LOUGHBOROUGH UNIVERSITY

Translational Requirements for Manufactured Dopaminergic Neurons for the Treatment of Parkinson's Disease

by

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Doctor of Philosophy

Abstract

After decades of research there is now a plethora of cell-based therapies traversing through different phases of clinical trials. However, there remains work to be done regarding optimising the quality and production efficiency of these therapies, as well as economic considerations to validate their commercial viability. This project focuses on the process development of a candidate human embryonic stem cell (hESC) based therapy for Parkinson's disease (PD) and the related health economics.

This work set out to identify the key translational requirements for successful, robust and reproducible manufacture of a cell-based therapy for PD, by optimising a preclinical validated differentiation protocol. Design of experiment (DoE) approaches were employed to investigate the effect of small molecule concentrations, seeding density and feeding regime on process outputs. Orthogonal analysis of flow cytometry, cell growth and metabolomics was used to ascertain the relationship between critical process parameters and their impact on critical quality attributes. The results demonstrated that higher seeding densities (15,000 cells/cm^2 and above) and specific small molecule concentrations such as 1 - 1.5 μM CHIRR99021 are required for cell survival and desired cell differentiation. Moreover, the results revealed an interaction between phenotype and feeding regime that may not be accurately reflected in growth rate but is linked to the specific metabolic rate. This emphasised the need for standardised protocols that control for culture procedures and feeding based on cell metabolic rate and growth dynamics.

In addition to the process development work carried out, a complimentary work-stream developed models incorporating economic evaluation, reimbursement price modelling and cost of goods modelling to ascertain the commercial viability and potential cost-effectiveness of the hESC-based therapy for PD. The results illustrated that a hESC based therapy would be a cost-effective treatment for PD at prices ranging from £180,000 to £255,000, with the potential to be cost saving and significantly reduce the economic burden of PD. Furthermore, such a therapy has the potential to generate revenue and provide profits while affording health gains presently unattainable through current treatments. Thus, the present work has exhibited that a hESC-based therapy for PD has the potential to provide value to multiple stakeholders, providing it achieves the translational requirements to ensure robust evidence generation for successful regulatory and reimbursement approval.

Acknowledgements

I wish to acknowledge my supervisors Dr Mark McCall, Dr Samantha Wilson and Professor Robert Thomas and to thank them for their support throughout the duration of this work, their supervision throughout this research, and their guidance on the direction of this work. Mark was supportive and yet provided me with the autonomy and confidence to pursue my own research direction. Sammy provided invaluable support in my project and the writing of this thesis. Rob's incredibly insightful assistance with regards to DoE methodology and data interpretation has been instrumental to the progression of the work. Together they have never failed to keep me challenged and inspired. I could not have asked for a better trio of supervisors and it has been a pleasure to have worked with them all. In addition, I would like to acknowledge the priceless support of Dr Preeti Holland, who I worked closely with in the final year of this project. The support and camaraderie Preeti provided both technically and personally was a major contribution and helped me to deal with the inevitable moments of hysteria that came with long days/evenings in a lab and biological safety cabinet.

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Table of Contents

List of Figures

List of Tables

List of Frequently Used Abbreviations

AGP	Annual Gross Profit
ATMP	Advanced Therapy Medicinal Products
ATTC	Advanced Therapy Treatment Centre
CADTH	Canadian Agency for Drugs and Technologies in Health
CGTC	Cell and Gene Therapy Catapult
CTI	Cell therapy industry
CTP	Cell therapy product
CNS	Central nervous system
CCRM	Centre for Commercialization of Regenerative Medicine
CHART	Challenges in the Adoption of Regenerative Medicine Therapies
cDNA	Complimentary deoxyribonucleic acid
CoGs	Cost of goods
CMA	Critical material attribute
CQA	Critical quality attribute
CPP	Critical process parameter
DBS	Deep brain stimulation
DoE	Design of experiments
DALY	Disability adjusted life years
DA	Dopaminergic
EMA	European Medicines Agency
FMEA	Failure mode and effects analysis
FCS	Flow Cytometry Standard
FDA	Food and Drug Administration
FCS	Forward Scatter
gDNA	Genomic deoxyribonucleic acid
HTA	Health Technology Assessment
HrQoL	Health-related quality of life
HRG	Healthcare resource group
H&Y	Hoehn & Yahr
hESC	Human embryonic stem cell
ICER	Incremental cost effectiveness ratio
LB	Lewy bodies
MEA	Managed entry agreement
MedFI	Median fluorescence intensity

MbD	Medicine by Design
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
PD	Parkinson's disease
PCR	Polymerase chain reaction
Pd	Population doubling
QALY	Quality adjusted life years
QbD	Quality by design
QTPP	Quality target product profile
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
SSC	Side Scatter
SGR	Specific growth rate
SMR	Specific metabolic rate
SbD	Summer by Design
THETA	Toronto Health Economics and Technology Assessment
UPDRS	Unified Parkinson’s Disease Rating Scale
vm	Ventral mesencephalon/midbrain
vmDA	Ventral mesencephalon/midbrain dopaminergic
WTP	Willingness to pay

List of Journal Publications

Published:

- Ventral midbrain dopaminergic neuron differentiation from human embryonic stem cells: Key manufacturing recommendations for the cell therapy industry.

Submitted:

- An investigation into protocol standardisation using the 2102Ep reference cell line - under review

Manuscripts in Progress:

- Pluripotent cell culture and protocol standardisation using H9 human embryonic stems cells: obtaining better defined input cells for differentiation.

- White Paper: Challenges in the Adoption of Regenerative Medicine Therapies

Chapter 1:

Introduction

Chapter 1. Introduction

1.1. Background

Parkinson's disease (PD) is an incurable and debilitating neurodegenerative disease. The current forms of treatment including medication therapy aim to increase the amount of dopamine in the brain, which is a mode of treatment first used the 1950s[1–3]. Although new drugs/medications have been developed, they still do not slow or stop the progression of the disease. There has been limited progress in the past half century since current therapies presently only alleviate/control the symptoms experienced by people with PD. Furthermore, medication therapies often cause unwanted side effects including dyskinesia due to the unregulated influx of dopamine[4–7]. The limited success of the current modes of treatment have necessitated the development of alternatives that can truly change the course of the disease. Clinical trials using foetal tissue sourced ventral mesencephalic dopaminergic (vmDA) neuroprogenitors cells have illustrated that transplanted cells can re-innvervate into the striatum and restore dopamine synthesis[8–12]. This is a magnitude of improvement for patient outcomes in comparison to medication treatments, as patients who have undergone cell transplantation therapy have had restored dopamine synthesis. In addition, some patients have also regained cognitive function and experienced reversal of their symptoms, even after they have stopped taking their anti-Parkinsonisian medicine. However, due to issues such as cell source sustainability this mode of treatment has resulted in varying levels of efficacy[13–17]. In addition, the ethical issues associated with a therapy derived from foetal tissue does not place the therapy in a favourable position from a regulatory standpoint. Therefore, a global effort has been directed at producing vmDA cells from human embryonic stem cells (hESCs) as a standardised cell source, that has a lower ethical burden[9,10].

In the latter years of the 20th century cell and gene-based therapies were promised to be the treatments of the future, however almost three decades later they have failed to become mainstream modes of treatment. This is due to the complex nature of their product development cycle, which has proven to be more complicated than initially perceived, especially in comparison to traditional pharmaceutical and biologics product development. This has led to the retardation of products reaching the market, which has been exacerbated by significant failures in the field, particularly those that have resulted in adverse effects including patient mortality[18–21]. Thus, it is apparent that many challenges need to be addressed in order to successfully cross the product development 'valley of death'. Current limitations of cell therapy products (CTPs) include the need for in depth product understanding that ensures that the products are potent, safe and free of any impurities. This has required a paradigm shift in thinking since unlike biologics manufacturing, the cells are both the process *and* the product. Furthermore, new tools have had to be developed and validated in order to address issues such as scale up of cell

production, in addition potency, safety and identity assays have had to be developed, which is necessary for process control, product validation and release[2]

This project is set in the context of the onset of cell therapy products traversing the product development pathway. A plethora of work has been carried out in academic institutions and small biotechnology companies, this has led to innovative cell and gene therapies that have the potential to revolutionise current modes of treatments and even potentially cure a wide range of disease indications. Regarding the present work, research regarding cell-based therapies for PD has advanced in recent years. Refined differentiation protocols have resulted in the successful and efficient generation of transplantable vmDA cells derived from hESCs as a therapy for PD[9,22–25]. Prior to these cells being used as a PD cell replacement therapy, it is important to gain detailed understanding of the cell production process, in order to implement manufacturing process changes. The detailed understanding is essential in providing knowledge that facilitates process control, which in turn provides understanding of the key process parameters. These parameters once understood form the basis of the tolerances of the process, allowing for the potential of an adaptive manufacturing process that is validated and robust within the defined set of parameters that allow for greater process control.

Prior to clinical realisation, there is a requirement for robust well-defined protocols to ensure comparability and reproducibility, however in many cases effective comparisons of data can be encumbered by the lack of standardised culture protocols. This lack of conformity often results in variation of the measured process outputs, as a result obtaining regulatory approval becomes a challenge that is not easily overcome. This requires translational work to be carried out, in order to understand complex cell dynamics and to standardise protocols for the manufacture of cell therapy products. These protocols need to provide robust and reproducible manufacturing processes that can withstand the high scrutiny of regulators such as the Food and Drug Administration (FDA) and European Medicines Agency (EMA).

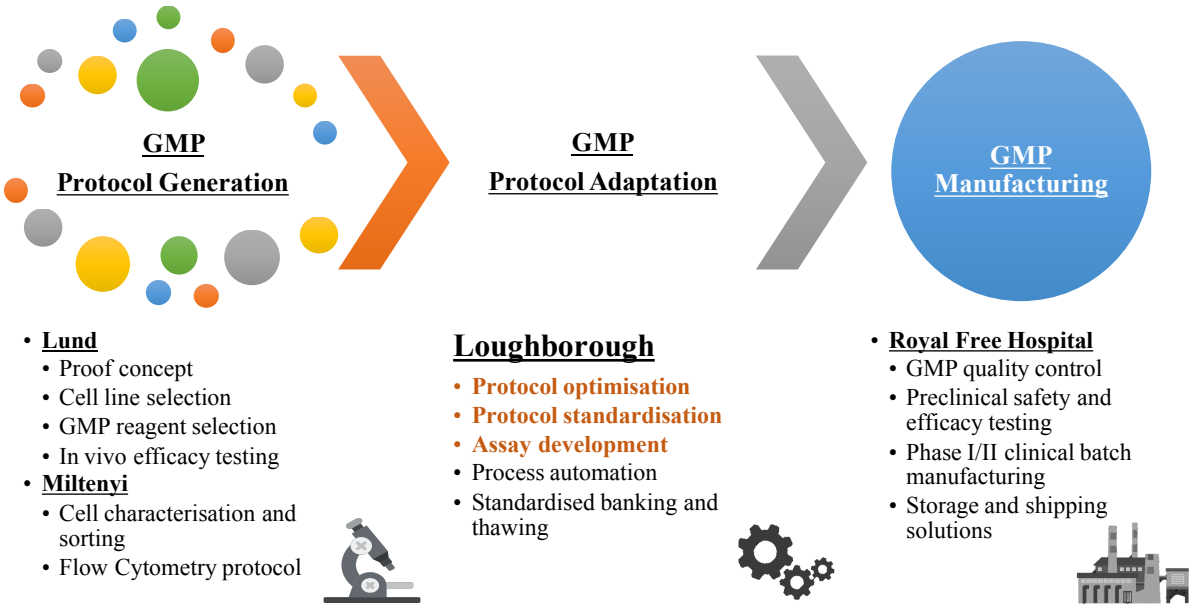
Although fulfilling regulatory requirements is an accomplishment for developers, in isolation it is not adequate to ensure the success of a product being adopted and reaching patients. In addition, it is important to understand the commercial viability of the product, in order to avoid cases such as Glybera, where the product was scientifically sound, yet it was deemed not to be cost-effective or affordable[26]. Therefore, this work also discusses the value of early health economic evaluations and their ability to act as a decision tool for a wide range of stakeholders. For developers and investors, being equipped with the concepts of health economics and a firm understanding of their product, its costs and the impact of its health outcomes can increase the commercial viability of their product. For payers, health economics provides valuable information regarding the cost-effectiveness of a therapy and its impact on their budget. This highlights the multifaceted nature of successfully translating research from

'bench' to 'bedside'; developers must endeavour to swiftly traverse this translational space in order to get their products onto the market so as to benefit the patients that need them.

This work sets out to identify the key translational requirements for successful, robust and reproducible manufacture of a cell-based therapy for PD, via the optimisation of a preclinical validated differentiation protocol provided by Lund University in Sweden (referred to as the candidate therapy). Analysis of the density and metabolite relationship of the cells during both expansion and differentiation phases is pivotal to understanding their roles in cell system growth dynamics. This has been achieved through orthogonal analysis using metabolic analysis, flow cytometry and cell growth analysis. The aim of these analyses was to implement the information obtained to develop robust in-process assays that can be linked to both the biological function and critical quality attributes of the CTP. This is to better understand the product so that process control and product quality can be obtained using a robust protocol. The lack of robust protocols has encumbered the success of CTPs to date, as many of these protocols are currently reliant upon human intervention, resulting in the endemic issue of variation in cell manufacturing. This is due to the subjective nature of the protocols, since typically critical decision-making processes are left in the hands of the operator; as operators change the variation in product output is compounded. This is an issue that has been highlighted in the present work as efforts have been made to show the importance of standardisation and protocols that minimise operator intervention as much as possible.

1.2. Objectives

The overall objective of this project was to investigate the translational requirements necessary to facilitate the "bench to beside" translation of the prescribed protocol for obtaining transplantable vmDA cells. A dual work-stream approach was used with the aim of progressing the preclinical protocol into a defined and standardised protocol suitable for manufacturing. One stream was a lab based experimental program helping to develop process understanding of the differentiation procedure and the differentiated cells through metabolic analysis, flow cytometry and cell growth rate analysis (**Schematic 1**). While a complimentary desk-based work-stream aimed to develop reimbursement models for the candidate therapy by incorporating economic evaluation, reimbursement price modelling and cost of goods modelling.



Schematic 1. From left to right, progression of the candidate therapy's development from protocol generation to manufacturing. The focus of the present work is on the middle section and in particular, the points in orange.

The key undertakings:

One of the initial undertakings of the project was to use a simple cell line to train the author and to develop protocols and skills to use for the clinically relevant cell line. To this effect, work was carried out using the embryonic carcinoma EC 2102Ep reference cell line to produce a standardised cell culture protocol.

The next stage of the project was to obtain a working cell bank of the clinically relevant H9 cell line and carry out experiments to understand the culture and expansion dynamics of the cells in their pluripotent state, prior to differentiation.

- This included analysis of:
    - Cell growth after the cells were thawed from cryopreservation.
    - Optimal seeding density for cell growth without inhibition.
    - Different feeding regimes during pluripotent expansion.
    - The stability of cell characteristics over a range of time.

For the differentiation process it was necessary to fully understand the protocol prior to conversion into a robust and translatable protocol for commercial scale manufacturing. This meant identification of and understanding of which parameters were important for the Quality Target Product Profile (QTPP). Therefore, failure mode and effect analysis (FMEA) and Design of Experiment (DoE) approaches were used to try and elucidate which protocol parameters were the most important to control in order to obtain a robust, reproducible protocol for efficient hESC to vmDA differentiation. High resolution growth dynamic and phenotype analysis was carried out to understand the differentiation process and the impact of the different parameters on cell differentiation.

1.3. Thesis Structure

1.3.1. Chapter Summaries:

Chapter 1 - General Introduction

This chapter provides the context and background in which this work is set, the general introduction also details the overall objectives of the project.

Chapter 2 - Literature Review

Chapter 2 provides a comprehensive review of the existing literature related to the work presented in this thesis. The review is presented in four distinct, yet interrelated sections; firstly, a review of PD detailing the epidemiology, current modes of treatment and PD cell-based therapies, including the use of dopaminergic neurons. Secondly, dopaminergic neurons are described in further detail, providing information of the *in vivo* and *in vitro* neurulation and patterning processes, specifically for ventral midbrain dopaminergic neurons. The third section in the review alludes to key translational requirements that must be considered for cell-based therapy manufacturing; these include defining and determining product purity, identity and potency. Finally, a discussion of the translational requirements from a commercialisation standpoint are discussed; specifically, health economic considerations and the current state of reimbursement and adoption for cell therapy products.

N.B. Nomenclature for genes, transcription factors and proteins:

Genes – capitalised and italicised e.g. *FOXA2* the gene

Transcription factor – small case and italicised e.g. *foxa2* the transcription factor

Proteins/antibodies/morphogens – just capitalised e.g. FOXA2 the protein expressed/antibody used/morphogen secreted

Chapter 3 – Material and Methods

Detailed procedures used for the experimental work carried out are provided in this chapter. Due to the prevalent focus of the importance of protocol standardisation that is presented in this body of work, very detailed accounts of the cell manipulations carried out are provided to ensure ease of translation by making the processes transparent. Background information regarding the techniques and the analytical tools implemented are provided

Chapter 4 - EC 2102Ep

EC 2101Ep cells were utilised in the studies presented in this chapter due to their 'robust' nature. This made them an ideal candidate for training the author in cell culture techniques whilst also allowing for the development of appropriate analytical skills and techniques that would be applied to the clinically relevant cells line. From the training, informative data regarding protocol standardisation and cell system dynamics was obtained, forming the basis of the work presented in this chapter. As the work carried out in this chapter was iterative, the methods and results sections are accompanied with narratives for each experiment to give context to both the experimental design and results obtained with each iteration.

Chapter 5 – H9 Pluripotent Understanding

This chapter focuses on the cell dynamics of H9 cells which are a clinically relevant hESC cell line that have been used by collaborators in both Sweden (Lund University) and Germany (Miltenyi Biotec). This chapter is comprised of a series of experiments of H9 cells in their pluripotent state during expansion, prior to differentiation. This is important as having standardised and well characterised input cells should provide less variation within the differentiation process. The results from this chapter are pivotal to understanding the intrinsic behaviour of the cells. This understanding is vital to providing a standardised bank of pluripotent cells that can be used for further, more complex experimentation, including differentiation. Specifically, the differentiation of H9 cells to vmDA cells, i.e. the cell therapy product

Chapter 6 – H9 Differentiation Understanding

Chapter 6 utilises the defined H9 cells banked in chapter 5 to adapt a research-based differentiation process into a reliable and reproducible manufacturing process. This chapter applies a highly process-development-based approach therefore, akin to chapter 4, the methods and results sections are accompanied by a narrative. The purpose of the narrative is to contextualise the meaning of the data obtained and how it was used to design the following experimental designs.

A range of multivariate experiments and DoE approaches were used to ascertain information regarding optimum protocol conditions, specifically the derivation of the precise small molecule concentrations that are required to obtain the desired ventral midbrain dopaminergic neuroprogenitors. Flow cytometry protocol optimisation is also discussed as an in-house panel was created to compliment the experimental designs utilised.

Chapter 7 – Challenges in the Adoption of Regenerative Medicine Therapies

Chapter 7 discusses UK and Canadian healthcare systems, in particular the mechanisms of HTA and reimbursement. The key challenges for CTP adoption are also discussed in the context of a workshop organised by the author entitled 'Challenges in the Adoption of Regenerative Medicine Therapies' (CHART).

Chapter 8 – Headroom Assessment

This chapter builds upon the commercialisation requirements discussed in chapter 2 as it looks into assessing CTP commercial viability. The headroom method was used to assess the reimbursement potential of the candidate cell therapy product. The results provide information on the cost-effectiveness, adoption and gross profit potential of the candidate cell therapy product.

Chapter 9 – Conclusions

This chapter provides an overall summary of the work that has been carried out. Here, the translational requirements that have been identified, investigated and presented in this body of work are reviewed.

Chapter 2:

Literature Review

Chapter 2. Literature Review

2.1. Parkinson's Disease

Parkinson's disease (PD) is an incurable, chronic neurodegenerative disease named after James Parkinson, who published the first detailed description of the disease in 1817 in 'An Essay on the Shaking Palsy'[3,27]. PD is one of the most common neurodegenerative diseases, particularly in western and developed countries where the aging population is higher[3,27]. As such, PD is mainly seen in the elderly, as the aging population increases it is estimated that the cases of PD will also increase. In the UK 1 in 550 adults are diagnosed with PD over the age of 60, and worldwide more than six million people are affected[28,29] (**Figure 1**). Dorsey *et al* (2007) reported "the number of people with PD will rise from 4.1-4.6 million in 2005 by two times to 8.7-9.3 million in the year 2030" based on studies of the ten most populous countries[30,31].

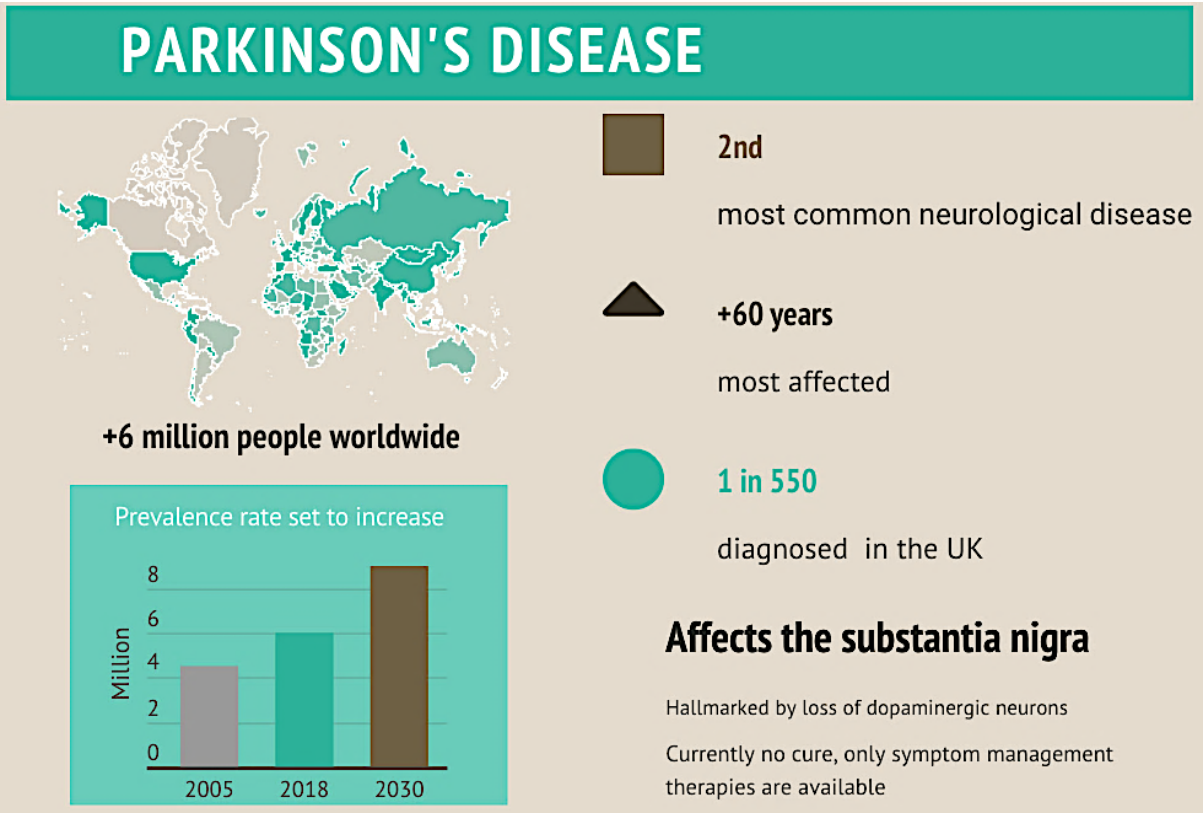


Figure 1. Broad overview of Parkinson's disease epidemiology. Data obtained from the literature[28,29].

PD is hallmarked by the discriminatory depletion of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) located in the midbrain[1,3,32,33]. Substantia nigra which translates to 'black substance' is so called due to its darker appearance as a result of higher neuromelanin levels compared to its neighbouring areas[27,34]. The SNc is part of the basal ganglia which controls movement, forming

part of the nigrostriatal pathway, which functions as a regulator of both the direct and indirect pathway of movement[32]. A decrease in the density of DA neurons results in diminished function of the nigrostriatal pathway and the concomitant motor problems such as hypokinesia, rigidity and tremors that are associated with PD (**Figure 2**).

There are different types of PD, namely vascular, drug induced parkinsonism and idiopathic Parkinson's, the latter being the focus of this work due to it being the most common type of PD (**Table 1**). Although most cases of PD are idiopathic; age, genes, gender, exposure to toxins and ethnicity can increase the risk of developing PD. The disease does not directly cause death, however due to complications, co-morbidities and risks of falls it does increase mortality rates. Typically, those diagnosed with PD have near normal life expectancy, however their quality of life is significantly diminished due to the debilitating results of the symptoms including tremors, muscle rigidity and the eventual onset of dementia in up to 75% of patients[4]. The pathology and symptoms of PD are summarised in **Figure 3**, along with the treatment options that are employed at the different stages of the disease.

Table 1. Details of the different types of Parkinsonism and their presentations

Type	Details
IDIOPATHIC	Idiopathic Parkinson's disease - or Parkinson's - is the most common type of parkinsonism. Idiopathic means that the cause is unknown. The main symptoms of idiopathic Parkinson's are tremor, rigidity (stiffness) and slowness of movement. (Shulman *et al*, 2011)
VASCULAR	Vascular parkinsonism (also known as arteriosclerotic parkinsonism) affects people with restricted blood supply to the brain - usually older people who have health issues such as diabetes. (Kalia *et al*, 2015)
DRUG-INDUCED	Neuroleptic drugs (used to treat schizophrenia and other psychotic disorders) which block the action of dopamine are thought to be the biggest cause of drug-induced parkinsonism. The symptoms of drug-induced parkinsonism tend to be static. Only in rare cases do they change in the manner that the symptoms of Parkinson's do. Symptoms are reversible upon cessation of the drug that is the cause. (Parkinson's UK, 2019)
MULTIPLE SYSTEM ATROPHY (MSA)	Both multiple system atrophy and Parkinson's cause stiffness and slowness of movement in the early stages. People with multiple system atrophy can also develop symptoms such as incontinence, difficulty with swallowing and dizziness, these symptoms are unusual in early Parkinson's. The condition used to be known as striatonigral degeneration, Shy-Drager syndrome, or olivopontocerebellar atrophy. (Parkinson's UK, 2019)
PROGRESSIVE SUPRANUCLEAR PALSY (PSP)	Progressive supranuclear palsy affects eye movement, balance, mobility, speech and swallowing. It is sometimes called Steele-Richardson-Olszewski syndrome. (Parkinson's UK, 2019)
NORMAL PRESSURE HYDROCEPHALUS	The symptoms of normal pressure hydrocephalus mainly affect the lower half of the body. The common symptoms are walking difficulties, urinary incontinence and memory problems. Removing some cerebrospinal fluid can help with these symptoms in the short term. If there is improvement after this procedure, an operation to divert the spinal fluid permanently (known as lumbar puncture) can help in the long term. (Parkinson's UK, 2019)

2.1.1. Clinical Features

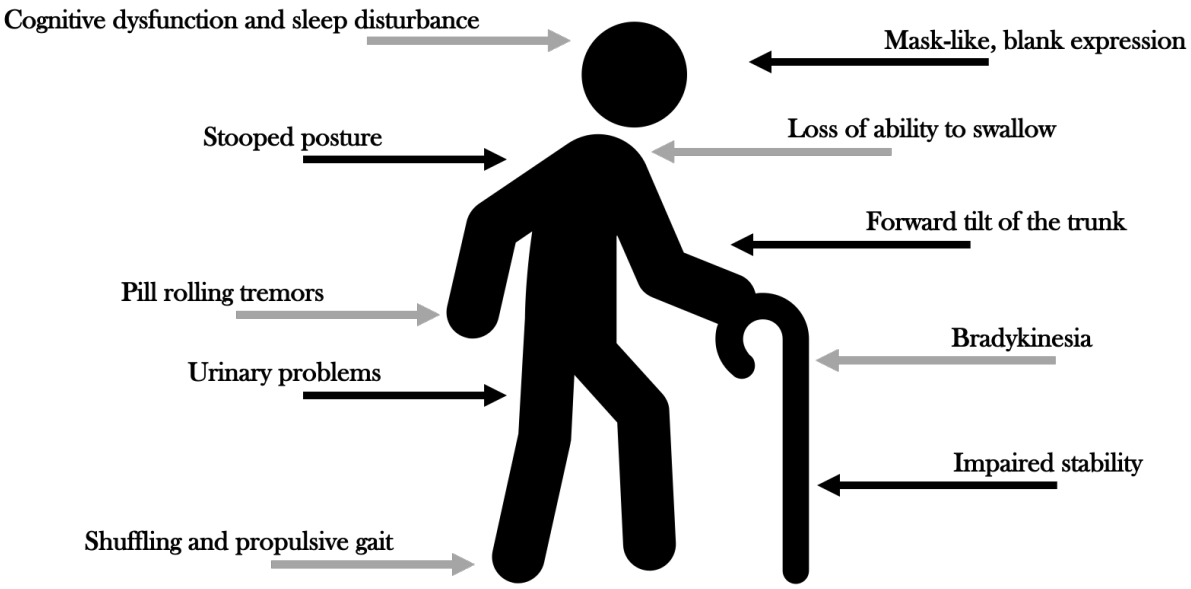


Figure 2. Schematic representation of the most common motor and non-motor PD symptoms

Patients of PD suffer from disturbances in regular movement; this includes bradykinesia which is a term used to describe a slowness in the execution of movement. In some severe cases, decreased activity of the direct pathway of movement results in akinesia, which is the inability to adequately initiate movement[5,35,36]. Tremors due to involuntary shaking are usually present at rest, with the tremor often becoming more distinguished as the disease progresses, although this symptom tends to diminish with movement[1,32]. Rigidity is experienced during movement due to stiffness in the limb causing resistance during movement, and as the disease progresses the rigidity becomes more widespread until it eventually impairs the individual's ability to move[32,37]. Postural instability affects the balance of PD patients, causing them to have a characteristic lean or stooped posture[4,38]. This symptom is usually observed in the latter stages of the disease and often leads to frequent falls due to the imbalance which is experienced. Furthermore, lack of facial expression, change in gait and dysphagia are other motor related symptoms that can be experienced as well as depression, dementia and sleep disturbances[32,39–41].

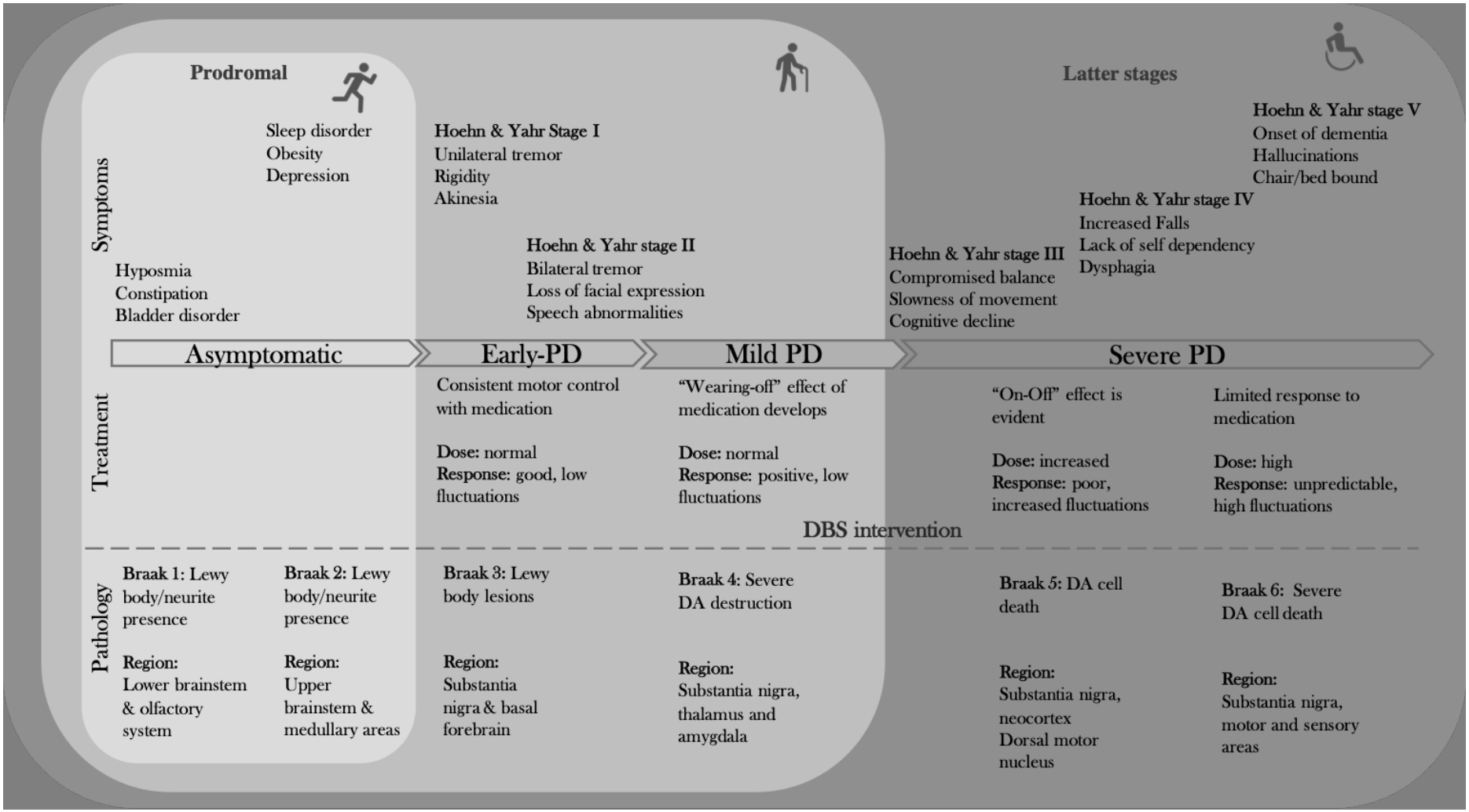


Figure 3. Summary of pathology, treatment and symptoms of PD.

2.1.1. Dopamine

Dopamine is a neurotransmitter that is produced by dopaminergic (DA) neurons, it is involved in a range of functions such as neuromodulation, reward behaviours and important to PD, motor utility of the direct and indirect pathway of movement[35]. It has been reported that a high flux of dopamine can be associated with alterations in behaviours as experienced in diseases such as schizophrenia and attention deficit hyperactivity disorder (ADHD)[34]. Dopamine is produced in the body but its production is mainly restricted to neuronal cells and the adrenal glands in the kidneys[34]. Of importance to PD reduced levels of dopamine result in deficiencies in motor pathways within the brain, this is mainly due to the loss of dopamine production by the A8, A9 and A10 cell groups which are found in the pars compacta of the substantia nigra[3,27,34]. Dopamine replacement in the brain is one of the predominant strategies currently used to treat PD as the depletion of dopamine is a key aspect of the disease. This is mainly through the use of medical drugs such as levodopa, dopamine agonists and glutamate antagonists that increase dopamine and/or stop its degradation[34].

2.1.2. Proposed causes & Risk Factors of PD

Although the putative loss of dopaminergic neurons is known to be linked to the progression of the disease, there is currently no consensus with regards to the aetiology of PD, despite a plethora of research. Specifically, disaccord comes from the mode in which the specific cell death of the DA neurons is initiated[42]. There is a solid body of work that considers the molecular causes of PD such as the genes and transcription factors involved in PD pathogenesis [2,43,44]. In addition metabolic botheration such as mitochondrial dysfunction has been considered[27,45,46], in addition protein phosphorylation[47,48], protein accumulation[3,49–51] and oxidative stress have also been implicated in the pathogenesis of PD[48]. Apoptosis and autophagy have been proposed as mechanisms of cell death in DA neurons, although the triggers are not well understood and this is an area of extensive research[50,52–55]. Current methods of detecting cell death such as cell staining, cell morphology microscopy, immunolabelling and DNA-labelling, are not conclusive enough to determine the cause of PD cell death[52].

PD is normally observed in the elderly particularly those over the age of 60, however there are rare cases of early-onset PD. Early onset PD is commonly seen when there is a hereditary link to PD, in these cases genes play a role and increase the chances of developing PD. These include but are not limited to mutations to the *DJ-1, α-synuclein (SNCA),* parkin (*PRKN*), leucine-rich repeat kinase 2 (*LRRK2*) and PTEN-induced putative kinase I (*PINK1*) genes[1,31,56]. Current research has also shown that males are more likely to develop PD than females at a ratio of 3:2, however the reason for this disparity is yet to be established[29,31,57,58]. Other research suggests that there is a slight risk increase of developing PD due to long term exposure to pesticides and herbicides[31,46,58]. It must be noted that

currently the evidence of the roles of sex and exposure to toxins is limited or only a probability at most[31]. A study in Northern California found that Hispanics and Whites (non-Hispanic) had higher incidences of PD compared to Asians and Blacks, however more research needs to be carried out on a larger and geographically diverse scale[57].

2.1.3. Genes and Transcription Factors

Although most cases of PD are idiopathic, there have been studies that have correlated PD with genetic predisposition[31,48,56]. In the case of idiopathic PD the roles of transcription factors has been of considerable interest both historically and more recently, as it has been illustrated that the timings and levels of essential factors for dopaminergic neurogenesis during development can have a subsequent effect on the onset of DA cell death; in some cases causing premature DA cell death[42]. This is also likely to be the case for factors such as nuclear-receptor-related-1 (*nurr1*), Fibroblast Growth Factor 8 (*fgf8*), Sonic hedgehog (*shh*) and Pentraxin 3 (*ptx3*), particularly *nurr1* which is essential for DA viability under both normal and stress conditions[48].

There are both dominant and recessive forms of the mutations which occur in specific genes that are linked to hereditary forms of PD[42]. Barzilai and Melamed (2003) highlight some of these genes, for instance *Parkin* which is important for the ubiquitin-targeted pathway, which was discovered in Japanese families with autosomal recessive early-onset PD[43,59]. Other predisposed genes include *α-synuclein (SNCA)*, *Ubiquitin C-terminal hydrolase L1(UCH-L1)* and *DJ1* which have all been alluded to involvement in oxidative stress response[42]. A review by Kaczmar *et al* (2006) also explores genes thought to be associated with PD including *LRRK2*, *PINK1* as well as *SNCA and DJI* as mentioned above [48]. Whilst there are many facets that contribute to the onset and progression of PD including the aforementioned genes, the focus of this work will be on strategies for the replacement of dopamine.

2.1.4. Proteins

In a wide range of neurodegenerative diseases, a common hallmark is the presence of Lewy bodies (LBs), which are irregular protein aggregates observed in the nerve cells, which cause displacement of other cell components[3,50,56]. However, it remains to be elucidated whether the LBs cause PD in a pathogenic manner; or conversely if LBs are a consequence of an unknown pathogenic factor of PD[48]. What is known is that, LBs can be comprised of α-synuclein, ubiquitin and neurofilament protein, however the mechanisms that cause the fibrils of these proteins form LBs are currently unknown[50]. LBs persist within the cells as they are insoluble, which impairs the normal function of the ubiquitin–proteasome system (UPS). The UPS is an intracellular mechanism for the removal of abnormal proteins in eukaryotic cells, again whether or not this is the cause of DA cell death, remains unknown[42].

2.1.5. Diagnosis

Currently there are no tests that allow for definitive diagnosis of PD. Diagnosis of PD is a combinatory process that is performed by clinical specialists who carry out comprehensive medical and neurological testing. In the early stages of disease progression, it is difficult to distinguish PD from other types of parkinsonisms as the initial presentations and symptoms are all similar. In many cases the symptoms that allow for specific diagnosis to be made, only become apparent as the disease progresses. The most common mode of diagnosing PD is evaluating a patient's response to dopamine-replacing medication such as levodopa[3,32,60]. If a patient's symptoms improve as a result of taking the medication it is likely that they have PD (idiopathic), furthermore, if cessation or reduction of their dose makes the symptoms reappear it is an additional indicator that the patient has PD. Generally, patients that have non-PD parkinsonisms will not respond well, or at all to dopamine-replacing medication. However, since this is not always the case, other tools are required in combination to aid the diagnosis process. A dopamine transporter chemical (DaT) scan using single-photon emission computed tomography (SPECT) scanning can be employed to assess the levels of dopamine in the brain[3,32,60]. PD clinical specialists will also examine a patient's ability to write, draw, walk, freely move their limbs, speak and move their face to create facial expressions when diagnosing for PD: these diagnostics are used to inform the course of treatment.

2.1.6. Current Treatments for Parkinson's Disease

Most of the treatments for PD tend to only aid with the symptoms, as none of the current treatments stop the neurodegeneration process[32,61]. As a result, current strategies focus of increasing dopamine for signalling or in the case of deep brain stimulation (DBS), counteracting the aberrant signalling that is observed in the pathways that regulate movement.

2.1.6.1. Anti-parkinsonian medication

Treatment strategies primarily concern exogenous dopamine replacement; with extensive pharmaceutical therapies available; most aim to provide cells with dopamine by either: **increasing** levels, **preventing** degradation; or **stimulating** receptors. Current medication therapies are unable to modify the disease state in terms of slowing, stopping or reversing progression.

Levodopa (L-DOPA) is an amino acid based drug that is used to provide cells with exogenous dopamine[32,61,62]. Naturally L-DOPA is the precursor of dopamine, L-DOPA is prescribed instead of dopamine because dopamine is degraded peripherally before it can cross the blood-brain barrier. Typically, L-DOPA is co-administered with catechol-O-methyltransferase (COMT) and monoamine

oxidase B (MAO-B) inhibitors which help to prolong the presence of L-DOPA by preventing its enzymatic degradation. L-DOPA is often prescribed under the brand names : Madopar, Duodopa, Sinemet and Lecado which can be used at all stages of the disease[61]. Dopamine agonists may also be prescribed to stimulate dopamine receptors by mimicking the dopamine that would be produced under normal conditions with the aim of inducing an effect on the cells i.e. to facilitate motor functions. There is a wide range of dopamine agonists that can be prescribed these include but are not limited to: Bromocriptine, Pramipexoe and Ropinirole which can be used at all stage of PD[61].

Akin to all medication, PD medication has potential side effects including: liver toxicity, sleep disturbances, hallucinations, delusions, dyskinesia and sometimes impulsive/compulsive behaviours[32,63,64]. Furthermore, use of medication therapy results in polypharmacy to ease side effects such as anxiety and constipation[65]. As PD is progressive, pharmaceutical efficacy diminishes over time; resulting in increased strength and dosing frequency as the medication becomes inadequate and symptoms are more pronounced. Furthermore, the therapeutic window whereby the patient is 'on' decreases, resulting in more 'off' periods (when the drug effect ceases prior to the next dose) and occurrences of dyskinesia caused by dosage increases (**Figure 4**) [4,6,66–68].

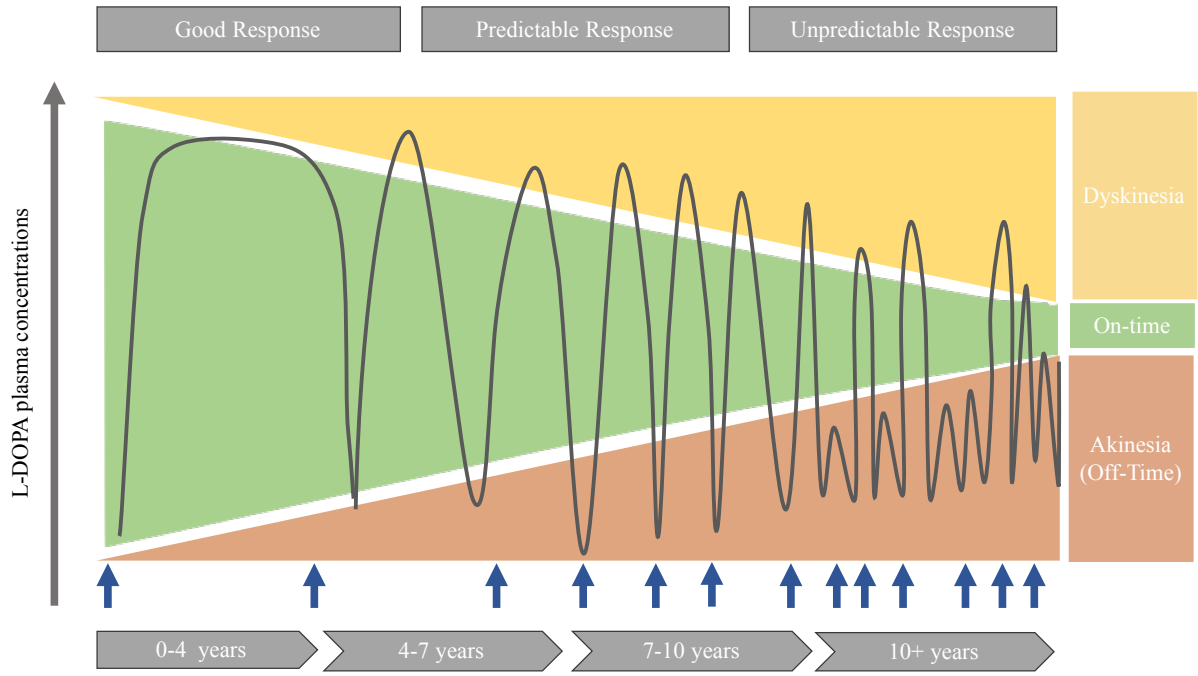


Figure 4. Illustrative example of the 'on/off' effect of PD medication, showing how the therapeutic window (green space) of the medication decreases as the disease progresses. In addition, more doses of medication (signified by the arrows) are needed to control the motor fluctuations however more time is spent in the 'off' periods and experiencing akinesia or dyskinesia as the disease progresses even with increased doses due to the loss of neurons. L-DOPA = levodopa, representing PD medication with a mechanism of action that provides L-DOPA. Image adapted from Worth, 2013.

2.1.6.2. Surgical Interventions to manage PD symptoms

Deep brain stimulation (DBS) is a surgical technique that is used to help with movement control; however, it is not curative. One of the reasons for choosing DBS as a mode of treatment, is that it can result in the patients being able to take less medication. This is ideal as it avoids some of the side effects that are associated with taking PD medication. However, mixed results for DBS treatment have been observed, as some patients do not experience any benefits from undergoing the operation[61]. During DBS a pulse generator is used to deliver high frequency stimulation, with the intention of producing an alteration to the irregular electrical signals due to the lack of the dopamine neurotransmitter that results in PD symptoms[61,62]. The procedure is non-destructive and can be reversible, as a part of the process patients get a programmable portable computer that is used to control the stimulations[61].

Other surgical procedures include lesioning surgery, unlike DBS lesioning procedures are irreversible as they involve selective damage to cells in a specific part of the brain. Electrodes are introduced to the target site, which is located using computerised tomography (CT) or magnetic resonance imaging (MRI) brain scans. An electric current is then passed through to cells that are involved in the control of movement, therefore potentially reducing movement problems. There are three main types of lesioning surgery used, thalamotomy, pallidotomy and subthalamotomy which involve making lesions in the thalamus, globus pallidus and the subthalamic nucleus respectively[61]. The latter is used in countries such as Northern Ireland and Wales were DBS is not available as a form of surgery.

2.1.6.3. Cell Therapy for Parkinson's

Although PD pharmaceutical therapies offer alleviation of some of the symptoms of PD, it is evident that medication has its limitations and in some cases complications when it comes to the treatment of PD[32,69,70]. As a result, there have been efforts in determining alternative sources to treat and potentially cure PD, with the concept of cell replacement strategies becoming more prevalent over the three last decades. This is due to early work in cell replacement strategies that showed sustained symptomatic relief and significant motor improvements could be realised in human subjects[71,72]. One of the strategies uses tissue grafting of ventral mesencephalic dopaminergic neurons into the putamen. Thus far, human allogenic grafts of ventral mesencephalic tissue have been shown to survive at the site of implantation for up to 24 years and improve motor function, in addition the engraftment restored dopamine synthesis and storage[71,72]. Consequent to such findings, interest in engraftment rose as it provides a quasi-natural form of producing and storing the depleted dopamine, which cannot be achieved with current treatments. Post-mortem analysis of a patient with PD who underwent dopaminergic neuron transplantation 24 years earlier showed "near-normal graft-derived dopaminergic reinnervation of the putamen"[71]. However, in the current state there is still a lot of work that needs to be done for cell

therapies to be first choice treatment for PD. Some of the hindrances include current regulations in the cell therapy industry (CTI), the cost of the therapies and most importantly the need for more in depth understanding of the ways in which the transplanted cells work, particularly, once engrafted. Furthermore, the CTI needs to establish how to produce reliable, reproducible and cost-effective cell based therapies[73,74]. Current results of cell replacement strategies show great promise and potential benefits for PD patients that cannot be currently achieved using pharmaceutical treatments and surgical interventions. However, it is worth noting that even after successful engraftment, the disease will still continue to progresses and will eventually have a detrimental effect on the grafted cells as well[71]. Nonetheless, it should be noted that this progression occurs over a prolonged period of time i.e. 14 years posttransplantation[71], thus providing the patient with a significantly improved quality of life during this timeframe. Therefore, part of the fundamental work that needs to be carried out in order to discover a cure for PD is gaining an understanding the underlying mechanisms that drive the pathogenesis of the disease. This mechanistic understanding will hopefully, with time, allow for researchers to focus their efforts on preventative measures to stop the progression of the disease. However, this is presently out of the scope of the current work. The following sections will discuss how cell replacement strategies and dopaminergic neuron protocols have developed and evolved. The work of Anders Björklund and Olle Lindvall has been used as the key exemplar, as they are the forerunners of the area of PD cell therapy and they are linked closely to the present work. It should be noted that this exemplar may not be applicable to all forms of PD.

2.1.7. The Lund method

The hESC based therapy that this work aims to contribute to was developed from collaborators at Lund University in the 1980s who pioneered the field of cell transplantation for the treatment of PD. The world's first ventral mesencephalic tissue cell transplantation for the treatment of PD was done in Lund in 1989 in work led by Anders Björklund and Olle Lindvall. Björklund *et al* showed that taking tissue from the human foetus, specifically ventral mesencephalic tissue that derives dopaminergic neurons, and transplanting it into a human subject had beneficial results for PD patients[12,75]. The transplantation of ventral mesencephalic tissue improved motor function and the engraftment restored dopamine synthesis and storage[71,72]. Prior to transplantation the patients had difficulty walking and were unable to make normal sized movements, typically they had smaller, slowed movements with obvious body rigidity. Post transplantation (five years after, during the foetal trial) walking eased and less rigidity was observed in patients, which was an unprecedented level of symptomatic relief, in comparison to pharmaceutical therapies such as L-DOPA[76–79]. In some cases, patients who have been followed up have ceased their use of anti-Parkinsonian medication for up to sixteen years post transplantation[80].

At present, over 100 patients have been grafted in Lund and other parts of the world that use cell transplantation (of vmDA progenitors) for PD treatment. Patient follow up prior to post mortem analysis has also shown dopamine synthesis post transplantation, using fluoro-DOPA (F-DOPA) positron emission tomography (PET) scanning[81–83]. Furthermore, it has been shown is that there no evidence of α-synuclein with the grafts up to 14 years post transplantation in human subjects. However, α-synuclein is present within the host cells, suggesting that although remarkable symptomatic relief can be achieved, PD disease progression still continues irrespective of the cell transplantation treatment.

2.1.7.1. How therapy is conducted

Different cell transplantation trials using foetal vmDA tissue, induced pluripotent stem cells (iPSc), parthenogenetic derived DA neurons and neuro stem cells have been conducted[84]. However, the focus of the present work will be on the Lund consortium trials as they are directly related to this work. In brief, the foetal based cell therapy requires three to five foetuses for each side in a bilateral transplantation, from elective abortion foetuses (six to eight weeks old). The foetuses have to be freshly obtained on the same day and utilised within four hours to maintain the viability of the tissue. The foetal neural tissue, specifically of the ventral mesencephalic region is treated to obtain the cells through a range of wash and purification steps[72]. The patients have to undergo immunosuppression the day before transplantation and remain on immunosuppression medication for at least one-year post transplantation. The transplantation is then carried out using minimally invasive stereotactic surgery, which uses three-dimensional coordinates to deposit the cells as a suspension into the putamen. Patients can be discharged 48-hours post-surgery, typically they followed up six and twelve months post-surgery. F-DOPA PET scan imaging is used to determine dopamine activity within the brain. A range of motor and behavioural assessments are also carried out to ascertain the level of clinical symptomatic relief afforded by the therapy. In summary, current trials have demonstrated that dopamine neuron cell replacement therapies work and can survive long term, i.e. up to 24 years in the host brain whilst efficiently re-innervating the ventral mesencephalon into the dorsal striatum, "near-normal graft-derived dopaminergic reinnervation of the putamen"[71]. This results in restoration of DA synthesis and release, which ultimately provides long-term clinical improvement, showing proof of concept. However, as it stands the results are variable even though many successes have been afforded from the ~ 100 patients that have gone through the transplantation procedure. One major factor of variation is the foetal source of the cell material which highlights the need to have cell preparations that are optimised and standardised. Furthermore, a foetal tissue-based therapy poses many challenges from regulatory, reproducibility and ethical stand points. As such, efforts have been made to produce the vmDA progenitors from a more sustainable and ethically less constrictive source, i.e. hESCs as opposed to foetal derived tissue. Efforts at Lund and internationally have resulted in the generation of protocols for deriving vmDA neuroprogenitors from hESCs that show comparative efficacy and potency to foetal tissue derived vmDA neuroprogenitors[85].

