but would benefit from standardized methods and data reporting, as discussed in detail in the methodology chapter.

5.2 The *in vitro* nutrient environment alters cell metabolism and drug response

The measurement of metabolism *in vitro* is important for fundamental research and to fully understand characteristics of cells cultured *in vitro*. *In vitro* culture is the first way to screen for cancer characteristics and responses before choosing to move forward with testing in more advanced models. We know more about culturing cells now than we did 70 years ago, when basic culture practices and media were first formulated. Many challenges from then have been overcome and it is now easier than ever to culture primary cells. When establishing cultures now, short doubling time should not be the goal, but maintaining physiological phenotypes as much as possible. Proper nutrient conditions are the main requirement for this. In Paper I, we show how much the glucose concentration affects metabolic phenotypes and drug response. From there, the effect of culturing in 3D versus 2D has a large effect on the metabolic phenotype (Paper II) and drug response (Paper III).

Perhaps intuitively, the level glucose in culture medium affects glucose metabolism and, by extension, drug response. We have used 1 g/L glucose (5 mM), as the average concentration of blood glucose in a fasted state is 3.8-5.5 mM [63]. Some groups haveused even lower glucose levels (e.g. 0.75 mM) to reflect much lower glucose concentrations present in tumor tissues [112]. In Paper I, there is a distinct difference in glucose sensitivity between the two colorectal cancer cell models used. SW1116 was more proliferative in low glucose (5 mM) than high glucose (25 mM) and did not have spare glycolytic capacity. Impaired glucose utilization has been linked to increased sensitivity to biguanides such as metformin [112]. However, that particular study examined much lower glucose concentrations than ours. We found that the relationship between glucose concentration and response to metformin was mainly due to metabolic differences exerted by the glucose levels, as glucose does not directly affect the function of metformin. The effect of glucose on GLUT1 levels presented in Paper I is an

example of how these conditions can make analysis of biomarkers difficult as well. For this reason, when carrying out the later studies (Papers II and III) only physiological glucose levels were used. Once the first study was done, no reason remained to continue to double the resources with irrelevant culture conditions. There are many other media components not addressed here, but have been implicated to also be important for more physiological *in vitro* culture: uric acid [87], amino acids such as citrate, acetate, and numerous minerals and cofactors [88, 89].

3D culture differs from 2D culture mainly in the presence of 360° cell-cell contact and natural gradients of nutrients and oxygen [113]. These have a downstream affect on metabolism, and further in a constant feedback loop on cell growth and drug response. We found higher ATP-linked respiration in standard nutrient conditions and higher non-aerobic ATP production in the absence of supplemented glucose (Paper II). Our finding that glycolysis is upregulated in spheroids, stands in agreement with what others have found [114–118], with some exceptions [119, 120]. However, not only do we find upregulated glycolysis, but also higher ATP-coupled respiration. This has not been reported before, but could fit with some other findings of the lower response of ATP-synthase to oligomycin in spheroids [104] and also a decreased number of ATP-synthase subunits [121]. The changes in protein expression were difficult to interpret, but deserve more analysis going forward, as both TOMM20 could serve as a marker of mitochondrial volume/function and MCT as an interesting marker of metabolic phenotype and perhaps nutrient shuttling [122]. This along with other results of higher ATP production without glucose, introduces complexity in metabolic changes and also fits with others' findings of 3D culture having much more variety of nutrient sources and metabolism [117, 119, 121, 123]. This is intuitive given the more heterogeneous nature of cells in 3D culture compared to 2D culture.

Additional culture conditions are also not addressed in the studies here but remain relevant. For example, the oxygen level in tissues is not as high as that in the air (21%, pO_2 = 149 mmHg), and varies considerably among different tissues: as low as 8 mmHg in the epidermis, 57.6 mmHg in the large bowel, and peaking at 108 mmHg in the alveoli [124]. Oxygen levels have an effect on the cellular metabolism and mitochondrial activity [6, 125]. By using 3D culture models, we have aimed to achieve this naturally via the spheroid's hypoxic core [113, 126–128]. The studies here have been focused on large single spheroids in order to model this effect. While we have not focused on studying oxygen profiles and the effect

thereof in the spheroids ourselves, there is much research being undertaken by others. In early spheroid studies, the metabolic shift and cell viability in spheroids was apparent but also noted to be more complicated than just due to nutrient and oxygen gradients alone [126], a finding which holds decades later [129, 130]. Oxygen and pH sensitive markers for fluorescent microscopy have been used to show significant nutrient gradients in spheroids, and this was the major cause of cell death, not oxygen depletion [117]. Oxygen mapping in spheroids have also been elegantly done using a small-molecule phosphorescent probe coupled with lifetime imaging, with potential for use in the clinic [131].

5.3 3D culture models are necessary for relevant *in vitro* drug screening

In Paper II, the effect of 3D culture on drug screening is clear. Results from 2D cultures both over and underestimate drug response at different concentrations of 5-FU. They overestimate in that lower concentrations trigger a maximum inhibitory effect that is not achievable in 3D. This is widely represented in many studies [132]. However, 2D cultures may also underestimate response, in part due to increased target expression in 3D culture [133]. Also, in typical high-throughput 2D culture for assays, the culture time may be shorter due to the culture vessels used and the growth dynamics of cells in a monolayer. This shorter exposure period does not allow the same maximum effect of a drug, regardless of concentration, that can be reached in 3D cultures over long-term culture with two treatment rounds. The multi-round 3D screening is able to show more robust response than standard 2D drug screening, including resistance to therapy. It seems this long-term culture and treatment regime is not common practice *in vitro* outside of toxicology models [134], and is a highlight of this work.

The ability to introduce more complexity in the therapy regime *in vitro* is one of the main benefits of screening with 3D culture. This would be enhanced even more by culturing under constant flow and media exchange to mimic more what is happening in the body with proximity to blood and lymphatic vessels. Some different microfluidic models have incorporated both seeding and culture of spheroids under flow are interesting examples for furture implementation [135–137]. To take this further, inclusion of a culture matrix is an important feature as well, considering the relation of the ECM to the cancer hallmarks [138], including metabolism.

With the extra dimension added going from 2D to 3D culture, extra consideration in analysis is required as well. Metrics such as confluency and absolute viability used in 2D culture do not transfer simply to 3D culture. In 3D, viability analysis requires more nuance, as presented in Paper III, with very healthy spheroids exhibiting strong dead cell staining due to large necrotic cores from significant growth. There, many measurements extracted from viability staining were integrated into principal components for the purpose of extracting trends from different cell types and treatment concentrations. The different viability staining patterns between cell lines points to the different organization and morphology of spheroids between cell lines and tumor sources. Beyond just necrotic core development, over this long culture period we see what has been referred to a spheroid cracking or tearing [139], whereby the spheroid loses its structure. This is a challenge in comparing controls to treated samples, when relying on size. The larger the spheroid grows and the more it is thriving, the sooner this may happen. This was only encountered in the CRC spheroids. What seems to be occurring is that the spheroid grows so large and the depth at which nutrients and oxygen infiltrate does not change, while the necrotic core grows larger. The ultimate structural failure has been theorized to be due to the loss of volume in necrotic cells while they still maintain adherence to other cells [130, 140]. This has been found to vary among cell types [130], depending on growth dynamics, which fits well with the differences seen here between the cell lines. Other indicators that become relevant in 3D culture include density [141] and refractive index [142].

Consistency is key in the success of any emerging method. In order for comparative and usable results to come to of 3D culture, full details of methods and anlayses used are necessary. A step in the right direction for this is the MIspheroID database [143] which is a repository for such data. Here certain information must be included to be entered into the database, forcing a minimum reporting for the purpose of comparison. In their analysis of all the metrics it becomes clear clear gaps lie in reporting of spheroid size and media formulation [143]. If detailed methods and variables are known, the effects of these can be deduced and it becomes more possible to collect quality data on drug response. This goes for both new drugs, well targeted using mechanism-informed drug discovery as discussed in the introduction, and for existing approved drugs which may be repurposed [144, 145] as anti-cancer agents if it can be seen they have adequate activity *in vitro*. Finally, with well-established methods, patient cells

can be introduced in the form of organoids [146] for the most relevant testing. Organoid cultures available commercially can be used in the method development stage and to understand any differences in response compared to established cell lines. Further, great potential lies in the information that can be extracted from treating samples from a patient's tumor specifically to understand the clinical relevance of the system, with the ultimate a goal of becoming a companion diagnostic tool to relay personal sensitivity to a therapy regimen.

5.4 Metabolic findings have translational potential

The purpose of working *in vitro* is to study cancer under highly controllable conditions, but the ultimate goal is to be implement findings for clinical translation. Modern technology has armed the field with more tools with which to analyse and interpret metabolism. The insight gained by complex methods is unquestionable but moving forward these have to be transferred into more usable techniques and monitoring for use in the clinic. One of the goals of this thesis was to gain some insight into how simpler biomarkers, such as protein expression, could be correlated with metabolic phenotype. As was presented here, it needs much more development and research. In Paper I, GLUT1 expression increased in both cell lines in physiological glucose conditions compared to high glucose, but there was no significant change or clear trend upon treatment with metformin. Thus, GLUT1 was not a indicator of either metabolic phenotype or metformin response. In Paper II, GLUT1 was too variable between experimental runs to draw any conclusions. TOMM20 expression was more consistent and decreased in all spheroid models compared to their 2D counterparts, which does agree with increased the glycolytic function also demonstrated in spheroids. It may have potential as a marker of mitochondrial function based on this and others findings [25, 26, 147]. Finally, MCT may act as a reporter of glycolysis via increased lactate transport needs. However, the data in Paper II is also quite variable and it makes it difficult to draw conclusions, especially considering the potential for MCT1 and MCT4 to be involved in either import or export of lactate [45].

When markers with well documented potential are found, options exist for clinical transition. The most likely would compound on already existing methods such as IHC in pathology analysis and biomedical imaging. If specific protein expression is the most relevant this will have to be through IHC, while flux of certain compounds like lactate and already standard glucose are best measured

through radio-labeled imaging.

How the drug response relates to metabolism was an important point to include in the final research paper. The relationship between the maximum effect of 5-FU and lower OCR/ECAR ratios, an indication of a more glycolytic metabolic phenotype, is interesting but was not significant. This is an essential relationship to investigate in later studies, as this has the most potential for translational impact from this type of drug screening study. Performing case by case metabolic phenotyping can enhance precision medicine by offering insight into effective drug combinations. Based on metabolism, more sensitive phenotypes can be produced. For example, pre-treatment with metformin could be used to shift the cancer cells to a more glycolytic phenotype (Paper I), perhaps making it more sensitive to 5-FU treatment (Paper III), or other drugs where oxidative metabolism relates to resistance [148, 149], such as proteasome inhibitors [150] and cisplatin [151]. As more glycolytic phenotypes have been found to be resistant to many different classes of drugs [152], dichloroacetate (DCA) is an alternative to push cancer cells more towards the oxidative metabolic phenotype [153]. The ability to phenotype different cancers in this way should also allow better sub-grouping of patients for analysis of response to metabolic drugs, like metformin, as discussed in Paper I. Many studies have found no significant affect upon treatment with metformin, but they also are not selectively treating cancers that may be most sensitive.