2.2. Dopaminergic neurons

2.2.1. Dopaminergic neurons

Dopaminergic (DA) neurons are located in the midbrain, with ~ 590,000 found in the adult human brain; this number is age dependent and depletes with increasing age[3,34,86]. Dopamine producing cells in humans are found in the A8, A9 and A10 cells groups on the substantia nigra (**Figure 5**)[27,34,42]. The intrinsic development of DA neurons is marked by a complex combination of secreted factors, gene expression and transcription factors. There are different transcription and soluble factors that are expressed and secreted dependent upon the stage of differentiation. Stages such as early midbrain patterning, specification of mitotic precursors, post-mitotic development, and functional maturation all have varying soluble factors and transcription factors associated with them[86–88]. By researching the developmental processes of the neural tube, specifically the processes that lead to the generation of DA neurons, it has been possible to carry out fate mapping studies studies[89–91]. These studies have guided the understanding of the cues and distinctive markers associated with DA neurons: highlighting precisely the factors and cues that drive the patterning and maturation of the cells both *in vivo* and *in vitro*[25,92]. This facilitates *in vitro* differentiation methods to afford the desired vmDA cells types. Hegarty *et al* (2013) and Arenas *et al* (2015) provide an in-depth review and primer, respectively, which offer comprehensive vmDA generation and development knowledge[93,94].

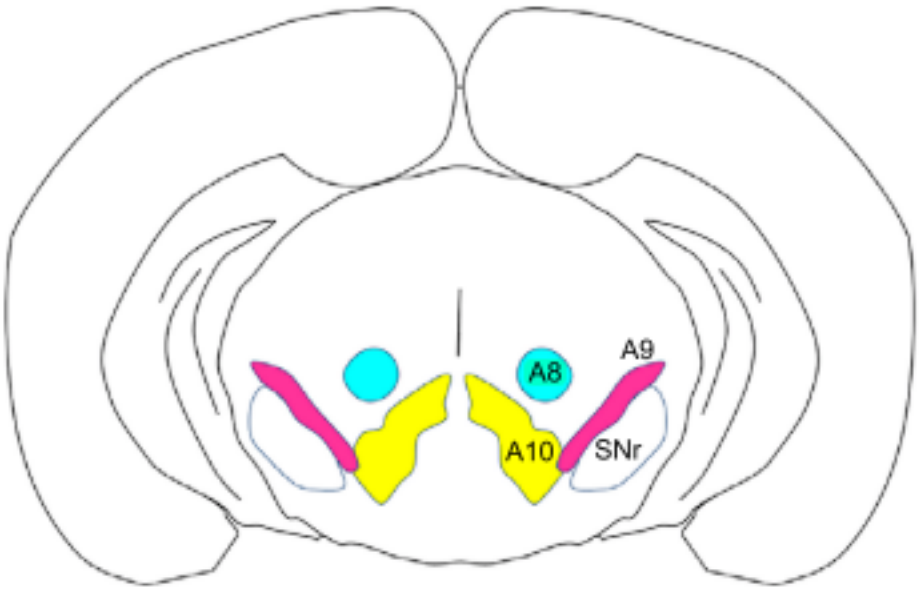


Figure 5. Coronal section view showing the A8, A9 and A10 dopaminergic cell groups. SNr = substantial nigra Image sourced from Arena *et al*, 2015.

2.2.2. The differentiation into vmDA neuroprogenitors

Understanding the development cues of the vmDA is key to the success of the therapy for the treatment of PD, as this allows for the derivation of protocols that reliably produce the desired vmDA for transplantation. The following sections discuss in a concise manner the key aspects of neurulation and development of the mesencephalon, specifically focussing on the development of the ventral mesencephalon which gives rise to the desired vmDA neuroprogenitors.

2.2.2.1. Neurulation

Once the three germ layers (endoderm, mesoderm and ectoderm) are formed as part of the gastrulation process, the central nervous system (CNS) through neurulation is first system formed during embryonic development. The development of the CNS starts around the third week of embryonic development[94,95]. The CNS originates from the ectoderm layer which thickens and forms neural plate through neuroectoderm cells, a process referred to as neurulation. Neurulation begins once the notochord is formed within the mesoderm layer. The notochord induces the thickening of the ectoderm in order to form the neural tube, by stimulating the invagination of the neural plate to form the neural groove and form the neural folds. The plate begins to fold inwards and forms what is referenced to as the neural groove which is in the centre and the neural folds which are on either side of the neural groove. By the end of the third week neural folds begin to fuse together forming the neural tube (CNS) and neural crest (peripheral nervous system) which eventually develops into the brain and spinal cord, respectively[25,96–98] (**Figure 6**). The protein sonic hedgehog (SHH), secreted by the notochord, is responsible for specification of the most ventral region of the neural tube which is the floor plate. With time (28 to 32 days) the neural tube closes, resulting in a bulging and bending of the neural tube[97,98]. This results in the formation of three distinctive areas known as the primary vesicles, comprising of the prosencephalon (forms the cerebrum), mesencephalon (forms the midbrain, deriving the substantia nigra) and metencephalon/rhombencephalon (forms the brain stem and cerebellum)[94,97]. At this point there are many pathways, factors, morphogens and signalling centres that simultaneously occur to allow for patterning of different regions of the brain[93]: As aforementioned, this work will focus on the development and patterning of the mesencephalon as it is the area which vmDA are derived from.

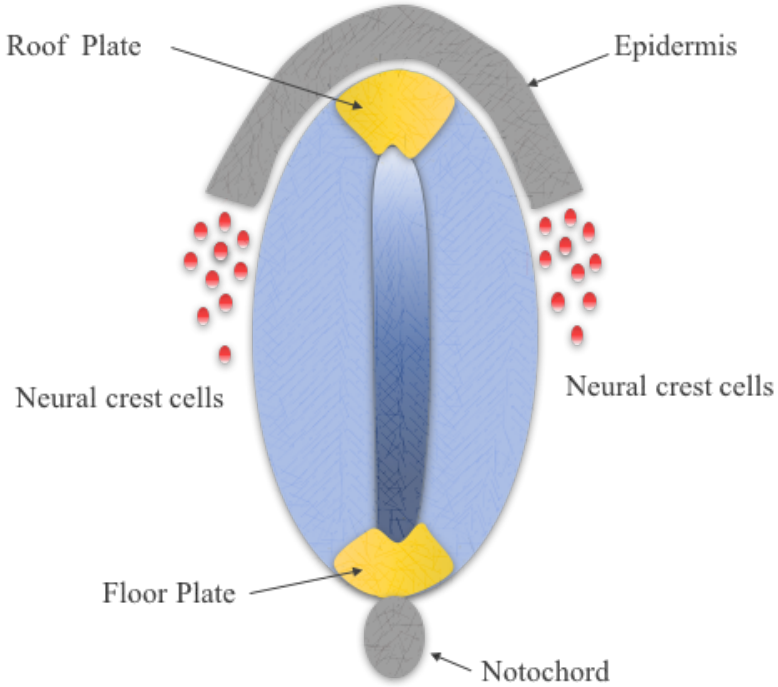


Figure 6. Depiction of the areas of the neural tube (blue) during neurulation showing the floor plate and roof plate regions (yellow), neural crest cells (red), the epidermis and notochord, both in grey. Image adapted from Green *et al,* 2015.

2.2.2.2. Patterning

The patterning of the mesencephalon from the neural tube is directed by two signalling centres and a series of morphogen gradients. One of the key gradients and centres is the roof plate-floor plate of the neural tube that directs either rostral or caudal ventralisation. This signalling centre involves gradients of bone morphogenetic protein (BMP) and SHH, both implicated in the patterning of the dorsal and ventral regions (**Figure 7**). High BMP concentrations at the roof plate drive towards dorsal regionalisation and high concentrations of SHH from the floor plate result in a ventralisation[86,95,99]. The second signalling centre is the isthmic organizer (IsO) which results in the midbrain-hindbrain boundary (MHB)[94,100,101]. Patterning of the MHB is mediated by *OTX2* and *GBX2* which regulate the expression of *WNT*, *EN1* (mesencephalon) and fibroblast growth factor-8 (*FGF8*) (metencephalon) using the resulting morphogen proteins[95,102,103]. Concentration gradients of FGF8 and WNT induce the IsO, higher concentrations of FGF8 result in the metencephalon, whilst lower gradients result in the mesencephalon[94]. WNT gradients pattern posterior to anterior, with higher concentrations resulting in the posterior/caudal regions[94,95,104]. Both centres (BMP/SHH and FGF8/WNT) through transcription factors and morphogens give rise to the regional identity of the ventral mesencephalon region.

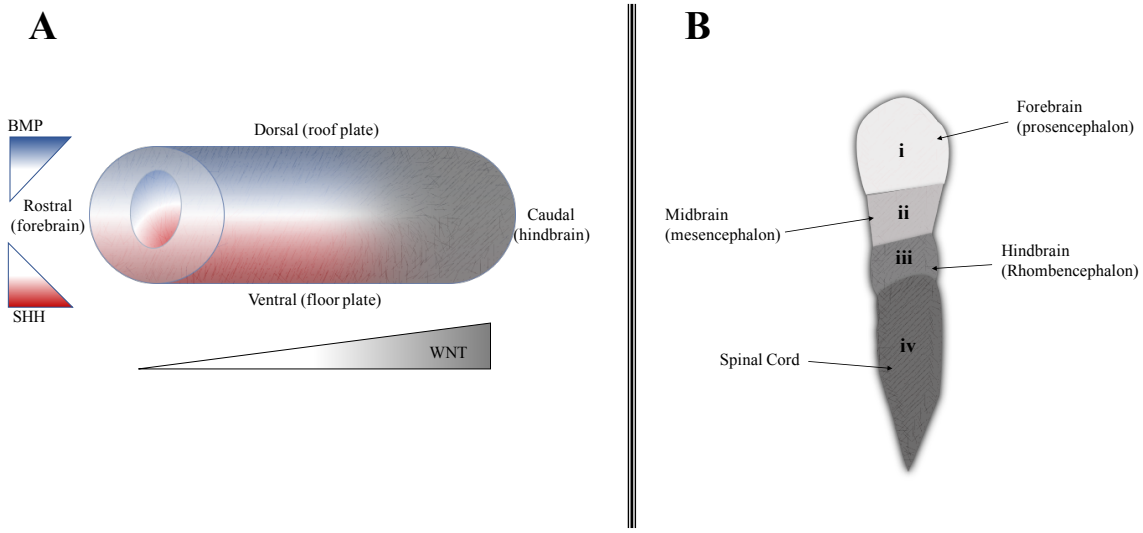


Figure 7. (**A**) Schematic of the concentration gradients of BMP, SHH and WNT involved in the patterning of the neural tube towards the three primary brain vesicles (**B**) forebrain (i), midbrain (ii), hindbrain (iii) and the spinal cord (iv). Image adapted from Kirkeby and Palmar, 2012.

For the patterning of the cells SHH plays an important role in the specification and patterning of the early neural tube. A gradient effect of SHH and WNT distinguishes between the ventral and dorsal regions, whereas gradients between SHH and BMP result in the floor plate and roof plate respectively[86,91,92,105]. FGF8 is also involved in patterning as it is a morphogen, specifically for the organisation and maintenance of the mid/hindbrain boundary[94,106].

2.2.3. *In vitro* differentiation of pluripotent cells into vmDA

The rational for using cell replacement strategies for the treatment of PD is based on a wide range of factors such as the evident benefits discussed in section 2.1.6.3 . However, currently these methods are hindered by ethical issues, reproducibility and cell source sustainability, availability and quality. Therefore, there is a demand in the area to find ways to obtain suitable cells for transplantation (of vmDA progenitors). This has led to the current plethora of work that provides protocols to easily, quickly and successfully produce neural cell lineages, from embryonic and pluripotent cell lines, that are stable and can be used as disease models as well as for transplantation[25,87,107]. The cells that are generated are similar to the foetal cells, in terms of their morphology, surface antigen and protein expression, functionality and have comparable potency[24]. In terms of transplantation, Grealish *et al* (2014) show that hESC derived cells are akin to their foetal derived counterparts; for instance, with regards to their ability to grow and sufficiently reinnervate allowing for the restoration of DA neurotransmission[85,108]. Neural differentiation has thus far been achieved through three key methods:

embryoid body (EBs) based differentiation, feeder based differentiation, and monolayer differentiation with dual SMAD inhibition[107,109].

Section 2.2.2 briefly describes the neurulation process, which has formed the basis of many differentiation protocols that aim to obtain vmDA. The protocols discussed in this section are specifically those that utilise hESCs for their differentiation process. It is worth noting that other cell sources for vmDA differentiation have been explored, such as somatic cell reprogramming (using *lmx1a, nurr1, foxa2, en1* and *pixt3*) [8,94,110,111] and neural stem cells[112–114], however both are out of the scope of this work which is hESC based.

It is important to emphasise that the brain is often considered to be the most complex entity in the universe. Therefore, it comes as no surprise that the differentiation of neuronal cell types is an onerous challenge that involves intricate nuanced combinations of signalling pathways, transcription factors and morphogens interacting a complex network. Understanding and controlling this network is crucial to successfully obtaining the desired neuronal subtype, as even the slightest of deviations will result in unwanted subtypes due to the infinitesimal proximity of cell nuclei groups in the brain and their sensitivity to signalling and factors. For instance, the A8, A9 an A10 cell nuclei are all dopamine producing and all found in the mesencephalon, however not all involved in the aetiology or potential cell-based treatments of PD.

In the present work the objective is to obtain DA neurons, specifically those in the substantia nigra of the mesencephalon (midbrain) originating from the ventral region of the mesencephalon, specifically from the FOXA2+/LMX1A+ floor plate. These vmDA are tyrosine hydroxylase (TH+) neurons that produce the catecholamine neurotransmitter dopamine. Furthermore, we need to specify the specific nuclei of cells as several groups of DA neurons exist in the brain, three of which (A8, A9 and A10) are found in the mesencephalon[115–117]. The A9 cell nuclei of the substantia nigra pars compacta is therapy-specific as they produce dopamine and importantly, the cells project axons to innervate the dorsal striatum, integrating into the nigrostriatal pathway which is responsible for motor function regulation. Loss of this A9 nuclei of vmDA neurons is the hallmark indicator of PD as loss of vmDA neuron innervation into the striatum is the pathological basis of PD. The following section highlight the key protocol develops in the area of vmDA differentiation.

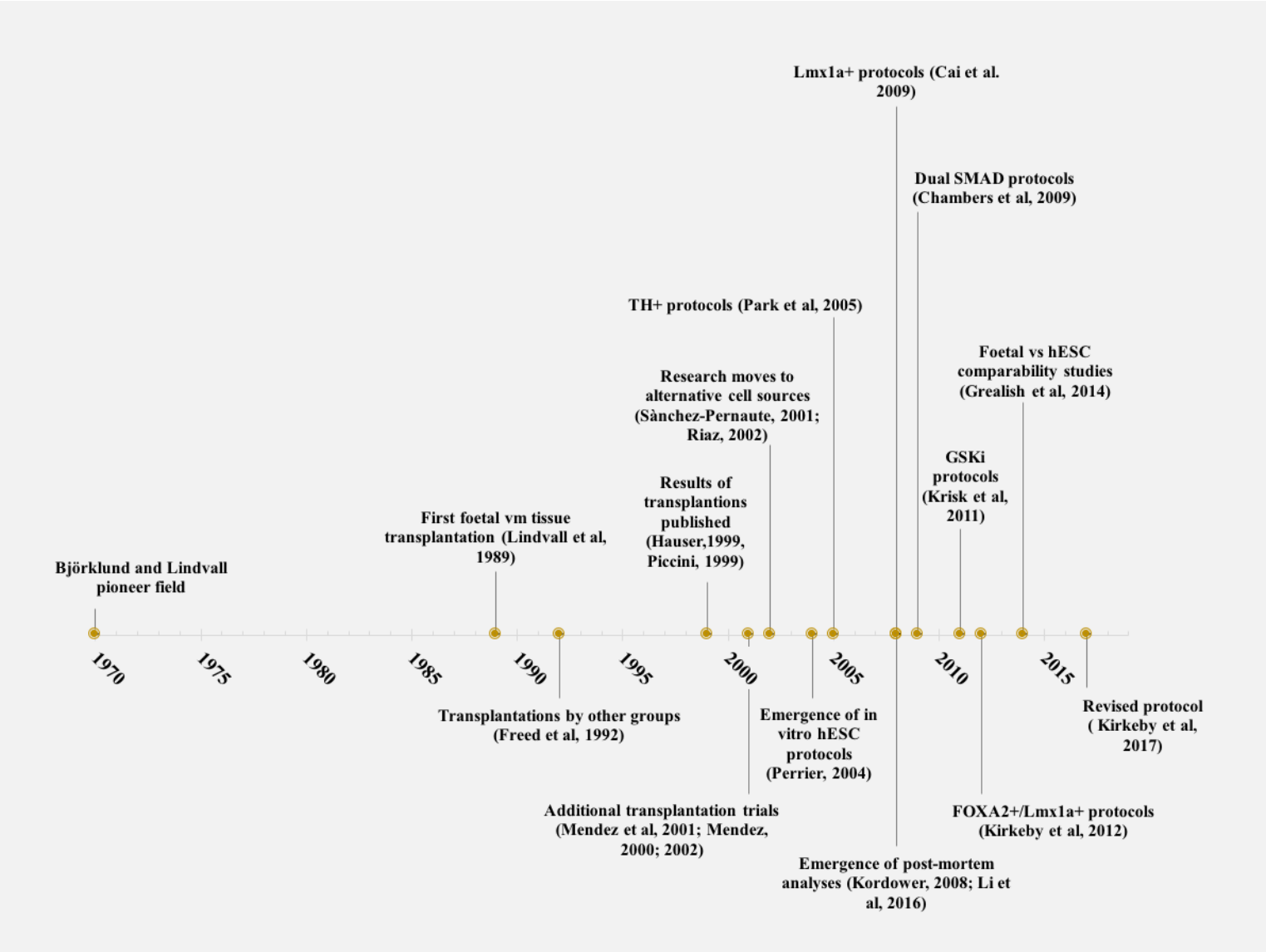


Figure 8. A timeline of vmDA cell transplantation progression over five decades. Highlights include the first transplantations in 1989 and 1992; different protocols that have been developed to produce transplantable vmDA progenitors including the dual SMAD inhibition protocol by Chambers *et al* (2009) and the latest protocol from Lund University (Kirkeby e*t al*, 2017).

2.2.3.1. The protocols

Initial protocols did not derive homogenous floor plate vmDA neurons, instead a mix of neuronal cells were obtained[94,95]. Indeed, these protocols produced TH+ cells however the cells were not authentic vmDA neurons that were suitably functional for transplantation. This work has endeavoured to produce a very concise review of the key protocol adjustments that have been made for differentiation of vmDA neurons over the last decade and a half, focussing on the latter ten years as this are the most useful protocols to consider (**Figure 8**).

2.2.3.1.a. Initial protocols

Initial protocols aimed to produce TH+ cells as this was believed sufficient for dopamine synthesis[118,119]. Although seemingly successful, it was apparent these cells were inadequately specific therapeutically, since other neuronal cells are TH+ and produce dopamine. Typically, these protocols were from a FOXA2-/PAX6+ neural stem lineage grown from EBs and feeder cells with activation of SHH and FGF8 pathways to mimic embryonic development. However the resultant cells had reduced viability post-transplantation and formed nonspecific axonal outgrowth in rats[94].This showed that just having dopamine producing cell (TH+) was not the only requisite for effective therapeutic benefit, as the cells being produced by these protocols were not adequately specified the into the desired vmDA A9 group that can innervation into the striatum, restoring the nigrostriatal pathway.

2.2.3.1.b. LMX1A protocols

The next generation of protocols took cues from the natural vmDA development during embryogenesis. These protocols homed on the necessity of LMX1A as an indispensable marker for appropriate vmDA lineage commitment as LMX1A suppresses basal plate cell fates within the floor plate[34,120]. Expression of *lmx1a* was achieved using hESCs in the presence of SHH and FGF8 to pattern for the ventral regionalisation. FOXA2, another important ventral patterning maker, suppresses lateral cell fates by inhibiting NKX2.2. In studies where FOXA2 was absent, NKX2.2 was not inhibited, resulting in both ventral and lateral mesencephalon cell fates[94]. This highlights that appropriate ventralisation is achieved by network interactions between WNT1-LMX1A and SHH-FOXA2 in the mesencephalon floor plate[95]. This network is not only important during the specification process but also for neurogenesis, differentiation and vmDA survival. The move to LMX1A+/FOXA2+ specification resulted in appropriate vmDA neuroprogenitors that proceeded to express late vmDA markers *LMX1B, NURR1, PITX3* and *DAT*. However, cells obtained were heterogeneous populations contaminated by glutamatergic subtypes resulting in low yields of vmDA progenitors[92,121].

2.2.3.1.c. Dual SMAD protocols

Taking influence from embryonic development, subsequent protocols were more rigorous in recapitulating patterning factors and morphogens, facilitating more efficient vmDA neuroprogenitors. Chambers *et al* (2009) introduced the dual SMAD approach which firstly ensures neural induction of hESCs followed by patterning of the neural stem cell towards the ventral mesencephalic fate[107,122]. These protocols typically avoided EB use. The dual SMAD approach inhibits BMP and TGFβ, resulting in feeder-free, efficient neural induction. The inhibition is dual as, blocking only one pathway is insufficient for effective neural conversion. The addition of both noggin and SB431425 blocks BMP and TGFβ signalling pathways, resulting in differentiation to the neuroectoderm by blocking SMAD transduction and decreasing pluripotency markers including OCT3/4 (POU5F1) and the presence of neurostem marker PAX6 at day 7-8[107,122]. This allows complete neural conversation of hESCs in adherent conditions in the presence of RHO kinase inhibitor (ROCK*i*), negating the need for stromal and astrocyte feeder layers.

2.2.3.1.d. WNT/β-catenin pathway protocols

The dual SMAD approach afforded neural induction however adjustments were necessary to ensure that the appropriate vmDA patterning trajectory. Krisk *et al* (2011) showed that although efficient neural induction was achieved using the dual SMAD approach, the midbrain specificity into vmDA was not correct as WNT/B catenin signalling was not initiated. Thus, an iteration introducing glycogen synthase kinase 3 inhibitor (GSK3*i*) allowed activation of the WNT/B catenin pathway, affording ventral-caudal patterning, resulting in cells with high co-localisation of LMX1A and FOXA2[94,123]. GSK3*i* and SHH concentrations are integral to ventral mesencephalic patterning; lack of GSK3*i* and SHH results in dorsal fate neurons and high concentrations result in hindbrain neurons, neither are TH+. Therefore, it is important to determine appropriate concentration balances to achieve correct patterning, especially as many protocols prescribe differing concentrations of GSK3*i*. Dual SMAD/GSK3*i* protocols have produced *in vitro* yields up to 75% vmDA-identity cells, resulting in functional recovery of motor behaviour in PD animal studies[75,94,107].

2.2.3.1.e. Preclinical and GMP adapted protocols

Since 2012 the dual SMAD/GSK3*i* approach formed the basis of vmDA differentiation protocols[75,87,122,124–127]. Grealish *et al* (2014) used this for preclinical studies and animal models, demonstrating appropriate vmDA specified cells, capable of innervation with no overgrowth or progeny[85,94,106,108]. The functional cells have long-term survival post-transplantation, produce dopamine and demonstrate improved motor behaviour in rat models[124,128]. Kirkeby *et al* (2012, 2014, 2017) have

since produced a series of protocol iterations based on the dual SMAD/GSK3*i* which have currently transitioned from EB-based to adherent culture on Laminin. Kirkeby *et al* (2017) ascertained that precise cell patterning with carefully timed FGF8 addition is required for high yields of vmDA neurons[129]. Moreover, Grealish *et al* (2014) demonstrated functional capacity of hESCs-derived vmDA neuroprogenitors using the SMAD/GSK3*i* approach is functionally comparable to foetal-derived vmDA neuroprogenitors[24].

2.2.4. Summary

Evidently mimicking embryogenesis is the most appropriate basis for contemporary hESC differentiation protocols. The challenge remaining concerns addressing translational requirements to achieve robust manufacturing processes capable of meeting clinical need and scale. Thus, it is important that protocols can be reliably scaled-up using defined components and reagents to ensure good manufacturing practice (GMP) compliance. This requires manufacturing process consideration to satisfy regulatory elements including quality, purity and adequate process controls, characterisation and validation. This is an onerous task for CTPs as they are a living entity and the cells themselves are the product and not just as part of the process, dissimilar to antibody/biologics production. The following sections highlight key considerations for process and protocol development to efficiently and effectively translate protocols into a manufactured CTP.

2.3. Manufacturing considerations

The area of regenerative medicine and the cell therapy industry (CTI) is growing exponentially both in terms of research and economically[130,131]. However, the full potential of the area has yet to be fully realised, in part due to the many process challenges that developers have faced along the way (**Figure 9**). As a result, the cell therapy market lacks products that provide the 'regenerative' properties to treat and cure 'all' diseases as promised during the conception of the area. The CTI and regenerative medicine field by their very nature are multidisciplinary, and as such there is a wide range of aspects that have retarded progression in the area; from a process and manufacturing development point of view, there a variety of bottlenecks that have a major impact. The following sections discuss some of the key aspects currently hampering protocol translation such as; cell purity, stringent identity assays and effective potency assays, that are established and validated during product development and used for manufacturing. Furthermore, considerations regarding potential adverse effects including tumorigenicity and assays should reliably ensure that product deviations are identified prior to release.

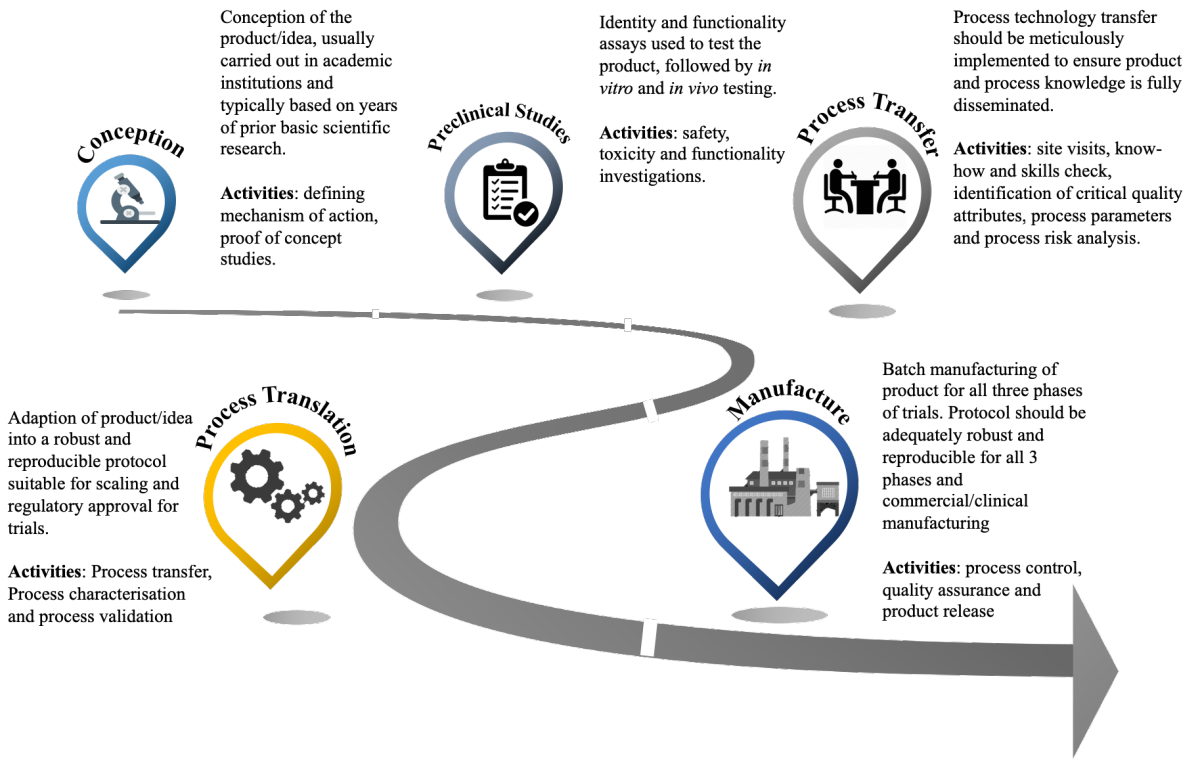


Figure 9. Process map of the key stages of CTP` development. Starting with conception of the product and ending with clinical/commercial product manufacture.

2.3.1. Purity

Traditional pharmaceuticals assure high purity levels, however, purity presents a greater challenge for CTPs[132–134], due to the inherent nature of cells which are a living material interacting with intrinsic and/or extrinsic factors. Unpredictable process variables pose problems where high purity yield is critical to functionality, and efficacy. For instance, only vmDA would be required to ensure efficacy and innervation of the correct cell type into the appropriate regions of the dorsal striatum. Understandably regulators expect proof of high purity, to assure that only the cells of interest are procured for patients[135,136]. The challenge remains in achieving high purity with such complex products i.e. ensuring all cells are differentiated or manipulated to the desired state and crucially, retain that state from bench to patient. This is important from a safety perspective and is scrutinised by regulators, since any undesired cells may potentially cause unwanted/unforeseen effects. This would be highly problematic and should be addressed through rigorous identity/purity testing and clear understanding of the mode of action. As a requirement, when a dossier is filed to the regulators, purity measures within the manufacturing process should be pre-established. In the case of vmDA manufacturing, appropriate cell identification and sorting assays should be considered throughout the whole product development cycle, resulting in robustly validated purity release criteria.

2.3.2. Impurities

Impurities present another bottleneck which can hinder clinical use and/or commercialisation of a CTP. Impurities usually fall into two broad categories: product-related impurities (e.g. cell fragments); and process-related impurities (antibiotics, cell culture reagents *etc.*) that need to be removed before regulators can deem a product acceptable for clinical manufacture[133,137]. Regulators tend to, understandably, have a strict view on all impurities and suggest that they should be addressed in the risk analysis[138]. In addition, impurities such as metabolites should be tested and/or have their removal demonstrated through validation. For the most part, the issue of impurities can be addressed by using monoclonal antibodies to remove the cells and leave behind the unwanted fragments using methods such as fluorescence-activated cell sorting (FACS). Furthermore, testing for the absence of bacteria, fungi and mycoplasma is essential, there are many techniques and organisations that can be employed to carry out such tests[132,133]. In some cases, if accredited, in-house tests can be used to screen the product and this can be integrated into the process quality management of the development and manufacturing process[139]. It is important to note that tests and interventions to remove impurities must consider that cells are a living material and therefore cannot undergo rigorous sterilisation and purification steps such as irradiation; that occurs during the manufacturing process of traditional pharmaceuticals. Thus acceptable limits have been established for (most) CTP process related impurities, however the onus is on the manufacturer to set specifications regarding product related impurities based on factors such: as pre-clinical and clinical safety studies, the capability of the process to remove the impurities and also related products/ historical data[138,140,141]. These specifications must be in line with the

existing guidelines and criteria of the regulators. As a result, the product developer/owner must submit impurity specifications that reflect an understanding of the individual phases of development, impurity risk and process capacity to produce a product with a safe impurity profile. Embracing such an integrated approach to dealing with impurities allows research and products to overcome this hindrance to CTP commercialisation and clinical use.

2.3.3. Identity

Knowledge of the identity of a product is required, it is crucial that the correct cells are identified and only those that are desired are used for the product. If undesired cells remain in the product and are administered to the patient, they may alter the product's mechanism of action or result in spontaneous and potentially tumorigenic differentiation i.e. with remnant pluripotent cells. Cluster of differentiation (CD) markers are one of the most prominent methods of identifying cells, *via* their cell surface molecules[138,142]. For instance, the co-expression of FOXA2 and OTX2 alludes to differentiation towards a vmDA cell linage. However, like many aspects of CTPs there are nuances that present some identification challenges. For instance, similar CD marker combinations can be found on very different cell types; and/or the marker expression profiles can be transient over time. Therefore, identity parameters should be established for the specific product based on validated evidence and tests. The European Medicines Agency (EMA) says that "identity of the cellular components should be based on phenotypic and/or genotypic markers"[143]. As a result, it means that the test methods used need to be specific for the cells of interest. Hence why, when addressing phenotype, relevant analyses should be used such as gene expression, antigen presentation and specific biochemical activity in an orthogonal manner [132,144]. For allogeneic CTPs, it is imperative that the identity profile should include histocompatibility markers, as the cells will be heavily scrutinised by immune system. For vmDA transplantation this is imperative as grafts could be rejected, resulting no innervation and lack of efficacy. In addition, morphological analysis for adherent cells may be useful, as morphology can have an important role on the function[133]. However, this should be in conjunction with other analyses as cells can exhibit similar morphologies under light microscopy which can be difficult to distinguish.

2.3.4. Tumorigenicity

Tumorigenesis presents trepidation with CTPs, particularly those using heavily manipulated or genetically edited cells, since they may undergo transformation, whereby chromosomal instability is possible[143,145]; thus, impeding authorisation. This relates back to the issue of purity, in the event that pluripotent cells are present in the end product, there is a risk that the cells would continue to proliferate and potentially become tumorigenic. For this reason, post mitotic vmDAs are desired for transplantations so that they cell do not continue to proliferate. When using unestablished cell lines, karyology tests should be considered to

investigate the tumorigenic potential of the cells[143,146,147], particularly when the cells themselves would be transplanted into the patients. Novel approaches to ensuring CTP safety are being devised, for instance the introduction of a suicide gene can reduce the risk of tumorgenicity for products differentiated from pluripotent cells[148,149]. The suicide gene can be activated in the cell product, thereby selectively removing undifferentiated cells from the product, thus increasing product purity and safety[149]. The challenge for CTPs is that they often require the use of pluripotent cells, extended cell culture periods, cell banking and the use of growth factors, all which have the potential to result in a tumorigenic profile. It is worth noting that, if a CTP cell line is found to have tumorigenic concerns the EMA states that "Use of cell lines known to be tumorigenic or to possess abnormal karyology should be evaluated in terms of risk-benefit for each product application"[147].

2.3.5. Potency

The challenges mentioned above relate to obtaining the right cells and having them as pure as possible. Potency according to the EMA "should be based on the intended biological effect which should ideally be related to the clinical response"[150]. With some CTPs this can be easy to prove, however when potency tests must also show clinically meaningful changes it becomes difficult for some CTPs to demonstrate potency prior to human clinical trial data. Clinical changes in health utility for PD can be measured by cognitive behaviour and motor assessments, through tools such as the Unified Parkinson's Disease Rating Scale and the Hoehn and Yahr scale. Due to the complex nature of CTPs prior to this data being available, developers may face unanswerable questions regarding product potency; however some answers can be achieved through *in vitro* and/or *in vivo* assays, or assays based on surrogate markers[132,138,151]. These can include but not limited to gene expression profiles, flow cytometry immunoassays and metabolite release assays. For example, dopamine release assays for vmDA neuroprogenitors can be used ascertain potential dopamine levels which can be related to improvements in motor symptoms. Such an assay would benefit from being non-destructive to the cells and the ability to be employed in real-time as dopamine release would be critical in the differentiation of vmDA for transplantation. An ideal potency assay would rapidly and reliably produce real-time data using inline manufacturing systems, permitting continuous process monitoring to facilitate informed proactive process changes and improved process control based on real-time data. In cases where potency can be easily addressed, it is due to the product's ability to clearly demonstrate its effectiveness when directly compared to other products currently on the market, however this is not the case for all CTPs[152]. For this reason, developers and regulators must work together to facilitate the creation of potency assays that provide valuable process information which can be utilised for quality control and release of manufactured CTPs.

2.3.6. Supply chain

Supply chain reliability is often overlooked; however reagents require batch validation with deviances being accounted for, or demonstrating non detrimental effects on the end product [132,134,138,153]. Therefore, it is important to consider reagents during process development to ensure manufacturing sustainability. Furthermore, when assays come in contact with the product, GMP-compliant reagents are essential as the end product will be introduced into the patient, therefore it should be free of any harmful substances. The GMP compliance of suppliers should be rigorously validated to ensure that reagents are truly GMP compliant[154,155]. This can be done by auditing suppliers, their premises, their staffs' GMP training and even their suppliers. Although it might seem tedious and laborious, it is an effective means of risk mitigation that would need be required by regulators to ensure appropriate compliance. Supply chain control and contingences are recommended to reduce variability, especially if contingencies are tested within the product's tolerance and design space[74,138]. This is pertinent for the differentiation of vmDA neuro progenitors as it requires a range of different supplements and small molecules (discussed in Chapter 6). Therefore, they should be simultaneously available for the process to work or appropriate validated substitutes should be identified as part of the product development process. The latter activity may help overcome comparability issues further down the product time-line, especially if suppliers become obsolete. Single supplier issues need to be addressed as they can render product development and manufacturing to a halt[155]. Therefore, the risk of raw material availability should be a key activity that is carried out during the development stages. It is encouraged that developers discuss with their suppliers about procuring future stocks that meet their projected needs in case of a shortage of key resources. Manufacturing process flexibility is important, allowing assay amenability throughout the product's lifecyle[134]. For supply chain management, product and assay development should be reliable and effective whilst considering associated costs, as hindrances to many CTPs is their cost-intensive development. This links to the need to consider reagents use and understanding the overall process unit costs[25].

2.3.7. Process Understanding

The aforementioned challenges are addressed or well mitigated in well-established industries including antibody and pharmaceutical production, thus providing learning platform for CTPs. For instance, the use of Juran's concept of quality by design (QbD) facilitates product and process development that is data, risk and knowledge driven; ensuring in-built quality from the outset[156–158] (**Figure 10**). QbD key aspects include defining a quality target product profile (QTPP) utilised to highlight the desired product specifications or critical quality attributes (CQAs). Having both defined QTPP and CQAs is the starting point for identifying critical material attributes (CMAs) and critical process parameters (CPPs). QbD concepts facilitate protocol and process development conceived with control strategies in place, aiming

to ensure that QTPP is met in every production run. This is *via* identifying and understanding the design space in which the CPPs result in the CQA and QTPP. The design space helps developers attain process controls given the CPPs, furthermore, the use of process analytical technology (PAT) allows for iterative, data rich development processes that are able to ascertain process capability.

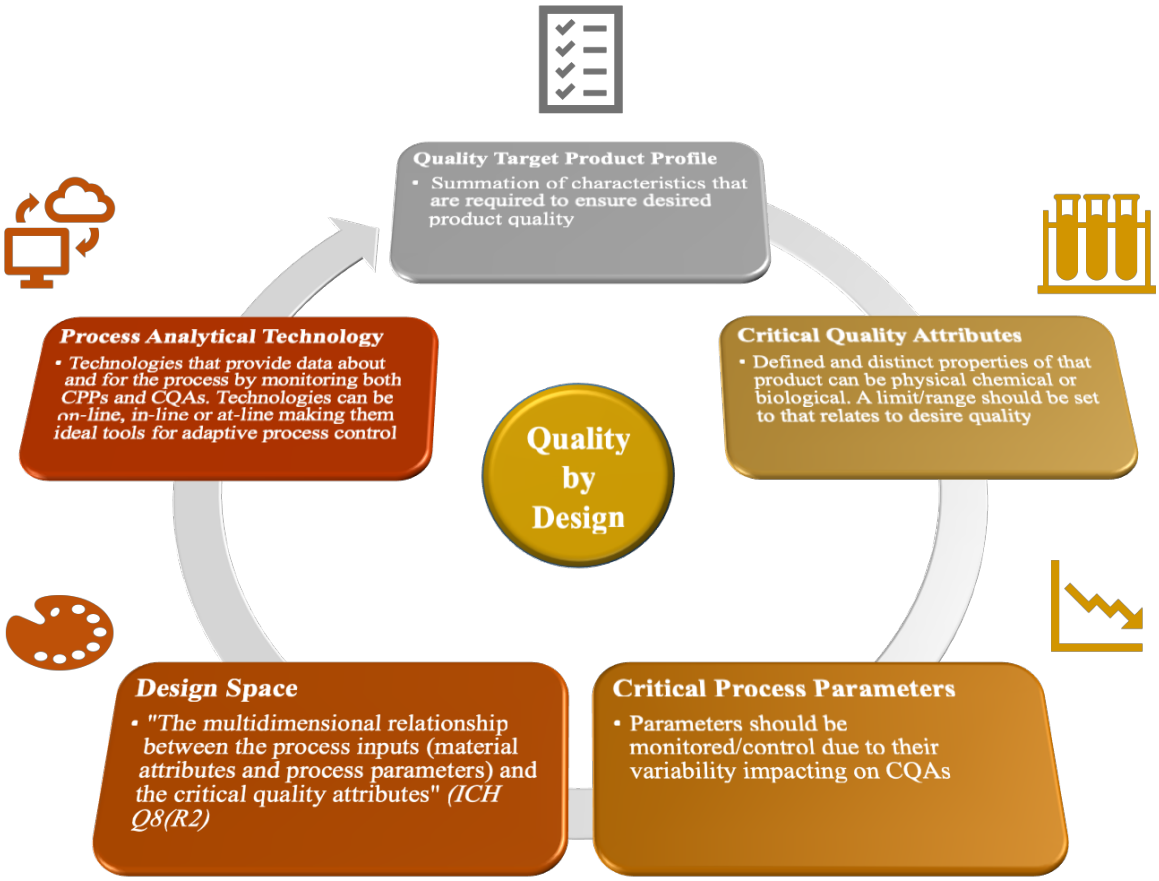


Figure 10. The elements of the quality by design (QbD) framework for product manufacturing.

Characterisation is fundamental to CTP manufacturing; it is important to characterise and identify both input CMAs and output cells of a manufacturing process. This facilitates input quality, ensuring materials are process appropriate; output characterisation evaluates product manufacturing success. Imperative to CTPs is that the cell characterisation can provide information regarding the functionality of the cells, therefore making potency assays a crucial tool in CTPs process development and manufacturing[132,133]. For instance, identification of a CQA cellular marker that could be linked to the function of a particular cell type. An example would be an assay that confirms the presence of CD19 on chimeric antigen receptor T cells (CAR-T cells, definition in section 2.4), since the presence of CD19 is linked to recognition and attachment to tumour cells which initiates destruction of the tumour[159].

This highlights the importance of CQAs to product understanding and the need to explicitly define a product's CQAs prior to approval. CQAs are functionality-based characteristics; these can be physical or chemical and linked to potency, identity and purity[160]. These characteristics can be identified by cell morphology, phenotypic markers and secreted factors, which can be potentially linked to functionality and clinical response i.e. detection of secreted dopamine[144,152,161]. It is important that developers are selective and robust in their characterisation, for instance, distinct marker profiles should be used to confirm identity. This is crucial for CTPs such as vmDA cells for transplantation as subtle differences appear between DA neurons and neighbouring neuronal cells. However, vmDA lineages restricted to the caudal ventral midbrain can be identified by *FOXA2, LMX1A, CORIN, OTX2* and *EN1* co-expression. [85,94]. Therefore, using high specificity characterisation criteria is a necessity for robust CTP assay development. Characterisation during product development can provide validated and quantitative data applicable to potency assay development. Flow cytometry and genetic analysis are robust methods for analysing identity, which can be used to track changes during expansion/differentiation. Molecular or chemical based methods including ELISAs can also be used to analyse secreted chemicals, which in some cases can be indicative of function[161,162]. For instance, detection of dopamine release by differentiated cells would imply successful lineage differentiation, moreover dopamine release is directly linked to the functionality and MOA of a dopaminergic cell based CTP[24,71,86]. Therefore, potency assays based on the observed MOA and concurred by the literature around PD treatment[109,132] could be used to characterise products prior to being released.

2.3.7.1. Phenotypic markers

Phenotypic markers can be used to ensure the CPPs and design space efficiently yield the desired CQAs. For instance, which small molecule concentrations and cell densities result in the desired cell markers and thus the CQAs. Intracellular and extracellular markers can be transient, thus are appropriate for monitoring process progression including differentiation, using techniques including flow cytometry or gene expression analysis. For the candidate therapy, hESCs are differentiated into vmDA neuro-progenitor cells, both cell types have distinctive identity profiles. Positive expression of SSEA-4 and OCT3/4 signifies undifferentiated cells, whilst decreased expression of these markers and transient presence of PAX6 demonstrates neural stem cell lineage progression[25,107163]. The desired differentiated end product can be identified by FOXA2/OTX2 co-expression[25,164]. Therefore, these markers can be assigned as process CQAs since expression corresponds to specific stages, permitting development of in-process assays set against these CQAs. Furthermore, markers such as EN1 and TH have been linked to vmDA function in animal models. If real-time information is obtained, it presents a powerful tool for process development monitoring and CPP capability. This is easily achievable for suspension cells as the culture vessels can have the ability to be directly linked to a flow cytometer, allowing for real-time analysis of the cells and process.

2.3.7.3. Secreted substances

Substances such as dopamine, hepatocyte growth factor and other growth factors are secreted from cells and can be utilised as a mode of assessing product potency and thus functionality. In addition, the secretome of cells can be used in process development as a mode of quality checking and ensuring that the cells are progressing towards the desired route of differentiation or cell state. In the case of the candidate cell therapy, there are specific factors that are secreted which have been linked to specific stages of DA neuron differentiation[86,109]. By applying the knowledge of the specific time point at which these compounds are released, it is possible to take samples to assess if the cells are proceeding in the desired manner. SHH and FGF8 are secreted during DA neuron development and have been shown to induce midbrain development in pluripotent cells[25,48,86,107]. Other soluble factors that are implicated in midbrain development processes include brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF)[25].This ability to map and assign factors to time frames is extremely valuable in a manufacturing setting as it can allow for detection of failure modes before the end product is tested, saving both economic and time resources. Fundamentally, these substances are released at distinct stages, therefore a failure to detect them can act as go/no-go tool for the progress of the cell differentiation/manufacturing process, allowing for process intervention to evaluate if the observation will have an impact on the final product's functionality.

2.3.8. Safety

Product safety is paramount and a regulatory requirement, it is one of the key aspects of phase I clinical trials and a key consideration by healthcare payers (definition in section 2.4). Prior to phase I clinical studies, there needs to be preclinical product safety testing and product characterisation. Product safety links to topics such as purity, potency and identity discussed above, which aim to ensure that the products meets the desired CQAs and is safe for administration. In addition to assays and activities such as chromosome karyotyping and tumorigenicity testing developers should also focus their efforts on the biodistribution and targeting of administered cells, as this can have potential side effects[165,166]. CTPs differ from other biological medicines as the cells have the potential to persist in the patient for their lifetime (which may or may not be desired – if desired long term safety studies should be conducted); they can replicate or to mature *in vivo* after they have been administered (which is a functionality that should be well understood) and the cells can migrate and distribute to tissues or organs other than the one intended[167–169].

Assessing and confirming the biodistribution, engraftment and persistence of the administered cells is required as cells respond to their environment and interact with other cells and extracellular fluids in ways which are not encountered with traditional pharmaceutical and biologics[167]. Biodistribution and

persistence tests can be done preclinical using appropriate animal and/or disease models, it is important to note that these tests should be scientifically and rationally validated to ensure that the evaluation criteria truly demonstrates meaningful data regarding product safety[165]. In combination to biodistribution tests, appropriate and maximal dosage levels should be ascertained to ensure that the potential adverse effects of cell administration are mitigated against[165,167,170]. This is pertinent in products such as CAR-T therapies which involve systematic administration of the product to the patient.

Early assessment of product safety is vital, preclinical safety studies in animal models are a great mode of acquiring safety data early on, however it is recognised that the value of these models can be limited due to species-specificity[167]. As a result, some responses might only be observed in certain species, thus a product that satisfies safety testing in an animal model might induce an adverse effect in humans. Therefore, this is a challenge that needs to be addressed, perhaps the use of human or patient specific disease models can alleviate the drawbacks faced with species-specificity[171,172].

The raw materials used in product development and manufacturing are a crucial element in product safety[155], thus it is mandatory for materials and reagents to meet the required levels of compliance and are tested for sterility, contaminants and microorganisms that could compromise the safety of a product[165,167]. Supplier validation and auditing, again, becomes a necessary activity to carry out. In addition, another area of importance is the safety of biologically active transformation agents such as such transgenes. Their MOA, sterility and safety should be addressed and verified, as well as the structure and characteristics of the transgenic construct, to safeguard safety through the whole supply chain and process[148,166].

It is worth noting that speaking to regulators as early as possible is imperative as regulators can give advice on the product safety requirements. In the development stage this a worthy activity to engage in as guidance on how to accrue appropriate safety data is invaluable, as insight on appropriate assays and activities can be gained, resulting in well-informed product development[160,170,173]. This can potentially de-risk the developer's regulatory approval submission as it would have been formulated and compiled with a clear understanding of how to meet the requirements of the regulator.

2.3.9. Technology

Innovative adaption and transformation of available technologies to meet new processes and product requirements is becoming commonplace. Instances include the creation of integrated systems resembling a modular process comprised of complimentary technologies[74,142,174]. These aim to provide essential CTP monitoring capabilities, production control and production capacity to meet potential clinical demand. Scale up technologies are necessary for efficient manufacturing however, adequate control and monitoring technologies are equally essential for downstream manufacturing process

optimisation such as cell sorting, purification and filling[142]. Technologies such as fluorescence-activated cell sorting and automated controlled rate freezing offer some solutions.

Truly integrated inline systems, capable of monitoring, characterising and sorting cells would allow for highly pure products to be obtained, facilitating both process control and quality assurance. Furthermore, non-destructive PAT that can analyse cell-secreted substances in real-time would allow for process mapping, as discrete secretome profiles are evident throughout the differentiation stages. Failure to detect can act as go/no-go tools for the progress of cell differentiation/manufacturing processes, allowing for process interventions to evaluate if the observation will impact final product quality and functionality. Furthermore, substance detection concomitant to product functionality would present a powerful potency assay. For the candidate therapy an assay confirming dopamine production would corroborate desired biological function[85,108]. This can be achieved with PATs directly linked to the culture vessel and detect the desired substance i.e. metabolite analysers; allowing for real-time process analysis.

2.3.10. Summary

The sections above detail the requirements and considerations that are integral to CTP process manufacturing, however even when equipped with this knowledge there are still other challenges that developers face. For instance, creating assays that are convenient, informative and non-destructive to the cells presents a major challenge. In addition to ease of use, an efficient assay should also be rapid in its production of data. An ideal assay should be able to produce real-time data using inline systems, as this would afford the opportunity to monitor processes continuously and facilitate informative proactive process changes based on real-time data.

In relation to process development, knowledge is certainly power, particularly for assay development as robust data is desired for their development. The combination of a QbD mind-set and development of in-process assays provides powerful toolsets for developers to understand their products and processes as they embark upon CTP manufacturing challenges. A proactive stance to assay development is encouraged, considering how the product works and batch analysis at an early stage can mitigate risks during later stages of process and manufacturing development. A plethora of information demonstrating a clear understanding of the process and product is likely to favoured by regulators, as it demonstrates due-diligence throughout the development stages. An in-depth understanding of both process and product design spaces makes the product amenable to change, where necessary, negating further comparability studies and the concomitant extensive paperwork. This fundamentally permits more informed, less hindered pathways to product authorisation as “Early and sustained investment in a bona fide potency program is essential for maximising a product’s commercial success”[138].

2.4. Translational considerations

Glossary:

Adoption – the procurement and implementation process of espousing health technologies in to the healthcare system. This covers a wide range of activities in including health technology assessment, price negotiations, personnel training and infrastructure considerations.

Amortisation – a mechanism in which the full payment is spread over a period of time and multiple instalments are made, typically over the expected duration of benefit.

Chimeric antigen receptor T-cell therapy (CAR-T therapy) – is a treatment for advanced B-cell acute lymphoblastic leukaemia (ALL), diffuse large B-cell lymphoma (DLBCL) and primary mediastinal B-cell lymphoma (PMBCL) cancers, that is used after other available treatments have failed. The treatment involves harvesting immune cells (T-cells) from the cancer patient and then genetic reprogramming them to specifically target their cancer by making them express the chimeric antigen on their surface. The process of making a CAR-T therapy is complex and involves apheresis, genetic engineering, cell expansion and then infusion back into the patient, this process takes approximately three weeks and can require patients to stay between one to one and a half weeks in hospital. Investigational work is still being carried out and all the possible side effects are still not known, however cytokine release syndrome and B cell depletion are two of the currently known side effects. Two CAR-T therapies are currently approved for use in the UK, Kymriah and Yescarta[175,176].

Drug plan - provincial and territorial organisations in Canada that are in charge of paying for prescription medication for eligible members of the population such as seniors, recipients of social assistance, and individuals with diseases or conditions that are associated with high drug costs[177].

Equity - the absence of avoidable or remediable differences among groups of people, whether those groups are defined socially, economically, demographically, or geographically.

Health technology - the application of organized knowledge and skills in the form of medicines, medical devices, vaccines, procedures and systems developed to solve a health problem and improve quality of life – definition pr. **N.B**. this term has been used interchangeably with the word *intervention*.

Health technology Assessment - the systematic evaluation of properties, effects, and/or impacts of health technology. It is a multidisciplinary process to evaluate the social, economic, organizational and ethical issues of a health intervention or health technology. The main purpose of conducting an assessment is to inform a policy decision making by considering the clinical and cost effectiveness and broader impact of healthcare technologies

Nation Tariff –a set of prices and rules used for the reimbursement of care delivered to patients.

Opportunity cost - the money or other benefits lost when pursuing a particular course of action instead of a mutually-exclusive alternative

Pay for performance – a payment mechanism in which healthcare providers are paid depending on the level of their performance or their ability to meet the defined outcome measures.

Payer – a body/organisation that pays for the provision of healthcare for an individual or for society. Payers are in charge of the financial and operational aspects of providing healthcare to ensure that the cost of health services are appropriately reimbursed.

Payment mechanism – a method of paying for health care services i.e. the way in which providers are reimbursed. There is no single best method and different countries use a mix of different payment mechanisms for different purposes. In general, the methods employed create different sets of incentives form the providers such as quality and efficiency.

Reimbursement – the act of paying hospitals, doctors, test centres and health technology suppliers for their services in the provision of care.

As mentioned in the manufacturing considerations section, there are many challenges that needs to be addressed in order to successfully translate developmental therapies to the market, especially as the CTI is an area that is growing both in terms of scope and investment[131]. Recently there has been a faction of CTPs and gene therapies traversing through various stages of clinical trials and over the last three years, with a number of cell and gene therapies gaining regulatory approval [26,74,178–181]. For the majority of CTPs, the concomitant scientific aspects have been fine-tuned and shown to be effective. However, economic and commercial aspects of CTPs have for the most part, been neglected; this has been predominantly due to the fact that developers tend to be based in academic institutions, where economic and commercial considerations are not necessarily development priorities [182,183]. Thus their products' ability to generate revenue and successfully compete on the market has either not been fully considered, or has been neglected until the latter stages of development[182]. In addition, the CTP industry is emerging and there is no/little set precedence, thus many developers have been simultaneously facing similar development challenges unbeknown to each other. However, with the growth of the CTI community it has become evident that commercial and economic aspects need to be considered at an early stage of the development timeline[182,183] (**Figure 11**). As a result, there has been a plethora of literature within the last ten years that highlights the importance of early assessment of healthcare technologies.

Health economics is a valuable tool in healthcare, it provides information for both developers and payers as well as any other interested stakeholders. For many developers, economic considerations are not considered a priority at the early stages of development, however with increasing competitiveness in the CTP field, economic considerations are becoming more and more important if the developers hope to have a product that is commercially viable. Early economic consideration is slowly becoming trend that is growing, particularly with novel interventions such as CTPs as their reimbursement landscape is, for the most part, unknown. Therefore, early assessment of potential return on investment and reimbursement of their products is vital.

A Health Technology Assessment (HTA) refers to an exercise that can be used to assess the 'readiness' of a product to be procured into the healthcare system. HTAs cover a wide range of intervention types to improve health, these can be pharmaceutical drugs, medical devices and surgical, transplantation or replacement therapies[182]. European Network for Health Technology Assessment (EUnetHTA) define HTA as "A multidisciplinary field of (policy) assessment"; essentially HTA assesses the clinical effectiveness of an intervention i.e. its patient health benefits compared to alternative interventions or the course of no intervention at all[184,185]. The economic impact of adopting the intervention or the alternative (or no intervention) is also analysed to evaluate what the cost to payers such as national health services, insurers and society would be. HTA considers the social and ethical implications involved with new interventions in addition to any potential health policy changes that may result[182,184]. Therefore, HTA as a process involves the examination of a diverse range of aspects associated with the

value of an intervention, which provides a platform for decision making on the adoption of a new intervention[182,184,186]. As a result, HTA is carried out independent of interested parties and must be performed in a systematic manner, with transparent results for effective and well informed decision making, based on the best current research and data[183,187,188]. In most countries HTA appraisals are performed by an agency that represents the ministry/department of health; in the UK the National Institute for Health and Clinical Excellence (NICE) performs HTA appraisals and provides clinical guidelines for the Department of Health.

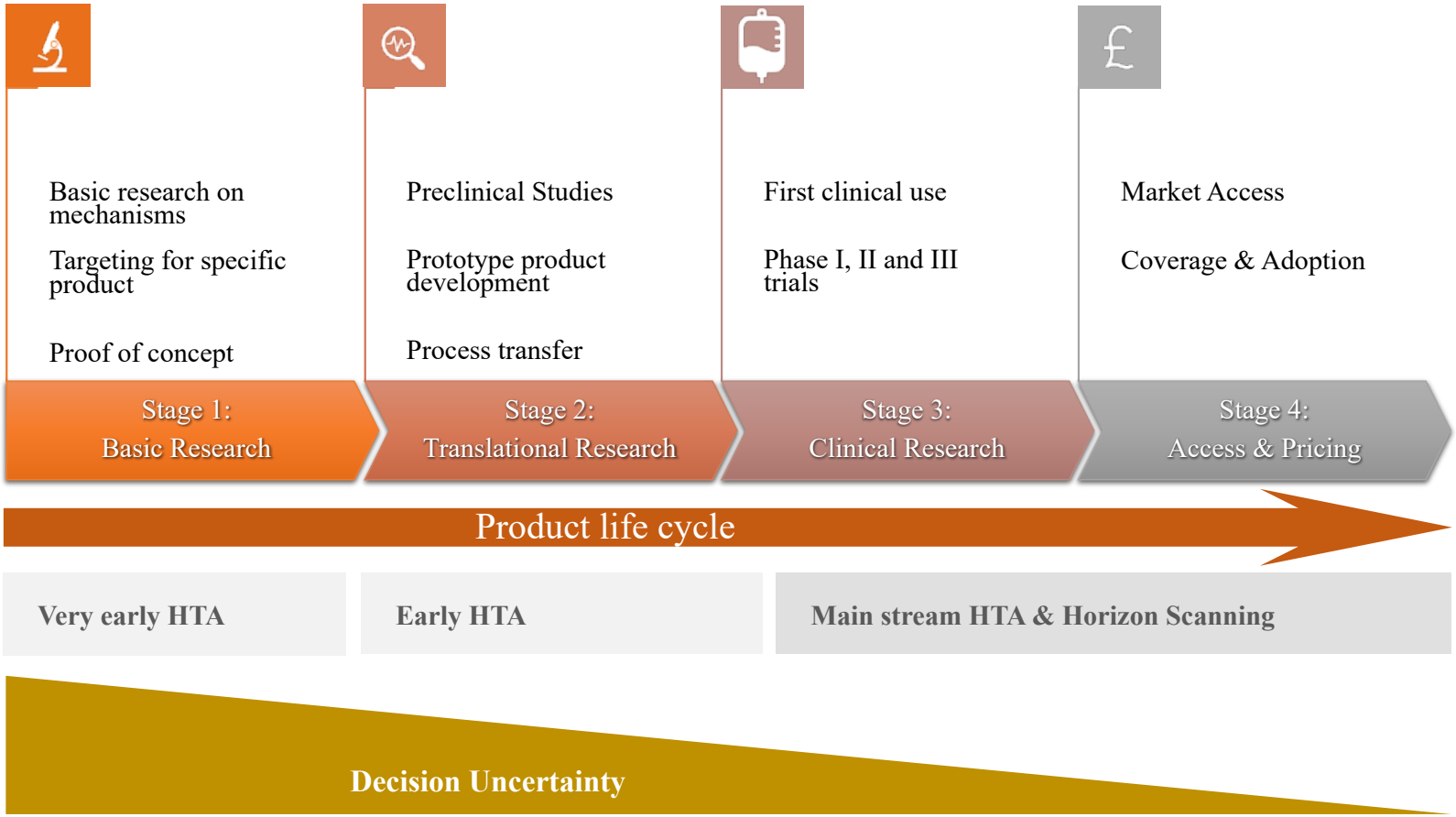


Figure 11. Schematic of HTA and decision uncertainty based on an illustrative product development timeline. Four stages of HTA analysis are shown with very early stage considered at the basic research phase. Most HTA is currently performed at stage 3 after the first clinical trials have been initiated. Considering HTA analysis only at stages 3 and 4 risks the successful reimbursement and adoption potential of a product, if the required evidence was not generated in the earlier phases of development. This can result in wasted time and resources efforts as the product will not get adopted if the evidence required by the payers is not adequate, regardless of the product receiving regulatory approval. Decision uncertainty refers the uncertainty of market access, post-market success and reimbursement decisions. Adapted from IJzerman and Steuten (2011)

2.4.1. Health economics

An important element of HTA is the study of health economics, which involves understanding the product's effectiveness and its associated economic impact. Health economics is particularly important to healthcare budget administrators as it is the study of the movement and allocation of resources to fulfil the health needs/wants of the society under scarce resources[188–190]. Health economic evaluations facilitate the assessment of the reimbursement potential on a product based on its comparable effectiveness both in terms of cost and efficacy[69,182,191]. Therefore, health economics can be used as a decision-making tool when it comes to the commercial viability and reimbursement of a new therapy[182]. There are many factors that need to be included when considering health economic evaluations such as clinical effectiveness, costs and overall health benefits of the intervention. The models produced as part of economic evaluation exercises inform both the developers and investors regarding the commercial and economic prowess of the product being evaluated, as well as informing the payer about the economic impact an intervention will have on their budget. The models employed use techniques such as incremental cost-effectiveness ratios (most appropriate for payers), the headroom method (most appropriate for developers) and return on investment (most appropriate for investors), all of which will be discussed in later sections[182,183,192]. There are some keys concepts that need to be considered when carrying out health economic evaluations, two integral aspects are the health outcomes and costs related to the intervention. These two aspects and some of the analytical methods that are typically used are discussed in the following sections.

2.4.1.1. Health outcomes

How a disease affects a patient's life is a very important consideration in health economic evaluations. If the disease burden is high and results in high healthcare costs, it is in the interest of the healthcare provider to treat the disease in the most effective manner in terms of both health outcome and costs. Positive health outcomes are important especially when they reduce morbidity, for instance, with PD health outcomes that allow patients to prolong their independence are desired from a quality of life and healthcare cost reduction perspective, for the patients and healthcare provider, respectively.

In the field of health economics, Quality Adjusted Life Years (QALYs) and Disability Adjusted Life Years (DALYs) are the common metrics used to measure health outcomes and to incorporate disease related reductions in length of life and the negative effects of morbidity[193–195]. However, they deliberate these from opposite aspects, QALYs evaluate health and higher values are desired: whereas DALYs evaluate disease burden and lower values are favourable (**Figure 12**). Thus, health interventions aim to gain QALYs whilst avoiding DALYs. Both metrics are considered to be good measures of assessing how well an intervention impacts health outcomes [196,197]. QALYs are calculated using health utilities

whereas DALYs are calculated using disability weights. This means that there are some fundamental differences in how the data for the values is obtained. Disability weights were derived in a single process for hundreds of diseases and disease states that are publicly available, which means that there is standardisation which facilitates comparison across different diseases and interventions[198–200]. Health utilities are widely used in literature, thus there are many utility values for various health conditions. However, they have been measured using different methods, which can result in different utility values[198]. Since QALYs have been heavily discussed in other literature[196,198,199,201,202] the current work will pay attention on DALYs.

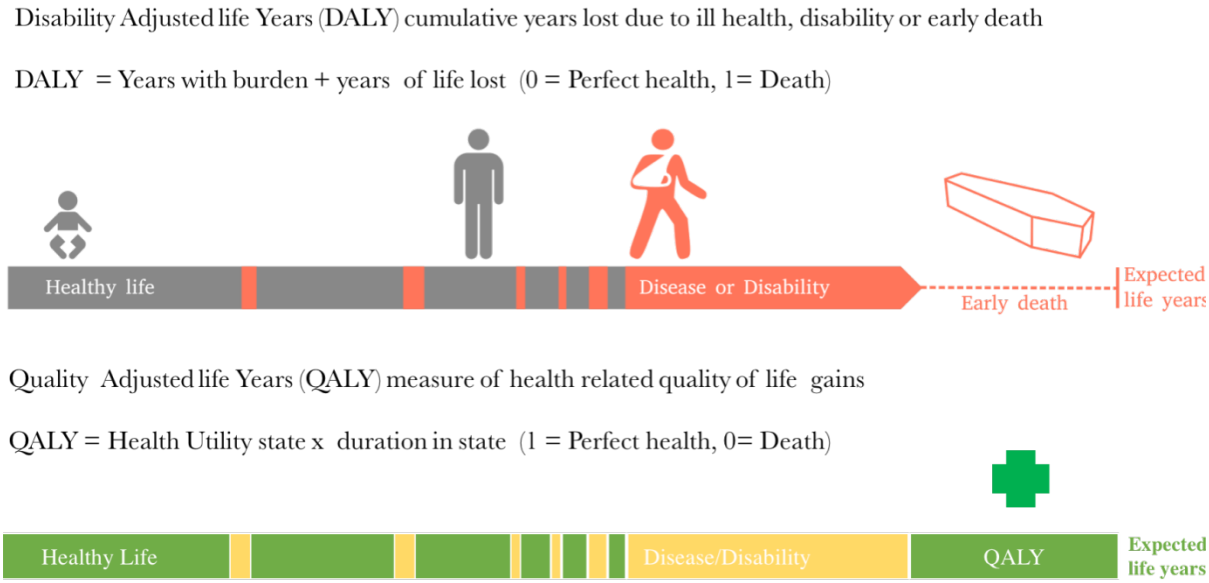


Figure 12. Schematic of how DALY and QALY values are calculated and how each metric aims to quantify health outcomes due to disease (DALY) and interventions (QALY). The orange and yellow segments show phases where an individual's disease burden is increased (DALYs) or their health state decreased (QALYs) as a result of illness or diseases through their life.

2.4.1.1.a QALYs

QALYs are a way of assigning a quantitative value to health states using health utility data and health questionnaire scores, meaning they can be used as a health metric to measure how well someone feels in terms of their health[198,199]. By multiplying the heath state with the period of time (years) it is experienced, a QALY value can be obtained. Values range from 0 to 1, with 0 being dead and 1 being perfectly healthy[196,198,199].

The Health Utilities Index (HUI) is commonly used for obtaining the health state of individuals. HUI-3 a system that measures eight health related attributes (vision, hearing, speech, ambulation, dexterity, emotion, cognition, and pain) and each attribute is measured on a range from level 1 to 6, with 1 being

the best health state for that attribute and 6 being the worst. Each attribute and level is given a coefficient that can be used in an equation to give the value of the overall health state of the individual[203]. Using the HUI-3 matrix (**Table 2**) a perfectly health individual with scores of level 1 on all attributes would have a health utility of 1. On the other hand, an individual with level 5 and 6 scores i.e. extremely poor health, would have a health utility of 0.36, this negative value would be considered as 0 as that is the poorest health state, which is death (**Table 2**).

These two examples are extremes used for illustrative purposes only, over a 10-year period the healthy individual would have a QALY of 10 (1 x 10) meaning that for each year they are in perfect health. In comparison, the individual with a negative/utility score of 0 would have 0 QALYs (0 x 10) over the same 10-year period i.e. they are dead. It is worth noting that QALYs and health utilities are dynamic as they will change throughout the course of normal life, disease and treatment, therefore a person will have multiple health states through their life.

Table 2. Matrix of health related coefficients used to determine an overall health state[203]. For each health state, the attribute level selected in the response is entered into the health utility equation (**Equation 1**). A health utility score of 1 represents perfect health while a negative health utility represents extreme poor health.

Health State Level	Vision	Hearing	Speech	Ambulation	Dexterity	Emotion	Cognition	Pain
1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
2	0.98	0.95	0.94	0.93	0.95	0.95	0.92	0.96
3	0.89	0.89	0.89	0.86	0.88	0.85	0.95	0.90
4	0.84	0.80	0.81	0.73	0.76	0.64	0.83	0.77
5	0.75	0.74	0.68	0.65	0.65	0.46	0.60	0.55
6	0.61	0.61		0.58	0.56		0.42	

$$U = 1.371 \times (Vision \times Hearig \times Speech \times Ambulation \times Dexterity \times Emotion \times Cognition \times Pain) - 0.371$$

Equation 1

2.4.1.1.b DALYs

Disability Adjusted Life Years (DALYs) provide a standardised metric that can be used to quantify the burden of different diseases[197]. This is important as it would otherwise be difficult to comparatively demonstrate how different diseases compromise the lives of patients. In particular, when there are differences such as the populations affected, physiology, complications and most importantly the strategies for treatment[196,197]. DALYs can be used as a tool to combine the negative effects morbidity on patient quality of life and early death[196,199]. DALYs represent the full burden that is imposed by a disease in a manner that allows for quantification of disease specific burden.

DALYs are comprised of mortality, which is the loss of life lost due to premature death caused by the disease; and morbidity, which is a measure of chronic disability experienced as a consequence of the disease. Mortality can be assessed by calculating the life years lost as a result of the disease, i.e. the average age of death of an individual with the disease is subtracted from the life expectancy of a healthy individual[199]. Morbidity is calculated by obtaining the disability weight (how much people are disabled by the disease) of the specific disease, which ranges from 0 to 1, with 0 being perfectly healthy and 1 being fully disabled. As the effects of the morbidity can be brief or extended, the duration of disability is factored into the metric[197]. Therefore, it can be used to compare different interventions and show how many DALYs can be averted, which is the health outcome of the given intervention. This means that the cost-effectiveness of an intervention can be measured using the benefits of the intervention achieved through reductions in the severity of disease burden.

In the literature regarding economic evaluation of healthcare interventions, DALYs are not often used as the health outcome metric, instead QALYs are the preferred metric of choice. Despite this DALYs present an equally valid and useful alternative and in some instances, for example in cases of incurable chronic illnesses, it is more appropriate to use DALYs. For example, an intervention might not afford an increase in health utility, yet its use would reduce disease burden experienced by the patients; resulting in no QALY gains but providing a decrease in DALYs. This is typically the case when evaluating PD, as it is a chronic incurable disease and current treatments do not necessarily increase health utility and quality of life but can decrease the burden of the disease by temporarily alleviating the symptoms.

The benefit of DALYs is that disease burden data and disability weights are readily available and were obtained in a standardised exercise unlike QALYs as discussed above[195,196,199]. Therefore, it is easier to compare results from different interventions and even different diseases, to assess cost-effectiveness based on a health outcome. This is important when healthcare providers have to assess the opportunity cost of choosing one intervention over another. Moreover, the robustness of DALY input data is

particularly useful when no efficacy data is available to provide utility scores; as DALYs can provide information on the potential reductions in disease burden and therefore the health benefit of an intervention. Furthermore, assessing reduction on disease burden can provide payers with more information on the cost-effectiveness of an intervention[189]. For example, the reduction in costs that can be afforded by the reduction in disease burden, compared against other interventions. This information can highlight that although an intervention might initially be more expensive, it can result in significant reductions in disease burden and reductions in future costs be i.e. further hospitalisations. In other words, if an intervention results in QALY gains and DALY reductions then it can save costs for the healthcare provider by health gains, reduced disease burden and avoiding future health costs

2.4.1.2. Costs

Costs are an important element of health economics, fundamentally when carrying out an economic evaluation of an intervention, it is important to know precisely what costs the intervention is comprised of. Therefore, it is critical to qualify and quantify the resources used and validate who pays for them, this is particularly important in healthcare systems such as an American/privatised system, as they do not operate on a single payer healthcare system like the UK's National Health Service (NHS). This is important when considering reimbursement, as different payers will have different priorities and budgets, which results in differences of opportunity costs[189,191]. Furthermore, there should be explicit information regarding the region and/or country that the costs are being analysed for, as studies have shown that there can be considerable differences between geographic regions[41,70]. This is predominantly due to differences in allocation of care and healthcare resource amongst regions and countries. Costs can be separated into direct and indirect costs, direct costs are usually easy to qualify and quantify[41,70]. Indirect costs however are more difficult; this is because there is a wide range of costs that can be considered. There is no consensus on what to include for indirect costs, which can lead to differences in economic evaluation results. Typically, direct costs include the cost of the procedure/test, the cost of the product/device, hospitalisation fees and any other consumables that are directly related to the use of the intervention for the payer[69,70]. Indirect costs can include but are not limited to: productivity loss, side effect costs, carer costs, outpatient care and prescription drugs (for co-therapies)[70,204]. Some authors include uncompensated care costs, costs to families, life style adaptation costs and in some cases mortality costs[204]. Evidently, the difference in the included costs can result in significant cost differences, some have reported up to 16 –fold variation depending on whether or not indirect costs are evaluated[69]. Additionally, costs should also reflect the appropriate stage of the disease, as severe and late stage patients have a propensity to be more resource intensive. This consequently increases the costs compared to early stage patients which is highly evident in diseases such as PD and Alzheimer's disease[191].

Assessing the true cost of an intervention needs to take multiple factors into consideration; in addition, the factors discussed in the previous sections, there must also be an assessment of the costs, savings and health benefits that can be afforded. It is also important to take note of any potential costs, both in the short and long-term, that might be incurred by any adverse effects such as product failure that is detrimental or fatal to the patient. Understanding the cost and benefits of an intervention should be done in a context that includes wider aspects of the disease and treatment, rather than just the immediate costs and benefits[178]. For example, a therapy might initially be expensive, but it the long term the therapy may reduce use of medication, hospitalisations and administration costs, which would offset the initial costs in the long-term. This highlights the need to need to analyse costs in a wider context and across different time horizons in order to understand the true value of the intervention.

2.4.1.3. Analysis

Healthcare providers aim to deliver high quality care; however, they have limited financial resources to do this. Therefore, they need clinical and health economic data that can allow them to assess intervention options, so that they can make informed decisions based on policies and products. There are various economic evaluation methods that may be utilised, all which have their merits and short falls. Economic evaluations are carried out with budget constraints in mind; therefore inventions being assessed must have costs that are deemed acceptable given the constraints. These budget constraints vary but usually include the payer's willingness to pay (WTP) for an intervention. In the UK the WTP is set between £20-30,000 per QALY; this means that for each year gained in better or full health, the maximum WTP is between £20,000 to £30,000. Interventions costing less than the WTP are likely to be recommended for adoption, especially if they are both cheaper and more effective than a comparator or the current gold standard. If an intervention exceeds £30,000 per QALY, it is unlikely to be recommended for adoption as it would not be deemed cost-effective. In such circumstances, further analyses or rationalisations would be required to justify its adoption i.e. it might be the only form of treatment available for a particular condition. This is typically the case with orphan disease therapies and mostly likely to be the case with cell and gene therapies in the near future, as budget impact and long-term cost off setting would need to be considered. Calculation of cost-effectiveness can be carried out by a range of different analyses, three of which will be discussed below. The three analyses include: cost benefit analysis (CBA); cost utility analysis (CUA) and cost-effectiveness analysis (CEA), with the latter being the analysis of focus.

2.4.1.3.a. Cost benefit analysis

Cost benefit analysis (CBA) is a classical economic/accounting technique that is used to assess the benefits of an action with regards to its total costs i.e. choosing a particular intervention over

another[190,205]. Due to its ties to classical economics, CBA utilises monetary units as a metric to compare between different actions[69,190,206]. Although sound as an analytical technique, its applicability in health economics is arguable as it is difficult to express outcomes such as health gains in a purely monetary manner[206]. Thus, CBA is rarely used as the sole mode of analysis in a health economic evaluation.

2.4.1.3.b. Cost Utility Analysis

Cost utility analysis (CUA) is a mode of analysis that encompasses different aspects of intervention by including the costs and the utility such as satisfaction of the consumer[190,201]. In health economics terms this means that that costs and the reported level of patient well-being, likely to be expressed in QALYs, are considered into the analysis[201,206,207]. Therefore, gains in utility with regards to the cost of different interventions are used to assess and inform decision making processes. The inclusion of a health outcome (utility) means that the impact on quality of life can also be considered in the analysis, which better informs decision makers as resultant significant/insignificant changes in quality of life can influence the potential adoption of an intervention.

2.4.1.3.c. Cost-effectiveness analysis

Cost-effectiveness analysis (CEA) assess the cost of interventions not only from the monetary aspect of the intervention but also the effectiveness of the health outcomes that are achieved by the intervention[178,184,208]. Effectiveness can be accessed via positive outcomes such reduced hospitalisations or less organs transplants required as a result of the intervention. These can be considered as a standardised unit of health, so gains or losses in the determined unit can be used to compare the effectiveness of different interventions, that aim to produce the same health outcome e.g. number of lives saved, complications prevented, or diseases cured. QALYs are often used in CEA to calculate what the incremental cost-effectiveness ratio (ICER) is when comparing two or more interventions[189,198]. Therefore, it is integral for ICER to specifically state the interventions being compared as intervention analysis cannot be compared in isolation. QALYs and DALYs can be used to assign a standardised unit to the outcome of an intervention. ICER values are positive as they show the cost of gaining a QALY or adverting a DALY. A negative value has no technical meaning, therefore the values are not reported, instead it is reported that the intervention with the negative value is dominated by the alternative[187,209,210]. ICER works on an incremental basis comparing each intervention to the next least-expensive (non-dominated option). When the WTP is combined with ICER values, the cost-effectiveness plane (**Figure 13**) can be used to determine how likely it is for the intervention to be recommended for adoption and reimbursement.

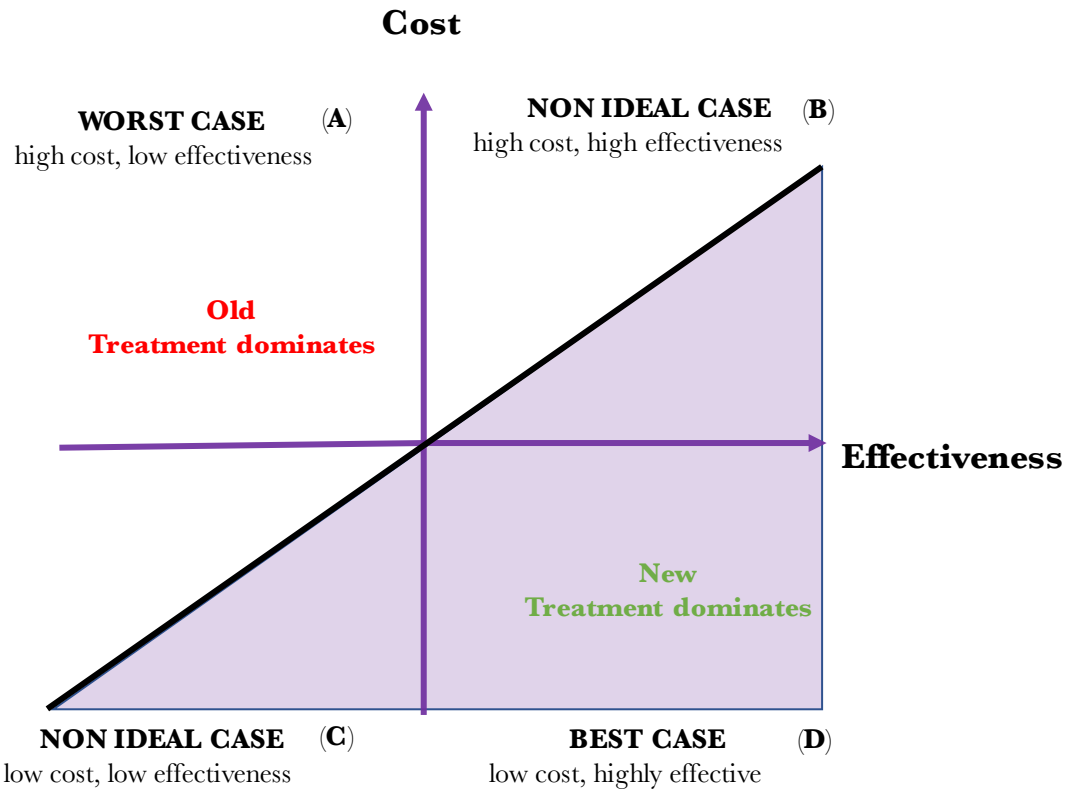


Figure 13. The cost-effectiveness plane is used to visualise how likely it is for an intervention to be adopted in comparison to other interventions compared in the ICER, in the context of the WTP. If an intervention's ICER plots below the black line (and shaded area) it is below the WTP which means it is likely to be recommended. The best-case scenario is for an intervention to plot on the bottom right quadrant (**D**) as it would be both low cost and highly effective, meaning it dominates in comparison to other interventions in the analysis. Typically, cell and gene therapies are in the top right-hand quadrant (**B**) where they are highly effective but are encumbered by high costs.

Cost-effectiveness analysis thus measures the incremental cost of achieving an incremental health benefit, expressed as a particular health outcome that varies according to the indication of the therapy[211]. Examples of ICERs using this approach are: the cost per extra patient improving in motor functions and cognition for PD patients or the cost per extra acute rejection episode avoided in patients with kidney transplants[212]. Assessing the value for money of using an intervention requires the extra health benefits achieved to be weighed against the extra net cost. This comparison is usually expressed as an ICER which is the net incremental cost (costs minus cost offsets) of gaining an incremental health benefit over another therapy[209,213]. The value of these analyses is that they provide developers with an understanding of the potential economic impact of the product, so it can be assessed whether the expected income will offset the development and production costs associated with the CTP[182,183].

ICERs provide a good basis to work from when it comes to the comparison of different interventions, however there some issues that need to be addressed with regards to their appropriateness. This is because there is a lot of uncertainty associated with the input variables for instance, the data sources of QALYs. These uncertainties become compounded in the ICERs, and as a result sensitivity analysis is necessary to deal with the uncertainties[187,210]. The uncertainty needs to be dealt with systematically, by considering each input independently and in combination with other inputs. This is necessary to show the robustness of CEA data i.e. that the basic conclusions are the same despite the uncertainties[187,214]. This is especially the case for pivotal uncertainties such as intervention effectiveness and life-time costs of a therapy. There are two types of sensitivity analysis, one way and multiway sensitivity analysis; both of which can help in the deconvolution of complex models, particularly in terms of the interplay of different inputs[210,213,215]. Using sensitivity analysis can reveal technical errors and can potentially uncover interesting dynamics in the outputs of CEA based on different inputs[187,216]. In addition, sensitivity analysis gives transparency to the model as the effect of the different inputs are assessed for their importance, which helps when using ICERs for decision making processes. Furthermore, this can highlight inputs that are poorly understood allowing for further research to be carried out to establish additional data which can be used for re-evaluation [187,189]. This can be an iterative process, carried out until a robust cost-effective value is obtained from the models and evaluations.

2.4.2. Decision models for health economic evaluation

The points discussed in the previous sections are integral to the study of health economics and health economic evaluations which are a complex process that is carried out using health economic decision models. In order to formulate robust and effective decision models, there are specific model characteristic and aspects that should be considered. These aspects include time horizons, data presentation, sources of input data, comparators, model choices and the analytic perspective[187,214].

2.4.2.1. Time

Timing is vital when it comes modelling for health economics, since the different time periods have an effect on the resolution and therefore the power of the information that the models produce. Time horizons must be chosen ad hoc, for instance for a rapidly developing illness, days or weeks would be an adequate measure to capture the information that would impact on the outcomes and costs of the intervention[178,217]. However, for a chronic illness, months and years would be more appropriate measure as the outcomes of the intervention would be realised over a longer period of time, typically covering a natural life span[188,210]. Having such information would add value to the results obtained from the models as payers would be able to extrapolate the long-term future costs and savings of different interventions. This information would be of greater value in comparison to the current models that have relatively short time horizons (one to five years), even for chronic illnesses.

2.4.2.2. Data presentation

Presentation of data and results is important for decision models and particularly useful for decision makers[187,210]. For instance, decision trees are convenient for CEA as they diagrammatically present the consequences of two or more different options. This is because decision trees can display potential action options, their consequences and the resulting consequences measured in costs and health outcomes[187]. The cost-effectiveness plane is also a convenient tool as it allows for visual representation of the ICER of the different interventions. Other useful presentations include one and two-way threshold analysis, ICER distribution graphs and cost-effectiveness acceptability curves (CEAC)[187,215,218]. Most importantly with regards to data, is that it must be transparent, as this allows decision makers to truly understand or at least have a good basis of understanding of how the models have been conceptualised.

2.4.2.3. Data sources

Continuing with the topic of data, models should be data rich in terms of therapy effects and costs, particularly in different scenarios as this results in more informed and robust models which and can be used for effective and efficient decision making. There are various sources of data available, ranging from trial data and synthesised evidence through methods such as cohort simulation; it is at the discretion of analysts to choose appropriate data for their models[213,219]. When creating models caution is necessary, as metrics that do not assume maximum utility are required, unless that is the realistic level to be obtained. This means that models that assume maximum utility need to be adjusted so that they are more representative of the actual effects of the therapy/product. Input data for CTPs presents a challenge as there are limited trials to provide information. Furthermore, due to the unconventional

nature of CTPs and their various mechanisms of action, traditional pharmaceutical data cannot be used as surrogate data.

2.4.2.4. Comparators

Since methods such as ICER cannot be performed in isolation, it is imperative to have comparators[214,220]. The gold standard and other effective interventions (as many as realistically possible) should be compared in order to fully evaluate the true cost-effectiveness of the intervention[188,205]. If relevant comparators are not included, the validity of the results could be brought into question as the cost-effectiveness analysis would not be fully representative of the interventions that are available, limiting the power of the information and consequently the reliability of the decisions made.

2.4.2.5. Model choice

Choosing the appropriate model is a key activity as the model should have the ability provide useful information relevant the decision that needs to be made. Key aspects to consider during model selection include defining the boundaries of the model and the availability of the data required to populate the model. Decision trees are useful for comparison of the different outcomes of two intervention options (

Figure 14). However, decision trees are limited in their scope as they are typically focussed on a single discrete time-period, therefore they cannot be effectively used to model long periods of time without becoming overly complicated and 'bushy'[187]. In such circumstances, Markov models can be used as they have the ability to model longer periods of time[219]. Markov models are based around stochastic process and transition probabilities (**Table 3** and **Figure 15**). Relating back to the importance of time horizons, Markov models present a useful tool to model long term treatment effects, which is imperative for interventions that target chronic diseases that would need to be modelled over an entire life span[198,214].

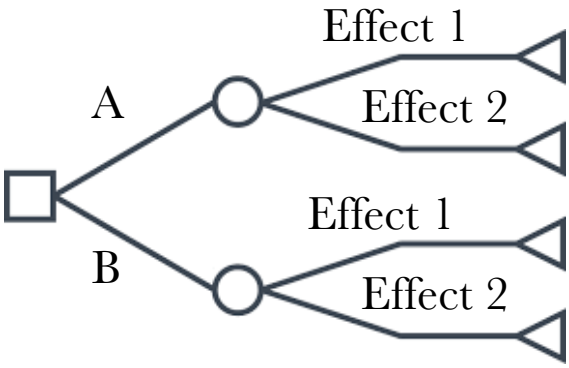


Figure 14. An illustrative example of a decision tree to demonstrate how the effects of two different decision pathways can be traced to give an outcome. The decision node represented by the square shows the decision being made, the chance nodes (circles) branch out to the different pathways that result due to the decision made. The end nodes (triangles) represent the payoff of each pathway of each decision. The payoffs are calculated using probabilities of given parameters such as costs and health utilities. Therefore, a populated decision tree can be used to model the different costs associated with using different treatments as an intervention for a disease.

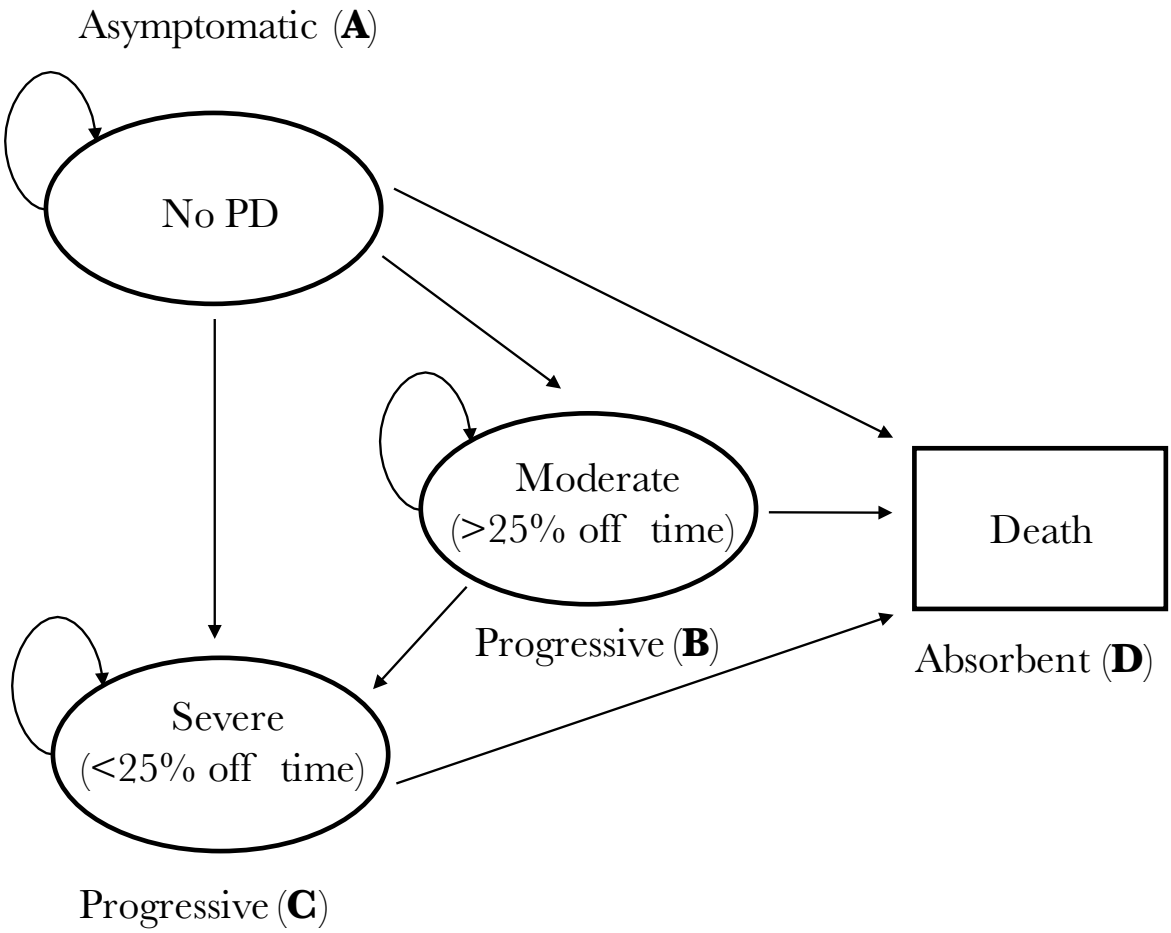


Figure 15. Markov models are based on stochastic processes which can be on a continuous timeframe. This allows them to be used for modelling the transition of an individual or cohort group from one state into another. The transitions are based on transition probabilities which can be weighted against health outcomes or costs, therefore Markov models can be used to model the costs and health outcomes related to a patient or group of patients being in a specific state or transitioning between states. In this illustrative example a patient can be asymptomatic of Parkinson's disease(**A**), have moderate Parkinson's disease(**B**), have severe Parkinson's disease (**C**) or be in the absorbent state of death (**D**).

Table 3. Transition matrix showing the different transition probabilities (tp) from one state to another. This matrix works on the principle of staying in the same state or progressing to the next state, but not being able to go back into a previous state – as indicated by the direction of the arrows in **Figure 15**.

Transition Matrix	A	B	C	D
A (Asymptomatic)	tpA2A	tpA2B	tpA2C	tpA2D
B (Moderate PD)	0	tpB2B	tpB2C	tpB2D
C (Severe PD)	0	0	tpC2C	tpC2D
D (Death)	0	0	0	1

2.4.2.6. Perspective

Finally, it is important to understand the analytical perspective of the evaluations being carried out. All parties, from a moral point of view, should have the health outcomes and effectiveness as the priority of their intervention[187,214]. However, monetary considerations are important as budgets are generally limited, expectedly developers and payers will have different perspectives. For instance, developers will want to be granted the highest possible reimbursement price whilst payers will want the lowest reimbursement price for the most effective intervention. Patients are also another stakeholder, their perspective (particularly in national healthcare systems) is to have access to the most effective interventions[136,187,191]. With all these different and at times conflicting stakeholder perspectives to consider, models need to be as informative and transparent as possible so that all parties are able to infer relevant information for their decision-making processes.

2.4.3. Health economics model and concepts summary

The above sections highlight just some the key aspects of health economics analysis and decision models. It is evident that this is a complex endeavour with many sources of uncertainty in terms of the data utilised. For instance, in most evaluations, meta-analysis of similar trial data or data extrapolation is used when the intervention being compared has limited efficacy data, which may not be an accurate representation of the intervention. This is an even greater drawback in evaluations of cell and gene therapies as there is often no similar trial data to use or the sample sizes used are small in comparison to drug trial data. Therefore, sensitivity analysis of the input data used for evaluations must be carried out to ensure the results are robust and useful for decision making.

In general, health economic evaluations are carried out in the latter stages of the product development process as accumulated efficacy data through the development timeline can be used. This results in early health economic evaluations being a futile task as there is not enough evidence generated in the preclinical stages to ascertain the clinical effectiveness of a product. Thus, there is insufficient data to populate the models to run credible analysis of the intervention. As a result, developers to do not have the ability to provide estimations at an early stage of the commercial viability and reimbursement potential of their product using traditional HTA methods.

2.4.4. Headroom method

The ability to obtain information about a product's reimbursement potential at an early stage would be a powerful tool for developers to use during their product development. Early economic evaluations can allow developers to make more informed decision about costs, pricing and market selection that is in line with the payer needs, making their product better placed for reimbursement and adoption. Furthermore, by carrying out early economic analysis and market selection developers can provide investors with estimates of the potential gross profits that they can expect.

If a product is deemed to not be commercially viable at an early stage, then it would be advisable to terminate further development or carry out more research. This would save costs further down the development timeline, rather than getting to a stage where a product gets regulatory approval after all the cost of clinical trials and development, only to be deemed not cost-effective and to be not recommended for adoption and reimbursement. This is a fate that has been suffered by cell and gene therapies; such as Uniqure with their product Glybera, which achieved regulatory conditional approval[26,221]. However, it was not deemed adoptable by payers due to the high price of €1,000,000 per dose, even under the promise of the treatment being a one dose therapy it was removed from the market[26,221].

The headroom method is a tool that can be used to determine the maximum potential reimbursement price of a new product at an early stage. The headroom method incorporates the demand and supply aspect the product. Therefore, it can be used to forecast potential revenues, as it is possible to predict if the developers can produce the product at the given imbursement price and development/manufacturing cost[186,192]. As such the headroom method is an early economic evaluation tool that can be used by developers to determine if they should proceed or abandon products based on their commercial viability. It is worth noting that headroom method analysis is only predictive and therefore should be used as tool amongst other considerations, such as the potential to expand into other areas, other non-health related preferences and the clinical and market context of the product[182,184].

There is a wide range of literature based concerning the use of headroom methods, although this tends to be heavily focused on the area of medical devices, however the concepts and principles can be applied to CTPs[186,192,222]. The addition of information about future deviations of outcomes into the headroom calculations provides more realistic conclusions as it takes into account the non-linearity of inputs and outcomes over time [136,220]. However, there are limitations as some of the fundamental inputs, such as the QALYs, WTP (sometimes) and the expected improvement in clinical performance, can only be capriciously assumed because of the uncertainties associated with an undeveloped product or technology, particularly in the CTPs industry[220,223,224]. Thus early evaluation models need to be done iteratively as more data is obtained, in order to become more robust so that the business models of CTP can facilitate confidence in the commercialisation of CTPs[174].

Research and models that combine the headroom method and gross profit have been developed, this combination produces "a feasible, useful, and informative tool for assessing the potential commercial viability of medical devices under development"[186]. The advent of economic evaluation in CTPs has led to such innovative methods to try and provide information as early as possible. Much of these analyses rely on accruing as much information both scientific and economic/commercial, as often possible, so that they can be as accurate as possible in their projections. However, it is important to consider that, the results are typically based on estimations and assumptions, particularly for CTPs were surrogate data is not readily available. Thus due diligence needs to be taken when choosing input sources for data, relevant and reliable sources must be sought after to ensure that the resultant outcomes are dependable for decision making purposes[186].

Using methods of analysis such as those mentioned above it is possible to predict the saleability and adoption potential of the product by the various healthcare providers. For instance, the cost-effectiveness plane, obtained from ICER results, can be used as quick tool to visualise the likelihood of the product (s) being adopted. The ideal situation for a new therapy in a competitive market would need to be in the lower right quadrant (**Figure 13D**), as it means that the therapy is more effective and economic in comparison to other options and the gold standard. For CTPs this a currently a difficult quadrant to enter as costs are currently very high for most CTPs thus, they tend to be found in the top right quadrant (**Figure 13B**). A major challenge for the CTI is to reduce costs to allow for favourable reimbursement and adoption of CTPs [186]. The value of health economic evaluations is the information it provides for investors and payers alike. For developers being equipped with the concepts HTA and a firm understanding of their product, its costs and health outcomes should set them up to be in a position to successfully traverse through the reimbursement pathway.

2.4.5. Health Economics in the context of PD

There some nuances when carrying out health economic evaluations for CTPs compared to pharmaceutical or other interventions. This is because clinically, there is limited information regarding the long-term effects of CTPs and unlike pharmaceuticals, surrogates and trial data do not yet readily exist for CTPs as previously mentioned. Health economic evaluations for other PD interventions such as anti-Parkinsonian medication have been carried out[39,69,206], however there is a lack of economic considerations from the CTPs interventions that are currently being developed. Current therapeutic medication does not result in significant improvements to quality of life in the long term, whereas cell therapy treatments have the potential to significantly restore native DA neuronal function and greatly improving patient quality of life[24,40,225]. As a result, cell replacement therapies have the potential to be highly effective and potentially cost-effective in cases where the patients are alleviated from symptoms, increasing their quality of life therefore negating the use of antiparkinsonian medication and rehabilitation.

As PD affects many people around the world it represents a high economic burden due to inpatient care (hospital and rehabilitation), outpatient care, antiparkinsonian medication and both formal and informal care, all of which are considered to be direct costs of PD[41,70]. The cost of treating PD varies from country to country due to differences in healthcare systems. Western countries on average spend more money on managing PD as higher percentages of their health budgets are spent on the geriatric population. Different countries cover different aspects of the PD management, in national healthcare systems such as the UK, the direct costs are absorbed by the healthcare provider. While in more privatised systems such as the United States of America (USA) these costs are absorbed through private health insurance or by the patient directly, out of pocket[69]. These costs have a significant economic impact, in a US study of over 40,000 people, it was found that people with PD had over double the direct costs per year associated with their health costs in comparison to people without PD[226]. With adjustment for comorbidities and demographics PD patients had costs of $23,101 whilst those without PD were $11,247[226].

Comorbidities and loss of productivity represent some of the indirect costs that are associated with PD[70]. These costs are incurred due to PD rendering patients incapable of carrying out their daily routines and lowering their quality of life, which requires patients to have assistance, particularly at the mid to late stages of the disease. The cost of care therefore makes up a large part of the overall cost of PD[41,69]. For instance, institutional and professional care attribute to increased overall cost of managing PD, reported to be 4.5 times higher than people who do receive care at home[69]. Various studies have evaluated the costs and economic impact of PD, typically the cost percentage distribution is similar between studies. However, the actual costs do differ, as mentioned above costs can vary between regions and countries,

which also the case in PD[70]. A study by von Campenhausen *et al* (2011) highlights that there are considerable differences in PD treatment costs even between European/Western countries[70]. As a result, this will affect reimbursement depending upon the country, however it can be inferred that reimbursement would be higher in western countries due to higher life expectancy and greater prevalence of PD. Therefore, such countries and their healthcare systems would greatly favour therapies such as CTPs that can slow down the disease and reduce costs, potentially for up to 24 years[71]. Work by Keranen *et al* (2003), illustrates that costs in PD do increase over time, the more severe the disease the costlier it becomes. Therefore, attenuation of the disease is a suitable way of reducing the overall costs and increasing patient QoL[41].

As discussed previously DALYs can be used for ICER analysis as they represent changes in disease burden, for PD the database is formed from retrospectively collected clinical information from a diverse PD population. This provides valuable and robust input data for modelling the transition from one Hoehn & Yahr (H&Y) stage to the other that can be linked to observed or modelled cost. Therefore, it is possible to produce decision models that are more dynamic as the data is based on real occurrences, making it possible to realistically assess a patient's time-period at a particular stage of PD. This can provide valuable information about the effectiveness of an intervention across the different stages of PD, allowing decision makers to evaluate when the intervention is most cost-effective. The data can also be used as a source of transition time and quality of life input for modelling purposes. This can provide information about the variability in costs and quality of life produced by an intervention based on how long a patient spends in a particular health state[195].

2.4.6. Considerations for developers

In any product development process obtaining approval from regulatory bodies such as the FDA and EMA are considered to be key milestones. However, further to this, developers have to traverse an adoption and reimbursement pathway; this is typically not considered until the later stages of development (**Figure 11**). Failure to consider this pathway can be extremely detrimental to the program progression and developers must consider additional time and funding to adequately provide the evidence that is required by the payer for adoption and reimbursement. This section briefly highlights key considerations for developers, in addition to the early commercial evaluation and analyses discussed previously in this chapter, and challenges that must be addressed in order to have an approved, adopted and reimbursed product. This is ultimately the end goal for any developer as they need to recuperate product development/manufacturing costs and have their product reach the patients to provide benefits.

Evidence is a key barrier that must be addressed as part of the commercialisation strategy; this is related to efficacy and safety evidence as shown in the clinical trials. Furthermore, evidence of long-term

clinical effects is required to justify the use of an intervention that is procured by the payer. While safety and efficacy are sufficient evidence for regulators, these criteria are not enough to satisfy payer requirements; payers are required to make decisions regarding funding interventions under significant fiscal pressures. Ideally, all intervention options would be provided to those in need but given the finite budget of healthcare services including the NHS, decisions have to be based upon cost-effectiveness and overall value. Therefore, for payers the key considerations alongside safety and efficacy are cost, value and comparable performance. Payers look for new interventions/technologies that decrease overall costs and alleviate their budgets while providing quality and value to the patients. How well a new intervention compares to the current gold standard and its usability given the current infrastructure are also significant adoption and reimbursement considerations.

If a developer is able to prove that their product meets the aforementioned considerations, there are still further translational barriers to overcome, particularly if the intervention is different from the current standard. Administrators would need understand how the new intervention would be implemented, budgeted for, where the money would come from within the current budget to pay for it, what currency codes would be applied, or if new codes need to be created (currencies and code are discussed in a later section - 7.1.1.3. The NHS payment and reimbursement system.). Another challenge is overcoming the dogma of current standards of care, developers need to have advocates for their interventions so that they are successfully adopted and utilised. Resistance to new interventions can result in product failure. Therefore, when traversing the adoption pathway, it is important to have key opinion leader (KOLs) on side and to engage with them as early as possible once the clinical need has been identified. Failure to anticipate and consider such aspects during early development stages will most certainly result in failure since resources and time will both have diminished, making late-stage changes nearly impossible to implement. By having early economic considerations and a defined adoption pathway as part of the development process, developers can be equipped with the translational tools they need to tackle the so called “commercialisation valley of death”.

Chapter 3:

Materials and Methods

Chapter 3. Materials and Methods

3.1 Introduction

This chapter provides details of the fundamental methods utilised in the present work. Due to the optimisation and process development nature of the work, deviations have been made in some experiments, where they differ to the methods described here; the exact methods used are stated within the methods employed section of each chapter. Protocol standardisation is an important element of this project in order to produce protocols that are translatable, therefore steps have been described in detail to minimise sources of error and variation. All reagents and consumables were obtained from Fisher Scientific, UK unless otherwise stated

3.2. Cell culture

Two cell lines were used within this work: EC 2102Ep and H9s. EC 2102Ep (GlobalStem, USA) are an embryonic carcinoma cell line provided by the National Institute for Biological Standards and Control (NIBSC). H9s (WiCell, cat# hPSCreg WAe009-A) is a human embryonic stem cell line obtained from WiCell Research Institute, Incorporated. All cell work and medium formulations were performed in a class II biological safety cabinet (BSC) using aseptic technique.

3.2.1. Culture Medium

Different media formulations were used to culture each cell line, H9 medium formulations differed for the expansion and differentiation process respectively. The formulations are described in the following sections.

3.2.1.1. Media formulation

3.2.1.1.a. EP growth medium

For the culture of EC 2102Ep cells, Gibco Dulbecco's Modified Eagle Medium (DMEM) high glucose with GlutaMax™ (cat#10569010), supplemented with Foetal Bovine Serum (10% *v/v,* cat#10100139) herein referred to as EP growth medium was prepared.

3.2.1.1.b. Wash medium

A wash medium was prepared using an appropriate amount of 10 % (*wt/vol*) Human Serum Albumin (Irvine Scientific, USA, cat#9988) added to DMEM/F12 for a final concentration of 1% v/v HSA. This medium was used for wash steps and resuspension of H9 cells upon resuscitation from cryopreservation.

3.2.1.1.c. H9 expansion medium

The expansion of pluripotent H9 cells was carried out using StemMACS™ iPS-Brew XF (cat# 130-104-368) supplemented with 10 mL of StemMACS™ iPS-Brew XF supplement (50X) to produce the medium herein referred to as H9 expansion medium. 40 mL aliquots of the H9 expansion medium were prepared and stored at -20 °C for up to eight weeks. Aliquots were thawed prior to use overnight at 4 °C, thawed aliquots were then stored 4 °C in between use, for up to fourteen days.

3.2.1.1.d. N2 base medium

For the differentiation of H9s a medium herein referred to as N2 basal medium was prepared at the start of the differentiation process and used for medium exchange on day 2, 4, 7 and 9 (**Table 4**). A range of small molecules were added into an aliquot of the N2 basal medium on the days of the medium exchange, details of which can be found in section 3.2.1.1.f. This medium was used for neural induction and patterning towards a ventral mesencephalic cell fate. 40 mL aliquots of the N2 basal medium were prepared and stored at 4 °C for up to two weeks.

Table 4. Components used in the N2 base medium formulation.

Reagent	**Concentration (% vol/vol)**	**Supplier**
DMEM/F-12	49	Invitrogen Cat# 21331-020
Neurobasal CTS	49	Life Technologies Cat# A13712-01
N2 Supplement	1	Life Technologies Cat# A13707-01
L-Glutamine	1	Invitrogen Cat# 25030-081

3.2.1.1.e B27 base medium

Akin to the N2 base medium prepared in section 3.2.1.1.d., a medium herein referred to as B27 base medium was prepared as a stock for use during the replating and medium exchange on day 11 (**Table 5**). A range of small molecules were added into an aliquot of this medium on day 11 and 14, details of which can be found in section 3.2.1.1.f. This medium was used to pattern the early caudal ventral mesencephalic progenitors into late stage caudal ventral mesencephalic progenitors. 40 mL aliquots of the B27 base medium were prepared and stored at 4 °C for up to two weeks.

Table 5. Components used in the B27 base medium formulation.

Reagent	Concentration (% vol/vol)	Supplier
Neurobasal CTS	97	Life Technologies Cat# A13712-01
B27 Supplement	2	Life Technologies Cat# A12587-010
L-Glutamine	1	Invitrogen Cat# 25030-081

3.2.1.1. f. Differentiation Media

The differentiation process used three different formulations split into sections:

- Day 0 to 7 (N2 differentiation medium),
- Day 9 (FGF-8 N2 differentiation medium),
- Day 11 to 14 (B27 differentiation medium).

The formulations are specific as they support the cells at their different stages of differentiation. Details of the roles of the small molecules are discussed in section 6.1.1.1.