Conclusions and Future Perspectives

In vitro culture of cancer cell lines are the first line screening in research. Getting the culture conditions right in this format provides higher quality data and potentially more transferable results when transitioning to more complex testing. Cells having different metabolic phenotypes show a clear differential response to metformin treatment based on glucose concentration (Paper I). This demonstrates the importance of physiological growth conditions for experiments or clinical studies involving metabolic drugs such as metformin. Culturing cells in 2D instead of 3D also have a major effect on metabolic phenotype as well, with significant differences in glycolysis and oxidative phosphorylation (Paper II). Intuitively, 3D culture is more complex and differs greatly from flat 2D culture, but these results show that it is not just with respect to morphology and growth dynamics, but also changes in bioenergetics. Culture method ultimately plays a role in the different models' response to anti-cancer drugs (Paper III). In conclusion, both culture method and nutrient conditions are important consideration for *in vitro* cancer models. Based on the results, there is good reason to not maintain *in vitro* cultures in artificially high glucose conditions. It can have downstream affects on drug response and likely other important metrics. Furthermore, assays should be carried in 3D. If not in everyday assays, at least to validate results. Finally, metabolism even in this small scope is complex in terms of phenotypic variation. This shows the importance of metabolic screening *in vitro* to better understand the effects of these small changes and be able to model how a specific tumor may behave based on its complex metabolism.

From these experiments, the next natural step would be to expand to more cell lines. Including a wider range of primary tumor types would add more insight into variation in metabolic phenotypes and drug response, and verify any correlation between different factors. However, the ability of cell lines to mimic actual tumor behavior can be questioned. For better understanding of the actual clinical insight offered by these models, culturing organoids from patient tumor biopsies is necessary and is an ongoing project in the group. To further understand the relationship between metabolism and drug response, it would be interesting to test the metabolism of cultures after treatment as well. Knockdown of different markers to see how this changes both metabolic function and drug response is another important experiment to analyze the direct role different proteins play in these metabolic phenotypes and response profiles. Drug testing would be better in more complex, physiological system embedded in a matrix and under continuous media exchange using flow, but then throughput becomes an issue. With all or just some of these in place, especially a wider range of samples analysed, using the metrics to create *in silico* models [117, 128, 139, 154] offers a further way to analyze the relationship between the different factors and make predictions on how changes to the system may affect phenotype and drug responses. Adding different protein markers to the panel and including different protein expression analysis such as IHC or immunofluorescence would improve the understanding of protein differences in different conditions and models. As GLUT1 was not a great marker, another option is GLUT3, also a high affinity glucose transporter. Sectioning of spheroids would remove the effect of potentially harsh dissociation on sensitive and dynamic markers. Relevant drug combinations with 5-FU should be included as well, such as FOLFIRI and FOLFOX, as 5-FU is rarely used as a monotherapy in the clinic. Finally, compounding on the results here, pre-treatment of cultures with metabolic drugs based on the metabolic phenotype to increase response would fulfill the ultimate aim of this thesis. This could potentially have a large effect for difficult to treat tumors with a high incidence of chemo-resistance.

Beyond the scope of this thesis, are other important characteristics of cancers, that potentially have large affect on the drug response and metastasis: epithelial-to-mesenchymal transition (EMT), biophysical forces [77] and other cells in the TME such as fibroblasts [155, 156] and immune cells. The first plays into more fundamental research in how markers of EMT can tell us something about potential cancer response and how metabolism is related to this as well. The

latter two would be naturally included in more complex models.

Ultimately, complex *in vitro* models need to be implemented in a higher-throughput processes in the same way 2D cultures have. This is the only way we can achieve the level insight needed to really have the desired impact on the cancer research landscape. I believe this is possible and will result in much better personalized treatments for cancer patients due to repurposing and targeting of current therapies, and improved drug discovery and screening. With the latter, the drug development process can be transformed, resulting in both therapeutic and economic benefits.

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Papers

Metformin treatment response is dependent on glucose growth conditions and metabolic phenotype in colorectal cancer cells.

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Metformin treatment response is dependent on glucose growth conditions and metabolic phenotype in colorectal cancer cells

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Cancer cells exhibit altered metabolism, a phenomenon described a century ago by Otto Warburg. However, metabolic drug targeting is considered an underutilized and poorly understood area of cancer therapy. Metformin, a metabolic drug commonly used to treat type 2 diabetes, has been associated with lower cancer incidence, although studies are inconclusive concerning effectiveness of the drug in treatment or cancer prevention. The aim of this study was to determine how glucose concentration influences cancer cells' response to metformin, highlighting why metformin studies are inconsistent. We used two colorectal cancer cell lines with different growth rates and clinically achievable metformin concentrations. We found that fast growing SW948 are more glycolytic in terms of metabolism, while the slower growing SW1116 are reliant on mitochondrial respiration. Both cell lines show inhibitory growth after metformin treatment under physiological glucose conditions, but not in high glucose conditions. Furthermore, SW1116 converges with SW948 at a more glycolytic phenotype after metformin treatment. This metabolic shift is supported by changed GLUT1 expression. Thus, cells having different metabolic phenotypes, show a clear differential response to metformin treatment based on glucose concentration. This demonstrates the importance of growth conditions for experiments or clinical studies involving metabolic drugs such as metformin.

Nearly a century ago, Otto Warburg described a commonality among many cancers that is still under intense study[1]. What Warburg described was the cancer cells' ability to consume glucose, even in the presence of oxygen, later termed the Warburg effect. Cancer cells that have perfected this ability are avid glucose consumers supporting a high proliferation rate, with many cell signalling pathways primed to maintain this rapid growth[2]. Thus, many studies show that calorie restriction and nutrient deprivation may be both cancer preventative and may enhance treatment response[3]. However, lowering blood glucose within fasting range (< 5 mmol/L) seems to not be enough to prevent cancer growth, as cancer cells express high affinity glucose receptors[4–6], which even at fasting glucose levels, as low as 1 mmol/L, are able to import glucose[7]. However, lowering glucose levels may compromise the metabolic flexibility of these cells under stress[8]. Therefore targeting cancer metabolism is an interesting avenue to pursue, and has prompted many recent studies testing the efficacy of metabolic drugs for cancer treatment[9]. One drug that has spurred great interest is metformin, normally used to treat type 2 diabetes (T2D). Metformin use in diabetes is associated with lower incidence in many cancer groups worldwide compared to diabetic patients not using metformin[10–13]. It has since been studied in pre-clinical settings using in vitro cancer cell models[14–16], animal models[17–21], and consequently over 300 clinical trials are found when searching "metformin AND cancer" in clinicaltrial.gov. The high number of initiated clinical trials reflect both the low cost of the drug and the extensive use with minimal side effects reported since it was approved as a drug nearly 60 years ago[22,23]. However, many of these studies have so far been inconclusive, and have not shown major treatment effects nor improved survival[23–26]. The low success rate may be due to many reasons, but lack of patient stratification, metformin dosage, and mode of delivery seem to be areas to address. A regular metformin treatment in diabetic patients starts at 500–850 mg administered orally every 12 h, increasing to, but no more

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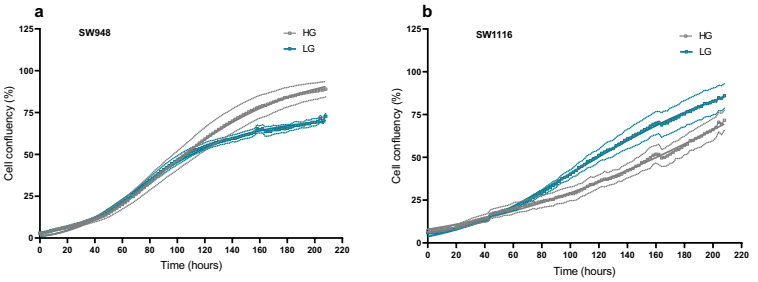

Figure 1. Cell growth recording of SW948 and SW1116 in HG and LG over 9 days continuous monitoring using Incucyte ZOOM. Cell confluency was analyzed in the Incucyte ZOOM software and plotted here as confluency (%) over time (hours). (**a**) SW948, initial seeding density of 10,000 cells/well. (**b**) SW1116, initial seeding density of 20,000 cells/well. Complete growth medium was exchanged at day 2 and 7 of the culture period. Grey solid line depicts growth in high glucose (HG) conditions, whereas blue solid line represents growth in low glucose conditions (LG). The dotted lines represent standard deviation from the average of N = 8 for each condition.

than 2550 mg/day, achieving a steady state concentration of 1.4 mg/L (10.8 μM)[27]. As an orally ingested drug, its highest concentration is found in the gastrointestinal (GI) tract, and GI derived tumours would most likely be affected[28], which further indicates that achieved drug concentration play a major role in tumour responses. This is recapitulated in its various mode of action ranging from influence on microbiota[29], immune modulation[30], and direct intracellular effect[31].

Metformin became an interest in the cancer field due to studies reporting that diabetic colorectal cancer (CRC) patients taking metformin had a better overall survival compared to those treated with other glucose lowering drugs[14,26,32,33]. This effect has largely been tied to the lowering of insulin growth factor and activation of AMPK[22,34–36]. Hence, the potential anticancer effect of metformin is most likely not due to expected lowering of blood glucose, but rather due to intracellular effects of metformin in the tumour cells[37]. Once inside the cell, the exact target of metformin has been difficult to pinpoint, although a change in mitochondrial function is one major effect seen[34–36], attributed to its inhibition of the electron transport chain (ETC)[37]. Metformin has also been shown to reduce GLUT1 expression alongside HIF-1α[38]. GLUT1 is a rate limiting transporter for glucose metabolism and involved in maintaining higher levels of glycolysis intermediates[39,40]. Clinically, its increased expression has been correlated with tumour aggressiveness and is correlated with increased proliferation activity and poor survival[5]. However, this increase could be either due to an oncogenic transformation in cells or indirectly due to high glucose consumption of cancer cells and resulting low intracellular glucose levels[40]. On the other hand, reduced glycolysis due to lower levels of GLUT1 has been associated with less malignancy[40]. Monitoring GLUT1 levels under the previously mentioned conditions could be an indicator of the adaptive mechanisms different metabolic phenotypes undergo in their response to glucose levels and metformin treatment.

In the last decades, there have been a few major breakthroughs in cancer drug discovery[41], however a fundamental issue for drug development is the discrepancy in drugs' effects once they reach efficacy testing in humans, despite promising results in earlier pre-clinical model systems[42]. One major challenge relates to the unphysiological metabolic conditions normally applied in cell cultures, often using supraphysiological levels of glucose in the growth medium. Moreover, most studies investigating metformin have applied higher concentrations than what is achievable in vivo[14,34,37,43]. Metformin is administered orally, typically achieving concentrations at up to 300 times higher in the GI tract than that found in plasma[44]. The aim of this study was therefore to test how colorectal cancer cells responded to metformin at a concentration typically found in the GI tract and grown in physiological glucose media. We show that using physiologically relevant glucose levels in cell growth media could provide a response to metformin that is more representative to in vivo conditions and that GLUT1 may be used as a metabolic biomarker for studying these responses.