Table 6. Components used in the media formulation for the differentiation process. Table details the small molecules added at specific time points of the process. * An appropriate volume for the conditions being cultured was used. ** Y-27632 is a RHO kinase (ROCK) inhibitor that increases cell survival following dissociation, therefore it was added only at day 0 seeding and on day 11 for the replating.

Time point	Reagent	Concentration	Supplier
Day 0, 2, 4 and 7 **N2 differentiation medium**	N2 base medium	*	Prepared as described in section 2.2.1.1.d.
	SB431542 (TGFβ inhibitor), StemMACS GMP	10 µM	Miltenyi Biotech, Cologne, Germany cat# 130-106-543
	Recombinant human Noggin, StemMACS GMP	100 ng/ml	Miltenyi Biotech, Cologne, Germany cat# 130-103-456
	CHIR99021 (GSK3 inhibitor), StemMACS GMP	0.7 µM	Miltenyi Biotech, Cologne, Germany cat# 130-106-539
	Human Sonic Hedgehog C24II, StemMACS GMP,	300 ng/ml	Miltenyi Biotech, Cologne, Germany cat# 130-095-727
	Y-27632 StemMACS GMP **	10 µM	Miltenyi Biotech, Cologne, Germany cat# 130-106-538
Day 9 **FGF-8 N2 differentiation medium**	N2 medium	*	Prepared as described in section 2.2.1.1.d.
	Recombinant human FGF-8b, Premium grade	100 ng/ml	Miltenyi Biotech, Cologne, Germany cat# 130-095-740
Day 11 and 14 **B27 differentiation medium**	B27 base medium	*	Prepared as described in section 2.2.1.1.e.
	Recombinant human FGF-8b, Premium grade	100 ng/ml	Miltenyi Biotech, Cologne, Germany cat# 130-095-740
	Recombinant human BDNF, GMP	20 ng/ml	Miltenyi Biotech, Cologne, Germany cat# 130-096-286
	Ascorbic Acid (Tocris 4055)	0.2 mM	Sigma A4403-100MG
	Y-27632 StemMACS GMP **	10 µM	Miltenyi Biotech, Cologne, Germany cat# 130-106-538

3.2.2. Coating flasks

The adherent nature of the cells requires them to be cultured on a surface, for the H9 cells a feeder-free and xeno-free natural cell niche is obtained by coating tissue culture treated vessels with human recombinant laminin protein.

Tissue culture plastic (TCP) were coated with Biolaminin-521 (cat# LN521, Biolamina, Sweden) for H9 expansion or Biolaminin-111 (cat#LN111 , Biolamina, Sweden) for differentiaton, diluted with phosphate buffered saline with calcium and magnesium ($PBS^{+/+}$) at a coating density of 0.5 µg/cm^2 or

1 μg/cm^2, respectively. Biolaminin 521 is used for pluripotent expansion as it is the natural laminin for pluripotent cells, whereas Biolaminin-111 is used of the differentiation process as it supports neural differentiation while disfavouring pluripotent growth. The laminin/PBS coating solution was added to the TCP and incubated at 37°C for 2 h, prior to seeding the cells. Following 2 h the coating solution was aspirated and the appropriate medium was added to prevent drying of the coating.

3.2.3. Thawing

Prior to thawing the appropriate basal medium was warmed using a water bath (Sub Aqua 26 Plus, Grant Instruments) at 37 °C for a minimum of 20 min. Cells were thawed from cryopreservation by placing the cryovial at the front of the middle shelf of an incubator (Panasonic, MCO-17MUVH-PE) set at 37°C, 5% CO_2 for 7 min or until thawed. A 1000 μL pipette was used to transfer the cell suspension dropwise into a 15 mL centrifuge tube. The cryovial was then washed twice with 1 mL basal medium to ensure maximum cell retrieval. The resulting cell suspension was centrifuged for 5 min at 350xG **(**Sigma Laboratory Centrifuges, 3-15).

To avoid dislocation of the pellet, the supernatant was carefully removed using an aspirator and pump (Welch, 2511 Dry Vacuum Pump). 1 mL of fresh pre-warmed medium (as appropriate to the cell line) was added to the pellet using a 1000 μL pipette; the pellet was resuspended by gently pipetting the solution up and down 10 times whilst avoiding bubble formation. An additional 4 mL of fresh medium was added to cell suspension, giving a total volume of 5 mL cell suspension for all cell counting and seeding.

3.2.4. Standard culture

After thawing a cell count was carried out to obtain the volume required to seed at the desired density (cell counting procedure is detailed on page - 75 -). The required volume was then transferred to a tissue culture vessel using an appropriately sized pipette, the cells were agitated and rocked gently in all directions to ensure even distribution across the culture surface. The methods described below are the standard conditions and parameters used to culture the cells, any deviation from the described methods are noted in the relevant chapters.

3.2.4.1. EP2102 expansion

All reagents/consumables were obtained from Fisher Scientific, UK unless otherwise stated. EC 2102Ep cells were thawed and cultured at 37°C, 5% CO_2 using EP growth medium (3.2.1.1.a.). For standard culture, cells were subjected to a medium exchange following 48 h and passaged following 72 h.

3.2.4.1.a. Cell passage

The procedure described here was followed to carry out a standard cell passage. EP growth medium, and Trypsin- Ethylenediamine tetracetic acid, 0.25 % (EDTA) (Thermo Scientific, UK, cat#25200072) were pre-warmed in the water bath at 37 °C for at least 20 min. The cells were microscopically (AMG, EXOS x1) inspected for growth, contamination and attachment before transferring to BSC. The spent medium was aspirated, and the cells were washed with PBS (-Ca^{2+}/-Mg^{2+}), 250 µL/cm^2. The PBS was aspirated and 40 µL/cm^2 Trypsin-EDTA was added to the culture vessel which was rocked gently to ensure the cells were submerged prior to incubation for 5 min at 37 °C, 5% CO_2. Following 5 min the cells were observed under the microscope to check detachment from the culture surface, signified by the cells rounding up. Wash medium was added at twice the volume of Trypsin-EDTA used, the surface of the culture vessel was flushed using a stripette 5 times before transferring the cells into an appropriately sized centrifuge tube i.e. 15 mL tube for volumes under 12 mL. The cell suspension was centrifuged for 5 min at 300xG. Following centrifugation, the supernatant was aspirated off ensuring that the pellet was not disturbed. The pellet was broken down by adding 1 mL EP growth medium to the cells and pipetting up and down 10 times using a 1000 µL pipette. A further 4 mL of EP growth medium was added. A sample of the cell suspension was taken for cell counting. From the cell count, the total live cell number was determined and used to seed the cells at a density of 66,667 cells/cm^2 with 200 µL/cm^2 of EP growth medium. The culture vessel was manually rocked in all directions to yield homogenous plating of the colonies and then placed in an incubator set at 37 °C, 5% CO_2.

3.2.4.2. H9 Expansion: Standard culture route

H9 cells were thawed from cryopreservation and cultured on Lamin-521 coated plastic-ware at 37°C, 5% CO_2 using H9 expansion medium with addition of ROCK inhibitor (10 µM) at every passage point. The standard expansion culture routine subjected the cells to a medium exchange every 24 h and a passage following five days (120 h) upon thawing and four days (96 h) for routine expansion culture.

3.2.4.2.a. Cell passage

The passaging of the H9 cells followed the same protocol as the EC 2102 Ep cells with the exception of H9 growth medium and wash medium replacing the EP growth medium and EDTA was used at the dissociation agent instead of Trypsin-EDTA. The wash medium was used to carry out the wash and resuspension steps prior to cell counting. The cells were seeded at a density of 10,000 cells/cm^2 on a Biolaminin-521coated culture vessel with 250 µL/cm^2 of H9 growth medium. The culture vessel was manually rocked in all directions to yield homogenous plating of the colonies and then placed in an incubator set at 37 °C, 5% CO_2.

3.2.4.3. H9 Differentiation

TCP was coated with Biolaminin 111 as described in section 3.2.2 at a density of 1 µg/cm^2. Cells were harvested following two passages of H9 expansion culture and cultured under standard conditions (37°C, 5% CO_2) using N2 differentiation medium with ROCK inhibitor. A combination of small molecules (**Table 6**) was added on each day of medium exchange. This medium was used to seed the cells on day 0 and for medium exchange on day 2 (250 µl/cm^2), day 4 (300 µl/cm^2) and day 7 (350 µl/cm^2). On day 9 the medium was changed to the FGF-8 N2 differentiation medium (400 µl/cm^2). The B-27 differentiation medium (**Table 6**) was used to replate the cells on day 11 (600 µl/cm^2) with ROCK inhibitor and for medium exchange on day 14 (**Figure 16**).

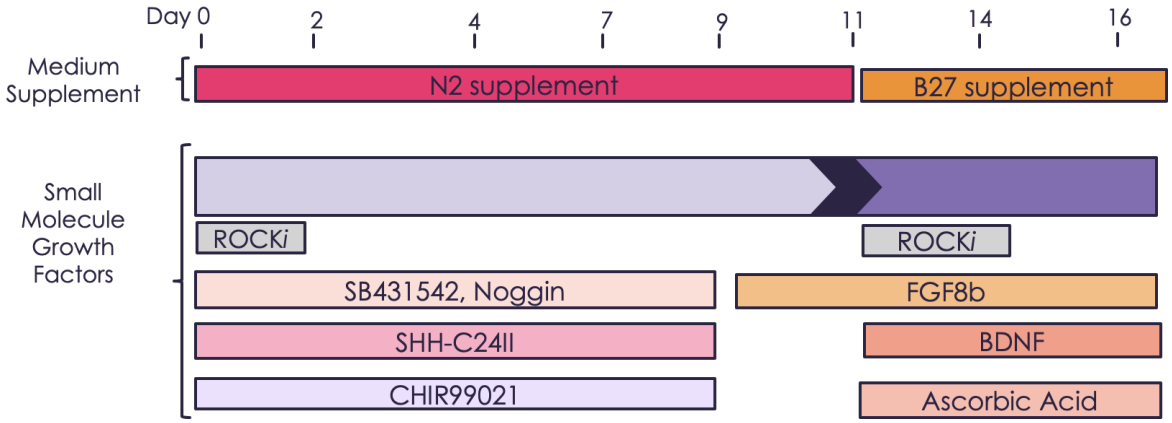


Figure 16. Schematic of the different media compositions used in the differentiation process.

3.2.4.3.a. Replating

The procedure used to replate the cells on day 11 and to harvest the cells on day 16 is described in section 3.2.4.3.a (page - 72 -) with the following changes implemented. The use of EDTA was replaced with the dissociation agent Accutase (Thermo Scientific, UK, cat#A1110501) for 10 min at 37°C and 5% CO_2, B27 base medium was used in place of N2 differentiation medium on day 11

3.2.5. Cryopreservation:

Cells were harvested and counted prior to resuspension in a solution comprised of Cryostor® CS10 (cat#C2874-100ML, Sigma Aldrich, UK) and growth medium (10% *v/v*) to obtain a cell suspension of 6x10^6 cells/mL for EC 2102Ep cells. For H9 cells, StemMACS Cryo-Brew (Miltenyi Biotech, Germany, cat# 130-109-558) was used to obtain a cell suspension of 1x10^6 cells/mL. 1 mL aliquots of cell suspension were aseptically transferred into cryogenic vials (Corning, USA, cat#430659) and stored in a Mr Frosty CoolCell® passive cooling device for 24 h at -80°C before being transferred to the vapour phase of liquid nitrogen where they were stored prior to experimentation.

Cell counting

3.3.1. NucleoCounter® NC-3000™

The NC-3000™ automated image cytometer (Chemotec, Denmark) was chosen as the method of performing cell counts as it reduces human bias. Fluorescent dyes are used to label and image cells for automated image analysis, this permits analysis of characteristics including cell number, average cell diameter and cell aggregation levels. Furthermore, the software autonomously performs cell viability counts and calculations using validated algorithms. The use of commercially available reagents, cassettes and slides significantly reduces errors in sample manipulation and loading. As such, the NC-3000™ was used for all counts since reproducibility and standardisation are fundamental aspects of the experimentation process in the present work. Traditional counting methods such as the use of a haemocytometer would not be able to achieve the same levels of reproducibility and standardisation, particularly between different operators. Furthermore, the software is formatted to alert the user if sample volumes at are out of the optimal range for accurate cell counts, ensuring reliable and standardised cell counts. The data is rapidly processed by the computer and results can be collected in a range of formats for analysis, including CSV, FSC and PDF reports.

Cell counts and viability were measured using the Cell Viability and Cell Count assay which utilises acridine orange base N,',N',N'-tetramethylacridine-3,6-diamine monohydrochloride (AO) uptake an' 4',6-diamidino-2-phenylindole dilactate (DAPI) exclusion. AO and DAPI work by staining the entire cell population and the non-viable cells, respectively. AO and DAPI are spectrally and biologically different dyes; AO is a cell permeable dye that binds to cellular material resulting in green fluorescence. DAPI strongly binds to adenine-thymine rich regions of DNA that results in blue fluorescence. Although DAPI is cell permeable, at low concentrations (such as in this case) it is inefficient at passing through intact membranes. Therefore, it strongly interacts with dead cells making it a good candidate for determining the number of non-viable cells. The assay counts can be accurately performed with low sample volumes which is advantageous when working with precious or limited samples.

3.3.1.1 Via1-Cassette™

Vial-Cassettes™ are calibrated single use cassettes that provide high precision cell counts (**Figure 17**). A sample is loaded by submerging the cassette tip into the cell suspension and depressing the piston, this action creates a vacuum that reliably and precisely draws the sample into mixing channels that are pre-loaded with AO and DAPI. Once the sample has passed through the channels, the cassette is inserted into the NucleoCounter® NC-3000™. The piston is automatically depressed further by the machine to deliver the now stained sample into the measuring chamber were the cells are imaged and analysed. The analysed sample volume is 3.2 µL.

Figure 17. Image of a Via1-Cassette™ from Chemometec detailing the different components of the cassette. Image sourced from NC3000 ™ product brochure[227]

3.3.1.2. NC-Slide A8 ™

NC-Slide A8 (**Figure 18**) use the same principles as the Via1-Cassette™ in terms of the use of AO uptake and DAPI exclusion to determine cell number and viability. However, the method differs from the Vial-cassette in that the slides are not pre-loaded with fluorescent dyes. Therefore solution 13 (Chemotec, Denmark, cat#9103013), a commercially available reagent (pre-mix of AO and DAPI) is mixed into the cell suspension at a ratio of 1:19 e.g. 10 µL of solution 13 added to 190 µL of cell suspension. 10 µl of the solution is then manually pipetted into each of the 8 individual chambers using a 20 µL sterile tip. Each slide chamber can be used to analyse a different sample, allowing for high throughput cell counting to be performed in a single run. The loading chambers are optically clear, allowing for the cells to be imaged and analysed based on their interaction with solution 13 and the resultant fluorescence signals.

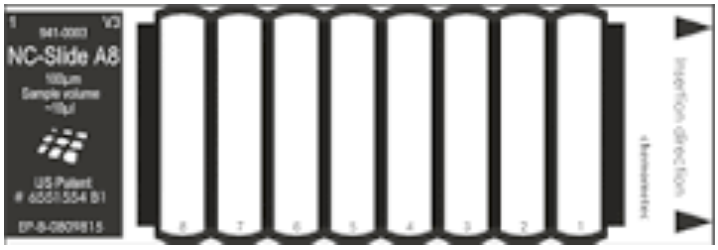


Figure 18. Image of the NC-Slice A8 ™ showing the 8 chambers that can be used to load the sample onto. Each chamber holds 10µL and an average of 2 chambers are required to gain an average count. Image sourced from NC3000 ™ product brochure[227]

3.3.2. Procedure and sample preparation

Following resuspension with 1 mL of the appropriate medium post centrifugation, the pellet of cells are gently pipetted up and down 10 times using a 1000 µL pipette. This step is performed to obtain a homogenous cell suspension that is as close to single cells as possible. The suspension is then further diluted with an additional 4 mL of the appropriate medium to obtain a stock cell suspension. For standard cell counting a sample of the stock cell suspension is taken and added in to a 1.5 mL Eppendorf tube, the sample volume used depended of the whether a slide or cassette was used. The sample was then vortexed (Barloward Scientific, Stuart Vortex Mixer) for three seconds, then pipetted up and down a further 10 times using a 20 µL pipette prior to mixing with Solution 13 for A8 slide counts or being loaded into a cassette. This procedure was adhered to for all counts performed as a means of standardising the cell counting protocol within the present work.

For Via-1 cassettes™ (Chemotec, Denmark, cat#942-0011), a 200 µL cell suspension is added to a 1.5 mL Eppendorf tube, vortexed for three seconds, pipetted using a 10 µL pipette and then loaded into the Via-1 cassette™ prior to analysis on the NucleoCounter® NC-3000™. Via-1 cassette™ were used for counts with less than eight samples. For counts with more than eight samples the NC-Slide A8 ™ slides (Chemotec, Denmark, cat#942-0003) were used instead. For the slides a 190 µL cell suspension is added to a 1.5 mL Eppendorf tube and mixed with 10 µL of Solution 13. This mixture was vortexed three seconds and then pipetted up and down ten times with a 20 µL pipette prior to loading into each chamber of the slide to breakdown the cell aggregates. A volume of 10.7 µl was used to load each chamber, as this was the most suitable volume to ensure coverage of the whole chamber and no air bubble formation, two chambers were used for each count of a condition.

The results were used to obtain the specific growth rate (μ) and Population Doublings (P_d) following **Equation 2** and **Equation 3** published by Heathman *et al.*, 2015[228].

$$\text{SGR value } (\mu) = \frac{\ln(C_x(t)/C_x(0)}{\Delta t}$$

Equation 2

Where μ is the net specific growth rate (h^{-1}), $C_x(t)$, and $C_x(0)$ are cell number at the end and start of the exponential growth phase, respectively and *t* is time (h).

$$\textit{Population Doubling, (Pd)} = \frac{1}{\log(2)} \cdot log\frac{C_x(t)}{C_x(0)}$$

Equation 3

Where $C_x(t)$, and $C_x(0)$ are cell number at the end and start of the exponential growth phase, respectively.

3.4. Cell metabolite analysis

Metabolite analysis can be used in manufacturing processes as a means of quality control since the levels of analytes such as lactate and lactate dehydrogenase (LDH) produced are putative indicators of glucose consumption rates and cell viability, respectively.

3.4.1. Cedex Bio HT Analyzer (Roche)

The Cedex Bio HT Analyzer (Roche) is an automated high throughput bio-analyser that was used to determine the levels of a wide range of metabolites/analytes (**Figure 19)**. This allows a user to understand the metabolic profiles of the cells from the spent medium. This system is capable of the analysis of electrolytes, carbohydrates, amino acids, specific proteins and enzyme activities, all on a single system.

There are a variety of different assay tests available that can be used to determine metabolite concentration levels; the assays require simple setup, whilst being automated, precise and capable of highly accurate reaction experiments; thus allowing for reliable and reproducible results. In addition, the system requires minimal manipulation from the user as it has an automated dilution function and programmed reagent mixing which minimises the risk of user error. This makes the Cedex an indispensable tool for metabolite analysis during process development. Furthermore, the system operates in a high throughput manner, with the capacity of loading 90 samples at any one time and running up to 200 individual tests per hour. Sample volumes as low as 2 μL can be used, permitting the same sample to be used for multiple assays.

To determine analyte concentrations the Cedex uses enzymatic photometric assays; a range of reagents appropriate for the analyte of interest are pre-loaded into the commercially available cassettes. Assay tests are performed by mixing the sample and the appropriate reagent cassette using the Cedex's two built-in probes which are capable of sampling intervals of 10.6 seconds (**Figure 19 A and B).** The sample and assay reagent products are delivered to the photometric module where they are analysed, concentrations are determined by the absorbance (endpoint or kinetic) of the reaction products. The data is recorded on a control unit computer, which can flag test results that are outside of the calibration and quality control limits set on the machine. This data can be reviewed, re-run, accepted or declined; accepted data can be collected for further analysis as a CSV file which includes all the test result and details.

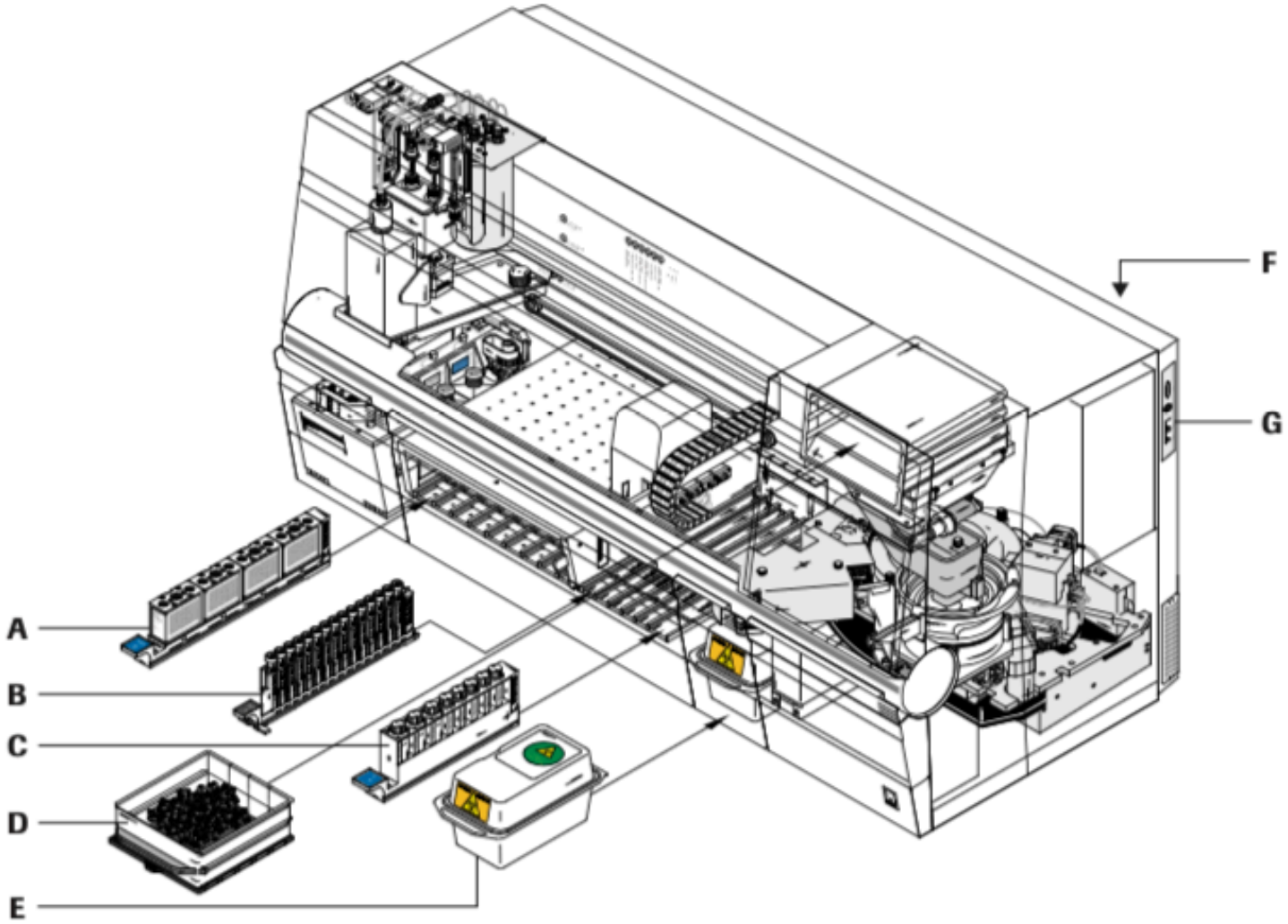

A
B
C
D
E
F
G

Figure 19. Depiction of the Cedex Bio HT instrument showing the main aspects. (**A**) Cassette rack with reagent cassettes. (**B**) Sample rack with samples in the holder chimneys. (**C**) Ion Sensitive Electrode (ISE) rack with ISE solutions, cleaners and diluents. (**D**) Reservoir for new cuvettes. (**E**) Disposable waste box for used cuvettes which is autoclavable. (**F**) Connections to the water and waste are at the rear panel, not shown in the image. (**G**) Data connectors that feed information to the computer control unit. Imaged sourced from Cedex Bio HT User Manual[229].

3.4.2. Sample preparation

Spent media samples, 500 µL, were collected in 1.5 mL Eppendorf tubes then stored at -20°C. Prior to analysis, samples were thawed at room temperature (RT), vortexed for five seconds and loaded into the sample rack prior to loading on the machine (**Figure 19B**). The samples were analysed for either glucose, lactate, glutamine, ammonia, lactate dehydrogenase or any combination of these metabolites, using the Cedex Bio HT Analyzer (Roche, Germany). The results were used to obtain the Specific Metabolite Rate mmol.cell^{-1}.d^{-1} (SMR) following **Equation 4** published by Heathman *et al.*, 2015[228].

$$\text{Specific metabolite rate (SMR)} = \left(\frac{\mu}{C_x(0)}\right) \cdot \left(\frac{C_{met}(t) - C_{met}(0)}{e^{\mu t} - 1}\right)$$

Equation 4

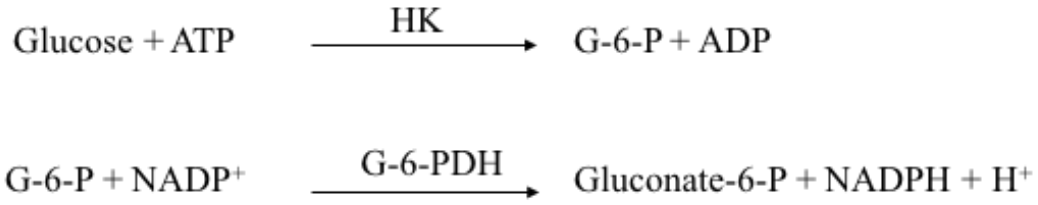
Where SMR is the net specific metabolite consumption or production rate, μ is specific growth rate (h^{-1}), $C_X(0)$ is cell number at the start of the growth phase, $C_{met(t)}$, and $C_{met(0)}$ are the metabolite concentrations at the end and start of the exponential growth phase, respectively and *t* is time (hours).

3.4.3. Metabolite test assays

This section uses a glucose assay as an exemplar to explain the principles of the CEDEX test assays. Although each analyte is measured with different reagents in an analyte specific cuvette, the principles of the assays are the same in that they use a catalyst to react the analyte with other reagents, resulting in a product that is measured photometrically.

The Glucose Bio HT cassette (cat#06608418001) contains adenosine triphosphate (ATP), hexokinase (HK), Mg2+, nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate dehydrogenase (G-6-PDH), 2-(N-morpholino) ethane sulfonic acid (MES) buffer and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer).

A reaction between glucose and ATP is catalysed by HK producing glucose-6-phosphate (G-6-P) and ADP as a by-product, as shown in **(Equation 5)**. The G-6-P is then oxidised by NADH in the presence of G-6-PDH to form gluconate-6-P, a hydrogen ion and NADPH. Glucose concentration determination is directly proportional to the rate of NADPH formation which is measured by UV-photometry.



Equation 5

3.5. Cell phenotyping

3.5.1. Flow cytometry

Flow cytometry is one of the most prevalent means of particle characterisation, the technology is commonly laser light based. Flow cytometry is used to analyse the properties of individual particles, including cells within a heterogeneous population of cells[230,231], which is the focus of this work. As a technique flow cytometry is a quantitative mode of analysis which can distinguish individual cells based on properties such as cell shape, size and fluorescence. Flow cytometry offers a comparably quicker mode of cell characterisation in comparison to real-time polymerase chain reactions (RT-qPCR), furthermore it can be used to simultaneously measure multiple parameters in a comparably more quantitative manner than immunocytochemistry (ICC) microscopy.

In principle, cell-laser light interactions and label-laser interactions are used to measure properties such as cell size/complexity and protein expression (phenotype), respectively. Label-laser interactions rely on the antibody targeting the protein of interest by bind to protein specific epitope, the antibody is labelled with a fluorescent marker which interacts with the laser light. Detection of a fluorescent signal from the marker corresponds to the presence of the target protein, under optimal conditions; detection of the fluorescence signal can be used to identify and quantify distinct cell populations. The fluorescent marker can be directly conjugated to the antibody or can be added on to the antibody through a primary-secondary system. The ever-expanding library of antibodies and ability of a single laser to excite several different fluorescent markers allows for flow cytometry experiments that can simultaneously measure the expression of several proteins.

3.5.1.1. Flow cytometer

Flow cytometers are complex analytical instruments, the key components of a flow cytometer are a fluidics system; lasers; optics and detectors[230,232]. The following sections provide a brief summary of the role that each of these components has within a flow cytometry experiment.

3.5.1.1.a. The fluidics system

Fluidics are an integral component element of flow cytometry and the basis of the ‘flow’ part in flow cytometry, the fluidics are used for hydrodynamic focusing of the cells sample in to a single line of flowing cells (**Figure 20**). The fluids system allows for the sample to be injected into a central nozzle that is surrounded by an outer sheath of fluid, which is typically saline. Both the sample and sheath

fluid are directed towards a nozzle which increases the fluid velocity, focusing the cells into a single file through the Bernoulli effect[230,232]. Laminar flow prohibits the mixing of the sample and sheath fluid.

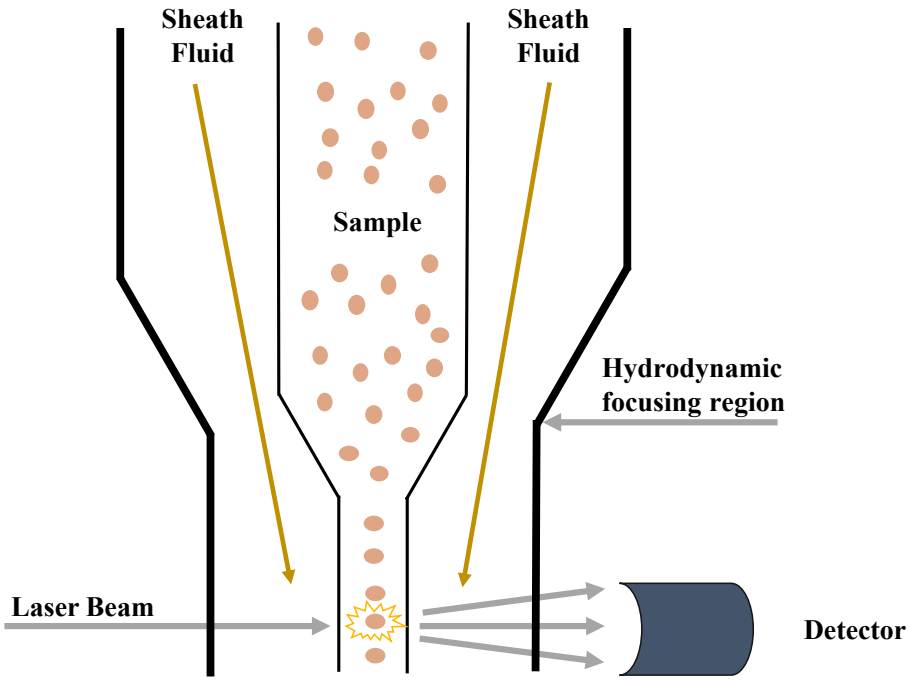


Figure 20. Illustrative depiction of the fluidics system creating a single line of cells after the hydrodynamic focusing region. Once the cells are in a single file, they travel to the interrogation point where they interact with a light source, in this case laser light, the cell-light interact is then detected and processed.

3.5.1.1.b. Lasers

The lasers provide the light source in a cytometry experiment, each laser produces a single wavelength of light within the visible spectrum of the electromagnetic spectrum; the number of lasers varies dependant on the flow cytometer model and manufacturer. The point at which the flow of single cells interacts with the laser light is called the interrogation point, the interaction results in the individual cell passing through the laser (s) to scatter light and fluoresce, in the case of fluorophore labelled cells or cell autofluorescence. Light scattering can be in the forward or side direction; forward scattered light is used to determine the size of cells passing through; side scattered light is putatively linked to cell complexity e.g. the granularity of the cell. By combining Forward Scatter (FSC) and Side Scatter (SSC) cells can be distinguished in a heterogeneous population based on their size and complexity[230,232]. Fluoresce interactions detect the presence of biomarkers (surface protein or intracellular molecules) through fluorescently conjugated antibodies that target the biomarker of interest. The emitted light from

the FSC, SSC and fluorescence are collected by photomultiplier tubes (PMT) which amplify the signals of the light interactions[233,234].

3.5.1.1.c. Optics and detectors

The light emitted from the interaction of the cell with the laser is then detected and measured using different channels of the PMTs. The optical filters, lens and mirrors (optics system) gather and direct the light that is emitted to control the specificity of the detection by the PMTs (**Figure 21**). The optics system comprises of three major dichroic filters; long pass, short pass and band pass filters, which block and transmit different wavelengths (either above/below a set wavelength or within a wavelength band width) allowing for selective light detection at the PMT detectors[230,232,234]. The light selectivity directs only specific light to the PMTs, which each PMT is set to detect i.e. forward scatter is detected by the FSC PMT detector and fluorescence light (FL) emitted at ~530 nm is detected by the 530 nm FL PMT detector; therefore, each detector measures a different parameter. The signal from the PMTs is then digitized allowing for quantification of the parameter being measured through the height, width and area values of the signal detected by the PMT. These values can be plotted for each parameter as histograms or dot plots allowing for analysis of population differences based on fluorescence intensity, size and complexity of the cell population sample.

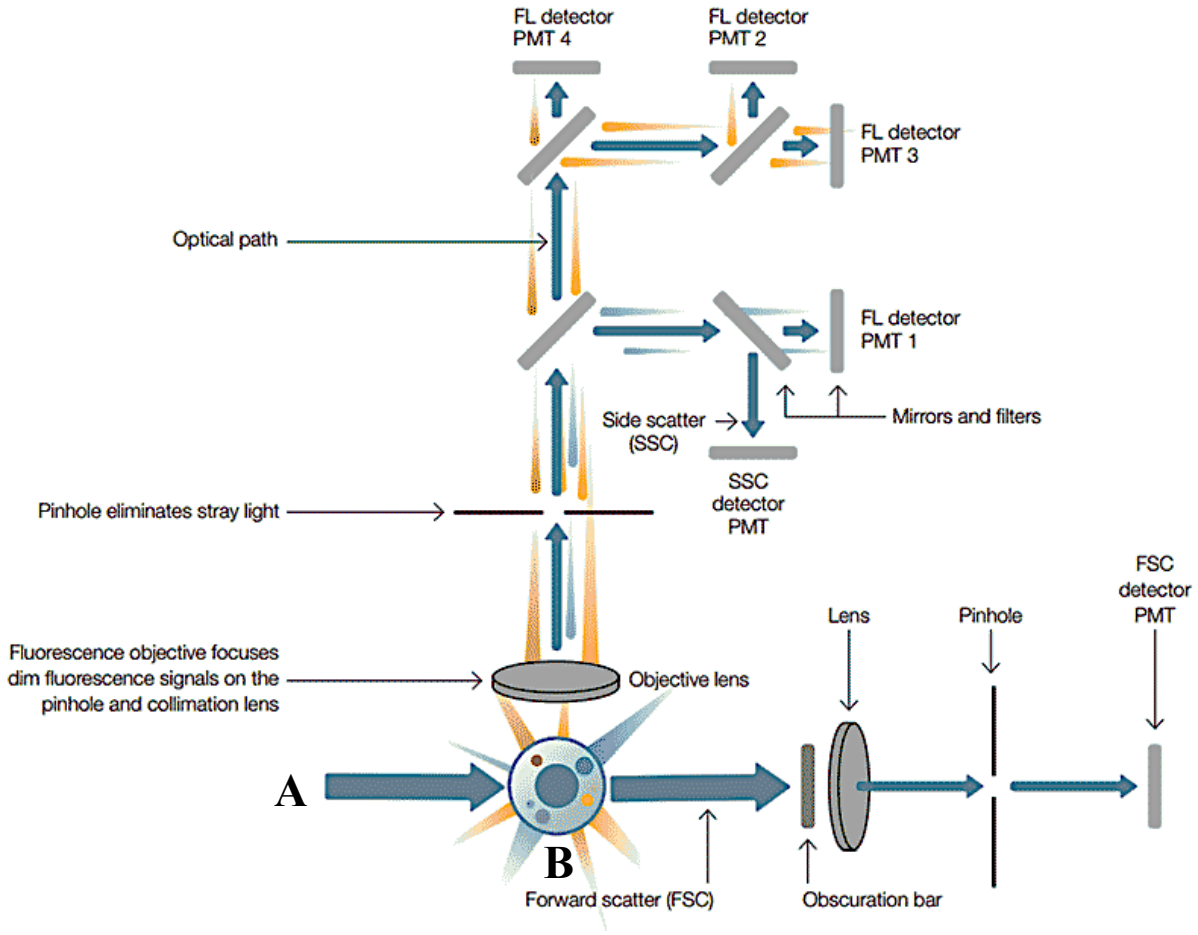


Figure 21. Depiction of the optics system, showing the different detectors FSC, SSC and PMT. Left blue arrow (**A**) shows a laser beam passing through and interacting with a cell (**B**), which results in light being scattered in different directions and detected by the various detectors in the optics system Image sourced from Bio-Rad Laboratories[234].

3.5.1.2. Controls

An integral aspect of flow cytometry is the use of an appropriate control to help accurately distinguish the target cell population and its respective protein expression. Controls help to reduce the likelihood of falsely identify signals as a positive reading. Unstained cells are one form of control in a flow cytometry experiment, these are cells being analysed in the experiment but not stained with the antibody-fluorophore. The lack of staining provides information regarding the background or autofluorescence of the cells, which allows for analytical gates to be set that can distinguish positive fluorescence signal from the background signal.

Isotype controls are another form of control used in flow cytometry, these are antibodies that are raised against an antigen that is not present in the target cell population. As a result, they provide information of any non-specific binding, this allows for confidence that the fluorescence signal being detected is

due to the specific antibody interaction with the protein of interest. The same fluorescent marker is used for both the binding antibody and the isotype antibody to ensure that any differences are not due to the fluorescent marker. Additionally, the isotype should be of the same immunoglobin class and host species as the primary antibody.

Fluorescence minus one (FMO) controls are used to determine which fluorescence signal should be used for analysis. FMOs are a sample of the cells of interest stained with all but one (minus one) of the fluorescent markers as shown in **Table 7.** This allows for the fluorescence spread of the other markers in the detection channel of the minus one channel to be determined, again allowing for more appropriate analytical gates to be set so that any signal from the other fluorescent markers being analysed are accounted for[230,232,208] .

Table 7. An example of how a FMO matrix is setup, the example shows an FMO matric for a three colour panel. Separate samples are stained with a staining mix that is missing one of the fluorophores.

FMO type	**Staining Mix**
APC	FITC-PE
FITC	APC-PE
PE	APC-FITC

Biological controls are particularly useful for determining differences in cell shape and size, as both these attributes can change due to cell treatments such a differentiation process and be distinguished by the different signals from their interaction with laser light. Furthermore, biological controls can be used to determine cell that are positive or negative for a protein. For instance, pluripotent cells can be used as biological control to distinguish between cells that have successfully differentiated and those that have not: the differentiated cells should not express pluripotency proteins such as OCT3/4. As such the differentiated cells would not have the appropriate antigen to bind the OCT3/4 antibody so no signal would be detected.

3.5.2. Procedure and solution preparation

A minimum of 1×10^6 cells were obtained from a harvested cell suspension as described in sections 3.2.4.1.a, 3.2.4.2.a and 3.2.4.3.a. The cells were centrifuged at 300xG for 5 min, the supernatant was removed and treated as described below (Fisher Scientific, accuSpin Micro 17). Resuspension of cells

was performed by pipetting the cell suspension up and down 10 times using a 1000 µL pipette. The specific antibodies, fluorophores and reagents used within the present work are described in further detail in the relevant chapters. The samples were analysed on a Becton Dickinson (BD) FACSCanto™ II flow cytometer (BD Biosciences, USA); the resulting FSC files were obtained and analysed using the FlowJo™ software (version 10.4.2, USA)

3.5.2.1. EP buffers and solutions

BD Cytofix™ fixation buffer (cat#560477) and BD Cell stain buffer (cat#554656) were used as provided. To achieve the appropriate working concentration for safe permeabilization of cells, the BD Perm/Wash™ (10X) buffer (cat#560477) was diluted 1:10 with distilled water before use (i.e. 1 mL of Perm/Wash™ (10X) buffer plus 9 mL of deionized/distilled water).

3.5.2.2. EC 2102 Ep cell sample preparation

The cells were resuspended in 100 µL of BD Cytofix buffer per 10^6 cells and incubated 20 min at RT away from light (all incubation steps were performed at RT and away from light), to preserve them in their biological state at the time of harvest. Following 20 min, the cells were washed twice with 1 mL of PBS and centrifuged for 5 min at 500xG. Following the second wash the cells were resuspended in a permeabilisation ion buffer and left to incubate for 10 min in order to permeabilise the cell membrane to allow for intracellular staining. Following 10 min the conjugated antibodies were added and incubated for 30 min. Following 30 min the cells were washed twice with 1 mL permeabilisation buffer and centrifuged for 5 min at 500xG. Following the final wash, the cells were resuspended in cell stain buffer and analysed on the flow cytometer.

3.5.2.3. H9 buffers and solutions

All buffers and solutions were prepared fresh on the day of analysis to prevent ion chelation with EDTA in the PEB buffer and degradation of both the permeabilization buffer and fixation solution.

3.5.2.2.a. Fixation solution

To achieve the appropriate working concentration for safe fixation and permeabilization of cells, the Fixation/Permeabilization Solution 1 (Miltenyi Biotec, Germany, cat#130-093-142) was diluted 1:4 with the Fixation/Permeabilization Solution 2 (Miltenyi Biotec, Germany, cat#130-093-142) (i.e. for 10^6 cells use 0.25 mL of Fixation/Permeabilization Solution 1 plus 0.75 mL of Fixation/Permeabilization Solution 2).

3.5.2.2.b. PEB Buffer

Solution prepared with PBS, pH 7.2, 0.5% v/v HSA, and 2 mM EDTA (Thermo Scientific, UK, cat#155757-038) (i.e. 10 mL solution: 10 mL PBS, 50 µL HSA (0.5 %) and 40 µL EDTA (2 mM)).

3.5.2.2.c. Permeabilization Buffer

To achieve the appropriate working concentration for safe permeabilization of cells, the 10× Permeabilization Buffer (Miltenyi Biotec, Germany, cat#130-093-142) was diluted 1:10 with Endotoxin-Free water (Thermo Scientific, UK, cat#SH3052903) distilled water before use (i.e. 1 mL of 10× Permeabilization Buffer 9 mL of distilled water).

3.5.2.4. H9 cell sample preparation

The cells were resuspended in 1mL of fixation solution per 10^6 cells and incubated 30 min at 4°C away from light (all steps of the process were performed away from light), to preserve them in their biological state at the point of harvest. Following 30 min, the cells were washed with 1 mL of cold PEB buffer and centrifuged for 5 min at 300xG. A second wash step was performed with 1 mL of permeabilization buffer. Following the second wash the cells were resuspended in 110 µl of the staining master mix and left to incubated for 30 min at 4°C in order to permeabilised and stain the cells. Following 30 min the cells were washed with 1 mL permeabilisation buffer and centrifuged for 5 min at 300xG. The cells were then resuspended in PEB buffer and analysed on the flow cytometer.

3.6. Cell genotyping

Cell genotyping was performed through quantitative gene expression analysis using quantitative real time polymerase chain reactions RT-qPCR

3.6.1. Sample preparation –EC 2101Ep

$1x10^5$ cells were obtained from a cell suspension and centrifuged at 300xG for 5 min, the supernatant was aspirated, and the resulting pellet was frozen at -80 °C. Gene expression quantification was then carried out using qRT-PCR. All procedures were performed on surfaces and using equipment that had been decontaminated with RNaseZap solution (cat# 10708345, Fisher Scientific, Loughborough, UK) and were performed on ice to prevent sample degradation.

3.6.1.1. RNA extraction and isolation

Total ribonucleic acid (RNA) was isolated using the RNeasy Mini Kit (Qiagen, Cat# 74104) according to the manufacturer instructions. RNA yield and purity were determined using the NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, UK), measurements at an absorbance wavelength of 260 nm were carried out and the 260/280 nm absorbance ratio was used to assess purity; a value of ~2 was putative of RNA. RNA integrity was assessed using an Agilent 2100 Bioanalyser (Agilent Technologies, Germany) which performs automated electrophoresis to assess the RNA samples[235].

3.6.1.2. cDNA synthesis

1 µL of total RNA was reverse-transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Cat# 205311) according to manufacturer instructions. A mix of genomic DNA (gDNA) Wipeout buffer, template RNa and RNase-free water was incubated for 2 min at 42°C then stored on ice, this step eliminated residual gDNA. Reverse transcriptase, reverse transcription (RT) buffer and RT Primer mix were added to the gDNA eliminated RNA template. The solution was incubated 15 min at 42 °C and then for 3min at 95 °C (Veriti Dx Thermal Cycler, Applied Biosystems, USA, cat#4452299. Complementary deoxyribonucleic acid (cDNA) yield was determined using the NanoDrop™ 2000 Spectrophotometer and cDNA samples were stored at -80 °C.

3.6.1. qRT-PCR

1 µL of the cDNA synthesis reaction was used as template for each real-time PCR using VeriQuest Fast SYBR Green qPCR Master Mix (cat# 756901000RXN, Fisher Scientific, Loughborough, UK), a

forward primer, reverse primer and ultrapure PCR water (cat# 11538646, Fisher Scientific, Loughborough, UK), which were all vortexed for 2 min at 350xG. PCR was run in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) for 40 cycles of 95°C for 3 seconds to denature, and 60°C for 30 seconds to anneal. The relative amounts of PCR product were quantified using the relative threshold cycle (ΔΔCt) method corrected for efficiency for each amplification. The gene quantities for each sample were normalised against the geometric mean of expression of the housekeeping genes. The primers used for each experiment are detailed in the relevant methods section.

3.7. Cell Adhesion

In order to measure cell adhesion and obtain quantitative results, the xCelligence® RTCA was used in combination with standard light microscopy.

3.7.1. xCelligence® RTCA

The xCelligence® (San Diego, USA) is a real time cell analyser (RTCA), capable of performing cell assays, the technology is based on monitoring cell behaviour changes that results due to changes in electrical impedance[236]. The system continuously monitors the cells in real time, providing information regarding changes in morphology, proliferation and viability. The xCelligence® provides a quantitative mode of accessing cell behaviours that is not possible through visual microscopy whilst also providing a direct comparison between wells and plates in a standardised manner. The system is label-free, allowing for cells to be in their natural physiological state, which provides the ability to monitor live cells in real time, resulting in experimental data that is significantly better than assays that use dead or fixed cells[237,238]. Moreover, cells analysed by the xCelligence® can be used for further experiments or other modes of analysis as the assay is not end-point based.

The system is comprised of three parts which are the E-Plate RTCA SP station, RTCA Analyzer and a RTCA Control Unit computer. Bespoke microtiter plates called E-Plates® are used in combination with the system. The E-Plates® have gold plated microelectrodes that are integrated onto the bottom of the plates, covering 70 or 80 % of the surface area. 70% coverage is due to a subtype of E-Plates® that have a viewing strip, allowing for cells to be imaged through microscopy, whereas 80% coverage is on non-viewable plates. The RTCA SP plate station provides electrical potential to the E-Plate® (22mV) through the contact pins on the cradle of the station which connect to conductive pads on the E-Plate®[239]. The resistance experienced by the microelectrodes on the E-Plate® is measured by the RTCA analyzer which digitises the analogue signal and processes it for analysis on the computer control unit. The analyzer can measure a whole 96 well E-Plate in 15 seconds.(approx. 150 ms per well).

Use of growth medium which has ionic components provides a conductive solution that passes electrical potential from a negative terminal to a positive terminal. The voltage applied across the microelectrodes on the E-plate® is small, therefore little resistance experienced on an empty surface. The settling down of cells towards the surface and their adherence disturbs the flow of electrons and ions on the electro-medium interface, resulting in impedance which is measured as resistance in Ohms (Ω) (**Figure 22**). The impedance that is produced is reported as Cell Index (CI), where CI = (impedance at time point n – impedance in the absence of cells)/nominal impedance value of 15 Ω. The CI reported is an arbitrary unit that can be used to compare differences between wells and plates of different experiments.

Graphical plots of the CI can be used to show the adhesion and detachment profile of an experiment as shown in the illustrative example in **Figure 23**.

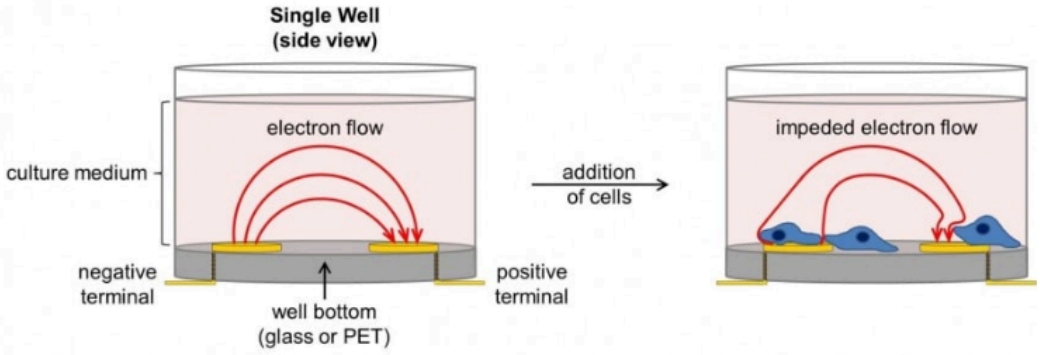


Figure 22. Cell are added to the plate which has integrated gold micro electrodes, the growth surface is tissue culture treated and can have a matrix such as laminin added onto it. Upon seeding the cells attach and grow, as the cells proliferate the measured impedance experience by the micro electrodes will increase over time. Changes in cell morphology, adherence and viability will result in a change in impedance which is continuously monitored by the RCTA analyser which is converted into cell index readings by the computer control unit. Imaged sourced from ACEA Bio[239].

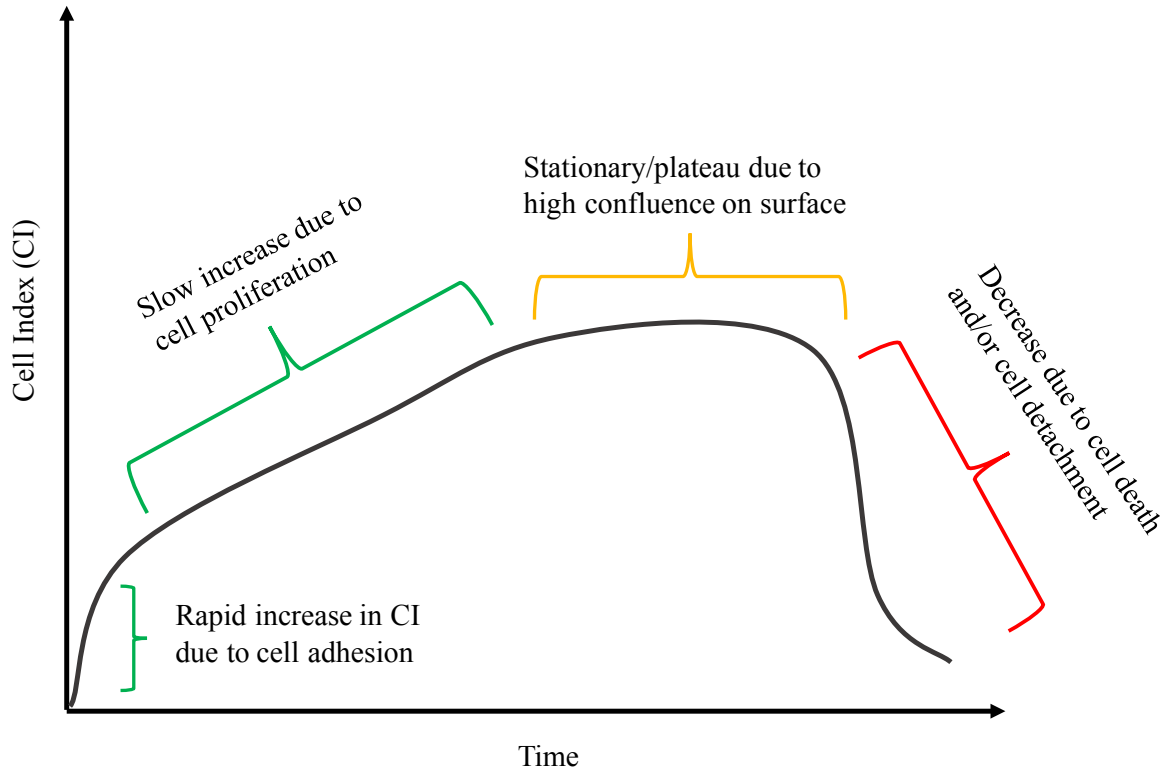


Figure 23. Illustrative example showing Cell Index measurements during a generic exponential cell growth and cell detachment experiment. *Imaged adapted from ACEA Bio*[239]

3.7.2. Procedure and sample preparation

E-Plate® microtiter plates (ACEA BIO, USA, cat#6472451001) were coated with either Biolaminin-521 or Biolaminin-111 (0.5 and 1.0 µg/cm^2, respectively) by adding a laminin and PBS solution to the well plate and incubating for 2 h at 37°C. Following 2 h the coating solution was removed and 50 µL of culture media was added to obtain a baseline impedance reading. A cell suspension was obtained as previously described (see 3.2.4. Standard culture), 100 µL of the suspension of a known density was used to seed the cells onto the plate resulting in a total volume of 150 µL. The plate was then left to equilibrate at RT for 45 min, after which the plate was placed on the xCelligence® RTCA SP station in an incubator at 37°C. Readings were recorded continually for different time intervals, based upon the experiment, details of which are described in the relevant chapters. Data from the analyser was plotted using the RTCA 2.1.0 software on the computer control unit.

3.8. Cell time

Cell time can be used to quantify the capability of a given volume of medium to sustain the growth of a given number of cells for specific period. Cell time (*CT*) permits the analysis of cell growth medium capacity/medium exhaustion. *CT* is expressed in cell hours/days.

$$CT = \left(\frac{N_o e^{kt}}{k} - \frac{N_o}{k}\right)$$

Equation 6

.

Where N_O is the initial cell density, k is the specific growth rate and t is the time period of culture (hours/days). It is the area underneath the curve of a [cell] *vs.* time graph and it can be obtained using **Equation 6.**

CT permits for analysis of media utilisation (*MU*) within a cell culture system. The total *MU* obtained is averaged as the initial volume of media has less cells to support compared to the latter volumes. *CT* is the same as the *MU* but for equivalent conditions, *MU* is useful as it considers changes in volume. Essentially *MU* is the *CT* supported per unit volume and can be obtained using **Equation 7**:

$$MU = \frac{CT}{V}$$

Equation 7

Where *V* is the volume of media that has been used for the culture period.

With the progression of time in a cell culture system the *MU* will be high, resulting in an incapability of the media to sustain cell growth and the growth rate. This provides evidence that the media would be unable to sustain the given number of cells at that point of time and density.

CT and *MU* facilitate calculation of densities to seed cells at, for a given growth medium volume without having to feed. This can provide an optimal seeding density (σ) at which a minimum intervention protocol with no interim feeding prior to passaging/harvesting could be used. σ is obtained at outlined below.

Equation 8 can be used calculate the supportable cell hours (γ), using the CT and the input volume (ω) e.g. in a T75 cm^2 flask (15 mL)

$$\gamma = CT \times \omega$$

.

Equation 8

σ can be obtained using **Equation 9**, where k is the growth rate, γ is the supportable cell hours in the given media amount and *t* is the length of time before passage (hours/days).

N.B: *t* must be kept in the same units throughout

$$\sigma = \frac{k(\gamma + 1)}{e^{kt}}$$

Equation 9

3.9. Statistical Analysis

Unless otherwise stated, statistical significance was determined using Graphpad Prism 7 Version 7.0d (CA, USA). Statistical significance was assigned as indicated in the figure legends. “ns” indicates no statistical significance “*” indicates $p< 0.05$, “**” indicates $p< 0.01$, “***” indicates $p< 0.001$, and “****” indicates $p<0.0001$. A range of tests including but not limited to Tukey and Sidak’s multiple comparisons tests were used to compare means between groups. Specific details can be found in the corresponding legends and captions.

3.10. N number covering statement

Since some of the experiments performed were proof of concept or for experimental exploration purposes, only one replicate was used. Cell counts were run in technical duplicates (i.e. two counts for each condition). Specific details regarding the number of experimental biological replicates can be found in the corresponding legends and captions.

Chapter 4:

EC 2102Ep Cell Line Standardisation

Chapter 4. EC 2102Ep cell line standardisation

4.1. Introduction

The continuing progression of cell therapies from basic research to clinical products has prompted excitement in the field of regenerative medicine over the last ten years. However, there are currently many challenges that still need to be addressed prior to clinical realisation[24,106,118]. One such challenge is the characterisation of cell therapy products (CTPs). This is due to the innately complex biological nature of CTPs; as a result, their characterisation is equally complex. Unlike traditional pharmaceutical products which already have well established standards that can be easily produced and used to compare and control the quality of product batches, CTPs currently do not have such a capability[132,224,240]. Therefore, reference cell lines are, at present, being utilised as the closest proxy in CTP manufacturing. However, the problem lies in that cell lines are as equally complex and dynamic as the CTPs they are being used to assess. This challenge is further exacerbated by the current lack of consensus and standardisation of protocols used to culture reference cell lines; this inherently produces an additional level of uncontrolled variability to the product manufacturing quality control (QC) process[241,242].

To this end, the present work aims to highlight the need for standardisation of cell culture protocols, especially in the cases when they are used for QC of cell-based products. This work has been executed using the Embryonic Carcinoma (EC) 2102Ep cell line (EC 2102Ep) derived from primary human testicular teratocarcinoma[243]. An initial procedure provided by the National Institute for Biological Standards and Control (NIBSC) was followed. However, it was revealed that there were inconsistencies in cell growth including discrepancies in population doubling times and growth rates when this procedure was followed. Therefore, it is hypothesised that a major contributing factor to culture inconsistency was due to the ambiguity of an undefined protocol that is reliant on manual intervention. Furthermore, parameters including unspecific time points for medium exchange and passage were subject to user interpretation. This issue is concomitant with many published protocols[241,243–248], for instance a range of timings for passage, i.e. 3-4 days, was suggested for the cells to reach adequate confluency; observed confluency in itself being a highly subjective parameter which is user-dependent. Additionally, the use of defined seeding densities is absent in many protocols, instead split ratios are often prescribed, typically with a range of ratios i.e. 1:3 or 1:5[247]. These current methods of culture do not account for the dynamic nature of cell culture, resulting in inappropriate practices which are not based on what the cells are doing and need, but are instead based on user convenience.

The requirement to understand how cells grow and consume growth medium is important, since gaining greater control of the cell environment through manipulation of feeding regimes can result in increased reliability in terms of cell growth performance. When it comes to scaling up processes for

manufacturing, unnecessary feeding results in the redundant use of reagents and time resources; this rapidly increases the costs associated with CTP production[136,153]. There is evidence of existing protocols that design feeding regimes to specifically avoid working at weekends; therefore, cells are over- or double-fed to 'sustain' them outside of the operator's working hours. Although operator-convenient, in many instances there is no quantitative reasoning or evidence to support the use of double-feeding regimes. Furthermore, as evidenced by cell growth literature, cells innately grow exponentially[249–251], and consequently their essential consumption of nutrients would be expected to increase exponentially too. This seems to be a vital detail that is not taken into consideration for the majority of cell culture protocols, since typically the volume of medium used to culture cells is maintained throughout the entire culture chain[99,243,252].

The aim of this chapter was to develop a defined protocol in-house, with the existing NIBSC procedure being used as the initial benchmark. The optimisation work carried out in this chapter was carried out after the process/protocol had been transferred from the collaborators at NIBSC. The resulting in-house protocol applied a predefined seeding density with time-defined passage points. This was in an effort to produce a more standardised protocol, with minimised human-based sources of variation including observed confluency and uncontrolled parameters such as split ratios. The protocol was then used to realise the impact of different culture conditions on cell characteristics measured by cell metabolic rate, cell specific growth rates, flow cytometry and genetic analysis. A streamlined version of the in-house protocol, which removed the medium exchange step and used a different seeding density, was used to experimentally compare the effect of different culture conditions. The streamlined protocol is representative of a culture state that the cells enter if there were under fed as a result of using split ratios and observed confluency to determine culture periods.

4.1.1. EC 2102Ep selection

EC 2101Ep cells were utilised for all work described in this chapter since they were an appropriate, "robust" candidate for training the author to culture cells and develop the appropriate analytical skills and techniques that would be later applied to clinically relevant cells lines. Furthermore, the low cost of culture associated with EC 2102Ep cells meant that the cells were an economical training material. A series of experiments were designed to explore protocol parameters including seeding density, culture periods and feeding regimes, to investigate their impact on cell behaviour and analytical outcomes such as growth rates and cell phenotype.

4.2. Methods

4.2.1. *In Vitro* Cell Culture:

All reagents and consumables were obtained from Fisher Scientific, UK unless otherwise stated. EC 2102Ep (Passage 54, GlobalStem, USA) cells were thawed from cryopreservation and cultured in T25 cm^2 tissue-culture flasks to create a working cell bank. Each vial contained $6x10^6$ cells in 1 mL of Cryostor® CS10 (cat# C2874-100ML, Sigma Aldrich, UK) which were stored in liquid nitrogen. The cells were cultured at 37°C, 5% CO_2 using Gibco Dulbecco's Modified Eagle Medium (DMEM) high glucose with GlutaMax™ medium (cat# 10569010) and supplemented with Foetal Bovine Serum (10% *v/v,* cat# 10100139) herein referred to as growth medium. Unless otherwise stated, P54 cells were thawed and seeded at a density of 66,6667 cells/cm^2 and cultured for 72 h with 100% medium exchange following 48 h, prior to the start of the experiment. Where stated spent media samples were collected then stored at -20° C; PCR samples were collected upon cell harvesting and stored at -80° C until analysis. Flow cytometry immunophenotyping samples were collected and fixed on the day of harvest and analysed up to 24 h later.

4.2.1.1. Experiment 1

The first experiment was designed to assess how the cells would grow under a range of different seeding densities. EC 2102Ep cells (P54) were cultured in a T75 cm^2 tissue-culture flask, the growth medium was changed following 48 h. The cells were harvested and subculture after 72 h and seeded at four different densities (5,000; 10,000; 15,000; 20,000 cells/cm^2) into T25 cm^2 flasks. The flasks were sacrificial resulting in 32 flasks i.e. four days of culture, four densities and duplicated. The densities employed were chosen as they are in within the ranges of typical seeding densities for pluripotent cell culture, which is a key aspect of the present work.

4.2.1.2. Experiment 2

Experiment 2 explored a wider range of densities to investigate if seeding density would be a cause of cell growth inhibition. The six densities were chosen (5,000; 10,000; 20,000; 40,000; 66,667; 80,000 cells/cm^2). 66,667 cells/cm^2 was used as this is the density that is recommended by NIBSC; 80,000 cells/cm^2 was chosen in order to investigate the effect of a higher seeding density on cell growth. EC 2102Ep cells (P54) were cultured in a T75 cm^2 tissue-culture flask, the growth medium was changed following 48 h. The cells were harvested and sub-cultured after 72 h and seeded at the six chosen densities into T25 cm^2 flasks. The flasks were sacrificial resulting in 54 flasks i.e. three days of culture, six densities, in triplicate.

4.2.1.3. Experiment 3

Experiment 3 was a series of experiments designed to investigate the relationship between cell seeding density and nutrient availability, to ascertain which of the two parameters had the most significant effect on cell culture variation. Cells were seeded at three different densities under three different volumes of growth medium to systematically assess the impact of both parameters on cell growth, this was experimental passage 1. The conditions used were chosen to further stress the cells as previous experimental conditions did not seem to be sufficiently hostile to induce inhibition of cell growth. Additionally, metabolic memory was explored by resetting all the conditions to the initial protocol culture conditions (66,667 cells/cm^2, in 5 mL growth medium) after the passage period at different conditions, this was experimental passage 2 (**Error! Reference source not found.**). The experimental setup was to assess if the cells would retain the metabolic cell state induced by the hostile culture conditions or whether they would recover. Both the densities and volumes were set above and below the control protocol parameter of 66,667 cells/cm^2 with 5 mL growth medium and a full medium exchange following 48 h. This resulted in a series of four experiments that observed the effect of different density and growth medium volume combinations; the densities ranged from 20,000 cells/cm^2 to 93,000 cells/cm^2 (**Table 8**). The volumes used ranged from 1.5 mL to 7 mL, in all the experiments 66,667 cells/cm^2 with 5 mL growth medium and full medium exchange following 48 h was used at the control condition (**Table 8** and **Table 9**).

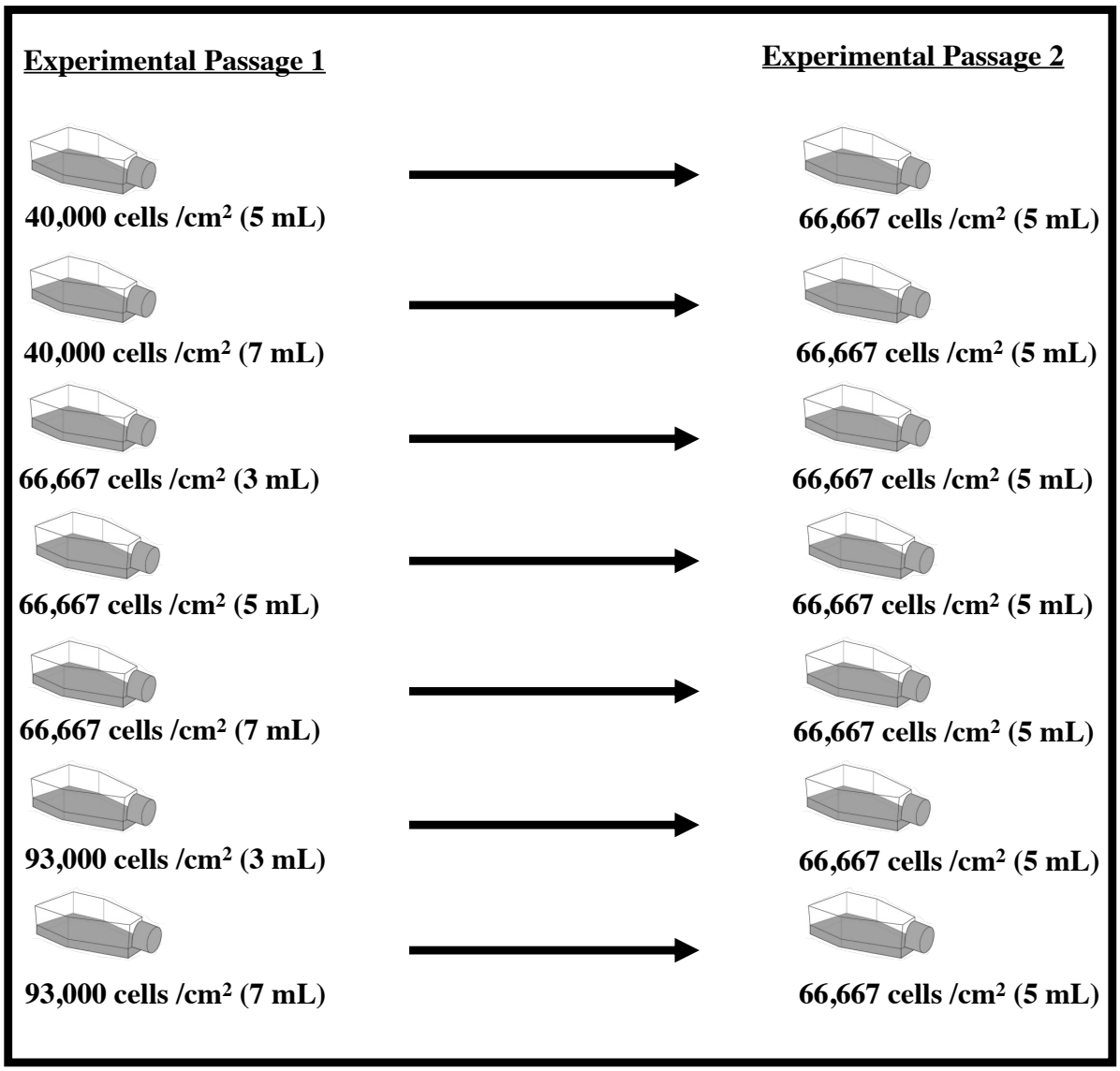


Figure 24.Schematic of experiments 3.1 to 3.4 and table showing seeding densities used.

Table 8. Culture details for experiments 3.1 to 3.4

Experiment	Pre-experiment culture period (h)	Culture flask (cm^2)	Culture Period (h)	Culture flask (cm^2)	Analysis
3.1	72	T75	72	T25	SGR and SMR
3.2	72	T75	96	T25	SGR and SMR
3.3	72	T75	96	T25	Flow Cytometry, SGR and SMR
3.4	72	T75	96	T25	Flow Cytometry, SGR and SMR

4.2.1.3.a. Experiment 3.1 Nutrients vs Density – 72-hour culture period

Cells were seeded at a density of 66,6667 cells/cm^2 and cultured for 72 h with medium exchange after 48 h. The matrix below (**Table 9**) shows the different conditions that were used to test a range of medium volume and density conditions.

Table 9. Parameters used in experiment 3.1.

	3 mL growth medium (Low)	5 mL growth medium (Middle)	7 mL growth medium (High)
40,000 cells/cm^2 (Low)	✗	✓	✓
66,667 cells/cm^2 (Middle)	✓	**Control Point**	✓
93,000 cells/cm^2 (High)	✓	✓	✗

4.2.1.3.b. Experiment 3.2 Nutrients vs Density – 96-hour culture period

The same experimental design from experiment 3.1 was applied to experiment 3.2, with the exception of the culture period increasing from 72 to 96 h.

4.2.1.3.c. Experiment 3.3 Nutrients vs Density – 96-hour culture period including lower seeding density with phenotype analysis.

Cells were cultured under similar conditions to experiment 3.2, except the condition of 93,000 cells/cm^2 with 7 mL growth medium was substituted to 20,000 cells/cm^2 and 1.5 mL. Flow cytometry analysis was carried out at both passage harvest points.

4.2.1.3.d. Experiment 3.4 Nutrients vs Density - 96-hour culture period with phenotype analysis.

This experiment followed the same experimental design as experiment 3.2; flow cytometry analysis was performed on this experiment.

4.2.1.4. Experiment 4: Longitudinal comparison of 2 protocol culture conditions

This experiment investigated the growth performance of the cells over ten experimental passages (E.P1-10), two routes of culture were used throughout the experiment (**Figure 25**). Route A1 was based on the NIBSC protocol conditions over 72 h, which was used as a comparison against a streamlined protocol that was based on previous experiments (Experiment 1, 2 and 3.3) and a cell time calculation (**Equation 6**). EC 2102Ep cells (P55) were cultured in T75 cm^2 tissue-culture flasks; the growth

medium was changed following 48 h. The cells were harvested and passaged following 72 h into 2x T75 cm^2 flasks for another expansion passage (P56). The cells were pooled then separated into 2 routes of culture in T25 cm^2 tissue-culture flasks, herein referred to as A1 and A2 (see **Figure 25**). *Route A1:* cells were seeded in triplicate at 66,667 cells/cm^2 in 5 mL of growth medium. Medium was changed after 48 h. The cells were harvested and passaged following 72 h (P57). Cell counts were performed in duplicate for each flask and cultured for a further 9 passages. *Route A2:* cells were seeded in triplicate at 20,000 cells/cm^2 in 5 mL growth medium. No medium exchange took place prior to passage. The cells were passaged following 72 h culture (P57). Cell counts were obtained in duplicate for each flask and cultured for a further 9 passages.

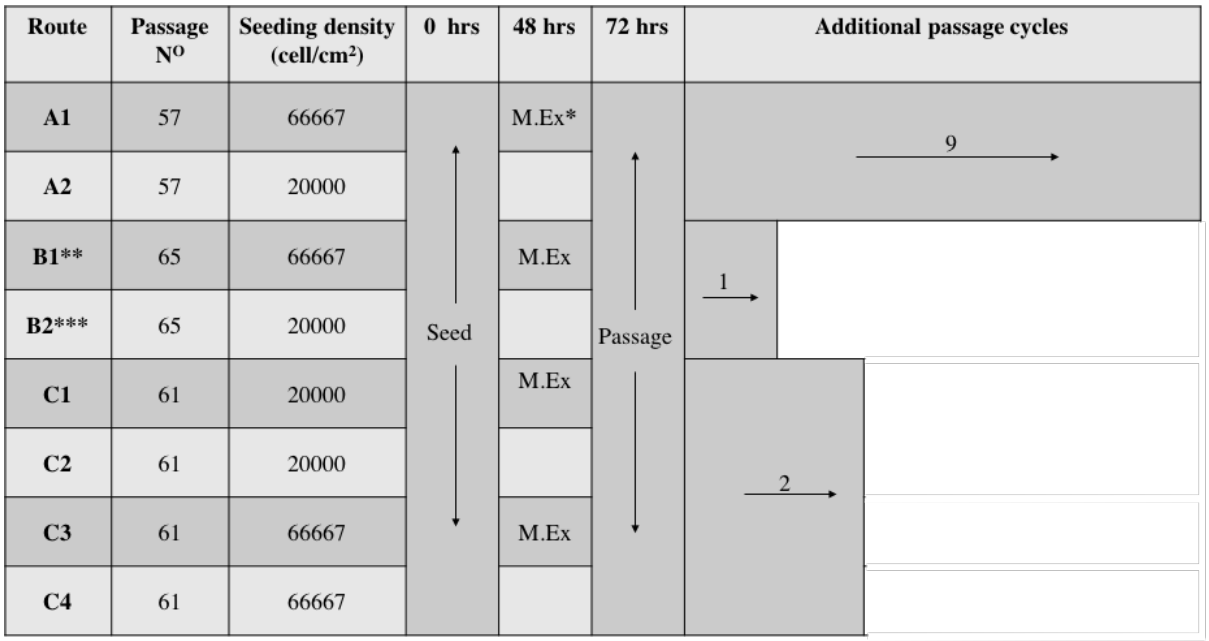


Figure 25: Schematic detailing the different experimental culture routes investigated. Cells in each route were seeded according to the density stated in the figure. Following 48 h route **A1, B1, C1** and **C3** were subjected to a 100% medium exchange. Following 72 h, all routes were passaged. Route **A1** and **A2** underwent a further nine passages (Experiment 4); Route **B1** and **B2** underwent one further passage (Experiment 4.1); Route **C1, C2, C3** and **C4** underwent a further two passages (Experiment 5). *M. Ex =medium exchange. **Cells from **B1** were obtained from passage cycle 8 of route **A2**. *** Cells from **B2** were obtained from passage cycle 8 of route **A1**.

4.3.1.4.a. Experiment 4.1: Inter-experiment reversal of protocol culture conditions

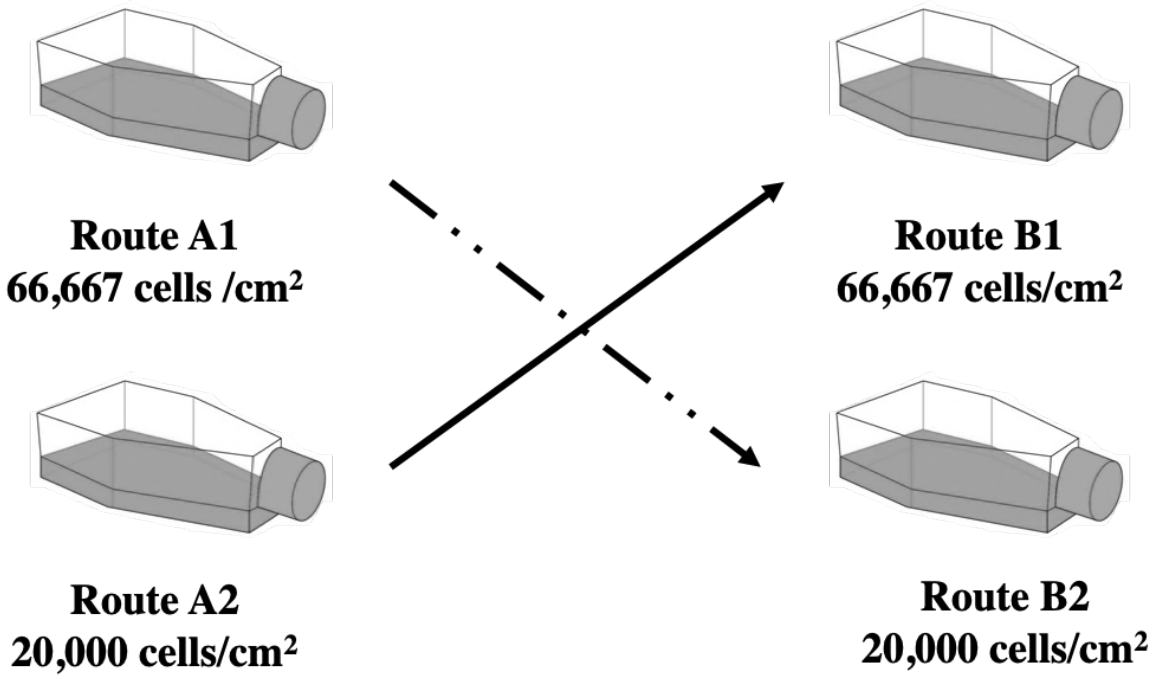


Figure 26. Cells from experiment A (see **Figure 25**) were passaged at cycle 8 for both route A1 and A2, the excess cells from route A1 were seeded to follow route A2 conditions, therein referred to as route B2. Excess route A2 cells were seeded under A1 conditions resulting in a switch of routes, therein referred to as route B1.

In order to investigate whether observed changes in experiment 4 were an artefact of the cells being cultured under route A2 or a divergence of the cells not linked to the routes, a reversal mini experiment was carried out. Cells that had previously been cultured at a density of 66,667 cells/cm^2 with medium exchange after 48 h (route A1) were harvested and used to continue experiment 4. The excess cells from the harvest of route A1 were then seeded and cultured at a density of 20,000 cells/cm^2 with no medium exchange after 48 h, resulting in a switch of culture conditions from the previous seven passages, leading to experiment 4.1 and the B2 culture route. These cells (now in experiment 4.1) were cultured in parallel with cells from experiment A for the remaining two passages of experiment A. The cells that had previously been cultured at a density of 20,000 cells/cm^2 with no medium exchange after 48 h (route A2) were also subjected to a switch of culture route in the same manner as mentioned above i.e. they were seeded and cultured at a density of 66,667 cells/cm^2 with medium exchange after 48 h, leading to experiment 4.1 and the B1 culture route as depicted (**Figure 26**).

4.2.1.5. Experiment 5: 4-way comparison of the effects of medium of exchange.

EC 2102Ep cells (P59) were cultured in a T75 cm^2 tissue-culture flask, the growth medium was changed following 48 h. Cells were passaged following 72 h and were seeded into 2 x T75 cm^2 flasks for a further expansion passage (P60). The cells were then separated into four different routes of culture (see **Figure 25**): *Route C1* and *C3,* cells were seeded in duplicate at 20,000 and 66,667 cells/cm^2, respectively in 5 mL growth medium. Following 48 h the growth medium was exchanged. The cells were then passaged following 72 h; cell counts were obtained in duplicate for each flask and cultured for a further two passages. *Route C2* and *C4,* cells were seeded in duplicate at 20,000 and 66,667 cells/cm^2, respectively, in 5 mL growth medium. No medium exchange took place prior to passage. The cells were passaged following 72 h culture, cell counts were obtained in duplicate for each flask and cultured for a further two passages.

4.2.2. Flow cytometry

All reagents were obtained from BD Biosciences (Oxford, UK) unless otherwise stated. Flow cytometry immunophenotyping samples were collected for the following experiments: experiment 3.3, 3.4, 4, 4.1 and 5. Samples were collected at experimental passage 1 and 2 for experiments 3.3 and 3.4. For experiment A, samples were collected at passage 1, 3, 7 and 9. For experiment 4.1 and 5 samples were collected at experimental passage 1 and 2. The following steps were all performed in the dark at room temperature (RT). A minimum of 1×10^6 cells were collected and fixed for 20 min (RT, BD Cytofix™). The cells were washed twice with PBS and centrifuged at 500 xG (Fisher Scientific, accuSpin Micro 17) for 5 min. The cells were permeabilised for 10 min (BD Perm/Wash™). Cell staining was performed using pre-conjugated antibodies OCT3/4-PerCp-Cy5.5, SSEA-1-PE and SSEA-4-Alexa 647, for 30 min, a sample with respective isotype controls was used to account for non-specific binding (**Table 10**). Following 30 min two washes were performed using permeabilising buffer. The cells were resuspended in cell stain buffer prior to analysis. 250µL of each sample was used for multiparameter analysis using flow cytometry (BD FACSCanto™ II, BD Biosciences, USA).

Table 10. Conjugated antibodies used for flow cytometry to determine pluripotency state of the cells

Marker	Relevant details	Desired expression
Oct3/4-PerCp-Cy5.5	Pluripotency marker	Positive
SSEA-1-PE	Differentiation marker	Negative
SSEA-4-Alexa 647	Pluripotency marker	Positive

4.2.3. PCR

PCR samples were collected upon harvesting and stored at -80^{o} C until analysis as dry pellets. For experiment 4 samples were collected at passage 1, 3, 7 and 9. Gene Expression quantification was performed using Quantitative RT-PCR. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Cat#74104) according to manufacturer's instructions. RNA yield and purity were determined using the NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, UK). RNA integrity was assessed using an Agilent 2100 Bioanalyser (Agilent Technologies, Germany). 1µL of total RNA was reverse-transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Cat#205311) according to manufacturer instructions. 1µL of the cDNA synthesis reaction was used as template for each real-time PCR using VeriQuest Fast SYBR Green qPCR Master Mix. PCR was run in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) for 40 cycles of 95°C for 3 seconds to denature and 60°C for 30 seconds to anneal. The relative amounts of PCR product were quantified using the relative threshold cycle (ΔΔCt) method corrected for efficiency for each amplification. The gene quantities for each sample were normalised against the geometric mean of expression of the housekeeping genes GAPDH and β-actin[243] (**Table 11**).

Table 11. The genes used for qRT-PCR analysis of the EC 2102Ep cells in experiment 4[243].

Gene	Full name	Relevant details
DNMT3B	DNA-methyltransferase 3 beta	DNA (cytosine -5)-methyltransferase 3 beta: abundantly expressed in ES cells but not detectable in differentiated cell and adult tissues
DPPA4	Developmental Pluripotency Associated 4	Developmental Pluripotency Associated 4: associated with inhibition of differentiation in ES cells
NANOG	Nanog Homeobox	Involved in maintenance of pluripotent state of ES cells and work in concert with pou5f1 and sox2 to marker ES identity
POU5F1	octamer-binding transcription factor 4	Pluripotency marker involved in the self renewal of undifferentiated ES cells, also know as Oct-4
REX1	Zfp-42	Pluripotency marker found in ES cells regulation of rex1 is essential to maintenance of pluripotent state of ES cells.
SOX2	SRY-box 2	Involved in the maintenance of self-renewal of ES cells and neural stem cells
TDGF	teratocarcinoma-derived growth factor	Link to signalling pathways that are essential to embryonic development and tumour growth
β-ACTIN	Beta actin	Putative housekeeping gene involved in cytoskeletal actin expressed in most cells
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Putative housekeeping gene found expressed in high levels in most cells and tissues

4.2.4. Metabolite analysis:

Spent media samples, 500 μL, were collected then stored at -20°C prior to analysis. For experiments 1 to 3.4 media samples were collected at two time-points: prior to medium exchange at 48 h and prior to cell passage. Experiment 4 and 4.1 samples were collected prior to medium exchange at 48 h (A1 and B1only) and prior to cell passage for all routes. In experiment 5, spent media samples were also collected on the medium exchange and passage time points for routes C1 and C3. Samples for route C2 and C4 were collected only on the passage time point. Spent media samples were analysed for glucose, lactate, glutamine, ammonia and lactate dehydrogenase using Cedex Bio-HT (Roche, Germany). The results were used to obtain the Specific Metabolite Rate mmol. $cell^{-1}$. d^{-1} (SMR).

4.3. Results

4.3.1. Experiment 1: Growth analysis of four seeding densities

Overall, all the conditions behaved similarly, with the exception of cells seeded at 10,000 cells/cm^2 at day 1, the specific growth rate (SGR) was similar between the different density conditions. Cells seeded at 10,000 cells/cm^2 were observed to have a much higher SGR on day 1 (0.029 h^{-1}) which dropped on day 2 to align with that other conditions (0.016 h^{-1}). At day 4 the observed growth curve illustrated that all the conditions were still in a phase of growth or just beginning to plateau, with the 10, 15 and 20,000 cells/cm^2 conditions all having similar rates from day 2 to day 4 (**Figure 27A**). For all four densities there were no detrimental effects on the viability of the cells, it was observed in all conditions except for the 5, 000 cells/cm^2 condition, that there was a higher percentage viability at day 4 compared to day 2 and 3 (**Figure 27B**). Metabolite analysis shows a relationship between SGR and specific metabolite rate (SMR) i.e. the cells consumed more glucose as the cell number increased (**Figure 27C**)

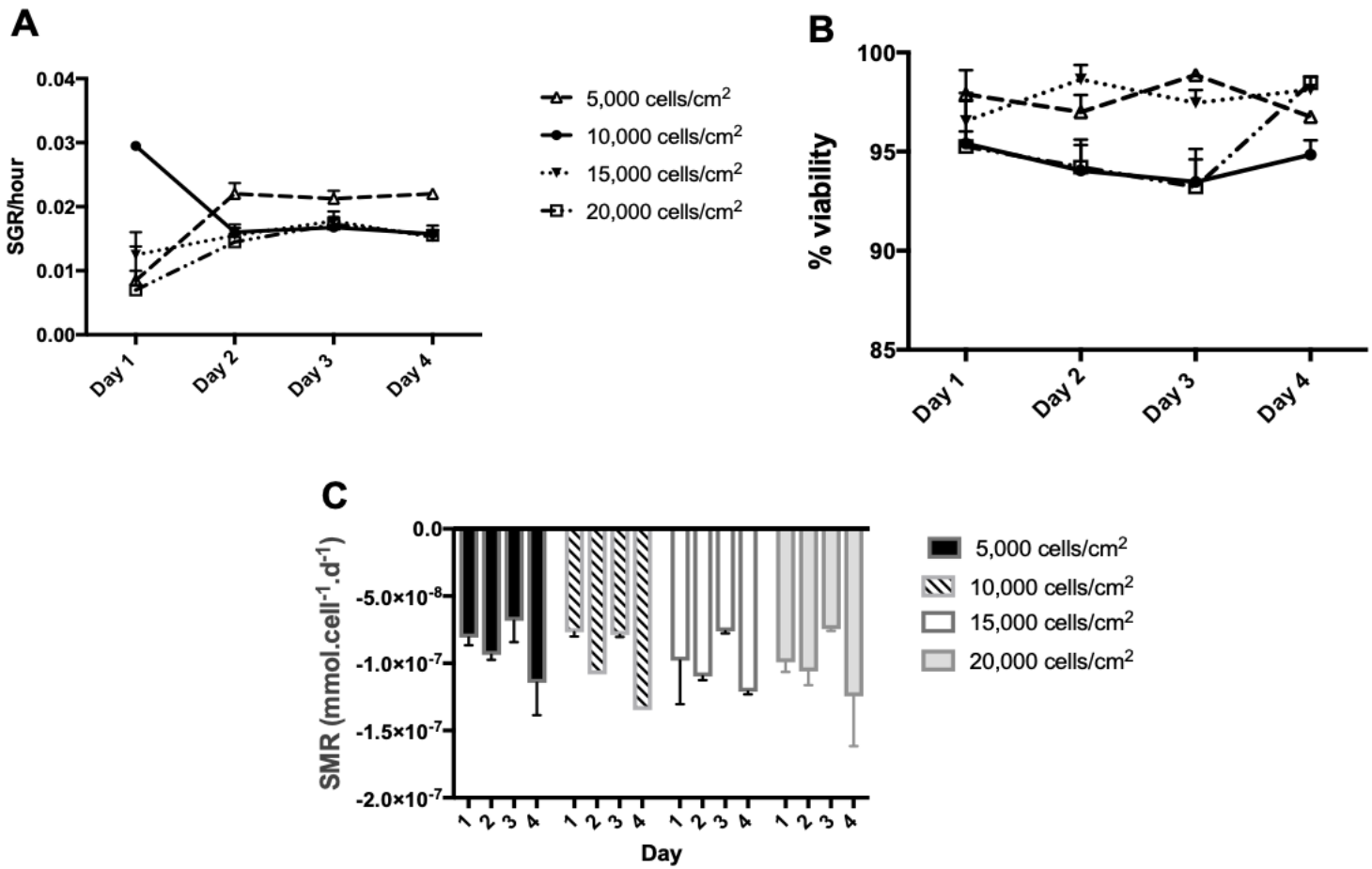


Figure 27. (**A**). Specific growth (SGR) trend for the four different seeding densities, n=4 error bars showing standard deviation, SD. The 10,000 cells/cm^2 condition was significantly different to the other conditions (P range = 0.01 – 0.0007), no errors shown for this condition on day 1 as the SD between replicates was too small (**B**) Cell viability trend over 96-hour culture period, all conditions were above 90 % viable throughout the culture period, n=4 error bars showing SD. (**C**) Glucose SMR for all four seeding densities, no significance difference was observed throughout the culture period or between different densities, n=2 error bars showing SD.

4.3.2. Experiment 2: Growth analysis of six densities

The three higher densities (40,000; 66,667; 80,000 cells/cm^2) had a decline in cell number resulting in a neutral/ negative growth rate (-0.019 to 0.006 h^{-1}), see **Figure 28A**. This is not likely to be due to cell death as the cell viabilities were all above 90% on day 1 (**Figure 28B),** this observation is most likely due to a seeding error at the beginning of the experiment, resulting a ~ 30% decrease in cell number from 0 to 24 h. The three higher density conditions behaved similarly to each other, whilst the three lower densities conditions also behaved similarly to each other, suggesting a grouping of cell growth behaviour dependent of seeding density. The SGR for the lower densities (5,000 and 10,000 cells/cm^2) were on average slightly lower (0.021 h^{-1}) over the 3-day period, whilst the higher densities had an average SGR of 0.022 h^{-1}. Overall the cells grew exponentially and do not hit a phase of decline or inhibition within the 72-hour culture period, independent of cell density as the SGR between conditions was not significantly different at day 2 and 3. The metabolite data revealed that main significant differences were between the 5000 cells/cm^2 condition and all the other conditions at day 1. At day 2 and 3 notable differences in SMR were only observed between the three lower densities and three higher densities (**Figure 28C)**, again highlighting a grouped behaviour pattern as seen in the SGRs.

N.B. The seeding error mentioned above resulted in the adjustment of the initial cell number used in the SGR equation for the three higher densities. The error in seeding was due to a different operator carrying out the manipulations of the higher density conditions, this operator unknowingly seeded at a lower density. By comparing the average SGR of the lower three densities at day 1 in the experiment, it was demonstrated that the higher three densities would need to have their initial seeding cell number adjusted by 30 % in order to come in line with the SGRs observed in the lower conditions. For instance, for the 80,000 cells/cm^2 condition, the initial seeding cell number should have been 2,000,000 cells in the T25 cm^2 tissue-culture flasks used, however in order to account for the seeding error this was adjusted by 30% to become 1,400,000 cells (56,000 cells/cm^2). Thus, the adjusted seeding densities for the three higher densities (40,000; 66,667; 80,000 cells/cm^2) were 28,000; 46,667; 56,000 cells/cm^2, respectively. Due to resource and time constraints this experiment was not repeated with correct seeding densities. However, .experiments carried out after this experiment using the correct seeding densities for higher conditions e.g. Experiment 3 and 4 also exhibit similar grouping between lower and higher densities, therefore it can be inferred that the results illustrated here are valid, despite the initial seeding error.

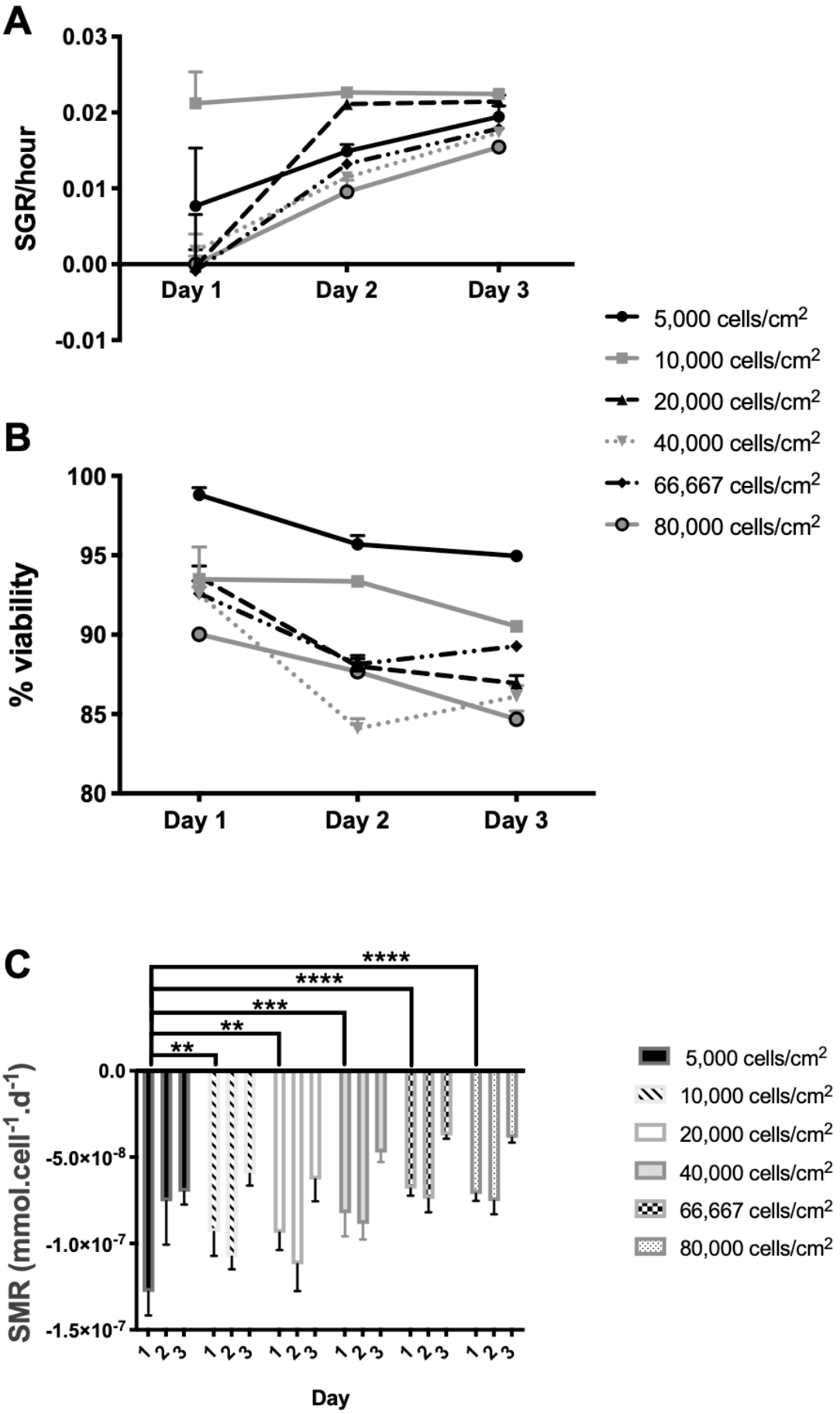

A
SGR/hour
0.03
0.02
0.01
0.00
-0.01
Day 1
Day 2
Day 3
5,000 cells/cm2
10,000 cells/cm2
20,000 cells/cm2
40,000 cells/cm2
66,667 cells/cm2
80,000 cells/cm2
B
% viability
100
95
90
85
80
Day 1
Day 2
Day 3
C
SMR (mmol.cell-1.d-1)
0.0
-5.0×10-8
-1.0×10-7
-1.5×10-7
****
****
***
**
**
Day
5,000 cells/cm2
10,000 cells/cm2
20,000 cells/cm2
40,000 cells/cm2
66,667 cells/cm2
80,000 cells/cm2

Figure 28: Data was collected every 24 h over a 72-hour culture period, six difference seeding densities were investigated in the experiment. Error bars indicate standard deviation, n=3 for each condition; n=3 for SMR data; n=9 for SGR and cell viability data. (**A**) SGR was shown to increase for all six conditions over the culture period. (**B**) Cell viability decreased uniformly for all conditions from day 1 to day 3. (**C**) Significant differences between conditions were at day 1, between 5,000 cells/cm^2 and the other densities. "**" indicates p< 0.01, "***" indicates p< 0.001, and "****" indicates p<0.0001

Table 12. Experiment 2 SGR. Tukey's multiple comparisons test of the mean SGR in each condition compared the means of the other conditions on that day. Summary shows only the conditions with significant differences, at day 1 it is evident that the 10,000 cells/cm^2 condition had a higher SGR than all other conditions. At day 2 only two of the conditions were different and no differences between conditions were noted at day 3.

Experiment 2 SGR		
Day 1	**Summary**	**P Value**
5,000 cells/cm^2 *vs.* 10,000 cells/cm^2	*	0.0119
10,000 cells/cm^2 *vs.* 20,000 cells/cm^2	****	<0.0001
10,000 cells/cm^2 *vs.* 40,000 cells/cm^2	****	<0.0001
10,000 cells/cm^2 *vs.* 66,667 cells/cm^2	****	<0.0001
10,000 cells/cm^2 *vs.* 80,000 cells/cm^2	****	<0.0001
Day 2	**Summary**	**P Value**
10,000 cells/cm^2 *vs.* 80,000 cells/cm^2	*	0.0168

Table 13. Experiment 2 SMR. Tukey's multiple comparisons test of the mean SMR in each condition compared the means of the other conditions on that day. Summary shows only the conditions with significant differences, at day 1 it is evident that the 5,000 cells/cm^2 condition had a higher SMR than the other conditions. At day 2 the differences observed in SMR were predominantly between the lower densities and the higher densities, highlighting the grouped behaviour shown in **Figure 28A** and **C**. The lowest density and the two highest densities were the only conditions noted to be different at day 3.

Experiment 2 SMR		
Day 1	**Summary**	**P Value**
5,000 cells/cm^2 *vs.* 10,000 cells/cm^2	**	0.0046
5,000 cells/cm^2 *vs.* 20,000 cells/cm^2	**	0.0054
5,000 cells/cm^2 *vs.* 40,000 cells/cm^2	***	0.0001
5,000 cells/cm^2 *vs.* 66,667 cells/cm^2	****	<0.0001
5,000 cells/cm^2 *vs.* 80,000 cells/cm^2	****	<0.0001
Day 2	**Summary**	**P Value**
5,000 cells/cm^2 *vs.* 10,000 cells/cm^2	*	0.0105
5,000 cells/cm^2 *vs.* 20,000 cells/cm^2	**	0.0027
10,000 cells/cm^2 *vs.* 66,667 cells/cm^2	**	0.0067
10,000 cells/cm^2 *vs.* 80,000 cells/cm^2	**	0.01
20,000 cells/cm^2 *vs.* 66,667 cells/cm^2	**	0.0017
20,000 cells/cm^2 *vs.* 80,000 cells/cm^2	**	0.0025
Day 3	**Summary**	**P Value**
5,000 cells/cm^2 *vs.* 66,667 cells/cm^2	**	0.0089
5,000 cells/cm^2 *vs.* 80,000 cells/cm^2	*	0.0136

4.3.3. Experiment 3.1: Nutrients vs. Density

On the first experimental passage (passage 1) the cells exhibited similar growth behaviour to that observed in the previous experiments. The SGR values appeared to be independent of cell density and in this instance nutrient availability also, as some values were the same for different culture conditions. On the reset passage (passage 2) the cells had very similar SGR values regardless of which condition they had been previous cultured from, illustrating that there seemed to be no retention or effect on cell metabolic state (**Figure 29A**). No significant difference was observed between conditions and/or passages. Three graphical representations have been used to demonstrate the different behaviours exhibited by SMRs for the different conditions. (**Figure 29B**) demonstrates the SMR of the conditions at passage 1 and 2 of the experiment. There were differences in SMR within conditions with the same

density and those with different densities, suggesting that both feed volume and density result in SMR variation. At passage 2, significant differences were observed between the two 40,000 cells/cm^2 conditions (5 and 7 mL) and all the other conditions (**Figure 29B**). The SMR data highlights a cycling trend across the four passage points (1 = passage 1 feed day; 2 = passage 1 harvest/reset day; 3 = passage 2 feed day; 4 = passage 2 harvest day). At passage point 1 all the conditions had a high SMR of glucose which dropped by ~ 25 % or more at passage point 2, then it reverted up by ~25% or more at passage point 3, to then decrease again at passage point 4 (**Figure 29C**). The SMR data in **Figure 29D** is presented in a way that better illustrates that during passage 1 the conditions exhibit variation, whereas when all the flasks are reset to the same culture condition at passage 2 there was no significant variation between the flasks regardless of the previous culture condition.

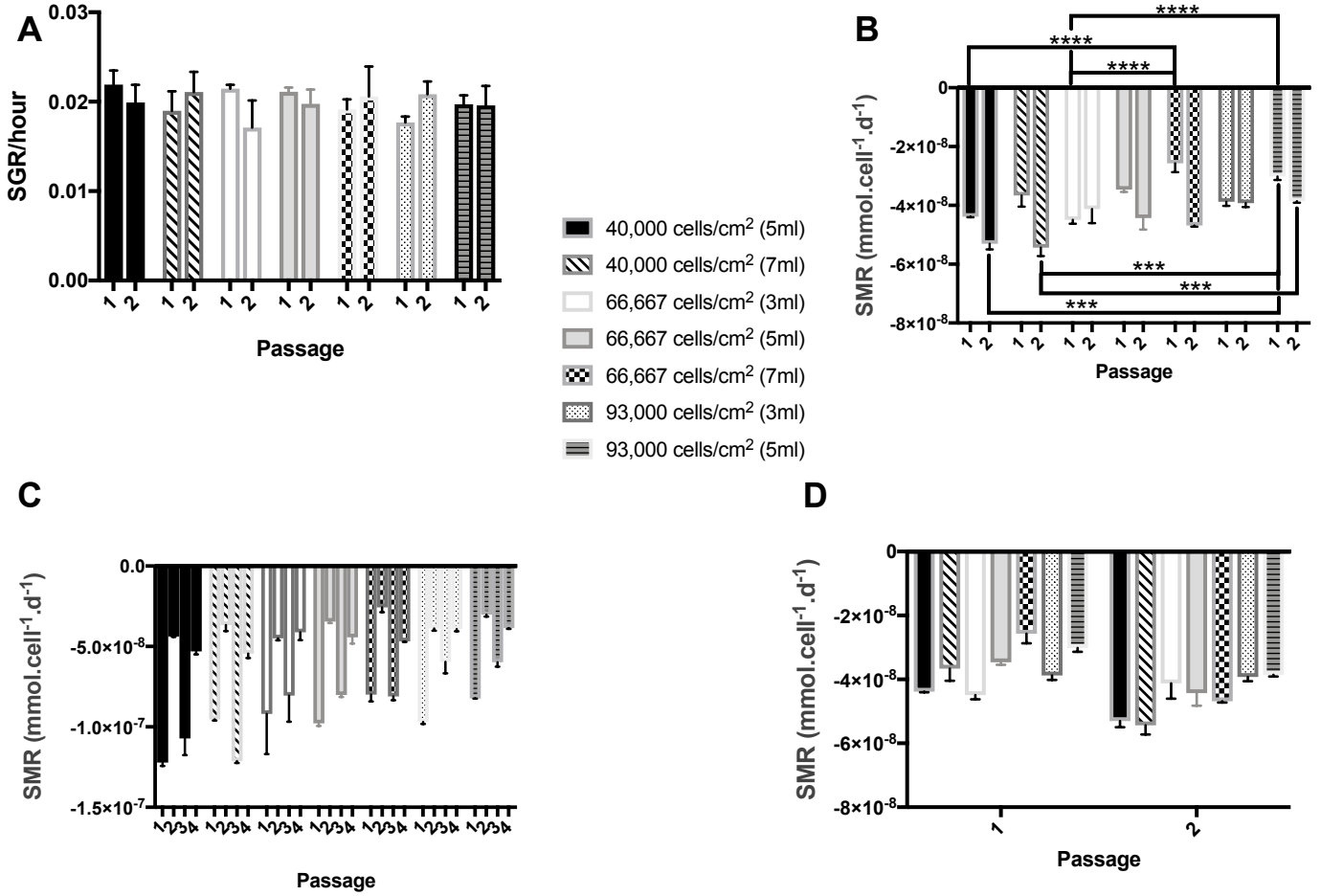


Figure 29: (**A**) SGR for the different conditions over the two passages, Error bars indicate standard deviation, n=2 for each condition; n=2 for SMR data; n=4 for SGR data. (**B**) Differences were observed between the conditions over the 2 passages. "**" indicates p< 0.01, "***" indicates p< 0.001, and "****" indicates p<0.0001. (**B-D**) Three graphical representations used to show the different behaviours exhibited by SMRs of the different culture conditions. Passage points shown in (**C**) correspond to: 1 = passage 1 feed day; 2 = passage 1 harvest/reset day; 3 = passage 2 feed day; 4 = passage 2 harvest day).

Table 14: Experiment 3.1 SGR. Tukey's multiple comparisons test of the mean SGR in each condition compared the means of the other conditions during that passage. The only difference observed was between the lowest density and highest density with normal and low feed volume, respectively. No differences in SGR were observed between conditions at the reset passage (passage2).

Exp 3.1 SGR		
Passage 1	**Summary**	**P Value**
40,000 cells/cm^2 (5 mL) *vs.* 93,000 cells/cm^2 (3 mL)	*	0.0388

Table 15. Experiment 3.1 SMR. Sidak's multiple comparisons test of the mean SMR in each condition compared the means of the other conditions during that passage. Differences between the majority of the conditions were observed at passage 1, whilst at passage 2 when the conditions were reset differences were only observed between the lowest density and all the other condition.