Results

Glucose concentration in culture media affect cellular proliferation rates. A glucose concentration of 25 mmol/L is commonly used for in vitro cell culture, and this is from here-on referred to as high glucose (HG). The physiological glucose concentration in blood at fasting state is around 5 mmol/L, here referred to as low glucose (LG). We found that glucose concentration in the growth media directly affects the proliferation rates of two cell lines SW948 (Fig. 1a) and SW1116 (Fig. 1b) with differential metabolic phenotypes (Fig. 3). By culturing them in HG and LG over 9 days with two full media changes (day 2 and 7), we found that the doubling time of SW948 is 25.1 ± 1.2 h and 25.0 ± 0.4 h in HG and LG, respectively. Whilst for SW1116 the doubling time is 74.0 ± 10.1 h in HG and 41.8 ± 2.4 h in LG ($p < 0.0001$). The glucose effect is not immediate in either cell line,

where in SW948 the effect on proliferation becomes apparent only when the stationary growth phase begins, thus showing lower cell confluency in LG. In contrast, glucose has a significant effect on the exponential growth of SW1116, with low glucose conditions resulting in increased proliferation.

Metformin predominantly suppressed the proliferation of SW948 and SW1116 cells under physiological glucose conditions in comparison to high glucose culture conditions. To see if the concentration of glucose in the growth media affects the cellular response to metformin treatment, we tested a range of metformin concentrations up to the clinically achievable range and higher only achievable in vitro. Since many proliferation assays measure metabolic activity (Alamar blue, WST-1), which may be affected by metformin treatment directly, the two assays we used were compared to nuclei count for verification of results. We found that after 48 h of metformin treatment, the SW948 cells (Fig. 2a) exhibit a concentration-dependent reduction in viability in HG up to 42% in 24 mM metformin treatment compared to HG control, which is consistent with corresponding cell counts. However, in LG, the CCK-8 measured viability of SW948 is reduced to 40% in comparison to LG control, even at the lowest metformin concentration (1.5 mM). At metformin treatments of 6 mM and lower there are discrepancies between the two viability measurements, whereas both cellular viability and cell numbers show more than 90% reduction in viability to control during metformin treatment of 12 mM.

There is no observed reduction in the viability of SW1116 cells (Fig. 2b) in HG using metformin concentrations up to 6 mM, while 12 and 24 mM concentrations result in viabilities of 86% and 72%, respectively. Cell numbers are lower than control across all treatments but follow the same trends as the CCK-8 assay results. In LG, the metformin effect is again found to be concentration-dependent with a reduction in viability from 61% (1.5 mM) to 11% (24 mM). The reduction in cell numbers is also consistent in a stepwise fashion with the increasing metformin concentrations and corresponds to CCK-8 viability results.

Metabolic phenotype plays a role in response to metformin. Metformin is thought to affect mitochondrial function, although its specific target in the mitochondria has been a source of debate. To investigate effects of metformin on mitochondrial respiration and glycolysis in the two cell lines, we measured the cellular oxygen consumption rate (OCR) and lactate production assessed via extracellular acidification rate (ECAR). Specific protocols involving sequential addition of modulators were used to test key functions of oxidative phosphorylation in the metabolic flux analyzer Seahorse XFe96 (see "Methods"). In SW948, there is no significant change in oxygen consumption rate (OCR) between high (7.953 ± 0.905 pmol O_2/min/µg protein) or low glucose (7.791 ± 0.407 pmol O_2/min/µg protein) medium (Fig. 3a). However, 48 h metformin treatment causes a significant drop in OCR levels in both glucose conditions (HG: 1.571 ± 0.216 pmol O_2/min/µg protein, $p < 0.0001$; LG: 1.862 ± 0.182 pmol O_2/min/µg protein, $p < 0.0001$) (Fig. 3a). In SW1116 cells there is a significant drop in OCR when cells are grown in low glucose compared to high glucose conditions (9.839 ± 0.598 and 7.175 ± 0.522 pmol O_2/min/µg protein, respectively, $p = 0.0018$). Like SW948, the metformin treatment in SW1116 also causes a drop in OCR in these cells under both glucose conditions (HG: 1.979 ± 0.342 pmol O_2/min/µg protein, $p < 0.0001$; LG: 1.737 ± 0.179 pmol O_2/min/µg protein, $p < 0.0001$) (Fig. 3a). Both cell lines exhibit similar normalized basal respiratory levels in control conditions and after 48 h with metformin treatment (Fig. 3a), when compared to one another. The ATP-linked respiration (Fig. 3b), revealed by oligomycin inhibition of ATP-synthase, was not significantly affected by high and low glucose concentrations in either cell line. While after metformin treatment, this is lower albeit not significant compared to the control. CCCP was used to uncouple the mitochondria and measure respiration under mild stress conditions. This CCCP-uncoupled respiration increases in both cell lines when grown in low glucose media and is further increased after metformin treatment (Fig. 3c). In SW1116, the increase in CCCP-uncoupled respiration (HG: $p = 0.0191$; LG: $p = 0.0314$) is significant compared to control. To see if HG or LG media and metformin influenced the cells' ability to use glycolysis, we performed a glycolysis stress test. We found that in SW948 there is no significant change in the cells' ability to use glycolysis under any of the conditions nor after metformin treatment (Fig. 3d), while metformin treated SW1116 cells show significantly increased glycolysis compared to control (HG: $p = 0.0027$; LG: $p = 0.0083$) (Fig. 3d). To see if either cell line had spare glycolytic capacity, we added oligomycin to block ATP synthesis in the mitochondria. We found that SW948 did not have increased glycolytic capacity, whereas SW1116 cells increased glycolytic capacity by over 170% in both high and low glucose control conditions. However, this glycolytic capacity is significantly lower after 48 h metformin treatment (HG: $p < 0.0001$; LG: $p < 0.0001$) (Fig. 3e). The absolute levels of normalized OCR and ECAR in each condition are included in supplementary information (Supplementary Fig. S2). The metabolic profiles based on basal levels of OCR and ECAR (Fig. 3f) show the differences and comparative shift of the cell lines in both glucose concentrations and metformin treatment. Untreated, SW948 are more glycolytic, while SW1116 are more aerobic. Following metformin treatment, both cell lines are less energetic, but perhaps more noteworthy is that SW1116 converges with SW948 at a more glycolytic phenotype.

Glut1 is affected by growth media and metformin treatment. We analyzed protein expression of one of the major glucose import proteins, glucose transporter protein 1 (GLUT1), and how it correlates to the mitochondrial and glycolytic changes found in the metabolic flux analysis. GLUT1 significantly increases in SW948 cells under low glucose conditions compared to high glucose conditions. The corresponding change after metformin treatment is, however, not significant in these cells and follows the same pattern as glucose response (Fig. 4a). Similarly, in SW1116 grown in low glucose concentration, the GLUT1 expression increases compared to high glucose. Furthermore, no further increase is seen after metformin treatment (Fig. 4a). To verify location of GLUT1 expression and mitochondrial detection throughout the different growth conditions and metformin treatment we performed a multi-stained confocal analysis (Fig. 4b,c) for both cell lines, identifying GLUT1 in

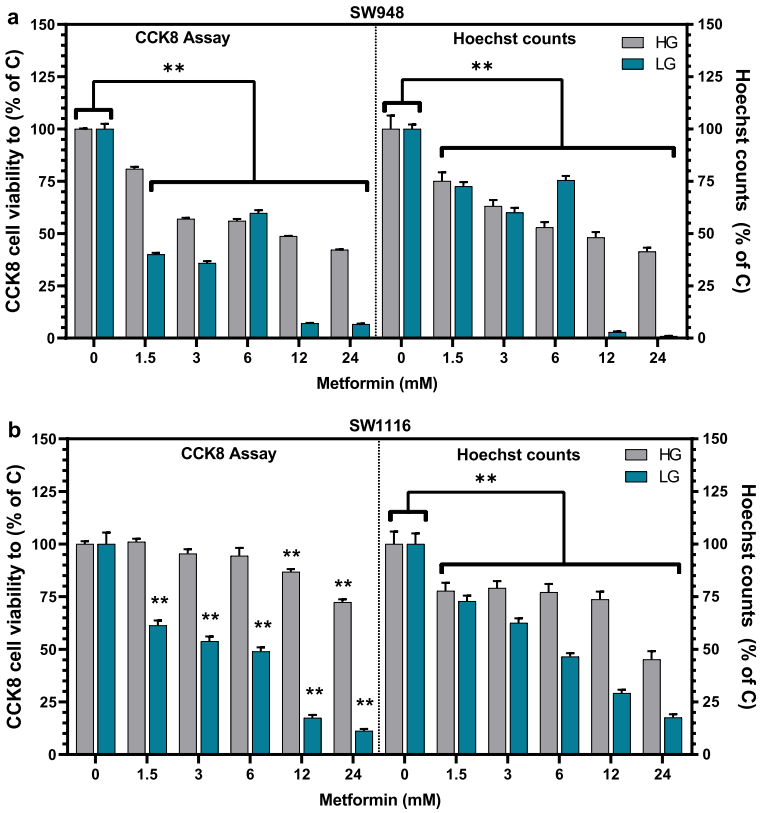

Figure 2. Viability assays of SW948 and SW1116 after metformin treatment for 48 h. The effect on the viability of (**a**) SW948 cells and (**b**) SW1116 in HG (25 mmol/L) and LG (5 mmol/L) is presented. Viability was calculated using CCK-8 (left) and cell counts were scored using fluorescent microscopy on Hoechst stained cells (right). CCK-8 absorbance values and the calculated cell numbers for all metformin treatments (1.5–24 mM) were compared to high glucose control (25 mmol/L, 0 mM Metformin) to show relative viability and cell numbers. Error bars denotes s.e.m, and statistical analysis was calculated using two-way ANOVA (** is $p < 0.05$) in GraphPad Prism (N = 3). *C* Control, *HG* high glucose, *LG* low glucose.

the cell plasma membrane during all conditions. We found no apparent change in mitochondrial morphology, nor in intracellular localization across the different treatments in either of the cell lines.

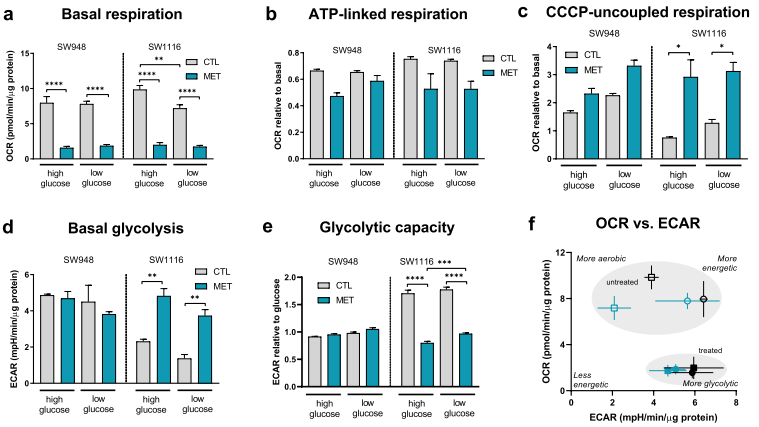

Figure 3. OCR and ECAR were measured in the Seahorse XF96 instrument, with injections of sequential compounds and concentrations according to the assays detailed in the methods section. Mitochondrial stress test assay: (**a**) Basal respiration, before any injections. (**b**) ATP-linked respiration, after oligomycin injection, shown relative to basal OCR level. (**c**) CCCP-uncoupled respiration, shown relative to basal OCR level. Glycolysis stress test assay: (**d**) Basal glycolysis, after glucose injection. (**e**) Glycolytic capacity, after oligomycin injection, shown relative to glycolysis level after glucose injection. Error bars denote s.e.m. Statistical analysis was performed using one-way ANOVA in GraphPad Prism (*$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$) ($N=2–8$) (**f**) OCR vs ECAR from the mitochondrial stress test assay. Squares: SW948; Circles: SW116. Metabolic phenotypes of untreated (open symbols square, circle: control) and treated (closed symbols circle, square: 3 mM metformin) samples. Black: high glucose (25 mmol/L); Teal: low glucose (5 mmol/L). Error bars denote s.d. *OCR* Oxygen concentration rate, *ECAR* extracellular acidification rate, *CTL* control 0 mM metformin, *MET* metformin 3 mM pre-treatment for 48h.

Discussion

Here we show how growth conditions influence the metformin response in different cell lines, specifically concentration of glucose in the cell growth media. Using clinically achievable levels of metformin and changing the glucose concentration in the growth media for colorectal cell lines, SW948 and SW1116, resulted in changes in cell growth, metabolic flux, and protein expression. The glucose concentration directly influenced the growth rate in SW1116, where doubling time decreased in low glucose (5 mmol/L) media compared to high glucose (25 mmol/L), suggesting that glucose itself imposes an inhibitory growth effect in these cells. Comparatively, for SW948 there was no change in doubling rate between high glucose versus low glucose media. Most standard growth medias are formulated with high glucose content to avoid nutrient deprivation over time, however the finding that high glucose levels may mask true drug responses is not well considered.

We found that SW948 and SW1116 colon cancer cell lines were distinguishable in their ability to use their mitochondria, as shown in results from metabolic flux analysis. SW948 is, under non-stressed conditions (basal), running at maximum glycolytic capacity, further supported by our finding that under low glucose growth conditions these cells increased their expression of GLUT1 receptors. This could be a compensatory response for keeping up the high glucose flux through glycolysis with the lower amount of glucose available in the media as previously described[45], thus supporting rapid proliferation. As SW948 resemble Warburg's phenotype, i.e. rapid proliferation and are avid glucose consumers, they show an inability to maintain rapid proliferation under the mitochondrial-inhibiting effects of metformin even with an abundance of glucose, since glycolysis alone cannot support a high proliferation rate without mitochondrial contribution of biomolecules[2]. This is contrary to SW1116 cells that utilize their metabolic flexibility to overcome the effects of metformin by upregulating glycolysis, supporting their lower proliferation rate. In both glucose concentrations, SW1116 cells exhibit low to no additional respiration under CCCP stress; however, when treated with metformin, the relative rates of CCCP-uncoupled respiration to basal OCR increases significantly.

The abundance of glucose in culture media seems to affect the way both investigated metabolic phenotypes of cancer cells react to metformin. SW948 is more glucose dependent and able to thrive and grow exponentially in both glucose concentrations by altering their GLUT1 expression response to adapt to different conditions.

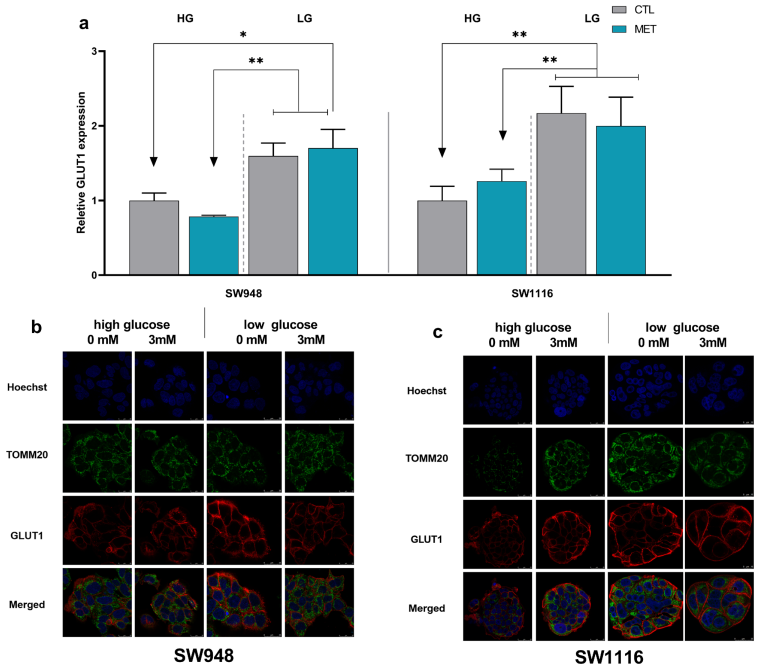

Figure 4. Protein expression analysis of GLUT1 in response to metformin treatment under different glucose culturing conditions. (**a**) Bars represent the relative GLUT1 fold inductions, measured by flow cytometry after 48 h metformin treatment in SW948 and SW1116, compared to respective controls at same glucose culturing conditions. Representative confocal images of (**b**) SW948 and (**c**) SW1116 using Hoechst for nuclei staining in blue, TOMM20 for the mitochondria in green, and GLUT-1 Antibody in red. Error bars denotes s.e.m. and statistical analysis was calculated using two-way ANOVA (*$p < 0.05$, **$p < 0.01$) ($N = 3$). *MET* Metformin, *CTL* control, *GLUT1* glucose transporter 1.

SW1116 is more metabolically adaptable to glucose concentration considering its GLUT1 and metabolic flux response. Here we found that after treatment in high glucose concentration, SW1116 show a slight increase in GLUT1 which could be due to their attempt to shift their metabolism towards glycolysis upon mitochondrial stress induced by metformin. SW948 had reduced GLUT1 expression in high glucose with treatment. Both cell lines had increased GLUT1 expression in the low glucose concentration, however SW1116 had a much larger increase, in line with their significant increase in basal glycolysis. In general, SW948 keep their glycolytic dependency in low glucose compared to high glucose, but the cell proliferation ultimately slows, which could mean they have less adaptability to glucose concentration compared to SW1116. In high glucose and with metformin treatment, there is a significant drop in OCR due to the inhibition of the ETC in both cell lines; similar results have been shown elsewhere[46]. Inhibition of complex I causes a drop in the ATP-linked respiration under metformin treatment. This could mean there is an unmet need of ATP production, which could cause a drop in proliferation or an increase in glycolysis. A higher uncoupled respiration in the metformin treated samples points to remaining functionality in the mitochondria, but it is reserved for acute stress, as induced by the mitochondrial uncoupler CCCP. The SW1116 cells showed a decreased response to metformin when cultured in high glucose media. This could be due to a metabolic shift towards glycolysis in a likely attempt to compensate for the metformin-driven inhibition of the ETC and TCA and resulting decrease of ATP-production in the mitochondria. However, in

both cell lines physiological glucose levels reveal an underlying concentration-dependent response to metformin. Three different assays were used here to assess the metformin response in our cell lines, presented by CCK-8 assay and direct cell counts (Fig. 2), and an Alamar blue assay (Supplementary Fig. S1). The Alamar blue assay, also known as resazurin assay, is commonly used to assess viability, however in our experimental set up did not correlate with counted cell numbers from the same assay wells. The CCK-8 assay which also measures metabolic activity, however with a different chemical reaction, showed good correlation to actual cell counts. We would thus advise caution when using viability assays that rely on metabolic activity to measure responses of metabolic treatments, without including a second confirming analysis as presented here by Hoechst cell counts (Fig. 2). This is well supported by previous studies who have found the same effect when using resazurin[47,48]. The sensitivity of cancer cells to drug treatments in different glucose concentrations is not only relevant for in vitro testing, but also clinically where many patients may present with T2D and elevated blood glucose levels, which has been associated with chemoresistance[49].

Not all cancer cells exhibit Warburg metabolism. This is becoming increasingly documented, both in vitro and clinically[50–52]. The results we see here of the difference in response of cell lines with different metabolic phenotypes, could explain the lack of response in the many clinical trials studying metformin. If only metabolically compatible cancers will respond and there are no criteria for treatment based on this, then it is unlikely a clear response would be seen when studying a mixed-phenotype clinical cohort. We found that metformin treated SW1116 shift toward a more glycolytic profile resembling that of the SW948 cell line. Fast proliferation is what is being targeted by cytotoxic chemotherapy drugs and SW948 is documented as being more susceptible to these, exhibiting lower IC50 to both 5-fluorouracil and oxaliplatin (GDSC2)[53]. However, cytotoxic drugs do seem to be targeting more than just proliferation[54], with one possible target being metabolic reprogramming. If this is the case, the metabolic shift of SW116 to be more like SW948 could then also result in increased vulnerability to chemotherapy. In this vein, metformin may be used a neoadjuvant agent in an effort to increase response[55–58]. If patient tumours are assessed for their metabolic phenotypes, either by metabolic analysis of biopsy tissue[59] or advanced tumour imaging[60–62], this can be directly translated to the clinic for improved course of treatment.

Methods

Cell culture, proliferation, and viability assessments. SW948 and SW1116 were purchased from ETCC and cultured under humidified conditions in a 5% CO_2 incubator at 37 °C. The culture media DMEM contained no glucose, (Corning, New York, USA) and was supplemented with 10% fetal bovine serum (FBS) (BioWest, Nuaillé, France), 2 mM (0.584 g/L) L-glutamine (Corning, New York, USA), penicillin (100 U/mL) and streptomycin (100 µg/mL) (Merck Millipore Corporation, Burlington, USA). For glucose experiments the DMEM media was supplemented with 25 mmol/L or 5 mmol/L glucose (Sigma-Aldrich, St. Louis, USA) concentrations for high glucose (HG) and low glucose (LG) culture conditions, respectively. The cells were acclimated to the glucose levels by being cultured and passaged several times in the respective glucose concentrations prior to metformin experiments. *Proliferation:* Cells were seeded in 96-well plates in 25 mmol/L or 5 mmol/L glucose supplemented media at a density of 20,000 cells/well or 10,000 cells/well for SW1116 and SW948, respectively. The plates were placed in the Incucyte ZOOM system (Essen Bioscience, Newark, United Kingdom) and monitored for 9 days with phase contrast images captured every 2 h. Media was exchanged on days 2 and 7. Growth was measured by analyzing the confluence of the cells over time using the Incucyte ZOOM software and reported as percent of image area covered. Doubling times were calculated during their respective log phases: 24–74 h for SW948 and 50–100 h for SW1116. Statistical analysis was performed as described in methods using an unpaired Student's t-test. *Viability:* Both cell lines were treated in 96-well plates at an initial seeding density of 10,000 cells/well using increasing concentrations of 1.5–24 mM of metformin hydrochloride (Sigma-Aldrich, St. Louis, USA) to determine the cellular viability after 48 h. Both Alamar blue (Resorufin) and CCK-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assays were carried out according to the manufacturer's protocol to estimate cell viability using fluorescence (Ex: 540 nm, Em:590 nm) and absorbance (450 nm) respectively via SpectraMax Paradigm plate reader (Molecular Devices, San Jose, USA). Cells in the Alamar blue plates were post-stained with Hoechst 33,342 (5 µg/mL) after fixation using 4% paraformaldehyde for 30 min, upon imaging using Leica SP8 Florescence microscope (Leica Microsystems, Mannheim, Germany). Image analysis using density counting of the nucleus was performed using ilastik[63]. Statistical analysis was calculated using two-way ANOVA.