Exp 3.1 SMR		
Passage 1	**Summary**	**P Value**
40,000 cells/cm^2 (5 mL) *vs.* 66,667 cells/cm^2 (5 mL)	*	0.0399
40,000 cells/cm^2 (5 mL) *vs.* 66,667 cells/cm^2 (7 mL)	****	<0.0001
40,000 cells/cm^2 (5 mL) *vs.* 93,000 cells/cm^2 (5 mL)	**	0.0012
40,000 cells/cm^2 (7 mL) *vs.* 66,667 cells/cm^2 (7 mL)	*	0.01
66,667 cells/cm^2 (3 mL) *vs.* 66,667 cells/cm^2 (5 mL)	*	0.0171
66,667 cells/cm^2 (3 mL) *vs.* 66,667 cells/cm^2 (7 mL)	****	<0.0001
66,667 cells/cm^2 (3 mL) *vs.* 93,000 cells/cm^2 (5 mL)	***	0.0006
66,667 cells/cm^2 (5 mL) *vs.* 66,667 cells/cm^2 (7 mL)	*	0.0494
66,667 cells/cm^2 (7 mL) *vs.* 93,000 cells/cm^2 (3 mL)	**	0.0019
Passage 2	**Summary**	**P Value**
40,000 cells/cm^2 (5 mL) *vs.* 66,667 cells/cm^2 (3 mL)	**	0.0053
40,000 cells/cm^2 (5 mL) *vs.* 93,000 cells/cm^2 (3 mL)	**	0.0012
40,000 cells/cm^2 (5 mL) *vs.* 93,000 cells/cm^2 (5 mL)	***	0.0007
40,000 cells/cm^2 (7 mL) *vs.* 66,667 cells/cm^2 (3 mL)	**	0.0019
40,000 cells/cm^2 (7 mL) *vs.* 66,667 cells/cm^2 (5 mL)	*	0.0205
40,000 cells/cm^2 (7 mL) *vs.* 93,000 cells/cm^2 (3 mL)	***	0.0004
40,000 cells/cm^2 (7 mL) *vs.* 93,000 cells/cm^2 (5 mL)	***	0.0003

4.3.4. Experiment 3.2: Nutrients *vs*. Density

For both the SGR and SMR the significant differences are observed within passage 1 where the cells were cultured under different conditions (see **Table 16** and **Table 17**). At the reset passage (passage 2) no significant differences were observed for the SMR whilst some difference were observed for the SGR within passage 2 (**Figure 30A**). The SMR graphs (**Figure 30B**, **C** and **D)** demonstrate similar trends to the previous experiment (Experiment 1**, Figure 27**, **Figure 30B**). Differences in SMR were observed based on both density and feed volume within passage 1, at passage 2 no difference was observed between the conditions/flasks once they had been reset. (**Figure 30C**) The SMR data highlights a cycling trend across the four passage points (1 = passage 1 feed day; 2 – passage 1 harvest/reset day; 3 = passage 2 feed day; 4 = passage 2 harvest day). Within all the conditions the SMR rate decreased from passage point 1 to 2, increased from passage point 2 to 3 and then decreased again from passage point 3 to 4. In the graphical illustration the consistency of SMRs between flasks at passage 2 is evident, whilst it can be observed that at passage 1 there was variation between the flasks cultured under the different condition (**Figure 30D**)

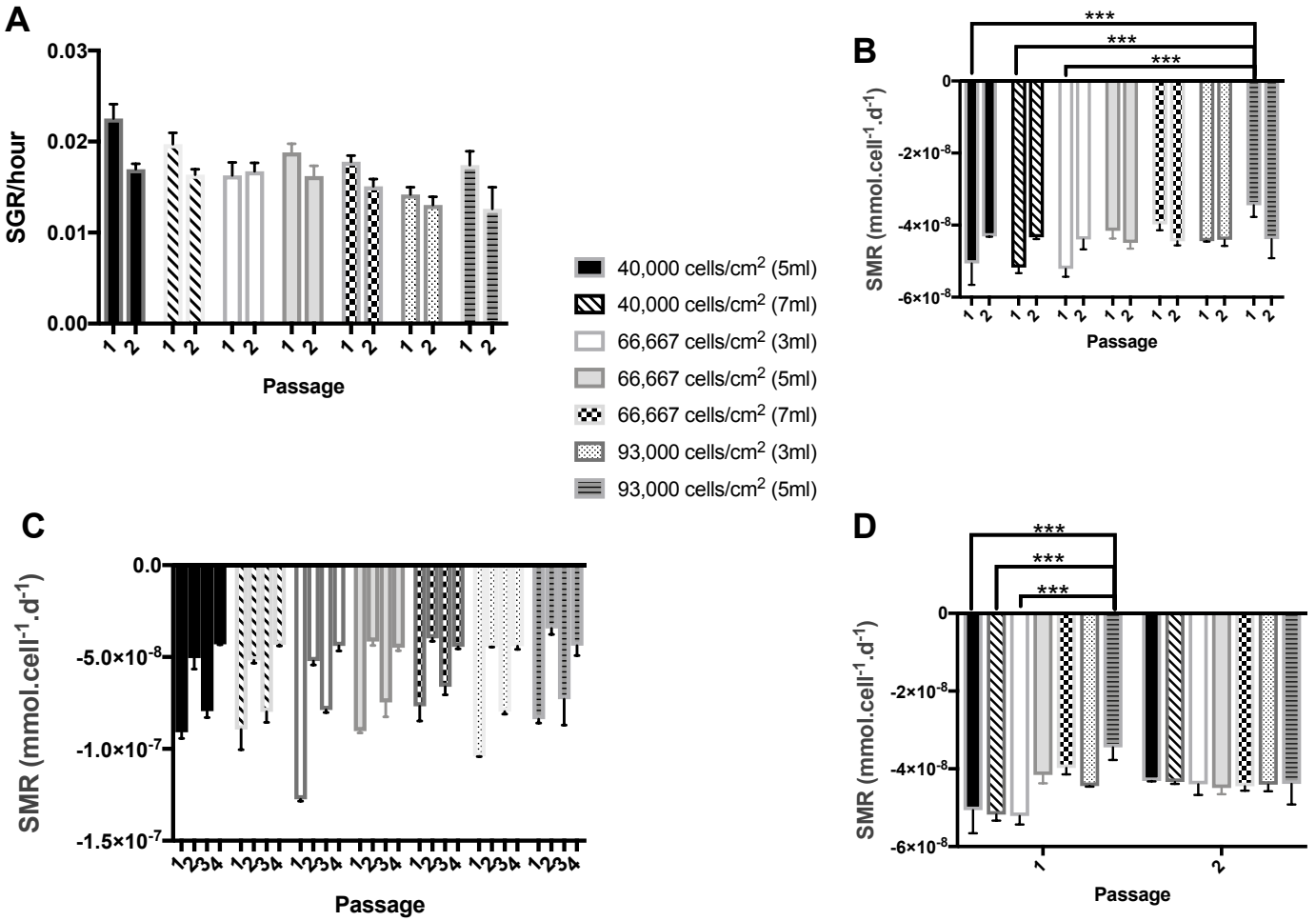


Figure 30: (A) SGR data revealed no retention of cell metabolic state as the SGR changes from passage 1 to passage 2 of the experiment (****, $p<0.0001$). Error bars indicate standard deviation, n=2 for each condition; n=2 for SMR data; n=4 for SGR data, "***" indicates $p< 0.001$, and "****" indicates $p<0.0001$. (**B-D**) Three graphical representations used to show the different behaviours exhibited by SMRs of the different condition. Passage points shown in (**C**) correspond to: 1 = passage 1 feed day; 2 = passage 1 harvest/reset day; 3 = passage 2 feed day; 4 = passage 2 harvest day).

Table 16. Experiment 3.2 SGR. Sidak's multiple comparisons test of the mean SGR in each condition compared the means of the other conditions during that passage. Differences were observed in most of the conditions at passage 1. At passages 2 regardless of being reset to the same culture condition the flasks demonstrated significant inter-flask variation of SMRs

Exp 3.2 SGR		
Passage 1	**Summary**	**P Value**
40,000 cells/cm^2 (5 mL) *vs.* 40,000 cells/cm^2 (7 mL)	*	0.0399
40,000 cells/cm^2 (5 mL) *vs.* 66,667 cells/cm^2 (3 mL)	****	<0.0001
40,000 cells/cm^2 (5 mL) *vs.* 66,667 cells/cm^2 (5 mL)	**	0.0017
40,000 cells/cm^2 (5 mL) *vs.* 66,667 cells/cm^2 (7 mL)	****	<0.0001
40,000 cells/cm^2 (5 mL) *vs.* 93,000 cells/cm^2 (3 mL)	****	<0.0001
40,000 cells/cm^2 (5 mL) *vs.* 93,000 cells/cm^2 (5 mL)	****	<0.0001
40,000 cells/cm^2 (7 mL) *vs.* 66,667 cells/cm^2 (3 mL)	**	0.0046
40,000 cells/cm^2 (7 mL) *vs.* 93,000 cells/cm^2 (3 mL)	****	<0.0001
66,667 cells/cm^2 (5 mL) *vs.* 93,000 cells/cm^2 (3 mL)	****	<0.0001
66,667 cells/cm^2 (7 mL) *vs.* 93,000 cells/cm^2 (3 mL)	**	0.0027
93,000 cells/cm^2 (3 mL) *vs.* 93,000 cells/cm^2 (5 mL)	*	0.0107
Passage 2	**Summary**	**P Value**
40,000 cells/cm^2 (5 mL) *vs.* 93,000 cells/cm^2 (3 mL)	***	0.0008
40,000 cells/cm^2 (5 mL) *vs.* 93,000 cells/cm^2 (5 mL)	***	0.0002
40,000 cells/cm^2 (7 mL) *vs.* 93,000 cells/cm^2 (3 mL)	**	0.0071
40,000 cells/cm^2 (7 mL) *vs.* 93,000 cells/cm^2 (5 mL)	**	0.0015
66,667 cells/cm^2 (3 mL) *vs.* 93,000 cells/cm^2 (3 mL)	**	0.0019
66,667 cells/cm^2 (3 mL) *vs.* 93,000 cells/cm^2 (5 mL)	***	0.0004
66,667 cells/cm^2 (5 mL) *vs.* 93,000 cells/cm^2 (3 mL)	*	0.0127
66,667 cells/cm^2 (5 mL) *vs.* 93,000 cells/cm^2 (5 mL)	**	0.0028

Table 17. Experiment 3.2 SMR. Sidak's multiple comparisons test of the mean SMR in each condition compared the means of the other conditions during that passage. Most of the conditions had different SMRs compared to each other at passage 1, whilst no difference in SMR is observed at passage 2 when all the flasks were reset to the same culture condition.

Exp 3.2 SMR		
Passage 1	**Summary**	**P Value**
40,000 cells/cm^2 (5 mL) *vs.* 66,667 cells/cm^2 (7 mL)	*	0.0292
40,000 cells/cm^2 (5 mL) *vs.* 93,000 cells/cm^2 (5 mL)	***	0.0008
40,000 cells/cm^2 (7 mL) *vs.* 66,667 cells/cm^2 (5 mL)	*	0.0441
40,000 cells/cm^2 (7 mL) *vs.* 66,667 cells/cm^2 (7 mL)	*	0.0127
40,000 cells/cm^2 (7 mL) *vs.* 93,000 cells/cm^2 (5 mL)	***	0.0004
66,667 cells/cm^2 (3 mL) *vs.* 66,667 cells/cm^2 (5 mL)	*	0.0346
66,667 cells/cm^2 (3 mL) *vs.* 66,667 cells/cm^2 (7 mL)	**	0.01
66,667 cells/cm^2 (3 mL) *vs.* 93,000 cells/cm^2 (5 mL)	***	0.0003

4.3.5. Experiment 3.3: Nutrients *vs.* Density

There is a notable difference in the SGR value of the lower density flasks (20,000 cells/cm^2) compared to the higher density, as its SGR values at both feed volumes were higher compared to the higher densities (**Figure 31A** and **Table 18**). Similarly, the SMR data exhibited the 20,000 cells/cm^2 condition had higher SMRs compared to the other densities, apart from the 40,000 cells/cm^2 (1.5ml) condition (**Figure 31B** and **Table 19**). The SMR values were comparable at passage 2 when the cells were reset to the same culture condition. The cells cultured under the different conditions at passage 1 had lower expression (63 – 86 %)) of OCT3/4 at passage which uniformly increased at passage 2 (80 – 97 %) resulting in a significant difference overall between the passages (**, p=0.001). (**Figure 31C)**. There was no significant difference in the SSEA-1 (**Figure 31D**) and SSEA-4 (**Figure 31E**), both within conditions and amongst conditions over the two passages.

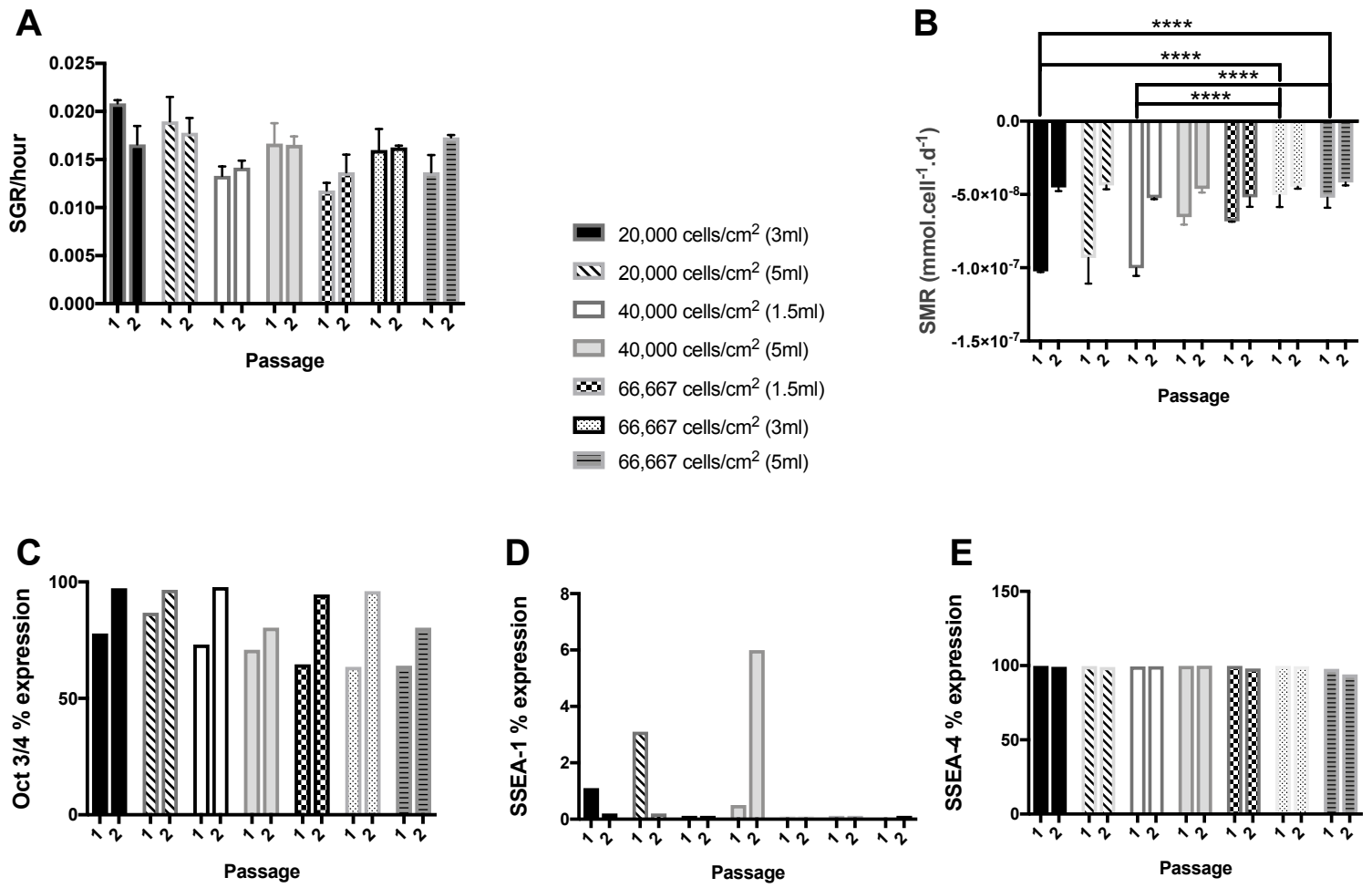


Figure 31: (A) SGR data for all the conditions, most of the variation was observed at passage 1. (**B**) SMR data over the two passages, significant difference observed with passage 1 only, "****" indicates p<0. 0001. Percentage levels of experience for the flow cytometry pluripotency maker panel used, (**C**) OCT3/4 positive marker, (**D**) SSEA-1 negative marker and (**E**) SSEA-4 positive marker. Significant difference between the two passages overall was only observed for the OCT/34 marker (**, p=0.001). Error bars indicate standard deviation, n=2 for each condition; n=2 for SMR data; n=4 for SGR data

Table 18: Experiment 3.3 SGR. Sidak's multiple comparisons test of the mean SGR in each condition compared the means of the other conditions during that passage. The main differences were between the lowest density 20,000 cells/cm^2 at both feed volumes and all the conditions at passage 1. Fewer differences were observed at passage 2 between the flasks upon reseeding at the condition 66,667 cells/cm^2 (5 mL)

Exp 3.3 SGR		
Passage 1	**Summary**	**P Value**
20,000 cells/cm^2 (3 mL) *vs.* 40,000 cells/cm^2 (1.5 mL)	****	<0.0001
20,000 cells/cm^2 (3 mL) *vs.* 40,000 cells/cm^2 (5 mL)	**	0.0061
20,000 cells/cm^2 (3 mL) *vs.* 66,667 cells/cm^2 (1.5 mL)	****	<0.0001
20,000 cells/cm^2 (3 mL) *vs.* 66,667 cells/cm^2 (3 mL)	***	0.0009
20,000 cells/cm^2 (3 mL) *vs.* 66,667 cells/cm^2 (5 mL)	****	<0.0001
20,000 cells/cm^2 (5 mL) *vs.* 40,000 cells/cm^2 (1.5 mL)	****	<0.0001
20,000 cells/cm^2 (5 mL) *vs.* 66,667 cells/cm^2 (1.5 mL)	****	<0.0001
20,000 cells/cm^2 (5 mL) *vs.* 66,667 cells/cm^2 (5 mL)	***	0.0002
40,000 cells/cm^2 (5 mL) *vs.* 66,667 cells/cm^2 (1.5 mL)	***	0.0008
66,667 cells/cm^2 (1.5 mL) *vs.* 66,667 cells/cm^2 (3 mL)	**	0.0057
Passage 2	**Summary**	**P Value**
20,000 cells/cm^2 (5 mL) *vs.* 40,000 cells/cm^2 (1.5 mL)	*	0.0283
20,000 cells/cm^2 (5 mL) *vs.* 66,667 cells/cm^2 (1.5 mL)	**	0.0073
66,667 cells/cm^2 (1.5 mL) *vs.* 66,667 cells/cm^2 (5 mL)	*	0.0288

Table 19. Experiment 3.3 SMR. Sidak's multiple comparisons test of the mean SMR in each condition compared the means of the other conditions during that passage. 20,000 cells/cm^2 at both feed volumes and 40,000 cells/cm^2 (1.5ml) were different to all the other conditions during passage 1, when all flasks were reset to the same culture condition at passage 2 no differences were observed between flasks.

Exp 3.3 SMR		
Passage 1	**Summary**	**P Value**
20,000 cells/cm^2 (3 mL) *vs.* 40,000 cells/cm^2 (5 mL)	***	0.0007
20,000 cells/cm^2 (3 mL) *vs.* 66,667 cells/cm^2 (1.5 mL)	**	0.0017
20,000 cells/cm^2 (3 mL) *vs.* 66,667 cells/cm^2 (3 mL)	****	<0.0001
20,000 cells/cm^2 (3 mL) *vs.* 66,667 cells/cm^2 (5 mL)	****	<0.0001
20,000 cells/cm^2 (5 mL) *vs.* 40,000 cells/cm^2 (5 mL)	*	0.0107
20,000 cells/cm^2 (5 mL) *vs.* 66,667 cells/cm^2 (1.5 mL)	*	0.0259
20,000 cells/cm^2 (5 mL) *vs.* 66,667 cells/cm^2 (3 mL)	***	0.0002
20,000 cells/cm^2 (5 mL) *vs.* 66,667 cells/cm^2 (5 mL)	***	0.0002
40,000 cells/cm^2 (1.5 mL) *vs.* 40,000 cells/cm^2 (5 mL)	**	0.0013
40,000 cells/cm^2 (1.5 mL) *vs.* 66,667 cells/cm^2 (1.5 mL)	**	0.0031
40,000 cells/cm^2 (1.5 mL) *vs.* 66,667 cells/cm^2 (3 mL)	****	<0.0001
40,000 cells/cm^2 (1.5 mL) *vs.* 66,667 cells/cm^2 (5 mL)	****	<0.0001

4.3.6. Experiment 3.4: Nutrients *vs.* Density

The SGR of the highest densities at both feed volumes and 66,667 cells/cm^2 (7 mL) were lower than the other conditions at passage 1, (0.011 *vs.* 0.015 h^{-1}). At passage 2 their SGRs increased to come in line with the other conditions, (**Figure 32A**). Differences in SMR between conditions were only observed at passage 1, the most significant of these are highlighted on the graph **(Figure 32B**), the rest are detailed in **Table 20**. The phenotypic marker expression profile did not change significantly from passage 1 to 2 for SSEA-4 (**Figure 32E**). SSEA-1 expression was observed, notably at passage 1 for the lowest densities and passage 2 for the highest densities. Expression was however below 2% in all cases and was deemed to be not statistically significant. In the case of Oct 3/4, difference in percentage expression is observed between passage 1 and 2 overall for all the conditions, from 85% to 95%, respectively (**, p=0.0011), **(Figure 32C)**

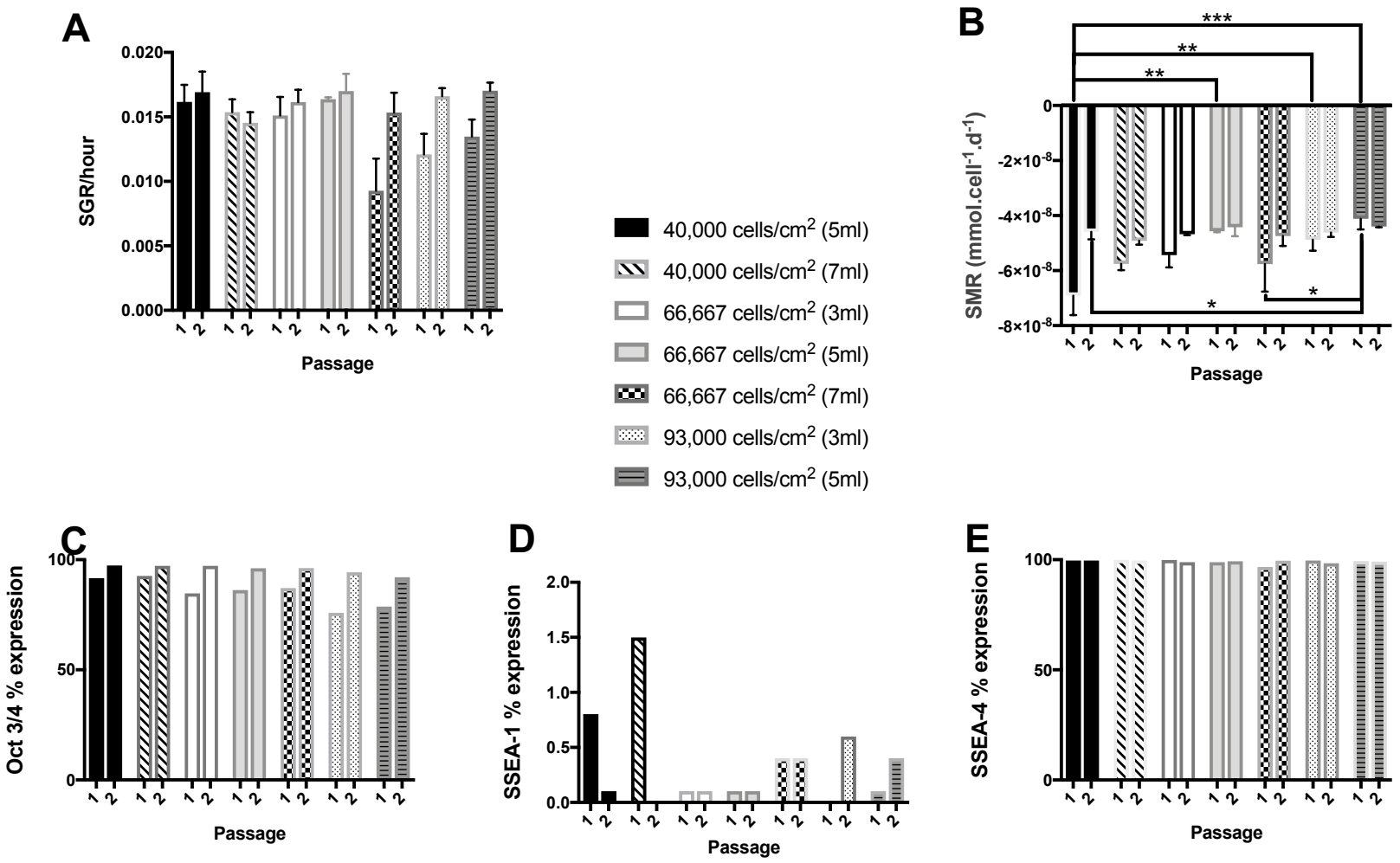

A
SGR/hour
0.020
0.015
0.010
0.005
0.000
Passage
40,000 cells/cm2 (5ml)
40,000 cells/cm2 (7ml)
66,667 cells/cm2 (3ml)
66,667 cells/cm2 (5ml)
66,667 cells/cm2 (7ml)
93,000 cells/cm2 (3ml)
93,000 cells/cm2 (5ml)
B
SMR (mmol.cell-1.d-1)
0
-2×10-8
-4×10-8
-6×10-8
-8×10-8
***
**
**
*
*
Passage
C
Oct 3/4 % expression
100
50
0
Passage
D
SSEA-1 % expression
2.0
1.5
1.0
0.5
0.0
Passage
E
SSEA-4 % expression
100
50
0
Passage

Figure 32: (**A**) SGR of the conditions investigated in the experiment. (**B**) Differences in SMR between conditions were only observed at passage 1, conditions with significant differences are highlight "**" indicates $p < 0.01$, "***" indicates $p < 0.001$. (**C**) Significant difference in % expression between passage 1 and 2 for OCT3/4 was noted overall for all the conditions in each passage, (**, $p=0.0011$). (**D**) Expression in SSEA-1 was observed most notably at passage 2, however this was not significant. (**E**)The phenotypic marker expression profile did not change significantly from passage 1 to 2 for SSEA-4. Error bars indicate standard deviation, n=2 for each condition; n=2 for SMR data; n=4 for SGR data**.**

Table 20. Experiment 3.4 SGR. Sidak's multiple comparisons test of the mean SGR in each condition compared the means of the other conditions during that passage. Differences in flasks under the various culture conditions were observed during passage 1, similar to previous experiments no differences were observed between flasks at passage 2.

Exp 3.4 SGR		
Passage 1	**Summary**	**P Value**
40,000 cells/cm^2 (5 mL) *vs.* 66,667 cells/cm^2 (7 mL)	****	<0.0001
40,000 cells/cm^2 (5 mL) *vs.* 93,000 cells/cm^2 (3 mL)	**	0.0017
40,000 cells/cm^2 (7 mL) *vs.* 66,667 cells/cm^2 (7 mL)	****	<0.0001
40,000 cells/cm^2 (7 mL) *vs.* 93,000 cells/cm^2 (3 mL)	*	0.0219
66,667 cells/cm^2 (3 mL) *vs.* 66,667 cells/cm^2 (7 mL)	****	<0.0001
66,667 cells/cm^2 (3 mL) *vs.* 93,000 cells/cm^2 (3 mL)	*	0.0484
66,667 cells/cm^2 (5 mL) *vs.* 66,667 cells/cm^2 (7 mL)	****	<0.0001
66,667 cells/cm^2 (5 mL) *vs.* 93,000 cells/cm^2 (3 mL)	***	0.0008
66,667 cells/cm^2 (7 mL) *vs.* 93,000 cells/cm^2 (5 mL)	**	0.0011

T**able 21**. Experiment 3.4 SMR. Sidak's multiple comparisons test of the mean SMR in each condition compared the means of the other conditions during that passage. Main differences observed between conditions at passage 1 were when between the lowest and highest densities within the experiment. No differences were observed during passage 2.

Exp 3.4 SMR		
Passage 1	**Summary**	**P Value**
40,000 cells/cm^2 (5 mL) *vs.* 66,667 cells/cm^2 (5 mL)	**	0.0013
40,000 cells/cm^2 (5 mL) *vs.* 93,000 cells/cm^2 (3 mL)	**	0.0049
40,000 cells/cm^2 (5 mL) *vs.* 93,000 cells/cm^2 (5 mL)	***	0.0002
40,000 cells/cm^2 (7 mL) *vs.* 93,000 cells/cm^2 (5 mL)	*	0.0304
66,667 cells/cm^2 (7 mL) *vs.* 93,000 cells/cm^2 (5 mL)	*	0.0296

4.3.7. Experiment 4: Longitudinal comparison of 2 protocol culture conditions

Data obtained demonstrated that over ten passages, culture route A2 on average had marginally higher SGR values (0.021 ± 0.004) compared to A1 (0.019 ± 0.004). The SGR values of both routes fluctuated throughout the 10 passages (**Figure 33A**) and the same can be observed of the Pd values. The fluctuation trend was similar for both routes with regards to the SGR values, Pd values and the cell viabilities. Route A2 had a marginally higher average cell viability (86.3 % ± 8.1) compared to route A1 (83.3 ± 8.8) over the 10 passages (**Figure 33B**). The metabolite data demonstrated that route A1 had a very consistent SMR for all measured metabolites over the ten passages, which appears to be independent of the SGR value (**Figure 33A** & **D**). Route A2 had more SMR fluctuation over the ten passages and in some instances, the trend correlates to the SGR value (passage cycle 1 to 4). Marker expression analysis using flow cytometry showed no significant change in SSEA-4 expression over the ten passages or between route A1 and A2 (p=0.07 and p=0.10, respectively). However, for route A2 there was expression of SSEA-1 which is a negative marker; this was observed from passage cycle 3 onwards. OCT3/4 expression in route A2 is shown to decrease by 52% and by ~ 30% in route A1 at passage cycle 7, which increased back to above 95 % in route A1 and only to 79.6 % by passage cycle 9 in route A2 (**Figure 33E** and **Figure 34)**.

The PCR performed for experiment A demonstrated that for a selection of the genes analysed, *DPPA4, POU5F1* and *REX1*, there is no significant difference between the two different routes throughout the 10 passages (**Figure 35**). However, *DNMT3B, TDGF* and *SOX2* exhibit significant differences between the two routes and between passage cycles as they have higher expression in route A2 **Figure 35A, C** and **F**). *TDGF* is the only gene that shows a significant difference at passage cycle 3, being much lower than the route A1 condition (** p = 0.018). *DNMT3B, NANOG* and *SOX2* show differences at passage cycle 7 and/or passage 9 between the two routes, in all cases route A2 having a higher fold change.

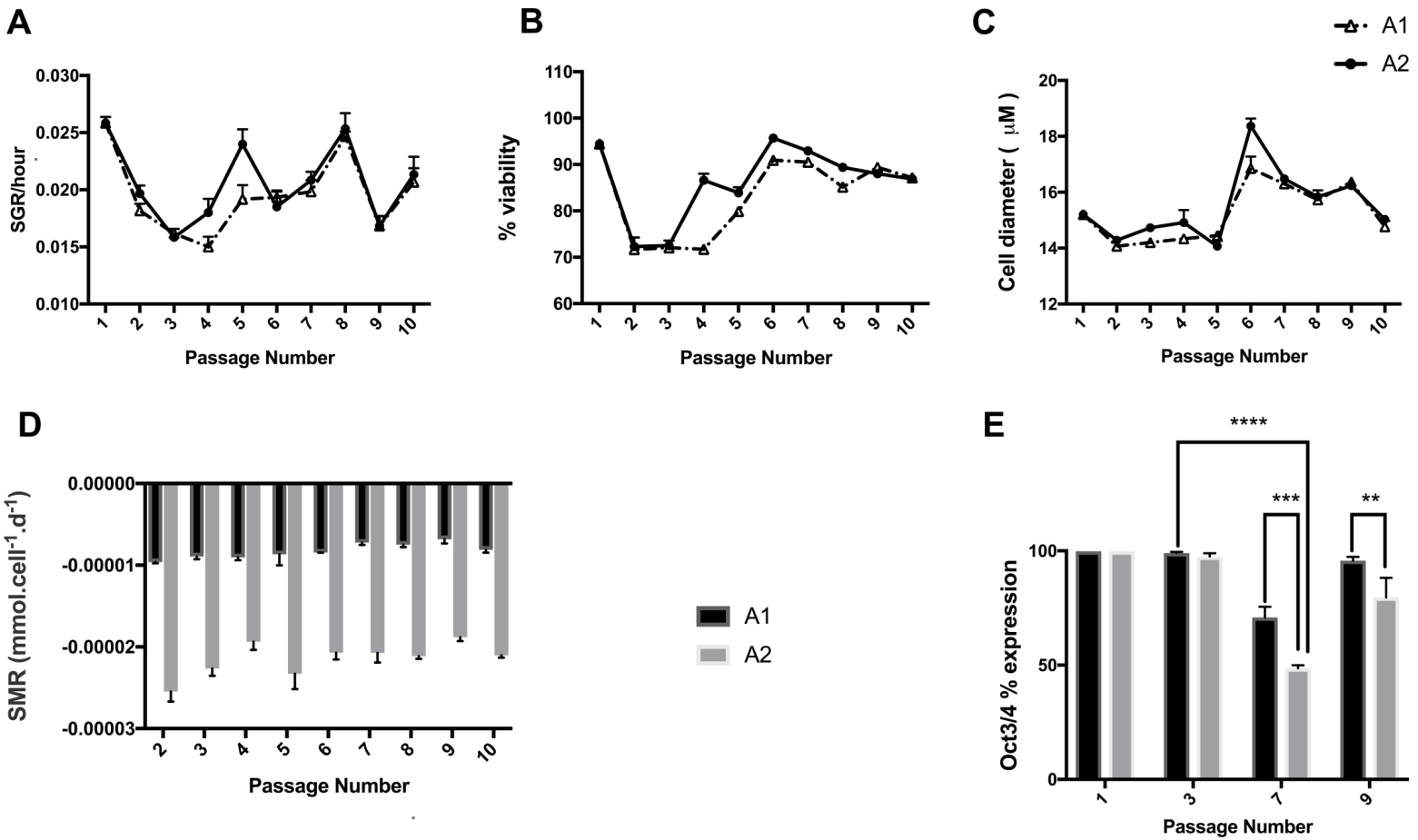
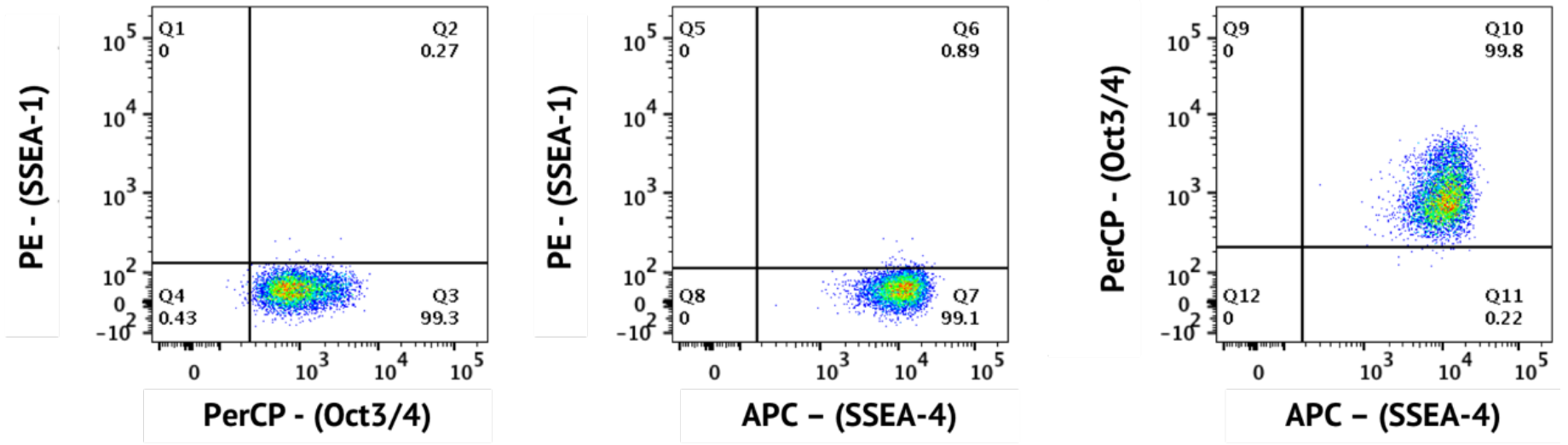

A
SGR/hour
0.030
0.025
0.020
0.015
0.010
1 2 3 4 5 6 7 8 9 10
Passage Number
B
% viability
110
100
90
80
70
60
1 2 3 4 5 6 7 8 9 10
Passage Number
C
A1
A2
Cell diameter (μM)
20
18
16
14
12
1 2 3 4 5 6 7 8 9 10
Passage Number
D
SMR (mmol.cell-1.d-1)
0.00000
-0.00001
-0.00002
-0.00003
2 3 4 5 6 7 8 9 10
Passage Number
A1
A2
E
****
***
**
Oct3/4 % expression
100
50
0
1 3 7 9
Passage Number

Figure 33. (**A**) Specific growth rate trend for route A1 and A2 over ten passages, showing a fluctuation in SGR throughout all ten passages that follows the same trend regardless of route, n=3 error bars showing standard deviation, SD. (**B**) Cell viability trend of route A1 and A2 over ten passages both routes follow a similar trend in viability throughout all passages cycles, route A1 is shown to have lower viability during five of the passage cycles (4 -8) in comparison to route A2, n=2 error bars showing SD. (**C**) Cell diameters of route A1 and A2 over ten passages showing synchronised trend; some difference between the two routes is observed at passage cycle 3, 4 and 6, , n=3 error bars showing standard deviation, SD. (**D**) Glucose SMR from passage cycle 2 to 10 showing a significant difference between passages (***$p<0.001$), at each individual passage cycle route A2 is significantly higher than route A1 (****$p<0.0001$) (**E**) OCT3/4 marker expression percentages for experiment Cat passage cycle 1, 3, 7 and 9 significant differences between passages (****$p<0.0001$) and significant differences between culture route A1 and A2 ($p=0.0004$, n=3).

A


PE - (SSEA-1)
PerCP - (Oct3/4)
Q1
0
Q2
0.27
Q4
0.43
Q3
99.3
PE - (SSEA-1)
APC – (SSEA-4)
Q5
0
Q6
0.89
Q8
0
Q7
99.1
PerCP - (Oct3/4)
APC – (SSEA-4)
Q9
0
Q10
99.8
Q12
0
Q11
0.22

B

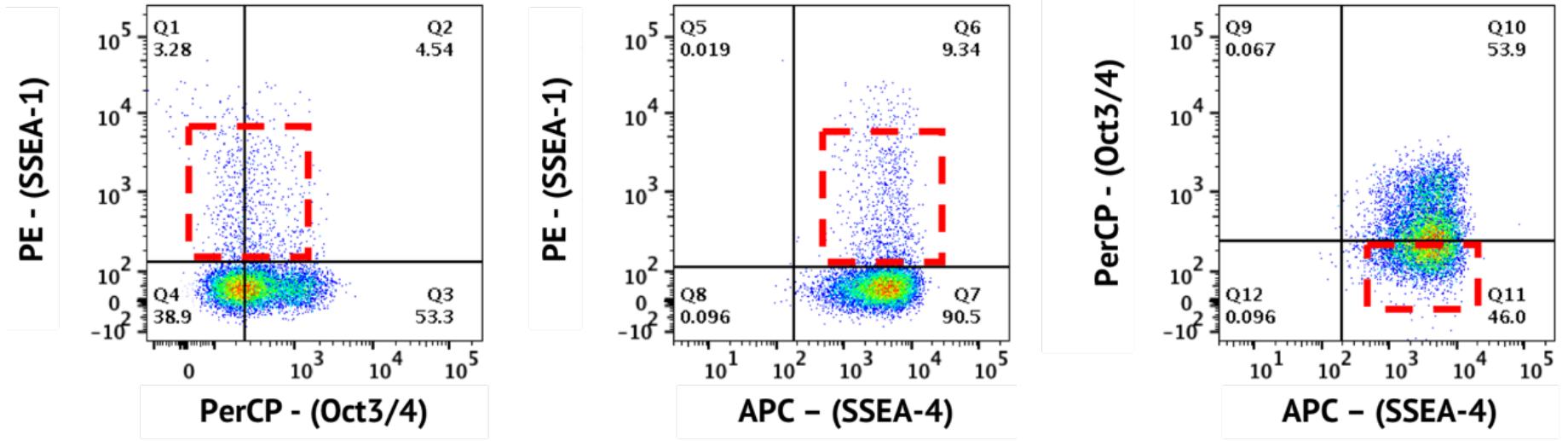

PE - (SSEA-1)
PerCP - (Oct3/4)
Q1
3.28
Q2
4.54
Q4
38.9
Q3
53.3
PE - (SSEA-1)
APC – (SSEA-4)
Q5
0.019
Q6
9.34
Q8
0.096
Q7
90.5
PerCP - (Oct3/4)
APC – (SSEA-4)
Q9
0.067
Q10
53.9
Q12
0.096
Q11
46.0

Figure 34.Flow cytometry dot plot profiles showing marker expression change from (**A**) passage cycle 1 to (**B**) passage cycle 7. OCT3/4 expression decreased from passage cycle 1 to 7 and an increase in SSEA-1 was observed, highlighted by the red hatched boxes. Oct 3/4 and SSEA-4 are positive markers for pluripotent human embryonic stems, SSEA-1 is a negative marker associated with pluripotency of human embryonic stem cells

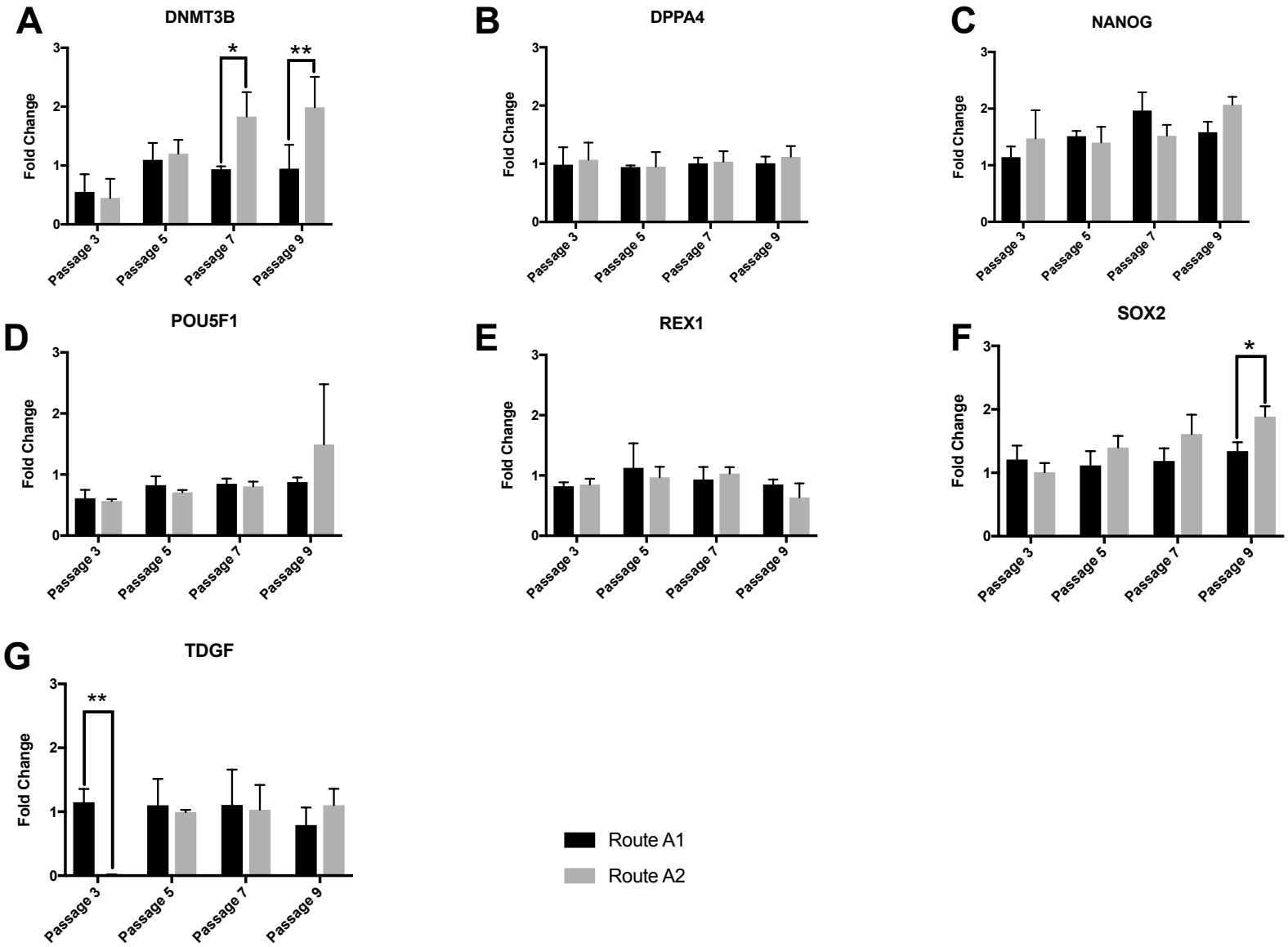

A
DNMT3B
B
DPPA4
C
NANOG
D
POU5F1
E
REX1
F
SOX2
G
TDGF
Fold Change
Passage 3
Passage 5
Passage 7
Passage 9
Route A1
Route A2

Figure 35: Gene expression analysis of experiment A at four passage points over the then passages, expression was measured by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Fold change was calculated using the relative quantity of each gene was calculated by the ΔΔCt method, using a correction for the amplification efficiency of that gene, and normalised to the geometric mean of two housekeeping genes: glyceraldehyde-3-phosphate dehydrogenase and β-actin. Significant differences in fold change at different passage cycles were seen only for **A** -*DNMT3B* (***, p=0.0007), *NANOG* (*, p=0.01) and *SOX2* (**, p=0.004). Significant differences between route A1 and A2 were seen only for **A -***DNMT3B* (**, p=0.0032) and **F**-*SOX2* (**, p-0.006). Differences between the two routes within a passage cycle were only observed for **G**-*TDGF* (passage cycle 3, **, p=0.018), **A** -*DNMT3B* (passage cycle 7, *, p=0.0221; passage cycle 9, **, p=0.0073), and **F**-*SOX2* (passage cycle 9, *, p=0.0191). Error bars indicate standard deviation (n=3 for all graphs).

4.3.8. Experiment 4.1: inter-experiment reversal of protocol culture conditions

As observed in the previous passages of experiment 4 cells (A2) that were under route B2 had slightly higher cell viability and higher SGR values whilst the converse was true for route B1 cells **(Figure 36)**. By the reversal harvest passage route B2 cells exhibited changes in marker expression as they started to express SSEA-1, which they had not expressed in the previous nine passages, whilst route B1 cells had a decline in SSEA-1 expression. However, the rate of conversion of B1 is seemingly slower compared to B2, as there is still SSEA-1 expression in route B1 cells (**Figure 37C**).

N.B. This experiment was carried out during the middle of the longitudinal study. Therefore, to maintain a reasonable workload the experiment was performed without any replicates, as it was principally an explorative experiment: to ascertain if the behaviours observed were due to the conditions the cells were being cultured under.

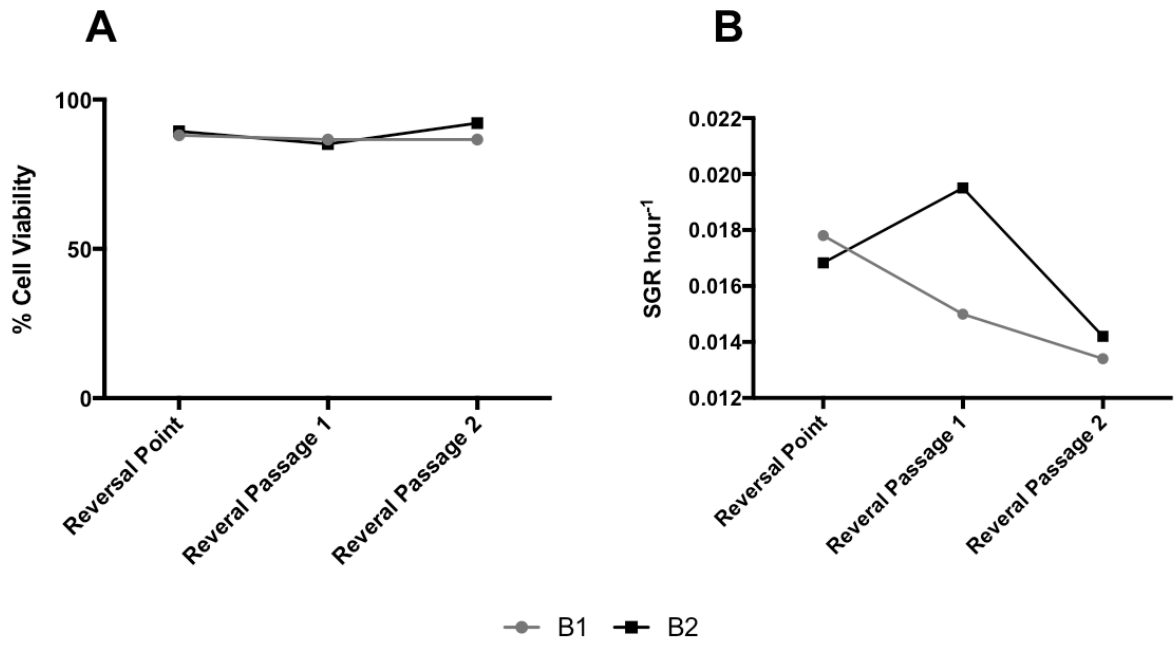


Figure 36. Growth data of the reversal route experiment over three analysis time points. B1 previously A2 overall has lower cell viability and SGR values compared to B2, previously A1, which had an increase in both cell viability and SGR.

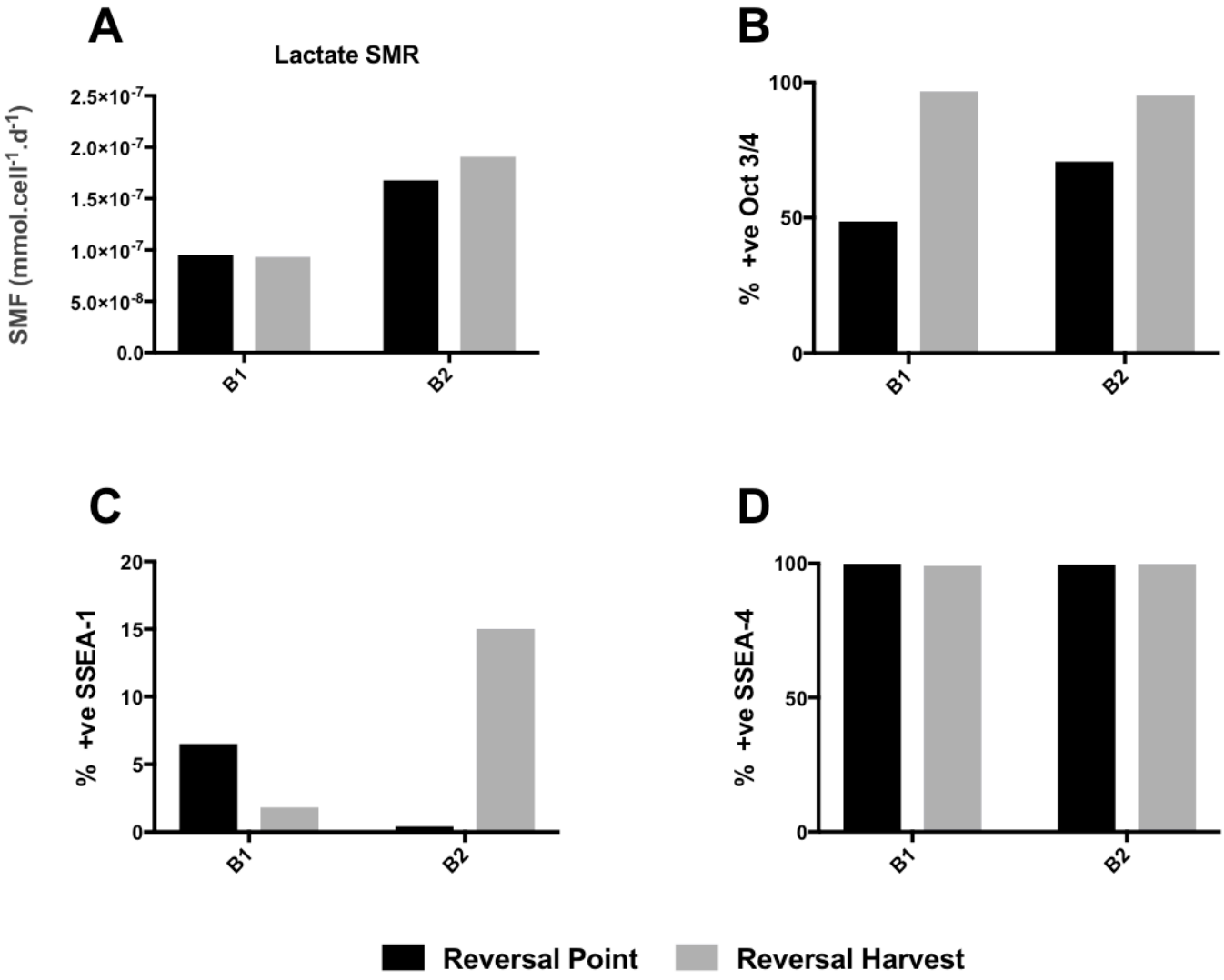


Figure 37. (**A**)Lactate SMR demonstrating a change in SMR due to culture route, B2 previously A1 has an increase in SMR. (**B**) Percentage expression levels at the reversal point and after 2 passages in the reversal experiment (reversal harvest), both B1 and B2 had an increase in OCT3/4 expression. (**C**) SSEA-1 percentage positive levels illustrating a decrease in expression for B1 and an increase for B2, demonstrating a change in phenotype express due to the exchange of culture conditions.

4.3.9. Experiment 5: 4-way comparison of the effects of medium of exchange.

SSEA-1 and SSEA-4 marker expression are both very stable with minimal variation throughout the three passages. The cells do exhibit variation in the OCT3/4 marker in all the conditions as expression decreased, most notably at passage 3, regardless of whether they subjected to medium exchange after 48 h or not; the same trend was exhibited across all of the conditions independent of the seeding density (**Figure 38C**). Akin to experiment 4, the route C2 condition exhibits a higher rate of metabolism (**Figure 38B**); other significant differences between routes are noted in **Table 22**. The SGR values were similar across all the conditions used, independent of the culture conditions and density, apart from C4 at passage 3 (**Figure 38A**).