Metabolic analysis. Mitochondrial respiration and glycolysis were measured using the Seahorse XF96e flux analyzer (Agilent Technologies, Santa Clara, USA). Cells were seeded in XF96e cell culture plates at a density of 20,000 cells/well or 10,000 cells/well for SW1116 and SW948, respectively. They were allowed to attach overnight before treatment with 3 mM metformin hydrochloride for 48 h. Prior to the mitochondrial respiration assay, culture media was exchanged for unbuffered, serum-free DMEM, composed of DMEM 8.3 g/L (D5030, Sigma-Aldrich, St. Louis, USA) pH 7.4, NaCl 1.85 g/L (Sigma-Aldrich, St. Louis, USA), 2 mM L-glutamine (Corning, New York, USA), and glucose (concentration dependent on condition as described in results) (Sigma-Aldrich, St. Louis, USA). For the glycolysis assays, the assay media contained no glucose. The plates were then incubated at 37 °C in a CO_2-free incubator for 1 h prior to running the assay. Oxygen Consumption Rate (OCR) and ExtraCellular Acidification Rate (ECAR) were measured over 100 min (15 mix and measure cycles), with compounds being injected every 3 cycles. For the mitochondrial respiration assays, the following compounds (Sigma-Aldrich, St. Louis, USA) were injected sequentially (final concentrations in the wells): Oligomycin (3 µM), CCCP (0.25 µM), Rotenone (1 µM), and Antimycin A (1 µM). For the glycolysis assays, the following compounds (Sigma-Aldrich, St. Louis, USA) were injected sequentially (final concentrations in the wells): glucose (10 mM), oligomycin (3 µM), CCCP[64,65] (0.25 µM), 2-deoxy-D-glucose (100 mM). Protein concentration

was measured in each well for normalization using a Pierce BCA assay (ThermoFisher Scientific, Rockford, USA) according to manufacturer's instructions. Statistical analysis was performed using one-way ANOVA.

Flow cytometry quantification of Glut1. SW948 and SW1116 cells were treated with 3 mM of metformin for 48 h at a seeding density of 1.0×10^6 cells per well using HG and LG media. After treatment, cells were trypsinized and washed twice with PBS before adding 4% Paraformaldehyde (PFA) fixation and incubating on ice. The fixed cells were subsequently incubated with GLUT1 primary antibody (Abcam, Cambridge, United Kingdom) at the concentration 1:500 for 1 h at room temperature. GLUT1 labelled cells were washed twice in PBS and labelled with Alexa fluor 647 conjugated Donkey anti-rabbit secondary antibody (Abcam, Cambridge, United Kingdom) for another 30 min before analysing with Accuri C6 flow cytometer (BD Biosciences, San Jose, USA).

Confocal imaging. Both cell lines were seeded in Ibidi µ-Slide 8-well chambered coverslips (Ibidi GmbH, Munich, Germany) at a density of 30,000 cells/well and were allowed to attach overnight in HG and LG media. 3 mM metformin treatment was added for 48 h before the cells were washed with PBS and fixed using 4% PFA. After 1 h of incubation with 20% FCS blocking solution, the cells were incubated overnight with GLUT1 Alexa fluor 647 conjugated antibody (Abcam, Cambridge, United Kingdom) (1:1000 in blocking solution). The next day, the wells were washed again and the cells permeabilized using 0.5% triton X in PBS for 15 min at room temperature**,** before another incubation step using TOMM20 antibody (Abcam, Cambridge, United Kingdom) 1:1000 in blocking solution overnight. The next day, cells were counterstained with Hoechst 33342 (ThermoFisher Scientific, Rockford, USA) (15 µg/ml solution) for 2 min, then washed 3 times with PBS. The cells were then imaged on a Leica TCS SP8 confocal microscope (Leica Microsystems, Mannheim, Germany)**.**

Statistical analysis. Statistical comparisons were made using GraphPad PRISM (version 8, GraphPad Software, Inc., USA) software with one-way or two-way ANOVA to determine significant differences between several treatment groups. Post-hoc corrections for multiple comparisons were applied according to recommendations by GraphPad for each experimental data set (viability: Dunnett; metabolic analysis: Sidak; flow cytometry: Tukey). A student's unpaired t-test was employed when only two groups were compared. The number of biological replicates (N) are given in the figures and legends. Values that follow ± within the results section are standard deviation (s.d.).

Data availability

The datasets generated during the current study are available on figshare, https://doi.org/10.6084/m9.figshare.13490271.

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Metabolic flux analysis of 3D spheroids reveals significant differences in glucose metabolism from matched 2D cultures of colorectal cancer and pancreatic ductal adenocarcinoma cell lines

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Enhanced drug screening of CRC and PDAC multicellular spheroids using long-term culture and multi-round exposure to 5-fluorouracil

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Review

Aging, Metabolism, and Cancer Development: from Peto's Paradox to the Warburg Effect

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ABSTRACT: Medical advances made over the last century have increased our lifespan, but age-related diseases are a fundamental health burden worldwide. Aging is therefore a major risk factor for cardiovascular disease, cancer, diabetes, obesity, and neurodegenerative diseases, all increasing in prevalence. However, huge inter-individual variations in aging and disease risk exist, which cannot be explained by chronological age, but rather physiological age decline initiated even at young age due to lifestyle. At the heart of this lies the metabolic system and how this is regulated in each individual. Metabolic turnover of food to energy leads to accumulation of co-factors, byproducts, and certain proteins, which all influence gene expression through epigenetic regulation. How these epigenetic markers accumulate over time is now being investigated as the possible link between aging and many diseases, such as cancer. The relationship between metabolism and cancer was described as early as the late 1950s by Dr. Otto Warburg, before the identification of DNA and much earlier than our knowledge of epigenetics. However, when the stepwise gene mutation theory of cancer was presented, Warburg's theories garnered little attention. Only in the last decade, with epigenetic discoveries, have Warburg's data on the metabolic shift in cancers been brought back to life. The stepwise gene mutation theory fails to explain why large animals with more cells, do not have a greater cancer incidence than humans, known as Peto's paradox. The resurgence of research into the Warburg effect has given us insight to what may explain Peto's paradox. In this review, we discuss these connections and how age-related changes in metabolism are tightly linked to cancer development, which is further affected by lifestyle choices modulating the risk of aging and cancer through epigenetic control.

Key words: Cancer, aging, mitochondria, metabolism, Warburg effect, Peto's paradox, epigenetics.

Human evolution has selected for somatic maintenance strategies that maximize reproductive success. However, the last century has provided us with a challenge where technology and lifestyle adjustments are outpacing natural evolutionary adaptation. Many of the previously life-shortening diseases, such as bacterial infections and viral diseases, can now effectively be treated, but other lifestyle-related diseases are increasing. Normal physiological responses are influenced by lifestyle habits such as high caloric diets, dysregulated sleep patterns, and toxic environmental factors; all common in modern Western civilization and known risk factors for

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developing cancer. The increase in life expectancy due to improved living standards and medical advances introduces the additional challenge of finding new treatments to treat the accumulation of age-associated diseases, including cancer.

As the aging population grows, cancer remains a fundamental health issue. Therefore, understanding its etiology and weaknesses to improve treatment is a major research focus worldwide. The lifetime risk of cancer has been associated with the number of stem cell divisions needed to maintain that tissue's homeostasis, suggesting that acquired somatic nuclear mutations over time due to "bad luck" are the primary causes of cancers in these tissues. However, such a model fails to account for key observations of early-life mutation accumulation (50% before full body maturation) [1], the size and scaling of cancer incidence with the lifespans of various animals (Peto's paradox) [2], and epidemiological research showing cancers of human populations differ based on geographical areas [3]. A more fitting model would be to ascribe the high incidence of cancers in tissues with more stem cell divisions to a buildup of bioenergetic dysfunction over time, which may confer a selective growth advantage in an aging tissue microenvironment. This could be affected, not only by inherited "bad luck" nuclear mutations, but more importantly, or external factors such as acquired epigenetic modifications, mtDNA mutations, or intrinsic asymmetric segregation of cargo during cell division.

Aging and metabolic control

Aging is defined as a physiological decline that leads to the loss of major organ function, ultimately leading to death. The question remains as to what is the cause of aging as, from an evolutionary perspective, an organism would benefit most from extended reproductive ability and lifespan. A possible answer is that aging is but a side effect of life progression and not a programmed occurrence. Supporting this is the fact that rates of aging are not fixed; for example, lower body temperatures tend to result in extended lifespan [4], possibly due to metabolic adaptations. Considering the environmental effects on rates of aging, it has become increasingly relevant in aging research to differentiate between physiological age and chronological age [5]. It is possible for two humans of identical or similar years of life, or even same genetic background as with monozygotic twins, to have very divergent states of health and lifespans [6]. The last decade of scientific research has dramatically improved our understanding of the aging process and that it is closely regulated by key metabolic proteins such as mechanistic target of rapamycin (mTOR), AMP-activated protein kinase (AMPK), and insulin/insulin growth factor (IGF) [7, 8], which are associated with age-related metabolic syndrome [9] and common to those found dysregulated in cancer [10]. The long-term causative effect of aging and how this relates to increased cancer risk therefore seems to be linked through metabolic control.

Alternative to the mutational theory of cancer, the metabolic theory of cancer development is that small undetected changes in genes regulating metabolism, or mitochondrial genome mutations, can reach a threshold over time whereby it effects whole cell metabolism and confers a selective advantage for growth of that cell in an aging tissue environment [11]. Aging is linked to both the accumulation of genomic defects and that of defective proteins and organelles such as mitochondria [12, 13]. Consequently, mitochondrial defects can become prevalent in dividing stem cells by asymmetrical segregation of cell cargo. On the other hand, inheritance of "good" cargoes can enhance cell health and responsiveness, whereby more of the dysfunctional cargo is delivered to the daughter cell, which will go on to terminal differentiation, thus protecting the original stem cell [12]. Both internal and external factors may affect the growing number of dysfunctional mitochondria, speeding up the physiological aging process and cancer risk (Figure 1). However, while chronological age is immutable, physiological age depends on lifestyle choices and can be shifted to exert a beneficial effect by extending length of life and reducing disease risk.

Discoveries made in the last decade showing that most of the known oncogenes and tumor suppressor genes are metabolic regulators has rekindled Warburg's discoveries made over a lifetime ago, highlighting the importance of metabolic control in any cell. The understanding that proteins and metabolites may be the instigators of aging and cancer development through epigenetic regulation is now a renewed research topic. This non-static mechanism of aging and cancer is gradually being accepted and helps explain why large long-lived animals with slow metabolism have a lower risk of developing cancer than humans. Of course, exposure to environmental factors are associated with increased cancer risk and can also contribute to changes in an aging system. However, these events will not be the primary focus here, since even in their complete absence, aging and cancer would occur. Therefore, this review focuses on the intrinsic events that may lead to aging and how they relate to cancer development, with a focus on the role of mitochondria and metabolism.

Mitochondrial role in cell metabolism

Mitochondria are remnants of an aerobic prokaryote that brought the selective advantage of using respiration for

higher yield energy production to a cell dependent on rudimentary substrate fermentation. The endosymbiotic event that occurred some two billion years ago is thought to only have happened once to give rise to all advanced lifeforms known today.

Mitochondria have retained some features of a prokaryote such as double membranes and their own DNA (mtDNA), which encodes for proteins and RNAs that are mostly involved in assembling components of the electron transport chain (ETC) [14]. Mitochondrial DNA mutations, deletions, and copy number changes can result in ETC dysfunction and are believed to accumulate with aging. The increase in dysfunction of energy homeostasis with age and increase in reactive oxygen species (ROS) has been the center of the free radical theory of aging [15]. Reactive oxygen species (ROS) have also been shown to affect ETC indirectly. In *C. elegans*, repression of the *PRDX-3* gene, involved in the detoxification of mitochondrial hydrogen peroxide in the ETC, did not alter the level of ROS or life length, but instead caused mitochondrial uncoupling and decreased adenosine triphosphate (ATP) production [16], suggesting an induced compensatory response. Continuous ROS exposure has been shown to affect mitochondrial oxidative phosphorylation (OXPHOS) and ATP production by lipid peroxidation of cardiolipins [17]. Cristae invaginations caused by the unique properties of cardiolipins is essential for efficient oxidative energy production and mitochondrial function [18, 19]. Defects in the cristae formation (i.e. lipid peroxidation due to ROS) can increase the leakiness of the inner mitochondrial membrane, consequently reducing the mitochondrial ATP production [20]. Alterations of cardiolipin through years of ROS exposure may lead to gradually reduced membrane potential and consequently depolarized mitochondria with less efficient ATP production. Another consequence is an imbalance in the metabolite levels in the aging cell which could affect gene regulation and transcription epigenetically. This nuclear-mitochondrial retrograde signaling, where gene expression is regulated by metabolic substrate levels, is important to respond appropriately to metabolic stress for restoration of cell homeostasis [21, 22]. One responder to this retro-grade signaling is the evolutionarily conserved human polymerase delta (*POLD1*) gene, which is involved in multiple forms of DNA repair, and found mutated in tumors and aging [23]. The expression of this protein is further dysregulated in diabetes [24] and can be modulated by enzymes involved in metabolism such as lactate dehydrogenase and 3-phosphoglycerate [25]. This retrograde signaling from mitochondria to nuclei is triggered in normal cells by changes in metabolite levels or altered proteostasis [22, 26, 27]. Response mechanisms can include upregulation of metabolic pathways producing more metabolites, including reactive oxygen species, to act as second messengers to tune signaling pathways in the cytoplasm or directly affect gene regulation through epigenetic events. Thus, the energy status of the cell is directly linked to its replicative and reparative functions, demonstrating how metabolic substrates and enzymes regulate cell turnover [28]. In support of dysfunctional metabolism controlling cell growth, laser capture of cancer cells from colon tissue, selected by the expression of a metabolic biomarker, were deep sequenced and shown to contain mtDNA mutations from the same lineage and progenitor cell [29]. This suggests that the cancer stem cell could be traced solely by mtDNA mutations, independent of any nuclear DNA mutations. However, tumors are functionally heterogeneous and harbor subsets of cancer cells with stem-like features. Consequently, mutations of the mitochondrial genome have been tightly linked to impairment of cellular energy conversion and tissue function [30-32], and further implicated in the pathophysiology of age-associated diseases and aging itself [33, 34].

Energy sensing mechanisms

Life is a physical system that maintains structure and avoids decay by feeding on negative entropy through metabolism [35]. Changes in metabolites and substrate availability are reflected in the energy output of the whole cell system in the form of the ATP and adenosine monophosphate (AMP) ratio, or other reducing equivalents such as nicotinamide adenine dinucleotide (NAD^+) vs NADH, which are detected by energy sensing mechanisms. Maintaining a constant ATP level within the cell is crucial, to the extent that all cells maintain a ΔG'ATP of approximately -56 kJ/mol [36], and any disruption of this energy balance will compromise cell function and viability [37].

Therefore, one of the central regulators of cellular and organismal metabolism in eukaryotes, and evolutionarily conserved across a multitude of species, is the AMPK [38], which acts as an integrator and mediator of several pathways and processes linking energetics to longevity. AMPK is activated by a high AMP to ATP ratio and then initiates energy producing reactions while inhibiting energy-consuming reactions as a rescue mechanism [38]. In *C. elegans*, changing the catalytic subunit of AMPK by increased expression, led to a lifespan increase of 13% [39], while a constitutively active truncated form of the protein increased life extension by 37.5 % [40]. In mammals, AMPK has a specialized function in metabolically active tissue such as the liver, adipose tissue and muscle, where it acts to integrate nutritional and hormonal signals to food intake, body weight, and

substrate homeostasis [41]. AMPK activation is further associated with inhibition of cell proliferation and is an attractive target in cancer treatment, as it shuts off metabolic cell processes needed to maintain cell proliferation [42]. A positive regulator of AMPK is the serine/threonine kinase LKB1, a known tumor suppressor, which phosphorylates the Thr172 in the activation loop of AMPK, thus inducing the downstream effects of AMPK. [43]. The Peutz-Jegher cancer syndrome involves an inherited mutant form of LKB1 and is one of the most commonly known mutations in sporadic human lung cancer [44], and more recently identified in 20% of cervical carcinomas [45]. The loss of LKB1 may therefore facilitate tumor growth under energetically unfavorable conditions. As an example, AMPK activation acutely inhibits fatty acid and cholesterol synthesis through direct phosphorylation of the metabolic enzymes Acetyl-CoA carboxylase (ACC) and HMG-CoA reductase (HMGR) involved in lipid production [46], whereas a defective AMPK sensor system would allow for lipid production even under low energy stress. Increased levels of enzymes such as fatty acid synthase (FASN) involved in cell lipid production have shown to be essential for the survival of a number of cultured tumor cell lines [47-49].

Beyond the lipogenic enzymes, AMPK can acutely modulate glycolysis through phosphorylation of multiple isoforms of phosphofructo-2 kinase (PFK2), a rate-limiting enzyme of glycolysis. PFK2 phosphorylation synthesizes fructose 2,6-bisphosphate, which is a potent stimulator of glycolysis [50], thus increasing the glucose demand. This is seen in response to hypoxic conditions [51], where ATP production from mitochondria drops and AMP levels increase activating AMPK. However, under normoxic conditions, a compensatory increase in mitochondrial volume could strengthen the mitochondrial capacity to produce more ATP. This is supported by findings that AMPK can regulate mitochondrial biogenesis via the p38-PGC-1α axis, maintaining cancer cell survival under glucose-limiting normoxic conditions [52]. Getting the cell back on track energetically may be the ultimate goal of AMPK, but also makes this pathway a crucial mediator involved in both cell proliferation and longevity. Metabolic drugs, such as metformin, resveratrol, and 5-aminoimidazole-4-carboxamide-1-D-ribo-furanoside (AICAR), that directly or indirectly activate the AMPK pathway, have been associated with pro-longevity and a reduced risk of developing cancer [53-58].

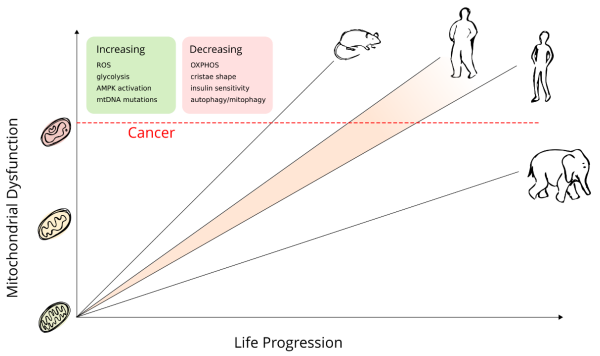

Figure 1. As animals age, there is an accumulation of dysfunction. This affects the mitochondria to a great extent and a higher metabolic rate provides further amplification, reflected by the slope in this line. Once the dysfunction passes a threshold and the cell can no longer compensate, a cancerous transition may occur. The difference in resting metabolic rate (RMR) and their relative cancer development can be seen between large and small animals, with large animals having a low RMR and late or nonexistent cancer development. While RMR may not increase in larger individuals within species, metabolic stress accumulates at a faster rate and the individual can reach the dysfunctional threshold at an earlier timepoint, as exemplified here by the obese human figure having a shifted cancer risk.

Theories of carcinogenesis and Peto's paradox

Aging and cancer involve many of the same cellular pathways in their progression, suggesting they are closely related in pathology. However, instead of following this lead, there is an ongoing pursuit to define cancers based on largely their mutational patterns. Meanwhile, the heterogenous gene expression pattern found in nearly all cancers begs the question of how critical each individual nuclear gene mutation is in the progression of the disease. Several theories exist to explain carcinogenesis, with the most prevalent being the mutation-centric theory stating that somatic nuclear mutations acquired over time eventually leads to a cancerous transition. Yet, this theory alone is unable to explain why a discrepancy exists between high cancer incidence in small, short-lived animals (i.e. few cells and less mutational events) compared to a low cancer incidence in larger, long-lived animals (i.e. many cells and more mutational events) (visualized in Fig. 1). This observation was named "Peto's paradox" after the epidemiologist Sir Richard Peto [2]. It highlights the discrepancy that if cancers are initiated by a series of somatic mutations acquired over time and cell number, then organisms with larger body size (more cells) and longer lifespans (more cell divisions) should have a cancer risk that is orders of magnitude greater than organisms of smaller size and shorter lifespan. Thus, in theory, humans should have a higher cancer incidence than mice. However, this is not confirmed in nature, as mice have a higher risk of cancer in their relatively short lifespan (about 4 years) [59], when compared to humans. The mutation-centric theory is established on a belief that there is a linear increase in mutations during cell division as we age. This is in contradiction to data that shows that a substantial portion of somatic mutations (up to 50%) accumulate early in life before full body maturation [1] and seem to slow when stem cells convert from body building to body maintenance [60]. A further challenge with mutagenesis models is the assumption that any mutation can affect cellular fitness, which is not supported by evolutionary theory. From an evolutionary perspective, only mutations that enhance cellular fitness above others in relation to the growth environment will be advantageous [61]. Thus, both mutations and the tissue microenvironment play a role in cell growth. Early-acquired mutations may stay latent and not provide a growth advantage to cells until the tissue microenvironment changes in aging organisms, further enhancing the relationship between aging and cancer risk. This was recently shown by using a computational stochastic model integrating real data on age dependent dynamics of hematopoietic stem cell division. The model demonstrated that previously acquired mutations only became advantageous in an aging microenvironment according to non-cell-autonomous mechanisms [11]. Identification of the most prevalent mutations in cancer and their clear link to metabolic regulations [62] therefore confers that they may be providing the cells with a selective advantage for growth in a changing microenvironment.