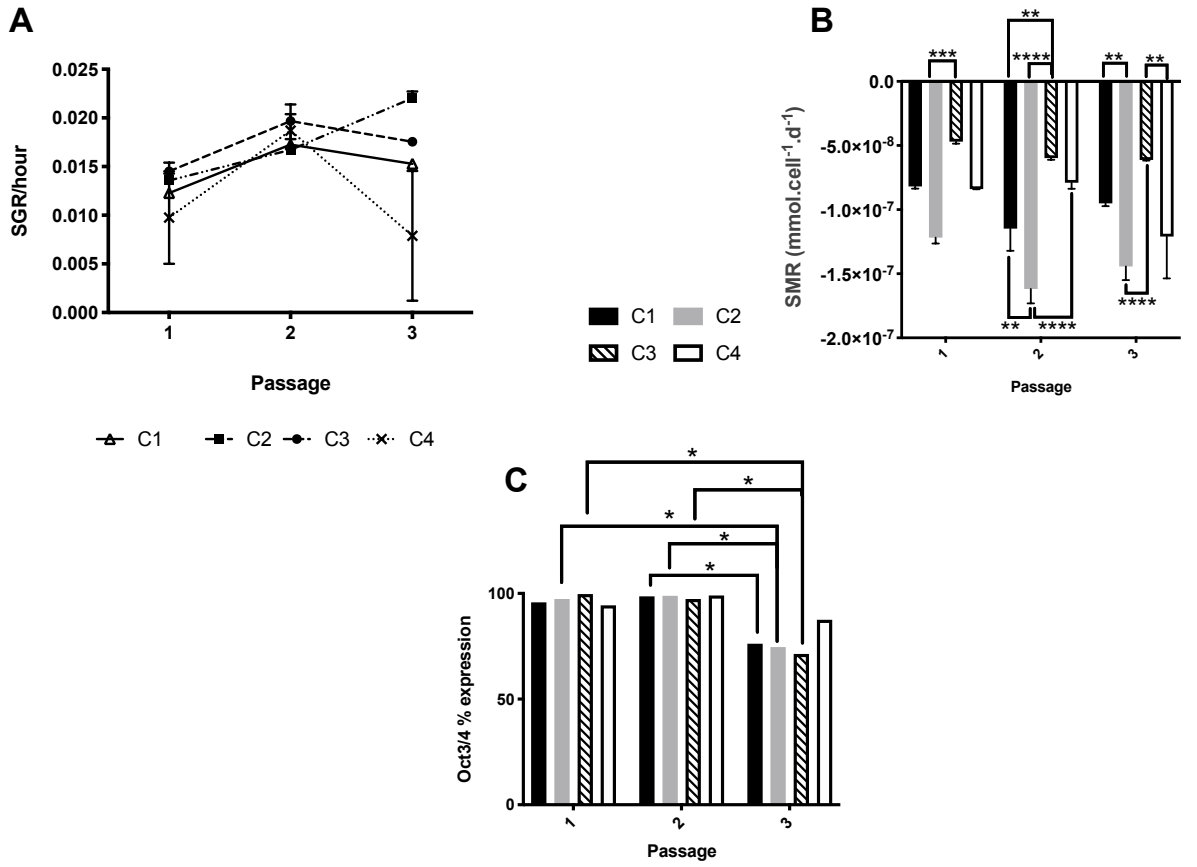


Figure 38: (**A**) SGR over three passages, similar values were observed in all culture routes, apart from C4 at passage 3 (n=2). (**B**) Glucose SMR over three passages demonstrating that route conditions C2 and C4 that had no medium exchange after 48 h had higher SMR compared to route C1 and C3. Significant difference between routes at each passage shown on the graph highlight that seeding density, feeding regime and a combination of both parameters resulted in a different SMRs (n=2). (**C**) OCT3/4 marker expression over three passages, expression levels are similar between routes, significant difference observed between passage 2 and 3 for all routes (*$p<0.05$) except route C4.

Table 22. Summary of the significance values at each passage, comparing the SMRs of the different routes using Tukey's multiple comparisons test. Both seeding density and feeding regime were shown to result in significant difference between the four routes. The highest significant differences between routes, within passages, were observed when both parameters were changed e.g. C2 vs C3, (n=2 for each condition).

		Passage 1		**Passage 2**		**Passage 3**	
Condition	**Parameter**	**Summary**	**P Value**	**Summary**	**P Value**	**Summary**	**P Value**
C1 vs. C2	Feed	*	0.024	**	0.0082	**	0.0059
C1 vs. C3	Density	*	0.0494	**	0.0026	ns	0.0573
C1 vs. C4	Feed & density	ns	0.998	*	0.0438	ns	0.1793
C2 vs. C3	Feed & density	***	0.0002	****	<0.0001	****	<0.0001
C2 vs. C4	Density	*	0.0327	****	<0.0001	ns	0.2435
C3 vs. C4	Feed	*	0.0364	ns	0.3936	**	0.0013

4.4. Discussion

In the context of the growing cell therapy industry, it is important to develop robust protocols that are easily translatable, transferable and comparable, to ensure high quality cell products that satisfy regulatory requirements without being burdensome on the manufacturing process[141,145,241]. As such, before truly automated and closed systems can be employed, it is important to have standardisation in the manual and semi-automated protocols that are currently in use. This chapter set out to demonstrate that defined, specific protocols that are barred to user interpretation are necessary to maintain consistency, particularly in the context of reference standards and reference cell lines[245,246,253]. The simultaneous culture of the EC 2102Ep cells using two different culture routes was utilised to explore the effect that changes in protocols have on cell culture process characterisation and outcomes.

Previous work within the lab by Dr Samantha L Wilson and Dr Maryam Shariatzadeh had shown that that even for embryonic carcinoma cell lines like EC 2102Ep, the growth is not consistent; however, it is unclear what causes the variability. It is our hypothesis that the root cause of the variability is due to the lack of controlled culture protocols; which results in differences in cell culture growth and characterisation outcomes. It is evident from the experiments that in terms of growth rate, the cells are robust to a wide range of seeding densities (5,000 to 93,000 cells/cm^2), illustrating that at least in the case of EC 2102Ep cells, seeding density is not a limiting factor of cell growth, within the ranges tested. Instead, it is assumed that the differences observed are resultant of the cell system dynamics, as a consequence of the protocol parameters such as undefined seeding densities due to split ratios and inconsistencies in feeding regime.

Density has been reported to be a major parameter that can influence undesirable cell differentiation, evidenced by the EC 2102Ep line and work carried out by Andrews (1982)[244,254]. Here the authors show that when seeded at a lower density (1,300 cell/cm^2) the cells start to change their phenotype as they expressed SSEA-1 which is a negative marker of EC 2102Ep cell line pluripotency[243,244]. Interestingly, preliminary results in the present work, scanning through a range of densities showed no difference in cell growth; morphology and specific growth rates (**Figure 27** and **Figure 28**). Therefore, additional cell characteristics such as gene expression, cell phenotype and SMR were measured to ascertain if density was the major driving force for differences previously observed. The experimental design employed explored feeding regime as an addition protocol parameter that could attribute to variation, either by itself or as a combined effect with seeding density. Akin to the work by Andrews (1982), it was observed that changes in seeding density did result in cell behaviour that suggests cell differentiation, however it was also observed that the feeding regime employed also resulted in other cell characteristic differences. These differences were assessed using secondary methods of appraisal that are based on rates and specific consumption/production of measured metabolites. These analyses

reveal that SMR is significantly affected by feeding regime regardless of seeding density, showing that density is not the only major factor that needs to be controlled, to ensure consistency in cell characteristics during cell culture. Although Andrews (1982) prescribe the route A1 density (66,667 cells/cm^2) as the optimal seeding density, it is evident that even at this density variation is observed through the passages and experiments carried out.

The results from the experiments presented highlight the need to understand process parameters within cell culture protocols, as greater understanding can result in increased process control. It is evident from experiments 1 to 5 that the cells in terms of growth are robust to a wide range of densities and culture conditions, illustrating that at least in the case of EC 2102 Ep cells, neither density or nutrient levels are a limiting factor of cell growth within the relatively wide ranges tested. The experiments carried out do show that there are some behaviours and instabilities observed based on cell density. The hypothesis that poor or non-ideal culture conditions i.e. starvation over a period results in the poor growth performance is not supported by the present work as the cells do not seem to maintain their cell state (i.e. metabolic rate) based on their previous culture state. This is highlighted in experiment 4.1 where the cells switched behaviour instantly even after being cultured under certain conditions for eight passages previously.

Experiment 4 was a longitudinal study that aimed to investigate the stability of the two routes and to assess the effect of feeding regime on the growth performance of the cells. The in-house defined NIBSC protocol condition, route A1, was compared to a streamlined protocol using a lower starting seeding density. The route A2 condition was chosen as previous experiments revealed that cells grown at 20,000 cells/cm^2 with 5 mL of growth medium performed well in terms of cell viability and SGR. The cell time for this condition was then calculated (**Equation 6**), a value of 2.22×10^7 cell hours per 5 mL of growth medium over 72 h was obtained, which was a more than sufficient medium capacity to sustain the cells over a 72 h culture period without medium exchange. From a manufacturing point of view this minimal intervention protocol is ideal as it allows for less human manipulation and use of reagents, which are simple means of reducing production costs[133,240]. The objective of experiment 4 was to elucidate whether the cell time defined protocol, route A2, would allow for cells to be cultured in a streamlined manner and still retain the same characterisation outcomes as the original protocol in terms of cell growth dynamics, phenotypic expression and metabolism. A paper by Josephson *et al* (2007) previously cultured the EC 2102Ep cells for ten passages and showed no notable characteristic changes in the cells, which made them an ideal human embryonic stem cell reference line candidate[243]. As such the experiment also cultured the cells for ten passages whilst tracking the growth and metabolic rates along with gene expression and phenotypic markers of the cells.

Over the ten passages of experiment 4, a fluctuation trend in growth rate and cell viability was observed. Interestingly this fluctuation is synched between the two different culture routes (**Figure 33**). This suggests that there is an artefact that synchronises the two routes in terms of cell growth and viability that is independent of the route of culture the cells are exposed to. It is unclear whether this is an innate feature of the cells or not, this synchronised behaviour is conserved throughout a range of experiments and even over ten passages of the same stock of cells, which alludes to the behaviour being an innate feature of the cells. Alternatively, although not considered to be the sole cause, it could be deemed that the synchronised behaviour is subject to a measurement system error. This notion is supported by observation of the behaviour across independent culture trains. However, different characterisation systems (cell counting, immunophenotyping, gene analysis and metabolic analysis) with different manipulation techniques in terms of sample handling were used. Therefore, it is unlikely that measurement error could be seen in all techniques used, suggesting instead, that the synchronised behaviour observed is an effect of the cells themselves; especially since some of the genes also support the synched behaviour that is seen in the SGR and cell viability. There is no difference in fold change between the two routes, and yet there were significant fold change differences between the passages for *DNTM3B, NANOG* and *SOX2* within each route. Conversely, there is no difference in gene regulation between the two routes for *DPPA4, POU5F1* and *REX1*, which all had consistently similar levels between routes and between passages (**Figure 33).** The high levels of consistency within the routes throughout the passages demonstrates that when a defined protocol is followed, less variation during cell culture is observed.

The notably higher SMR for route A2 (Experiment 4) was an unexpected observation as this disparity in SMR has not been previously seen to the same magnitude. This behaviour was only observed in experiment 4, 4.1 and 5, suggesting that the change in SMR is due to the cells being cultured on the streamlined protocol without medium exchange. Implying that there is an ability to affect cell SMR and phenotypic marker expression by the culture conditions i.e. nutrient availability based on feeding. This effect is observed to be reversible both in terms of SMR and the phenotypic markers that are expressed. The change in expression levels of SSEA-1 and OCT3/4 shows that culture conditions do have an influence on marker expression in a reversible manner. It is interesting to observe that changing the culture environment conditions by manipulating nutrient levels, had such a significant impact on the cells even if they had been cultured under certain conditions in previous several passages.

The change in expression levels of SSEA-1 was instantly noted following the first passage, illustrating that the culture feeding regime, can impact the metabolic behaviour of the cells and phenotypic marker expression (**Figure 33C** and **D**), but not necessarily the growth performance of the cells (**Figure 33A**). This implies that the cause of the different SMR is due to the lack of medium exchange following 48 h. This is substantiated by experiment 5, as conditions not subjected to medium exchange had higher

SMRs compared to the cells that were subjected to medium exchange. Evidenced through experiment 5 were the differences between the routes at the three passages, the most significant differences are indicated (**Figure 38B**), other significant differences are reported in **Table 22**. This highlights that differences in culture protocol based on seeding density, feeding regime or both parameters result in notable SMR variation within the same stock of cells cultured simultaneously. The highest significant differences between routes, within passages, were observed when both parameters were changed e.g. C2 *vs*. C3, where both the seeding density and feed regime are changed.

As the EC 2102Ep cells are destined as a reference line, it was assumed that there would be stability in the characterisation of the cells. In experiments 3.3, 3.4, 4 and 5 there were clear fluctuations in the expression of the OCT3/4 marker, implying that not only do the cells cycle in their SMR rate but also their phenotypic profile. However, it seems that the fluctuation can be exacerbated by the culture conditions. This was the case in conditions where 20,000 cells/cm^2 were seeded; higher fluctuations were observed compared to the other conditions in the previous experiments. It is evident that the cells within both routes were in a linked state of cycling in terms of the metabolic rate and the phenotypic expression of OCT3/4. However, the cycling between the SMR and marker expression, are independent to each other, as the cycles are observed at different passage points. Over the ten passages some unexpected behaviours were observed; of interest is the presence of SSEA-1 which is a negative marker for pluripotent stem cells and the decrease in expression of the positive marker OCT3/4. The presence of SSEA-1 and the significant decrease in Oct-3/4 (declined by up to 52% in route A2 passage cycle 7) implies that the cells are differentiating (**Figure 33E** and **Figure 34**) [243,244,254]. The significant differences in gene expression are observed at passage 7 and/or 9, which are the same passage cycles that demonstrate notable differences in phenotypic marker expression when cross validated through flow cytometry, particularly OCT3/4. It is unclear what causes the change in behaviour, in either cell SMR or marker expression[246,255,256].

All the experiments produced results that presented unexpected behaviours, of particular interest is the presence of SSEA-1 which is a negative marker for pluripotent stem cells and the decrease in expression of the positive marker OCT3/4 (up to 52% in route 2 E.P7). These results imply that the cells were differentiating which would not be expected as EC 2102Ep cells are a carcinoma cell line [243]. However, Andrews (1982) reports that when cells are seeded at lower densities they have a propensity to differentiate although this not substantiated by analytical evidence of cell differentiation assays[244,257]. However, it is still unclear as to what causes the change in behaviour, in either cell growth or marker expression.

4.4.1. Rationalisations:

The phenotypic change that was been observed in the experiments could be attributed to cell differentiation. This a possible effect of the culture conditions, the literature regarding embryonic and pluripotent cells suggests that seeding at low densities causes differentiation, much like the conditions that the cells were cultured under[256–258]. The gene expression fold changes observed for key pluripotency markers *DNTM3B* and *SOX2* suggest that the cells are differentiating. Furthermore, the experimental data shows evidence of higher metabolite rate and phenotypic change, which is often concomitant with cell differentiation and/or the presence of a different cell population. However, there is evidence within the experiments that is contrary to the cells differentiating, suggesting it is the protocol parameters i.e. medium exchange which cause variation. For instance, no morphological change was observed under light microscopy throughout the ten passages; additionally, NucleoCounter cell analysis showed no significant change in the measured cell diameters between the two routes (**Figure 33C**) and even cultures seeded at higher densities exhibit levels of phenotypic change.

Another proposed explanation of the observed phenotypic variation is that the change is linked to cell death. This rational is reinforced by the fact that the phenotypic change is not cumulative over time, suggesting that the expression presents just before the cells start to die, resulting in a non-cumulative expression of SSEA-1. However, cell viability and SGR data show evidence that is contrary to this, as cells in route A2 have on average, higher cell viability and growth rate which would not be expected with cell death **(Figure 33A** and **B**). Furthermore, at passage cycle 7 there is a drastic decrease in OCT3/4 yet in the next passage there is no significant decrease in cell number or cell viability.

Density had been reported to be a crucial factor in some cell culture protocols, particularly those using embryonic cell lines, as it is reported to have an influence on metabolic behaviour and both directed and spontaneous differentiation[87,125,257,259,260]. In experiment 4 the observed differences in behaviour could be attributed to the seeding densities used, since this was the main parameter that was altered between the two routes. Nonetheless, density is unlikely to be the only driving force, since the initial experiments showed no drastic difference in SMR and growth based on density (**Figure 29** and **Figure 30***)*. Furthermore, the results from experiment 5 demonstrated that growth medium exchange has a significant effect on the SMR on the cells regardless of density, implying that cell metabolism based on feeding regime had a notable impact on SMR and marker expression as well as seeding density.

An alternative rationalisation that has been considered is that the phenotypic change is linked to a cell ‘selection’ or ‘survival’ mode, which has no observed instances of support in the literature. It can be suggested that the cells are conditioned to metabolise nutrients differently under the excess nutrient

conditions which results in the marker expression change. Route A2 appears to be a favourable condition as the cells had higher cell viability and better growth performance. Therefore, it is unclear as to why the cells would default to selection or survival mode as they are in an optimal cell time range; thus, they should not experience any starvation or stress. Moreover, there is no observed response to excess nutrients in the metabolite data in experiments 3.1 to 3.4. Furthermore, if phenotypic change is a response to nutrient levels it would be expected that route A1 would also exhibit drastic changes in marker expression and/or SMR rate as fewer nutrients per cell were available in comparison to route A2 (due to a higher cell density in route A1). However, it is unclear if the feed/no feed regime is a potential driving force causing the observed change as no metabolic data is available until the passage day, resulting in unknown behaviour trends on the intermediate days of route A2. Furthermore, if the phenotypic change is linked to a cell 'selection' or 'survival' mode the most drastic change in marker expression during, E.P7 48.6 % (OCT3/4) and 6.5 % (SSEA-1), it would be expected to have occurred when the cells were performing at their best. This is not the case as the drastic change does not occur when the cells are at their highest rate of metabolism or growth rate.

The above rationalisations highlight that even in a cell line that is considered to be a stable reference point; differences can be observed due to changes in parameters and conditions, over time. These different conditions such as when to perform medium exchanges and the use of seeding densities that vary i.e. through the use non-standardised seeding densities or split ratios, are often left to the user's discretion in many protocols. Evidently, this results in noteworthy effects on cell behaviour and characterisation outcomes. Split ratios are not best practice as innately cells will grow differently from passage to passage. For example, a split ratio of 1:3 can be drastically different from passage to passage, particularly if the cells grow at significantly different rates at different passages, which is a concomitant feature of cells that are revived from cryopreservation. In addition, split ratios that are based on observed confluency, are also likely to cause variation in cell growth dynamics. This is due to the subjective nature of observed confluency and its lack of accurate representation of cell number, therefore potentially resulting in non-ideal metabolite profiles that can influence cell growth behaviour, producing further cell system inconsistency. Differing levels of variability and non-conformity are detrimental to the successful use of reference cell lines, especially for those intended to be a standardised QC reference[247,261]. This is of relevant importance as the use of reference cell line is likely to see prominence due to the increase in assays and product validation. The advent of chimeric antigen receptor T-cell (CAR-T) therapies, could potentially use reference cell lines as QC reference standards for flow cytometry. Interestingly, flow cytometry analysis revealed marker profile differences; even when the cells were at the prescribed density of 66,667 cells/cm^2, showing that even when cultured in a consistent manner there is inherent variation on marker profile through the ten passages.

This further emphasises the need to standardise parameters within protocols to minimise and control variation. This can be achieved by obtaining cell counts, ensuring that the input and output cells numbers are used to maintain optimal culture conditions for cell growth, without entering regions of metabolic instability. Consequently, this allows for greater control and consistency of the culture system as the seeding density and nutrient levels are predefined. The use of cell time, which is a concept that can be used to quantify the capability of a given volume of medium to sustain the growth of a given number of cells for a specific period, ensures that the culture system given the set density and nutrient availability will not enter a region of metabolic strain. This is important since imbalances in glucose and lactate levels have previously been shown to result in limitation of cell growth[241,260] and importantly for reference lines, metabolite imbalances have been shown to impact the stability of marker expression. Metabolic strain is potentially problematic if not controlled, as reference lines are used for QC of CTPs need to demonstrate their stability over time, using gene and phenotype marker expression.

4.5. Conclusions

This chapter has illustrated that cell system behaviour is affected by culture conditions; experiments 4 and 5 demonstrate that feeding regimes (medium exchange) also have a significant effect on SMR and phenotypic marker expression when compared to seeding density. This highlights that differences in culture parameters do cause variation, however, it is evident that when parameters are kept consistent less variation is observed. The initial experiments (Experiment 1 and 2) show that there is no significant difference in SGR between different seeding densities when feeding regime is kept consistent throughout the culture period. It has been demonstrated through a range of experiments that seeding density is not the sole factor that can cause variation especially when the flasks are seeded at the same density. Instead it is clear that other protocol parameter themselves or when compounded with seeding density result in variation, hence the need to ensure stringent control of protocol parameters. The present work has demonstrated that seeding density and the feeding regime employed, in terms of both the volume and frequency of medium exchange, have a major effect on cell culture variation. Lower densities have been shown to have distinctly different behaviours compared to higher density cultures in terms of their SMR. A combinatory effect of seeding density and feeding regime has been shown to cause variation in SGR, SMR, phenotype and expression of some genes. However, it is unclear under what mechanism medium exchange or lack of medium exchange influences cell characteristics such as SMR and marker expression. The present work demonstrates that culture conditions have an impact on cell characteristics, notably on specific metabolic rate and phenotypic marker expression. Thus, demonstrating that there is a complex interaction between gene expression, cell phenotype and the feeding regime that cannot be accurately represented by growth rate and cell counts alone. It is worth noting that the cells do not retain a detrimental cell metabolic state due to culture conditions as they can readily switch between culture condition dependent behaviours even after several passages under a certain condition, as shown in the reversal experiment (4.1).

The lack of robust, well-defined and standardised cell-culture protocols results in compounded variation in the culture systems. This is due to differences in inter-lab and individual decision-making processes based upon protocol parameters, such as observed cell confluency as a gauge of when to perform a cell passage. This highlights that use of non-standardised, ambiguous protocols can easily produce differences over time, further illustrating the need for standardised protocols not only from an uniformity point of view but also in terms of reproducibility consideration; which is integral to the pragmatic and successful use of reference cell lines in cell therapy manufacturing.

4.6. Technique optimisation and knowledge acquired from EC 2102Ep work

The work carried out in this chapter has highlighted that it is important to have an in-depth understanding of what the cells are doing within a cell culture system in order to have better-informed process control. Carrying out growth curve experiments both high resolution (everyday analysis such as in experiment 1& 2) and longitudinal provides information about how the cells grow and react to different conditions and how consistent their behaviour is at different time points. The EC 2102Ep experiments carried out have provided a framework that will be used in the following experimental chapters to explore the behaviours of the H9 cell line.

The matrix experiments (3.1 to 3.4 and 5) acted as screening studies to investigate the effect of different parameters within the cell culture systems. This will be a methodology used in the following chapters through design of experiment (DoE) approaches to assess the patterning of the H9 cells into vmDA neuroprogenitor cells. Akin to experiments in this chapter different conditions looking at varying concentrations of the important small molecules will be investigated. During the expansion phase of the pluripotent H9 cells, the feeding regime will be investigated as it had been shown here that different feeding regimes can be employed without having a detrimental effect on cell characteristics. Optimisation of the feeding regime of the pluripotent H9 cells, currently subjected to 100 % medium exchange everyday of culture, would be beneficial as reducing the feed days would subsequently reduce the overall resources and labour required. Thus, lowering the associated production costs which is favourable for the commercial viability of the product.

The work carried out in this chapter has also provided an opportunity to optimise techniques prior to carrying out the costlier and resource demanding work on the H9 clinically relevant cell line. The cell counting strategy used was developed within this chapter and has been standardised for use in the following experimental chapters, details of the counting procedure can be found in the materials and methods chapter section 3.3.2.

As the nature of this chapter became highly focused on standardisation, it was clear that the sample preparation and procedure used during analysis would need to be standardised as well, in order to minimise variation within the results obtained. Therefore, storage time periods following fixation prior to flow cytometry analysis were kept the same. In some cases, it was observed that sampled stored for more than 48 h post fixation did not pellet correctly resulting in sample loss. As a result, all flow cytometry data report in this chapter was obtained using cells analysed after no more than 24 h post fixation.

4.7. Chapter Bridge

This work has provided a strong basis to carry out work on the clinically relevant and destined cell lines. It is evident that there are a wide range of parameters that are often over looked that can influence the behaviour of the cells e.g. cell feeding regimes. Although it might not be possible to control them all, it is important that they are monitored to ensure they do not cause any significant changes to the cell product or reference cell line. The experiments on the EC 2102Ep have allowed for experimental designs to be established and for optimisation of techniques that will be used on the clinically relevant cell lines. The next chapter will focus on the process understanding of the H9 protocol, as evidenced in this chapter, protocol consistency is crucial within a cell-based process. Therefore, understanding the protocol is a key element that must be achieved prior to any optimisation and development work being carried out, in order to provide the robust consistency needed for a viable product.

Chapter 5:

Investigation of the Pluripotent State of H9 Human Embryonic Stem Cells

Chapter 5. Investigation of the pluripotent state of H9 human embryonic stem cells

5.1. Introduction

The focus of this chapter is on the expansion of the H9 pluripotent cells which are the input cell material for differentiation into ventral mesencephalic dopaminergic (vmDA) neuroprogenitors. The H9 cell line is a human embryonic stem cell line obtained from the WiCell Research Institute (Madison, USA). The H9 cell line was selected due to it being a clinically relevant cell line that can be used for vmDA differentiation for a Parkinson's disease (PD) cell therapy product. Understanding the culture dynamics of the pluripotent cells has been deemed important to the overall differentiation protocol, as input cell material can have an impact on the output cells[158,262]. The work in the previous chapter illustrates that expansion of embryonal cells is subject to variation, some of which can be controlled and measured *via* the implementation of robust and standardised cell culture protocols. Using the knowledge obtained from the EC 2102Ep cell line (Chapter 4), the work in this chapter aimed to understand the culture dynamics of the H9 cells in their pluripotent state, to produce a standardised protocol that can be used for the expansion phase prior to vmDA differentiation. The relatively low seeding densities (10,000 – 30,000 cells/cm^2) required to seed the cells for differentiation resulted in low levels of expansion prior to differentiation. Typically, one well of a 6-well tissue culture microtiter plate seeded at 20,000 cells/cm^2 (~177,000 cells per well) could yield an average of 1,600,000 cells per well (180,000 cells/cm^2), affording an almost ten-fold increase in cell number over three days of culture. The resulting pluripotent cells of each well can then be used to seed a further eight to nine well for differentiation, thus highly efficient in terms of cell usage. As such, the actual cell number yield was not a priority for optimisation, instead the consistency of obtaining the cell number yield, cell viability, cell phenotype and cell metabolic profile was the focus of the process optimisation of the present work.

The Lund protocol stated that the differentiation process should be started with "good-quality starting material[129]. However, there is no quantifiable data related to what this explicitly refers to. At most the protocol states that "*Before the differentiation is started, make sure that the hPSC [human pluripotent stem cell] colonies appear pluripotent by visual criteria (i.e., homogeneous-appearing colonies with clear borders and the absence of obvious differentiating zones). If you are using a hPSC culture system other than the one described here, manual removal of spontaneously differentiated colonies might be required. A desirable confluency of hPSC cultures is 70–90% of the well area at the start of differentiation*"[129]. The description above is open to individual interpretation making it highly subjective, non-standardisable and ultimately prone to operator variation. This is problematic for standardisation of manufacturing protocols as the lack of defined parameters and procedures ultimately

results in product variation; if this variation is out of the specification range, it can nullify batches, hinder product release and waste financial and labour resources.

As such the work in this chapter aimed to carry out characterisation studies that can provide information on what 'good-quality' starting cell material should be for the differentiation process. The optimisation work carried out in this chapter was carried out after the process/protocol had been transferred from the collaborators at Lund University. Using methods employed in the previous chapter (Chapter 4) such as metabolite analysis, flow cytometry and cell growth analysis, the present work aimed to produce a robust and informed understanding of H9 pluripotent cell behaviour. The ideal objective was to provide metrics including the minimum cell viability levels, specific growth rate (SGR) and levels of phenotypic markers such as Ki67 and OCT3/4, that are needed to qualify a pluripotent expansion culture as a source of 'good-quality' starting material. Adding these metrics would allow for quantifiable benchmarks that can be used to standardise the expansion protocol, which in turn would result in standardised input cell material for the differentiation process into vmDA neuroprogenitors.

5.2. Methods

5.2.1. H9 bank setup

A vial of H9 cells (P5) was thawed and seeded onto a Biolaminin-521 coated T25 cm^2 tissue culture flask as described in section 3.2.4.2. The cells were cultured for a longer period of time (eight days for the first passage and six days for the second passage) as they were being adapted for growth onto Biolaminin-521 from Matrigel (Corning, USA, cat#354320). Following two passages the cells were seeded into three T75 cm^2 tissue culture flasks which resulted in a total of 54 $x10^6$ cells following one passage (the third passage post thaw of the original vial); these were cryopreserved at a density of $1x10^6$ cells/ml producing a working bank with a total of 54 cryovials at P8.

5.2.2. H9 bank analysis

A single vial of P8 cells from the H9 bank created above was thawed and seeded onto a Biolaminin-521 coated T25 cm^2 tissue culture flask. The cells are cultured for two passages at a density of 10,000 cell/cm^2. At passage 10 a total of fifteen T25 cm^2 tissue culture flasks were seeded at a density of 10,000 cells/cm^2. Over five days, three of the flasks were harvested, counted and had their medium sampled, resulting in n=3 for the conditions harvested on each day. Following five days the remaining three flasks were passaged and used to seed an additional fifteen flasks which were treated as described above. Cells from passage 10 were sent for karyotype analysis at a third-party institution (TDL Genetics, London, UK).

5.2.3. Expansion: cell bank variability study

Three vials of P8 cells were thawed and seeded at two densities (10,000 and 20,000 cells/cm^2) onto Biolaminin-521 coated 6-well tissue culture microtiter plates. The cells were cultured for two four-day passages and analysed for their viability, SGR, SMR and phenotype at the end of each passage; using panel 1 of the Miltenyi protocol. The experiment was carried out in triplicate for each vial at each of the three densities (see **Figure 39**).

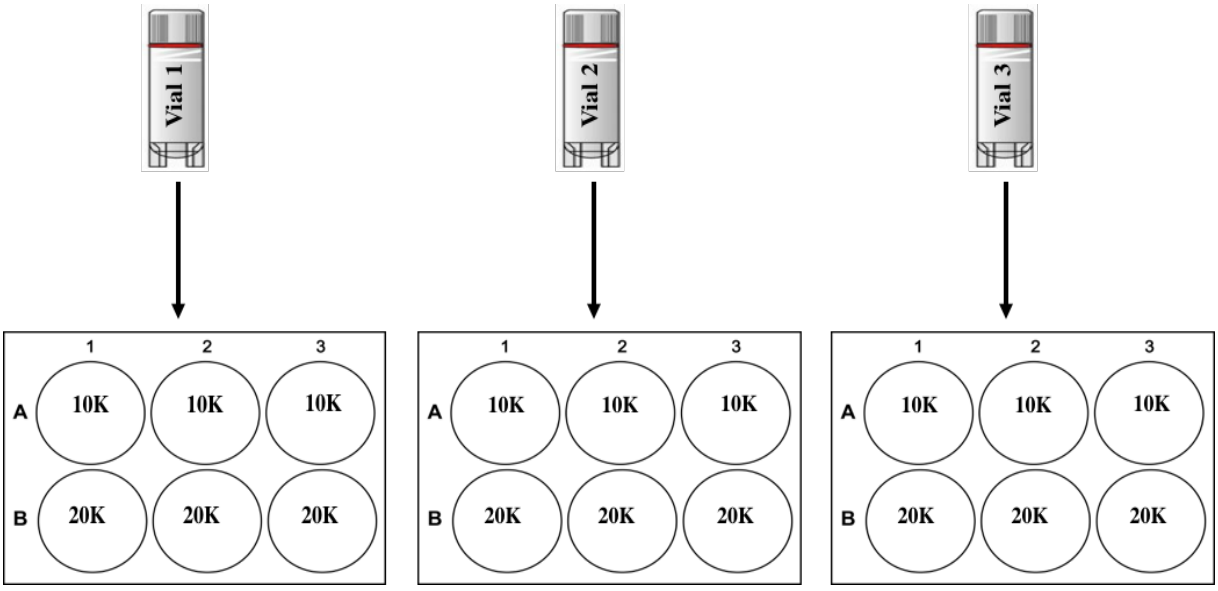


Figure 39. Schematic of the experimental design used to assess the reproducibility and variability of vials from the H9 bank of cells. Each of the three vials was thawed and seeded at 2 densities 10,000 cells/cm^2 (10K) and 20,000 cells/cm^2 (20K) and cultured for two passages: n=3 for each condition.

5.2.4. Expansion: the effect of ROCK inhibitor on attachment and proliferation

One vial of P8 cells was thawed and seeded at 20,000 cells/cm^2 into a Biolaminin-521 coated T25cm^2 flask. The cells were cultured for one passage and seeded onto Biolaminin-521 coated xCelligence E-Plate® 96-well microtiter plates at two densities; 10,000 and 20,000 cells/cm^2. The cells were cultured for three days at the two densities, using four different concentrations of ROCK inhibitor (ROCK*i*), as detailed in the plate layout (**Figure 40**). The xCelligence was scheduled to run sweeps and measure the Cell Index (adhesion/proliferation) as detailed in **Table 23** over the three-day passage.

	1	2	3	4	5	6	7	8	9	10	11	12
A			0 µM	0 µM	0 µM	0 µM	0 µM	0 µM				
B			5 µM	5 µM	5 µM	5 µM	5 µM	5 µM				
C			10 µM	10 µM	10 µM	10 µM	10 µM	10 µM				
D			15 µM	15 µM	15 µM	15 µM	15 µM	15 µM				
E			0 µM	0 µM	0 µM	0 µM	0 µM	0 µM				
F			5 µM	5 µM	5 µM	5 µM	5 µM	5 µM				
G			10 µM	10 µM	10 µM	10 µM	10 µM	10 µM				
H			15 µM	15 µM	15 µM	15 µM	15 µM	15 µM				

10,000 cells/cm^2

20,000 cells/cm^2

Figure 40. xCelligence® RTCA E-Plate® layout. Two densities, 10,000 and 20,000 cells/cm^2, were seeded onto E-Plate® 96-well microtiter plates with medium containing four different concentrations of ROCK*i* ranging from 0 to 15 µM: *n*=6 for each condition used.

Table 23. xCelligence® RTCA schedule used to measure the Cell Index.

Sequence	Step	Sweeps	Interval Time (mins)	Total Time (hh:mm:ss)
1	BACKGROUND	1	1	00:00:17
2	ADHESION	100	2	04:39:07
3	PROLIFERATION	30	60	21:25:52
4	DAY 1 POST FEED	100	2	24:44:11
5	DAY 1 PROLIFERATION	60	30	46:13:02
6	DAY 2 POST FEED	100	2	49:31:20
7	DAY 2 PROLIFERATION	60	30	70:43:57
8	DAY 3 POST FEED	100	2	74:02:09
9	DAY 3 PROLIFERATION	60	30	104:11:28

5.2.5. Expansion: the effect of cell seeding density

Seven P8 vials from the H9 bank were thawed and seeded at three densities (10,000; 20,000 and 30,000 cells/cm^2) onto Biolaminin-521 coated 6-well tissue culture microtiter plates (**Figure 41**). The cells were cultured for a single four-day passage and analysed for their viability and SGR on day 2 and day 4 of the passage. Cell phenotype was analysed at the end of the passage (day 4); using panel 1 of the Miltenyi protocol. The experiment was carried out in triplicate for each of the three densities.

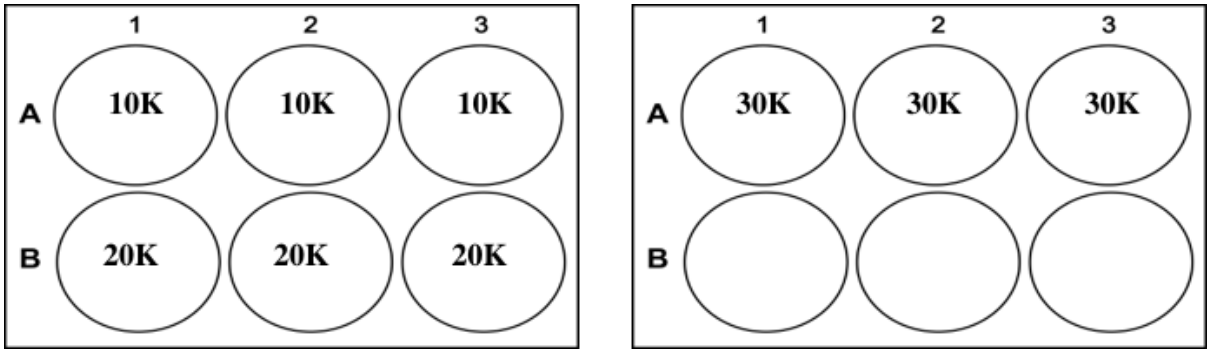


Figure 41. Schematic of the experimental setup used to determine the growth curve of three different seeding densities: 10,000 cells/cm^2 (10K); 20,000 cells/cm^2 (20K) and 30,000 cells/cm^2 (30K). *n*=3 for each condition was used.

5.2.6. Expansion: the effect of feeding regimes

One vial of P8 cells from the H9 bank were thawed and seeded at 20,000 cells/cm^2 onto Biolaminin-521 coated T-25cm^2 tissue culture flask and cultured for one passage. To investigate the effect of different feeding regimes the cells were seeded into four different routes of culture as detailed in **Table 24** and cultured for two passages in triplicate for each route condition. The P9 cells were harvested and reseeded at 20,000 cells/cm^2 onto Biolaminin-521 coated 6-well tissue culture microtiter plates. The cells were analysed for their viability, SGR, SMR and phenotype at the end of each passage; using panel 1 of the Miltenyi protocol in the first run of the experiment.

For the second run of the experiment, sacrificial wells for each of the four routes were seeded directly from thaw for daily harvests, the experiment was carried out in triplicate for each route. The cells were analysed daily for their viability, SGR and SMR: cell phenotype was analysed at the end of each passage; using panel 1 of the Miltenyi protocol. Four T25 cm^2 tissue culture flasks were also seeded at 20,000 cells/cm^2 and cultured in parallel to the four routes in the 6-well tissue culture microtiter plates, the cells from the flasks were used to seed cells for the second passage.

Table 24. Details of the different experimental culture routes investigated. **Control** was subjected to M. Ex daily; **route 1** was subjected to M. Ex on day 1 only**; route 2** on day 2 only and **route 3** was not subjected to a M. Ex throughout the passage. *n*=3 for each condition was used. M. Ex = medium exchange

Route	Day 0	Day 1	Day 2	Day 3	Summary
Control	Seed	M. Ex	M. Ex	Passage/Harvest	Daily M. Ex
1	Seed	M. Ex		Passage/Harvest	M. Ex on day 1 only
2	Seed		M. Ex	Passage/Harvest	M. Ex on day 2 only
3	Seed			Passage/Harvest	No M. Ex

5.2.7. Expansion: the effect of density and medium exhaustion

To investigate the effect of density and nutrient availability on cell growth inhibition, two conditions of culture were set up for a seven-day passage. The daily feed (DF) condition was subjected to medium exchange every 24 hours, while the one feed (OF) condition was only subjected to one medium exchange 24 hours following seeding (**Table 25**). Three vials of P8 cells were thawed and seeded at 20,000 cells/cm^2 onto Biolaminin-521 coated 6-well tissue culture microtiter plates. Sacrificial wells for each condition were seeded for daily harvests, the experiment was performed in triplicate for both conditions. The cells were analysed daily for their viability, SGR and SMR, cell phenotype was analysed on day 0, 6 and 7; using panel 1 of the Miltenyi protocol.

Table 25. Details of the two conditions used to investigate density and nutrient availability-based growth inhibition. The DF condition was subjected to M. Ex daily, while the OF condition was subjected to M. Ex on day 1 only. Cells were harvested and counted everyday: *n*=3 for each condition was used. M. Ex = medium exchange; DF = daily feed; OF = one feed.

Route	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Daily Feed	Seed	M. Ex	M. Ex	M. Ex	M. Ex	M. Ex	M. Ex	Endpoint harvest
One Feed	Seed	M. Ex						Endpoint harvest

5.3. Results

Initial efforts were focussed on creating a working bank of H9 cells, these cells were assessed for karyotype abnormalities, none were found *via* g-band analysis to stain the chromosomes. The growth and metabolic profiles of the cells were analysed to determine appropriate seeding densities, duration of culture period and optimal feeding regime to be employed for the H9 pluripotent cell expansion.

N.B. For the purposes of discussion, passages are referred to as; passage 1 or passage 2, while in fact it is passage 8 + *n*, i.e. passage 1 corresponds to cells at passage 9, passage 2 to cells at passage 10 and so on; unless otherwise stated.

5.3.1. Analysis of the bank:

The SGR values in passage 1 were either negative or low for the first two days. However, an increase in SGR from day 2 onwards was observed (**Figure 42A**). The cell viabilities at passage 1 were lower in comparison to passage 2 which had the lowest viability recorded to be 82% ±1.3 in comparison 70% ±2.1 at passage 1, day 2 (**Figure 42B**). A trend can be seen in the glucose SMR as the cells reduce their SMR as they increase in number over the five-day culture period (**Figure 42C**). The results from the karyotype analysis demonstrated that the cells had no karyotype abnormalities (**Figure 43**).

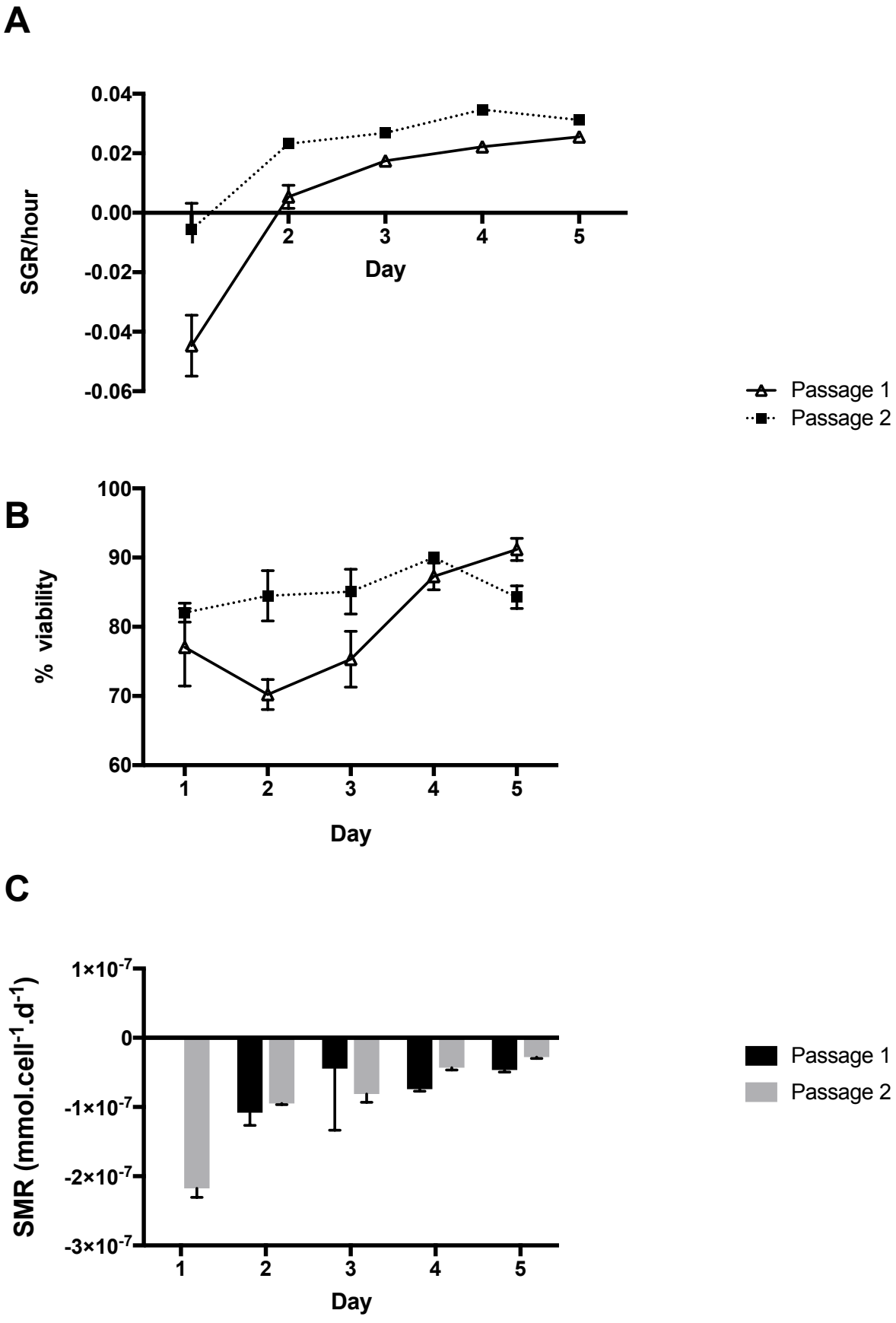

A
0.04
0.02
0.00
-0.02
-0.04
-0.06
SGR/hour
2
3
4
5
Day
Passage 1
Passage 2
B
100
90
80
70
60
% viability
1
2
3
4
5
Day
C
1×10-7
0
-1×10-7
-2×10-7
-3×10-7
SMR (mmol.cell-1.d-1)
1
2
3
4
5
Day
Passage 1
Passage 2

Figure 42. (**A**) Data was collected everyday over two five-day passages. SGR is shown to increase each day, with the greatest increases from day 1 to day 2. Cells at passage 2 started to decrease their SGR from day 4 while at passage 1 the cells still had an increase in SGR from day 4 to day 5. There is a significant difference overall in SGR between passage 1 and passage 2 on all of the days except day 5 $p<0.0001$ (****). (**B**) Overall, passage 2 cells had significantly higher cell viabilities that eventually dropped at day 4 ($p<0.0001$ (****), while passage 1 cells continue to increase in cell viability from day 2 to day 5. (**C**) Glucose SMR data of the cells throughout the five-day culture period of each passage. SMR is higher in passage 2 compared passage 1, however in both instances there was a general trend of a decrease in glucose SMR. As a result of the negative SGR on day 1 at passage 1, the SMR calculation for consumption was not carried out as it requires an increase in cell number, thus the data was omitted from the graph as it was erroneously showing production of glucose due to the negative SGR. Error bars indicate standard deviation, $n=6$ for SGR and cell viability data, $n=3$ for SMR data.

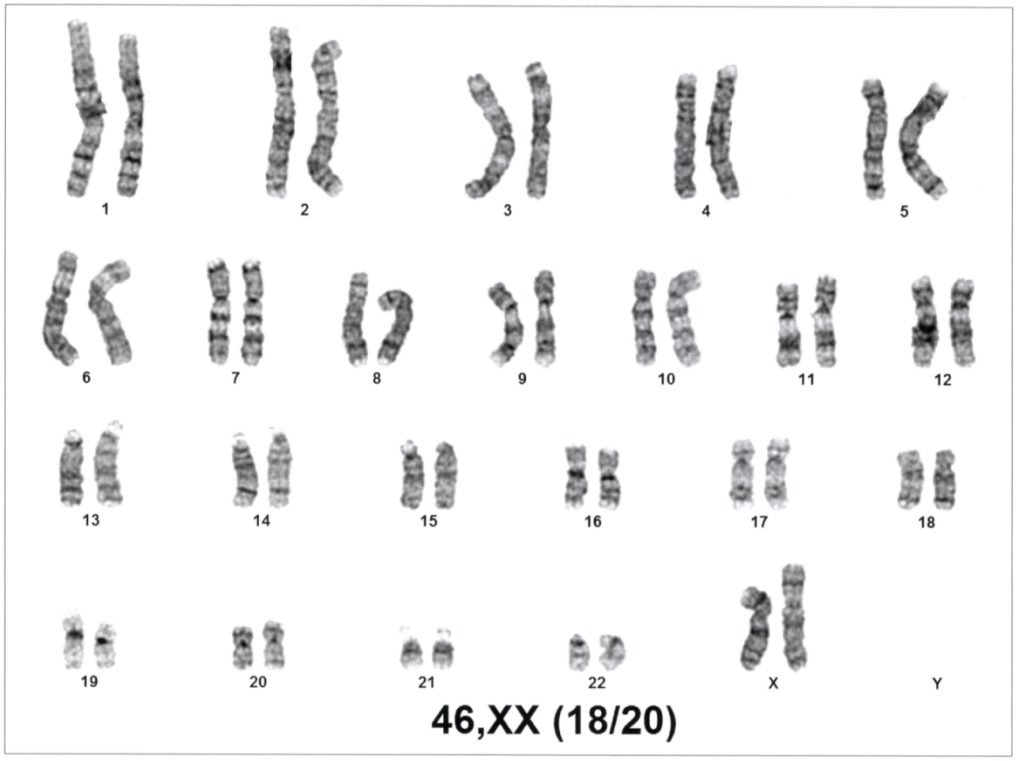


Figure 43. Karyotype analysis of H9 cells showing normal XX chromosomes, a sample of cells from passage 2 was used to produce this karyogram.

5.3.2. Expansion: cell bank variability study

The cell counts obtained prior to seeding the thawed vials demonstrated that there were differences in cell numbers banked and recovered from each vial, the cell numbers ranged from $8.9x10^5$ to $1.14x10^6$ cells (**Figure 45**). As expected, on the harvest days the cells seeded at 20,000 cells/cm^2 yielded greater cell numbers in comparison to their 10,000 cells/cm^2 counterparts. However, the cell number at the next passage was significantly higher but only in the 20,000 cells/cm^2 conditions and not in the 10,000 cells/cm^2 conditions as shown in **Figure 45C**. The SGR increased from passage 1 to passage 2 in all the conditions except for vial 1 at 10,000 cells/cm^2 (**Figure 45A**). Vial 1 and vial 3 both at 10,000 cells/cm^2 were the only conditions to have a significant drop in viability from passage 1 to 2, with the vial 1 condition dropping to as low as 45% ±1.9 cell viability in one of the biological replicates, 51.7 % ±5.3 on average (**Figure 45B**). The increased SGR from passage 1 to 2 was accompanied by an increase in LDH production SMR in all conditions except for the cells in vial 2 seeded at 20,000 cells/cm^2 (**Figure 44B**). Lactate SMR was only significantly different for cells in vial 1 at 10,000 cells/cm^2 (**Figure 44A**). The flow cytometry results demonstrated that there was a significant difference between the expression of Ki67 from passage 1 to passage 2 in all of the conditions as the median fluorescence intensity (MedFI) of Ki67 decreased (**Figure 46B** and **Table 26**). Similarly, there is a significant decrease in expression of OCT3/4 in all conditions, except for vial 3 at 20,000 cell/cm^2. However, PAX6 was lowly expressed in the cells at both passage 1 and passage 2
Figure 46C).

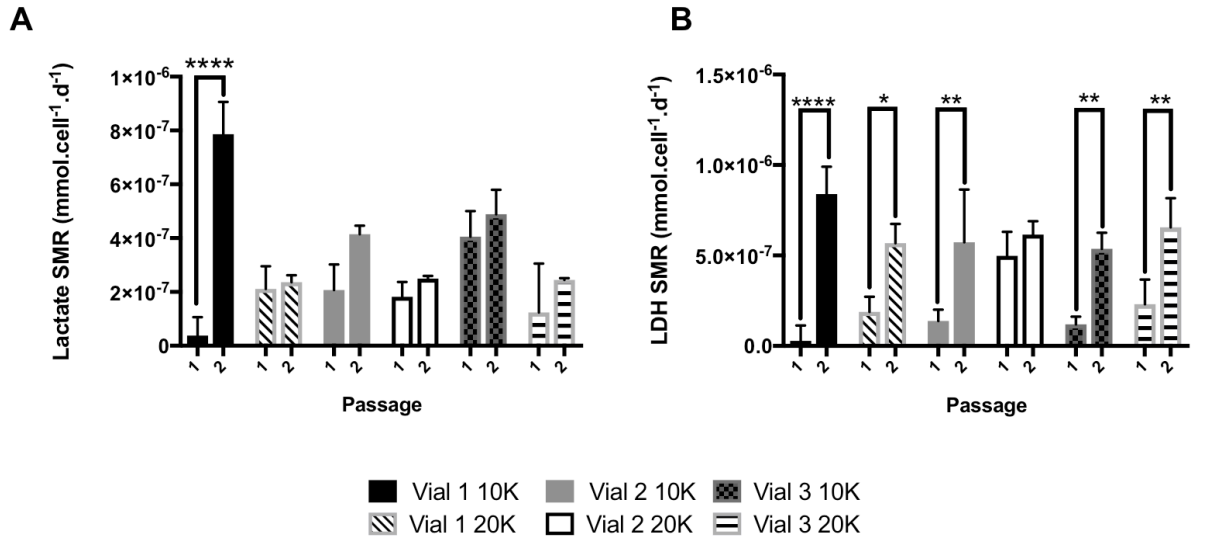


Figure 44. (**A**) Lactate and (**B**) LDH SMR data. Both lactate and LDH SMRs increased from passage 1 to passage 2. However, only vial 1 at 10,000 cells/cm^2 had a significant increase in lactate SMR, for LDH all conditions except vial 2 at 20,000 cells/cm^2 had a significant increase in LDH SMR. Error bars indicate standard deviation, *n*=3. "*" indicates p<0.05, "**" indicates p< 0.01, "***" indicates p< 0.001, and "****" indicates p<0.0001.