The Warburg effect and relation to Peto's paradox

Direct signals from mitochondria in the form of substrates, ROS, and other intermediates can affect cellular physiology via genetic and epigenetic mechanisms, and form the foundation for cancer development. The interplay between these metabolic changes, aging, and cancer development is illustrated in Figure 2. In support of the age-associated risk of cancer, tumors rarely occur following acute injury to cellular respiration and considerable time is required for non-oxidative energy metabolism (i.e. glycolysis, TCA cycle via substrate-level phosphorylation) to replace OXPHOS as the dominant energy generator of the cell. Substrate-level phosphorylation can compensate gradually for minor OXPHOS damages accumulated over time. Consequently, expansion of mtDNA mutations affecting ATP production can happen gradually [63] or by asymmetric segregation of cellular content during cell division [12]. This compensatory effect by the continued adaptation by substrate-level phosphorylation for energy production (i.e. increasing the uptake of glucose and glutamine to be broken down for ATP production) is a well-known hallmark of cancer called "the Warburg effect". Cells that undergo a Warburg transition and switch their metabolism to glycolysis and glutaminolysis produce increased levels of substrates, such as lactate from glycolysis, and succinate, alanine, and aspartate from glutamine or amino acid fermentation [64], that can regulate gene expression epigenetically.

Otto Warburg described the change in cancer metabolism as early as the 1950s [65]. He postulated that the change to this metabolic preference was due to defective mitochondria incapable of producing enough ATP to support cell growth. However, this may not be entirely true as proliferating normal cells, such as activated lymphocytes, also revert to aerobic glycolysis upon growth activation without showing any mitochondrial dysfunction [66]. The current understanding is that rapidly proliferating cells, both normal and cancer, revert to aerobic glycolysis to support the need for new biomass when producing a daughter cell [67]. Thus, in proliferating cells, the metabolic substrate turnover rate increases to support the cell with new cell components. If one considers Warburg's observations of increased metabolic substrate turnover in cells that are proliferating, it suggests that reduced metabolism should slow cell turnover, be cancer

preventative, and halt cell aging. Hence, in relation to Peto's paradox, larger animals may have evolved a slower metabolic rate that is both cancer preventative and increases longevity. Indeed, the "metabolic rate hypothesis" suggests that cellular metabolic rate and subsequently, oxidative stress, decreases with increasing body size and is protective for larger animals [68]. Slower metabolic rates have been found in large, long-lived animals such as whales and elephants [59, 69-71], compared with smaller animals. A common factor among long-lived animals is that they have no natural enemies and are at reduced risk of predation or death by other external factors, thus the "need for speed" is reduced and more energy can be put into maintaining protective somatic cell maintenance [72, 73].

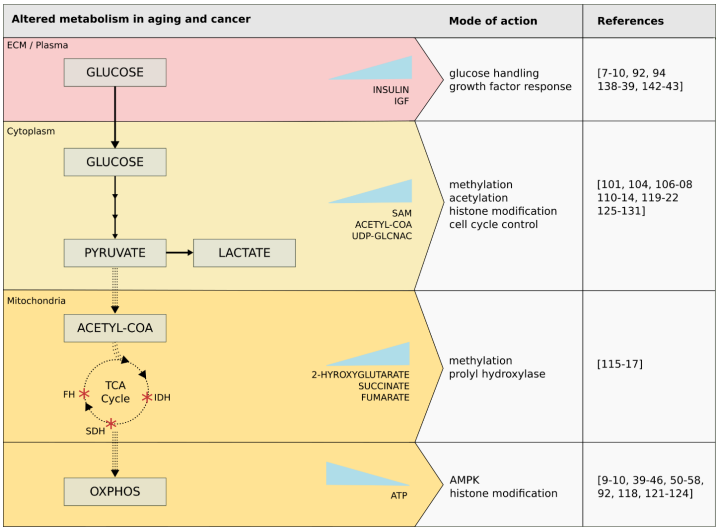

Figure 2. Tumors rarely occur following acute injury to cellular respiration and considerable time is required for non-oxidative energy metabolism (i.e. glycolysis, TCA cycle via substrate-level phosphorylation) to replace oxidative phosphorylation (OXPHOS) as the dominant energy generator of the cell. As minor OXPHOS damages accumulated over time, the cell uses substrate-level phosphorylation to compensate gradually for the energy debt. This compensatory effect, by increasing the uptake of glucose and glutamine to be broken down for ATP production, is a well-known hallmark of cancer called "the Warburg effect". Cells that undergo a Warburg transition and switch their metabolism to glycolysis and glutaminolysis produce increased levels of substrates that can have many downstream effects. Only glucose metabolism is highlighted here, with the solid arrows denoting the increased reliance on glycolysis and production of lactate, and dotted arrows denoting decreased activity in the remainder of the pathway. This translates to lowered production of acetyl-coenzyme-A (acetyl-CoA) from pyruvate, activity of the TCA cycle, and production of precursors necessary to carry out OXPHOS. Also, mutations of key TCA cycle enzymes commonly found in cancer are shown, such as isocitrate dehydrogenase (IDH), succinate dehydrogenase (SDH), and fumarate hydratase (FH), as well as substrates accumulated due to their alterations. Abbreviations: ECM, extracellular matrix; IGF, insulin growth factor; SAM, s-adenosylmethionine; UDP-GlcNAc, uridine diphosphate N-acetylglucosamine; ATP, adenosine triphosphate; AMPK, AMP-activated protein kinase.

Cellular maintenance in an aging system

The influence of metabolism on overall life span is still controversial. However, there must be an evolutionary trade-off to lower metabolic rates in larger animals to avoid cellular harm and increase life span. A possible answer is "somatic maintenance" [74], where the investment in gene repair will increase or decrease to maximize reproductive success in a changing environment. Therefore, lifespans of animals have evolved to maintain fitness and invest in somatic maintenance until reproduction is most likely to be achieved. This explains why most wild animals do not develop cancer, as they do not survive beyond reproductive age in the wild. Humans are the only species that have developed technological advances and designed their living environment to extend their lifespan far beyond reproductive age, thus exposing themselves to a variety of diseases associated with old age. This also holds true for animals kept in captivity, which live beyond their normal lifespan in the wild, and do develop cancer [75, 76].

The low cancer incidence in larger species could also relate to cell size. The difference in cell size among species has been well documented, with blood cell sizes ranging from 78 to 110 to 170 and 215 μm^2 in shrews, rats, humans, and whales, respectively [77, 78]. If cell size is considered when modeling cancer rates between species, then this skewed cancer risk almost disappears [68]. More importantly, in addition to the association of drop in cancer rate, larger cells exhibit a lower metabolic rate [79] and prioritize slow growth over division [80]. Furthermore, cell volume and metabolic rate scale with body mass in 13 different cell types [81], supporting the model in which metabolic rate and somatic maintenance play a crucial role in cancer development [82]. This phenomenon is only found between species and not within, as obese humans have an increased cancer risk [83] (Fig. 1). Nature's way of combating the inherent risk of living larger is exemplified in elephants which have an increased copy number of the tumor suppressor P53 [84], leading to an improved gene maintenance system with reduced cancer risk and a longer lifespan [69]. Some exceptions to the rule do exist. For example, the naked mole rat – a small, subterranean rodent – has been found to live over 30 years, while exhibiting no documented cases of cancer [85]. As with elephants, it was recently found that the naked mole rat had fibroblasts producing a type of high-molecular-mass glycosaminoglycan, which increase extracellular matrix tension and further signals to a cell cycle checkpoint p16INK4a that inhibits cell cycle progression [86]; thus supporting the notion that the tissue microenvironment plays a major role in cell cycle regulation.

Since continual growth and aging seem to be linked, with aging perhaps being directed by this unnecessary growth [87], evidence points to energy partitioning away from biogenesis and to cell maintenance as a way to extend lifespan [88]. Cellular maintenance is carried out in various ways and exists to combat the inherent mistakes our cells accumulate as they age. The main contributors of these are gene and protein errors, and while gene instability is hallmark of cancer, we are exploring factors beyond genetics in aging and cancer. Protein homeostasis (proteostasis) is vital for quality control of the cell proteome in an aging system. Altered proteostasis can occur upon the accumulation of dysfunctional proteins due to mutations and misfolded of proteins from lack of necessary enzymes or chaperones, incorrect compartmentalization, and problems in degradation systems for the clearing of these proteins, such as autophagy-lysosomal and ubiquitin-proteasome systems [89]. Cell-stress-signaling pathways regulate the proteostasis network and prevent the toxicity associated with misfolded proteins that could aggregate in subcellular compartments and tissues. The efficiency of the proteostasis network declines with age and this failure in protein homeostasis has been proposed to underlie the basis of common age-related human disorders [90].

Autophagy is a major driver of this housekeeping role whereby unwanted, excess, or damaged cytosolic components are self-degraded by the cell through lysosomal digestion. Autophagy is one of the programmed self-degradative processes that is important for balancing sources of energy at critical times in development and in response to cellular stress [91]. Many pathways are in place to detect nutrient stress (AMPK, mTOR, FOXO), hypoxia (HIF), misfolded proteins (unfolded protein response), immune response (NF-kB, MAPK), DNA damage (P53), mitochondrial stress (MMP, PINK) [92]. The selective removal of damaged mitochondria in particular has been termed mitophagy and dysregulation of this clearance is a risk factor for cancer development [93]. With decreased mitophagy, a slow accumulation of dysfunctional mitochondria may lead to accumulation of metabolic substrates causing epigenetic signaling changes and altered gene expression. In lifestyle diseases such as obesity and type 2 diabetes [94], dysfunctional mitochondria may not be cleared due to a constant excess of nutrient availability inhibiting the autophagic response mechanisms. Meanwhile, the strengthening of other metabolic pathways such as glycolysis to compensate for energy deficit provides the foundation for cancer cell transformation [95]. In mammals, one of AMPK's many targets is the UNC-51-like kinase 1 (ULK1), which regulates the formation of the autophagosome in response to energetic stress. This regulation is thought to be an important mediator of organismal aging [96]. Most longevity-promoting interventions require an intact

autophagic machinery; furthermore, reduced autophagic activity is associated with aging while evidence suggests that enhanced autophagy promotes longevity and delays age-related phenotypes [97].

Metabolism and epigenetic changes drive aging and cancer

Deregulation and loss of homeostasis is a driver of damage and dysfunction frequently encountered in aging and cancer, affecting all functions of the cell. Normal systemic operation requires the proper flexibility and functioning of gene and protein expression to respond adequately to intracellular and extracellular signals. This flexibility is made possible by epigenetic control over how the primary DNA sequence is expressed. Besides changes in bioenergetics and creation of cellular building blocks, metabolism can affect function through control of gene expression. Much of the enzymatic maintenance of epigenetic patterns occurs through information provided by metabolic substrates and metabolites, signaling when to grow and trigger transcription based on nutrient availability. The crucial role they hold in the support of cancer growth has been proven in elegant experiments in which enucleated normal cells were fused with nuclei from tumor cells to form cybrids. These cybrid cells did not form malignant cells, whereas tumor cells infused with normal cytoplasm had their tumorigenic potential in mice reduced from 92% to 51% [98]. The important feature of their study was that the non-transformed and transformed cells all originated from a cloned progenitor cell with a common nuclear and cytoplasmic background. When the experiments were conducted, the authors did not identify the nature of the observed effects, but with experimental results from a more recent study focusing on the role of mitochondria in this situation [99], it is highly likely that substrates and intermediates of metabolism could drive this effect via epigenetic regulation. These experiments and the increasing data showing that most of the common mutations found in cancers are related to changes in metabolism [62], demonstrate that there is more than just gene mutations driving cancer and aging. Compounds directly essential to the function of epigenetic enzymes are produced during metabolism, such as S-adenosylmethionine (SAM) and alpha-ketoglutarate (α-KG), nicotinamide adenine dinucleotide (NAD) and acetyl coenzyme-A (acetyl-CoA), and uridine diphosphate N-acetylglucosamine (UDP-GlcNAc). ATP is also vital for proper function but not rate limiting for these enzymatic reactions due to its relative abundance [100].

Methylation

SAM is produced in the cell from glycolytic intermediates shuttled to serine metabolism [101] and by addition of adenosyl to methionine from ATP. Histone methyltransferases (HMTs) and DNA methyltransferases (DNMTs) both use SAM as a methyl donor for transfer to the 5' carbon on cytosine, lysine or arginine residues of histones, respectively. α-KG is formed both in the TCA cycle from glucose-derived isocitrate by isocitrate dehydrogenase (IDH) and by transamination of glutamate [102] and is essential to the demethylation functions of lysine-specific histone demethylase 1 (LSD1) and JmjC-domain containing histone demethylase (JHDM) on histones, and ten-eleven translocation (TET) demethylase on DNA [103]. The effect of histone methylation depends on the specific proteins modified, and can have either repressive or enhancing effects on transcription [104], while DNA methylation results in reduced expression. There is a documented trend of methylation changes in aging, termed epigenetic drift [105], but it is difficult to tie it with specific functional implications absent of pathological symptoms. However, in a differential analysis of methylation in islet cells, genes associated with mitochondrial function and diabetes are targeted in this aging phenomenon [106]. In a study of 58 cancer cell types, DNA enhancer methylation was a strong predictor of cancer-related gene expression and of the 207 of hypomethylated/upregulated genes, two-thirds had function in metabolic processes [107]. Conversely, reduced expression of IDH and TET have been associated with decreased survival in chronic lymphocytic leukemia (CLL), but did not correlate with any measured change in global methylation [108].

Acetylation

One of the clearest links between metabolism and epigenetics is through acetyl-CoA. Acetyl-CoA plays a major role in cellular nutrient sensing is generated during oxidation of pyruvate and fatty acids in the mitochondria and from citrate in the cytosol and nucleus [109], and also serves as a substrate for lipogenesis in the cytosol. Acetyl-CoA levels directly affect the activity of histone acetyltransferases (HAT) as acetyl donors, and can act indirectly on histone deacetylation by Sirtuins due to its role in modulating NAD^+/NADH by availability to the TCA cycle [110]. Histone acetylation and demethylation generally result in an open structure allowing for expression of the region, while deacetylation produces a tighter chromatin structure and reduced expression [111]. Acetyl-CoA is dynamically regulated by glucose availability in cancer cells and the ratio of acetyl-CoA: coenzyme A within the nucleus modulates global histone

acetylation levels. Reduced sirtuin (SIRT7) activity (increased acetylation) is associated with stem cell senescence and mitochondrial unfolded protein response [112]. Additionally, expression of *ATG* autophagy genes are tightly controlled by acetylation and thus dependent upon acetyl-coA levels and nutrient sensing by the cell with the reduction of acetyl-coA synthesis, promoting autophagy and extending lifespan in drosophila [113]. Testing *in vitro* and confirmation *in vivo* has shown histone acetylation to be controlled by glucose availability and AKT-activation of ATP-citrate lyase (ACLY), the enzyme responsible for production of acetyl-CoA from citrate [114].

TCA Cycle Intermediates

In the TCA cycle, metabolites such as succinate and fumarate are essential for completion of the cycle, but they may also accumulate due to altered expression of succinate dehydrogenase (SDH) and fumarate hydratase (FH) inhibiting prolyl hydroxylases (PHD) and increasing HIF levels [115]. IDH mutants produce 2-hydroxyglutarate, similar in structure to α-KG, which interferes with the normal TCA cycle and other enzymatic reactions, such as demethylation, that depend on α-KG as a substrate [116]. Mutations in IDH, FH, and SDH are common in cancer and these metabolites contribute to cancer growth and survival by reduced expression of tumor suppression genes [117].

Chromatin modifications

Indirectly, modifications of enzymes responsible for these epigenetic changes can increase or decrease their activity. This occurs mainly through phosphorylation and O-linked N-acetylglucosamine (O-GlcNAc) glycosylation. Modification of histones can occur by the activity of AMPK through phosphorylation of histone protein H2B, resulting in expression of genes important to the cellular energy homeostasis [118]. Changes in histones by glycosylation (formation of O-GlcNAc) are catalyzed by O-GlcNAc transferase, but little is established on the function or consequence of this histone modification type. Recently, it has been shown to have a role in H3.3-histone cell cycle regulator mediated nucleosome assembly for transcription and also cellular senescence through its regulation of chromatin dynamics [119]. The O-GlcNAc glycosylations are directly affected by nutrient availability signals and activity is reliant upon UDP-GlcNAc as a donor substrate, a product of the hexosamine biosynthesis pathway (fed by 2-5% of imported glucose), upregulated in cancer [120]. Modification of histones through phosphorylation and O-GlcNAc glycosylation are implicated in cell cycle control [121, 122] with the two exhibiting inverse relationships during different cell cycle phases. Phosphorylation of histones has been found to be a significant marker of tumor grade and mitotic index in breast cancer [123] and proliferative marker in bladder cancer [124]. Increased O-GlcNAc glycosylation is consistently found in cancer as well (breast [125, 126], prostate [127], lung [128], colorectal [128, 129], liver [130], and nonsolid cancers such as chronic lymphocytic leukemia [131]) and has been correlated with increased metastatic potential. Unique modifications of histones for degradation also reveal the importance of histone turnover and homeostasis (and proteostasis) in epigenetic regulation [132]. In aging cells with DNA-damage signal activation, histone synthesis is reduced, demonstrating other ways in which histone control can affect cell homeostasis [133].

Lifestyle modulates longevity and cancer development

Cellular dysfunction and stress are recurring themes presented in this review for their likely role in accelerating aging and induction of cancer. This is largely due to increased metabolic activity, abnormal metabolism through diet or genetic/epigenetic modification, and inhibition of healthy cellular maintenance. These conditions are all capable of improvement through lifestyles changes.

In the developed world, food sources are constantly available and reduction of metabolic activity by prolonged fasting is rarely achieved. We no longer experience seasonal or periodic fluctuations in nutrient availability like our ancestors, but still have a vital and complex nutrient sensing system that can be severely affected by our modern diets. Reducing this excess consumed energy should result in a longer and healthier lifespan by decreasing metabolic activity and energy partitioning, ROS, and epigenetic-affecting metabolites. Calorie restriction was one of the first diets to show a direct relationship between metabolism and lifespan extension [134], and continues to be lauded as the best option for life extension and health [135]. The consumption of reduced calories, but not below nutritional levels, has been shown to reduce resting metabolic rate [136], and depends on mitochondrial function for its beneficial outcomes [137]. Calorie restriction and associated dietary restriction seem to exert their effects specifically through mTOR, AMPK, and glucose handling (IGF/insulin) pathways [138, 139] with outcomes such as lifespan extension, reduced inflammation and cancer. Taking the restriction further to a pure ketogenic diet has been shown in mice to reduce metabolic activity and increase uncoupling protein 2 and the ketone, beta-hydroxybutyrate [140]. Ketone bodies are important compounds in the body's response to restricted nutrients; as a precursor to acetyl-CoA it serves

as an energy source and donor for epigenetic modifications [141]. For more information on lifestyle modulations such as calorie and dietary restrictions, micro/macronutrients, and meal timing, Fontana *et al.* [142] provides a complete review. Diet is not the only negative modern lifestyle factor we have altered, however; sleep and circadian alignment are adjusted to fit societal norms and they also have large effects on metabolism and cell recovery. Most research in this area is performed on shift workers or through short-term sleep deprivation. While only shift work results in circadian misalignment, even a single night of sleep restriction can mimic its effects and both have been related to increased insulin resistance [143, 144].

Calorie and dietary restrictions in combination with regular sleeping patterns can lead to a more normal metabolism primarily through direct influx of nutrients and also regulation of cellular processes through healthy maintenance. The mild stress on mitochondria by preserving short periods of low-nutrients can induce retrograde stress responses and actually enhance cellular function by retaining the activity of autophagic processes. As mentioned before in relation to obesity and diabetes II, such activation of unfolded protein response and IGF has been induced by redox stress to mitochondria (mitohormesis) in muscle of Drosophila, resulting in increased mitophagy and lifespan [94]. These changes in lifestyle have persistent effects in major metabolic pathways, ROS, mitochondrial turnover, immune regulation, epigenetic control, and DNA repair; related to both aging and cancer.

Concluding remarks

Further investigation into the differentiating genetic or metabolic factors between species is a key to understanding the source of neoplasms and the mechanisms nature has adapted to fight them. With the explanations given here and even the outliers of Peto's paradox, a need to explain cancer formation using only the multi-stage mutational model lessens. The refitting of the model with respect to cell size and volume, differential tissue behavior, and the presence of long-living organisms with no cancer at all could have a unifying relationship within cell metabolism. Larger cells, less proliferating tissues, and long-living organisms all have slow metabolism and consequently slower cell growth. Upon analysis, carcinogenesis may be driven by a forced change in metabolism that is not tolerated by an organism or tissue and its adapted mechanisms.

To further expand the knowledge of how these factors are regulating aging and cancer development, there is a need to share and compare the accumulated evidence that underlies these mechanisms. The close relationship between aging, cancer, and metabolism across species can be addressed using accessible databases depositing published data. The era of computer science and technology which in many aspects seem to be outpacing our evolutionary adaptation to lifestyle changes, could very well be the technology that will help solve this puzzle. The ability to completely prevent aging and cancer is doubtful, but as said by Stauch *et al.*, "Aging is not necessarily pathogenic, and in healthy aging, organs, cells and subcellular organelles can respond to gradual age-associated stress" [145]. Armed with knowledge that our diet and behavior matter on a molecular level, we can make healthy lifestyle choices and allow our bodies to combat this gradual stress, while attempting to alleviate the modern prevalence of aging pathology and tumorigenesis.

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