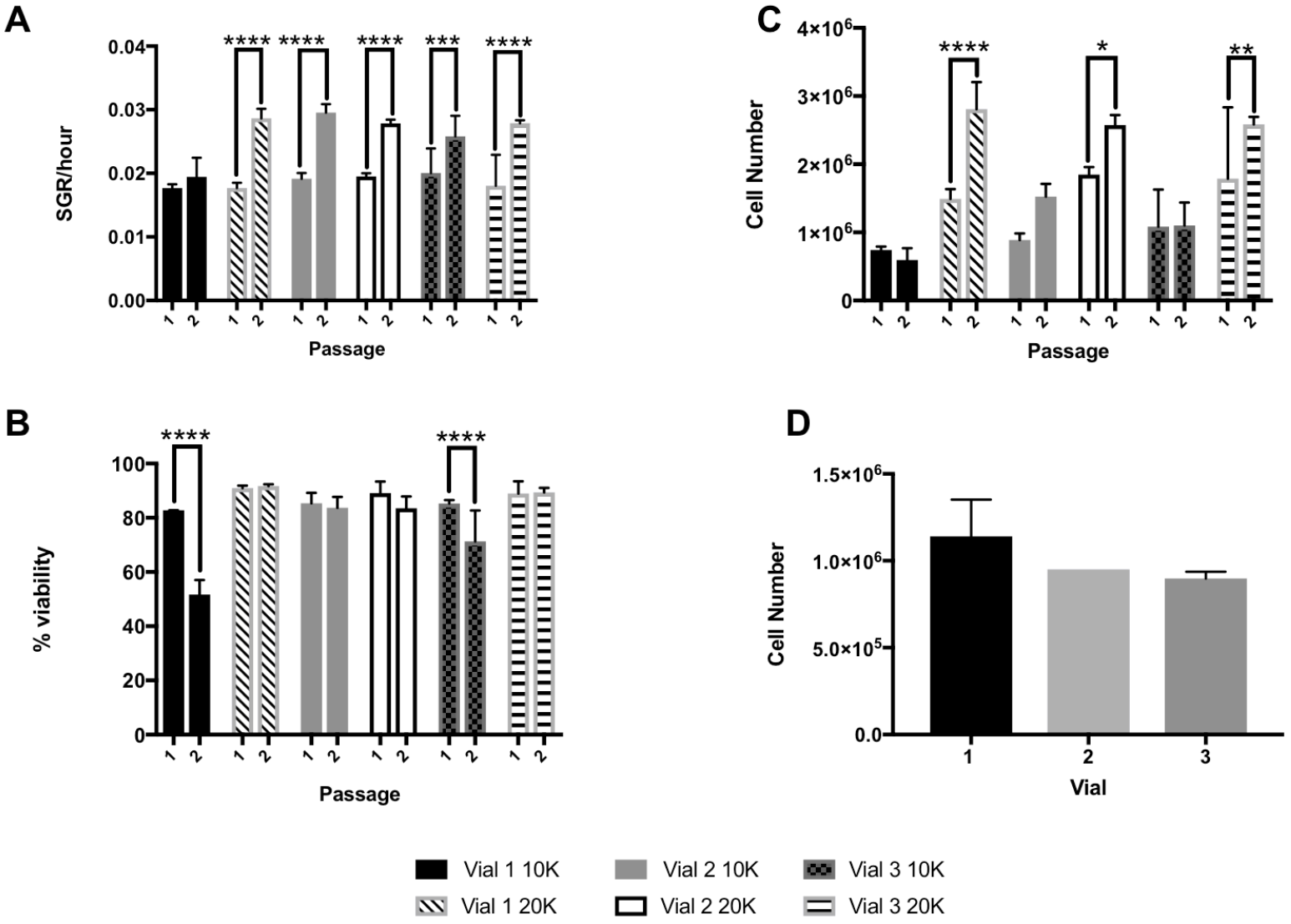

A
SGR/hour
0.04
0.03
0.02
0.01
0.00
****
****
****
***
****
Passage
B
% viability
100
80
60
40
20
0
****
****
Passage
C
Cell Number
4×10^6
3×10^6
2×10^6
1×10^6
0
****
*
**
Passage
D
Cell Number
1.5×10^6
1.0×10^6
5.0×10^5
0.0
1
2
3
Vial
Vial 1 10K
Vial 1 20K
Vial 2 10K
Vial 2 20K
Vial 3 10K
Vial 3 20K

Figure 45. Growth dynamics results. (**A**) SGR increases from passage 1 to passage 2, only vial 1 at 10,000 cells/cm^2 did not have a significant increase in SGR. (**B**) All of the 20,000 cells/cm^2 had a significant increase in cell number from passage 1 to passage 2. (**C**) Only vial 1 and vial 2, both at 10,000 cells/cm^2 had significant decreases in cell viability from passage 1 to passage 2. (**D**) vials 2 and 3 had similar cell numbers directly from being thawed after cryopreservation and before being cultured, however no difference was determined between the three vials. Error bars indicate standard deviation, n=6. "*" indicates $p<0.05$, "**" indicates $p< 0.01$, "***" indicates $p< 0.001$, and "****" indicates $p<0.0001$.

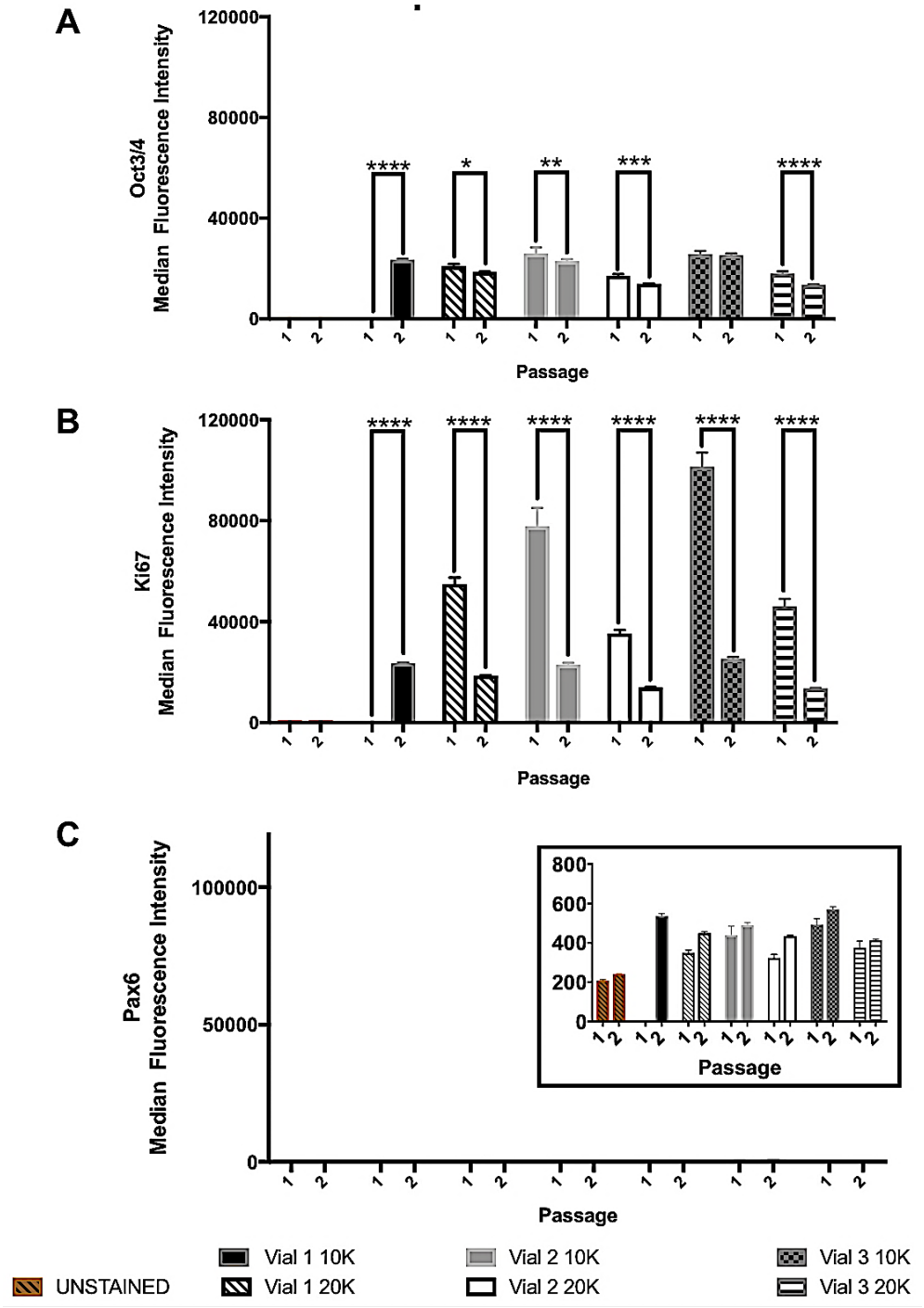


Figure 46. MedFI values for OCT3/4, Ki67 and PAX6, an insufficient number of cells to perform the phenotyping assay was obtained from the Vial 1 10K condition at passage 1, therefore no data was available. There is a decrease in MedFI from passage 1 to passage 2 in all conditions for both OCT3/4(**A**) and Ki67(**B**). PAX6 expression levels were low in comparison to OCT3/4 and Ki67, inset shows the very low levels of PAX6, all conditions are under 800 for their MedFI values (**C**). Error bars indicate standard deviation, *n*=3. "*" indicates p<0.05, "**" indicates p< 0.01, "***" indicates p< 0.001, and "****" indicates p<0.0001.

Table 26. Sidak's multiple comparisons test of the MedFI the two markers that were positively expressed by the pluripotent cells. The table indicates the conditions that were significantly different from passage 1 to passage 2.

	Oct3/4		Ki67	
Condition	**Summary**	**P value**	**Summary**	**P value**
Vial 1 10K	****	<0.0001	****	<0.0001
Vial 1 20K	*	0.02	****	<0.0001
Vial 2 10K	**	0.002	****	<0.0001
Vial 2 20K	***	0.0007	****	<0.0001
Vial 3 10K	ns	0.9976	****	<0.0001
Vial 3 20K	****	<0.0001	****	<0.0001

5.3.3. Expansion: the effect of ROCK*i* on attachment and proliferation

The results from the xCelligence readout revealed that the concentration with the highest Cell Index and therefore cell attachment was the 10 μM condition followed by 0 μM, 15 μM and then 5 μM, highest to lowest, respectively (**Figure 47**). When the readout was split into the two different densities, the 10,000 cells/cm^2 condition demonstrated that the 10 μM concentration results in the highest Cell Index. The results for 20,000 cells/cm^2 however show that irrespective on ROCK*i* concentration the cells are able to attach and proliferate, achieving a similar or higher Cell Index to those in the 10,000 cells/cm^2 conditions at equivalent ROCK*i* concentrations. For instance, the Cell Index for cells at 0 μM at 20,000 cells/cm^2 is higher in comparison to all the concentration at 10,000 cells/cm^2 (**Figure 48**).

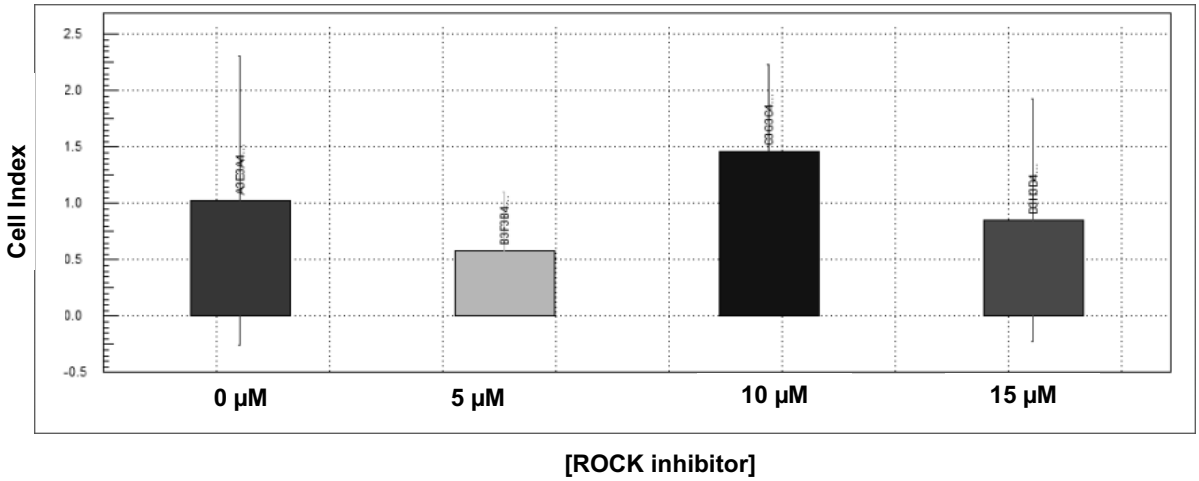


Figure 47. xCelligence® RTCA graphical summary of the Cell Index of values of the different ROCK*i* concentrations. The 10 µM condition had the highest overall Cell Index for the grouped data of both 10,000 cells/cm^2 and 20,000 cells/cm^2. Error bars indicate standard deviation, n=12.

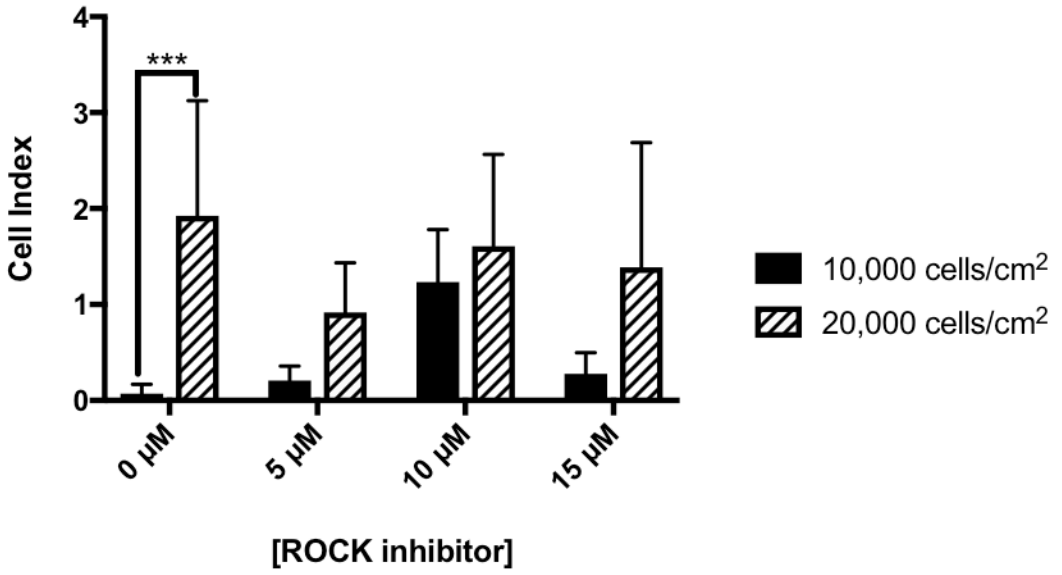


Figure 48. xCelligence® RTCA Cell Index of the two densities with four different concentrations of ROCKi. Only the 0 µM condition had a significant difference in Cell Index between 10,000 and 20,000 cells/cm^2. Overall, cells seeded at 20,000 cells/cm^2 had a higher Cell Index, across the four ROCKi concentrations that were investigated. Error bars indicate standard deviation, n=6. "*" indicates p<0.05, "**" indicates p< 0.01, "***" indicates p< 0.001, and "****" indicates p<0.0001.

5.2.4. Expansion: the effect of cell seeding density

There was no significant difference between the conditions in terms of their SGR from day 0 to 2. From day 2 to 4, there were significant differences in SGR when comparing the 10,000 cells/cm^2 condition to both the 20,000 and 30,000 cells/cm^2 conditions, respectively (**Figure 49A**). Furthermore, there was no significant difference in SGR overall between 20,000 and 30,000 cells/cm^2 at the harvest point on day 4. For each condition there was a significant increase in SGR from day 2 to day 4. In terms of cell number yield, only the 20,000 and 30,000 cells/cm^2 conditions had significant increases from day 0 to day 2 and day 2 to day 4 (**Figure 49B**). The cell viabilities across all conditions drops significantly from day 0 to 2 from ~80% to ~40 % and then increase from ~40 % back up to ~80 by day 4 (**Figure 49C**). The MedFI for Ki67 is the highest across the three markers analysed, there is expression of PAX6 observed in all three conditions and all conditions highly express OCT3/4 (**Figure 50A**).

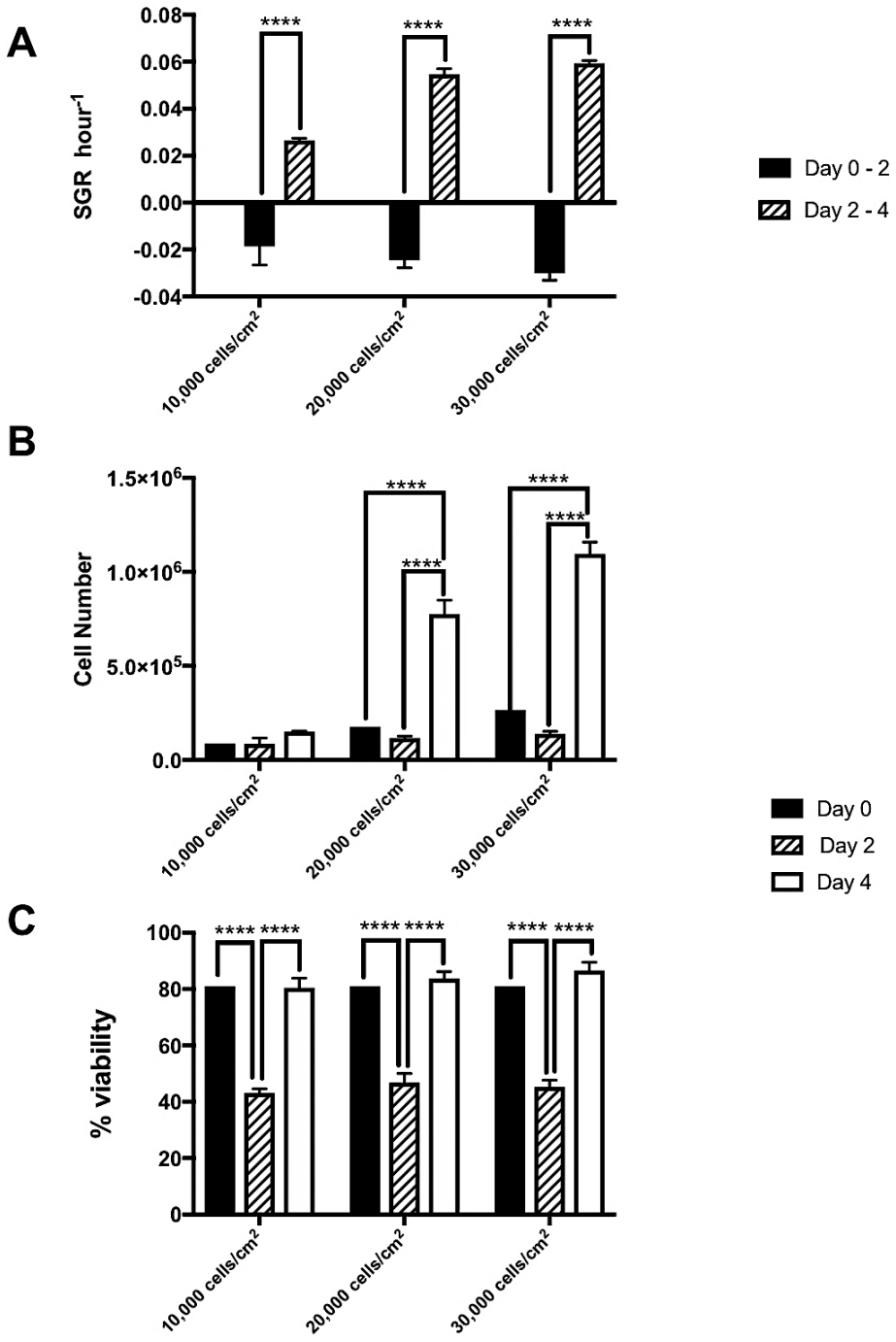


Figure 49. Growth dynamics. (**A**) SGR increased from negative SGR to positive SGR from day 2 to day 4 for all three densities. (**B**) Cell number decreased from day 0 to day 2 and then increased from day 2 to day 4, the 10,000 cell/cm^2 density does not have significant increase in cell number during the 4-day passage. (**C**) All three densities had a decrease in cell viability from day 0 to day 2 which increased from day 2 to day 4. Error bars indicate standard deviation, n=3. "*" indicates $p<0.05$, "**" indicates $p< 0.01$, "***" indicates $p< 0.001$, and "****" indicates $p<0.0001$

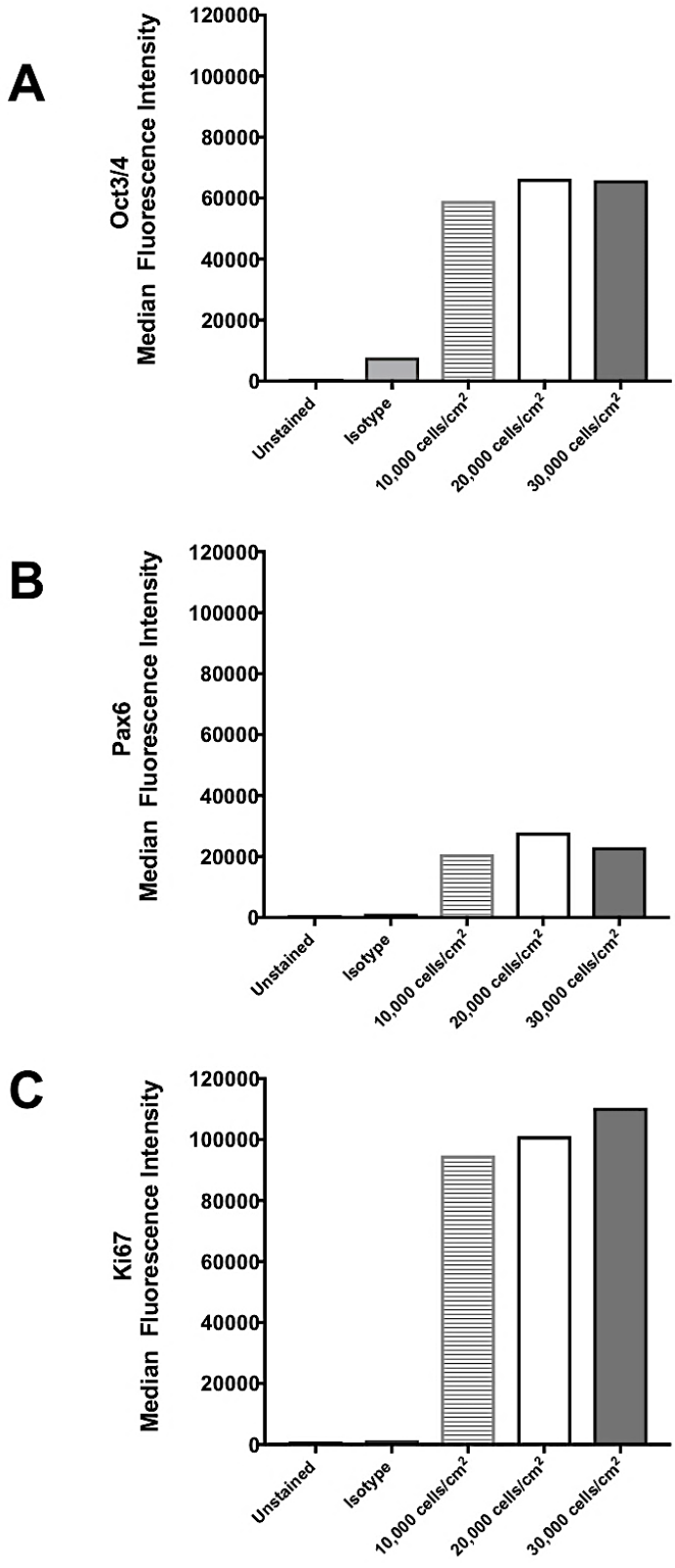


Figure 50. MedFI values for OCT3/4, Ki67 and PAX6. OCT3/4(**A**) and Ki67(**C**) were both highly expressed. (**B**) PAX6 expression levels are lower in comparison to OCT3/4 and Ki67.

5.3.5. Expansion: the effect of feeding regimes

Overall there was a significant difference in the SGR of the different routes with the exception of the control and route 1. Between passages each route differed in SGR from passage 1 to passage 2 with the exception of route 2 (**Figure 51A**). At passage 1 the significant differences were only between route 3 and the other conditions, however this changed at passage 2, with differences being between the control and route 2. Similarly, there was no significant difference in cell number yield for route 2 between passage 1 and passage 2, while significant differences are observed for the other routes; the control and route 1 both had a decrease in overall cell yield, while route 3 had an increase (**Figure 51B**). Both Ki67 and OCT3/4 were highly expressed in all conditions, while there is no expression of PAX6 (**Figure 52B**). The control and route 3 both had a decrease in OCT3/4 and Ki-67 from passage 1 to passage 2. Conversely, route 1 and route 2 both had increases in OCT3/4 and Ki67 from passage 1 to passage 2 (**Figure 52A** and **C**).

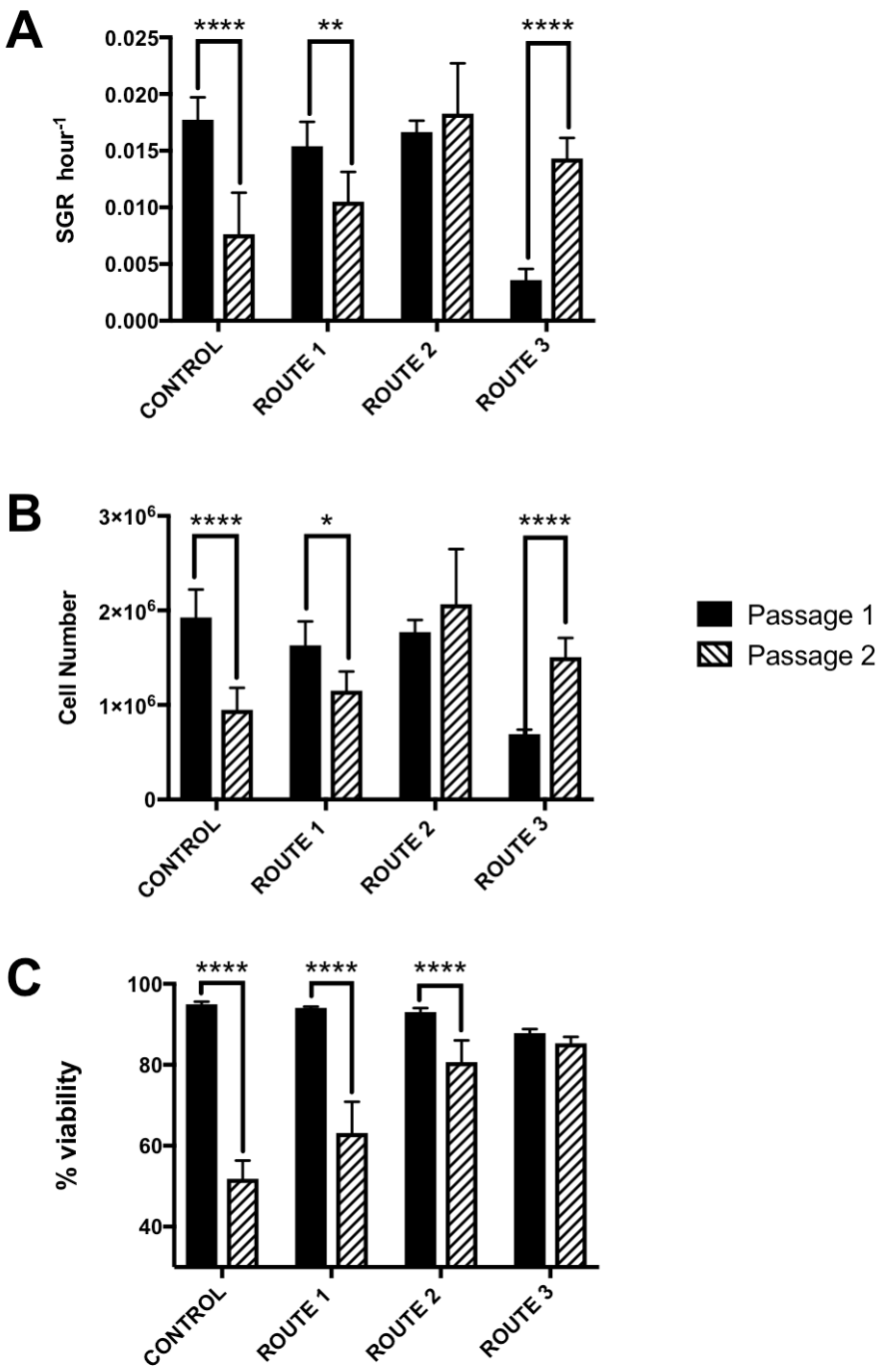


Figure 51. Growth dynamics. (**A**) SGR decreases from passage 1 to passage 2 for the control and route 1 conditions, no significant difference was observed for route 2 while route 3 significantly increased from passage 1 to passage 2. (**B**) The same trend observed in the SGR was observed with the cell number yields from passage 1 to passage 2 i.e. only route 2 did not have a significant change in cell number between the two passages. (**C**) The control, route 1 and route 2 all have a decrease in cell viability from passage 1 to passage 2, which was not observed for route 3. Error bars indicate standard deviation, n=3. "*" indicates $p<0.05$, "**" indicates $p< 0.01$, "***" indicates $p< 0.001$, and "****" indicates $p<0.0001$.

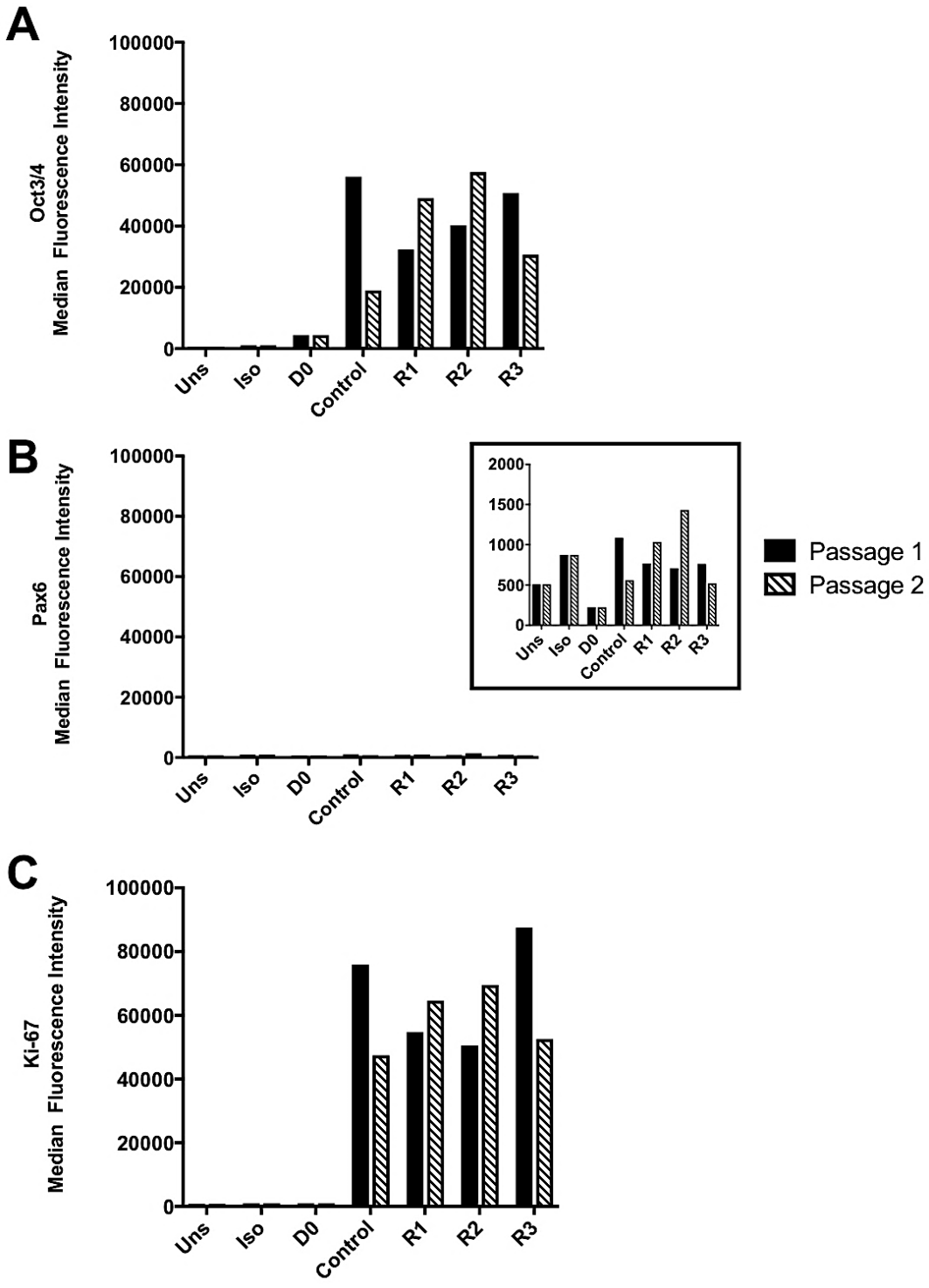


Figure 52. MedFI values for OCT3/4, Ki67 and PAX6. OCT3/4(**A**) and Ki67(**C**) were both highly expressed. (**B**) PAX6 expression levels were very low in comparison to OCT3/4 and Ki67, inset shows the very low levels of PAX6, all conditions are under 1500 for their MedFI values. Uns = unstained; Iso = isotype; D0 = cells at day 0; R1 = route 1; R2 = route 2; R3 = route 3.

In the second run of the experiment at a higher resolution, out of thaw the cells had low SGR and total cell numbers at each harvest, which were lower at passage 1 compared to passage 2 (**Figure 53A** and **D**). At passage 1 there was a difference in SGR between the different routes with the exception of the control with route 1 and route 2 with route 3, which appeared to group together i.e. the control and route 1 exhibited similar behaviours to each other and; route 2 and route 3 exhibited similar behaviours, thus the difference in SGR was between the respective set of conditions. Route 3 had the highest cell yield at the end of passage 1 (**Figure 53E**). Passage 2 showed route 3 had a significant difference compared to the other conditions, as it had both the lowest SGR and total cell number yield. In general, there was no difference in SGR from day 2 to 3 at passage 2. The viabilities were similar in passage 1 across the conditions as no significant differences were obtained, however, at passage 2 the conditions had significantly difference cell viabilities from day to day and overall (**Figure 53C** and **F**). Both route 2 and route 3 dropped in SGR from day 1 to day 2 and then increased from day 2 to day 3. PAX6 is not expressed in the different routes and there was no significant difference between the routes and the passages for all three markers, all the conditions highly expressed OCT3/4 and Ki-67 (**Figure 54**). The rate of lactate production increased from day 1 to day 2 and then decreased from day 2 to day 3 in passage 1 for route 2 and route 3 (**Figure 55A**). The rate of LDH production was at its highest in route 3 from day 2 onwards at passage 2 (**Figure 55**). There was no significant difference in the SMR for both LDH and lactate across the routes on day 1, however on day 3, route 3 was significantly higher than the other conditions for both metabolites, except in passage 1 for lactate SMR (**Table 27** and **Table 28**).

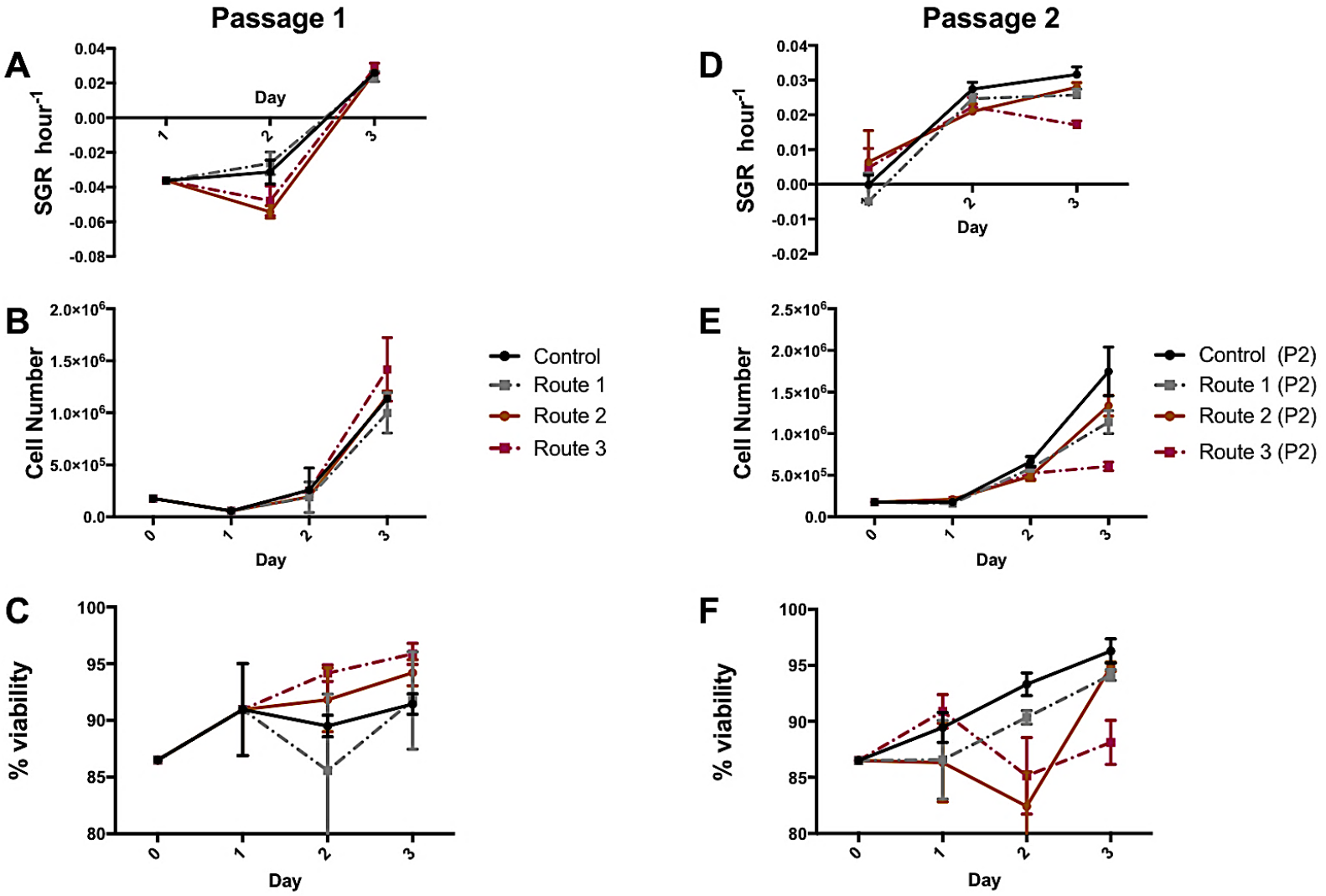

Passage 1
Passage 2
A
B
C
D
E
F
SGR hour-1
Day
Cell Number
% viability
Control
Route 1
Route 2
Route 3
Control (P2)
Route 1 (P2)
Route 2 (P2)
Route 3 (P2)

Figure 53. Growth dynamics (run 2). (**A**) Overall the SGR is lower at passage 1, with all four conditions having negative SGRs until day 3 of passage 1. (**D**)At passage 2, route 2 and route 3 had a positive SGR from day 1 onwards, only route 3 had a significant decrease in SGR by day 3 of passage 2. At both passages the cell number increased from day 0 to day 3, however at passage 1 (**B**) there was a decrease in cell number from day 0 to day 1, not observed at passage 2 (**E**). Generally, cell viability increased throughout passage 1, except for route 1 which had a decrease on day 2 (**C**). At passage 2, route 2 and route 3 had variable cell viabilities from day to day, while the control and route 1 had increased cell viabilities from day to day (**F**). Error bars indicate standard deviation, n=6.

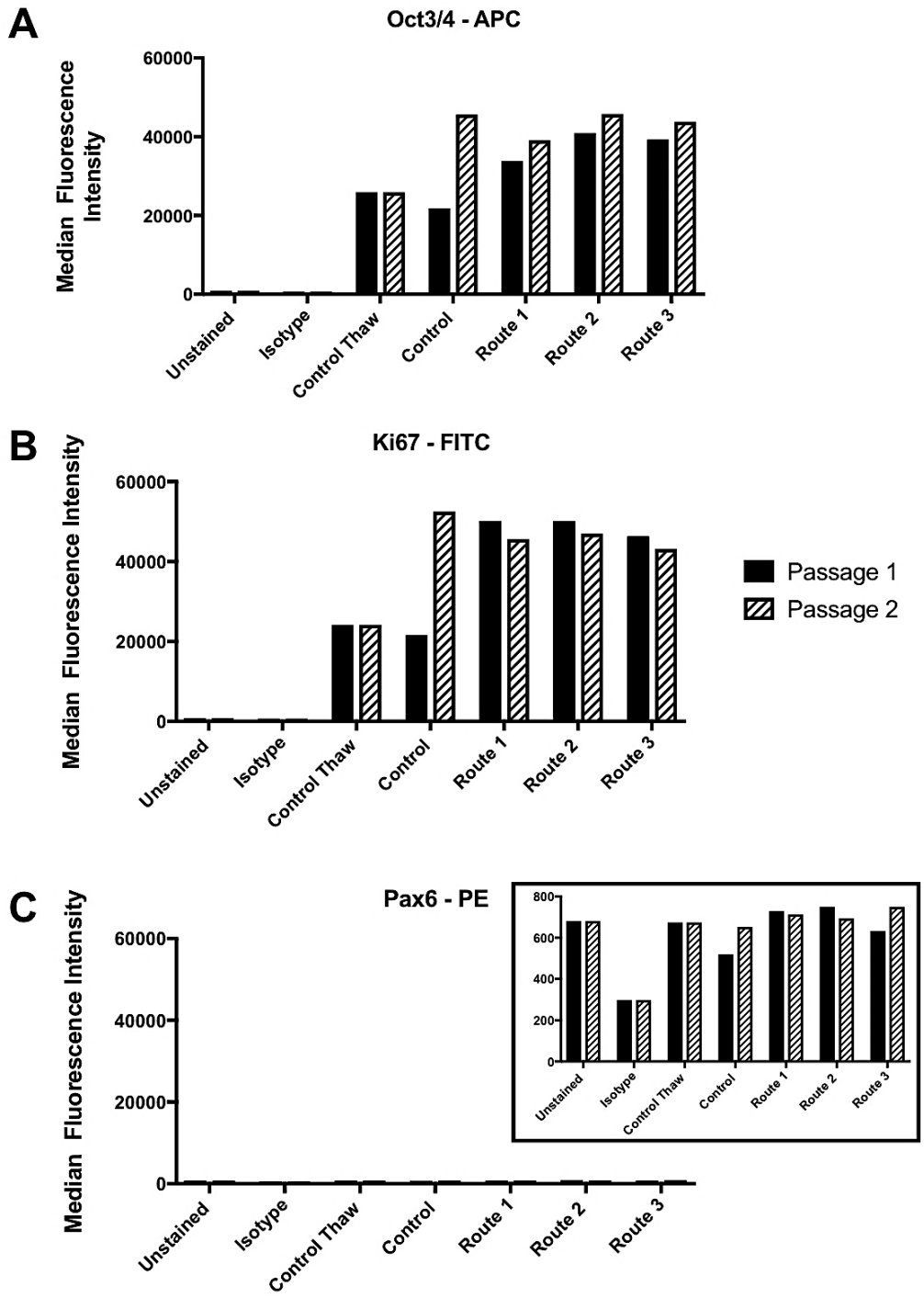


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Figure 54. MedFI values for OCT3/4, Ki67 and PAX6 (run 2). OCT3/4(**A**) and Ki67(**B**) were both highly expressed. (**C**)PAX6 expression levels were very low in comparison to OCT3/4 and Ki67, inset shows the very low levels of PAX6, all conditions are under 800 for their MedFI values. Generally, OCT3/4 increased from passage 1 to 2 and Ki67 decreased from passage 1 to passage 2, however the changes were not determined to be statistically significant. **N.B.** Control Thaw = cells analysed for cell phenotype immediately following thawing from cryopreservation, without being cultured.

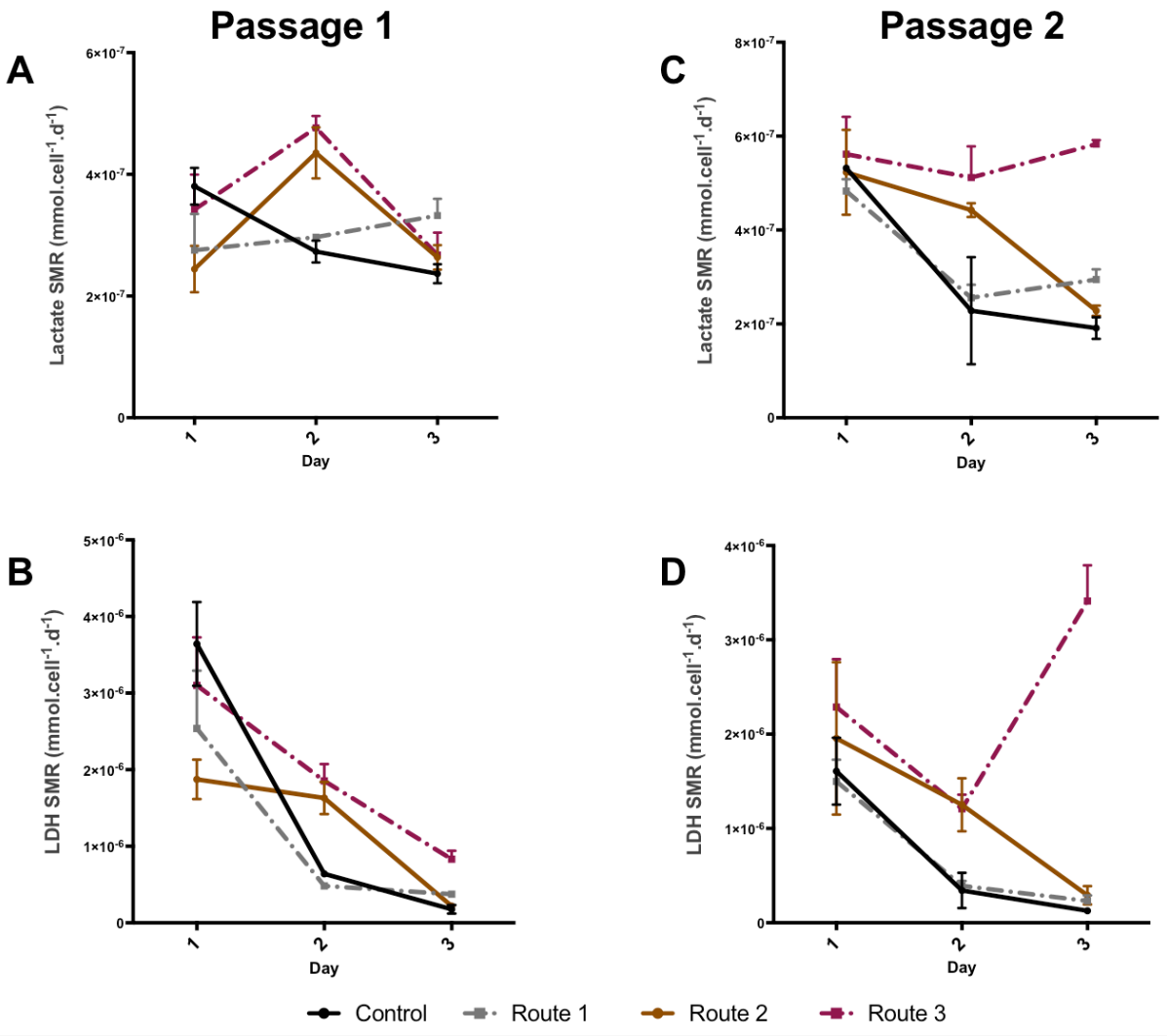


Figure 55. Lactate and LDH SMR data (run 2). Lactate SMR increased from passage 1 (**A**) to passage 2 (**C**), at passage 2 lactate SMR decreased for all routes except route 3. LDH SMR is highest for route 3 at passage 1 (**B**) and passage 2 (**D**), generally LDH SMR decreases for all routes except route 3 at passage 2. Error bars indicate standard deviation, n=3.

Table 27. Tukey's multiple comparisons test of the mean lactate SMR in each condition compared the means of the other conditions during that passage. Differences were observed mainly between route 3 and the other conditions, however at passage 2 most of the conditions were different to each other.

Lactate SMR				
	Passage 1		Passage 2	
	Summary	P Value	Summary	P Value
Control vs. Route 1	ns	0.8799	ns	0.7092
Control vs. Route 2	ns	0.4933	*	0.0198
Control vs. Route 3	**	0.0031	****	<0.0001
Route 1 vs. Route 2	ns	0.8963	ns	0.1806
Route 1 vs. Route 3	*	0.0133	****	<0.0001
Route 2 vs. Route 3	ns	0.0504	****	<0.0001

Table 28. Tukey's multiple comparisons test of the mean LDH SMR in each condition compared the means of the other conditions during that passage. Differences were observed in most of the conditions at passage 2, at passage 1 differences were only between route 3 and the other conditions.

LDH SMR				
	Passage 1		Passage 2	
	Summary	P Value	Summary	P Value
Control vs. Route 1	ns	0.9637	ns	0.9997
Control vs. Route 2	ns	0.9872	*	0.0327
Control vs. Route 3	**	0.0091	****	<0.0001
Route 1 vs. Route 2	ns	0.9984	*	0.0402
Route 1 vs. Route 3	**	0.0021	****	<0.0001
Route 2 vs. Route 3	**	0.0023	****	<0.0001

5.3.6. Expansion: the effect of density and medium exhaustion

On day 1 and 2 both the daily feed (DF) and one feed (OF) conditions had a negative SGR which increased significantly from day 2 to day 3 (**Figure 57A**). The two conditions diverged in terms of total cell number yield from day 3 onwards, the DF condition started to plateau at day 4, as there was no significant difference in SGR and cell yield from day 4 onwards (**Figure 57A** and **B**). Meanwhile, the OF condition significantly dropped in cell number yield from day 5, which was also accompanied by a decline in cell viability down to 65 % ±2.1 by day 7 (**Figure 57C**). The MedFI of Ki67 and OCT3/4 decreased overall from day 0 to day 7 and from day 6 to 7 in both conditions, the biggest decrease in MedFI was from day 6 to 7 of the DF condition for OCT3/4. There was very low expression of PAX6 in the conditions across the sampling points, while expression of Ki-67 also decreased from day 6 to 7 for both DF and OF (**Figure 58**). The rate of lactate and LDH production decreased over the 7 days for the DF condition (**Figure 56**). A similar trend was observed for the OF condition until day 5 when the rates of production significantly increased from day 5 to day 6 and then stabilised from day 6 to 7 **Figure 56.**

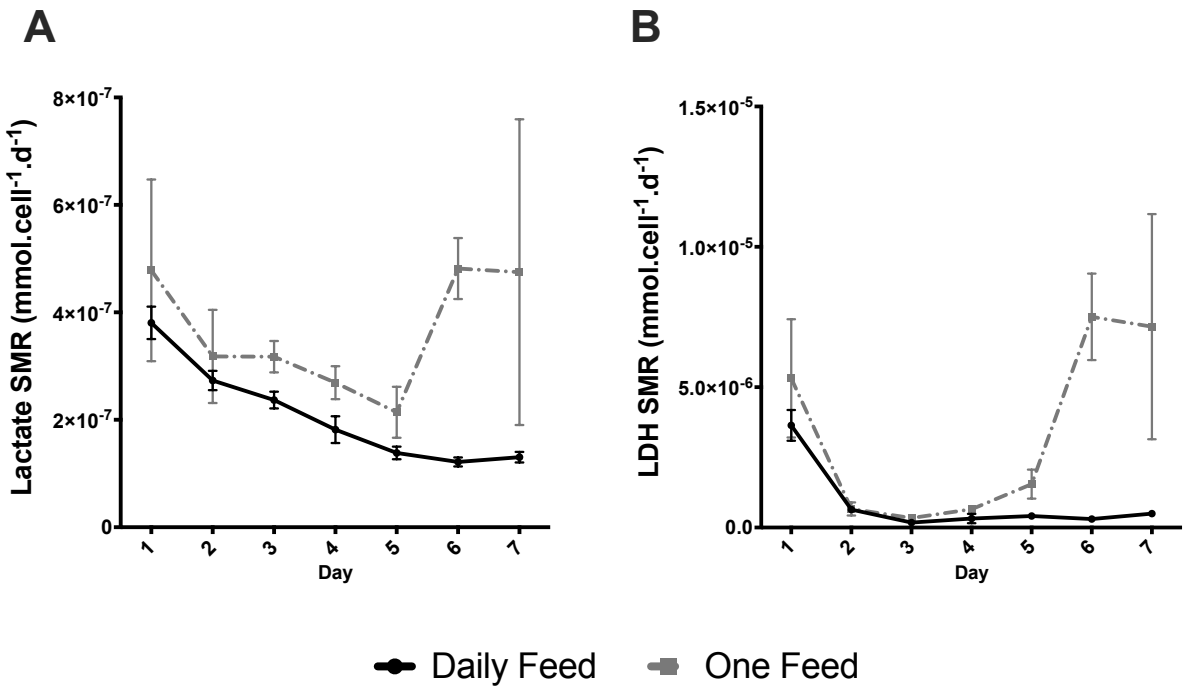


Figure 56. Lactate and LDH SMR data. Lactate SMR decreases throughout the passage for DF, OF decreases until day 5 to day 6 where it increases significantly (p=0.0079) (**A**). LDH SMR is highest from day 5 to day 6 for the OF condition, the DF condition has low levels of LDH production over the 7-day passage (**B**). Significant differences in SMR between the two. conditions for both lactate and LDH are observed at day 6 ($p<0.0001$) and day 7 ($p<0.0001$). Error bars indicate standard deviation, $n=3$.

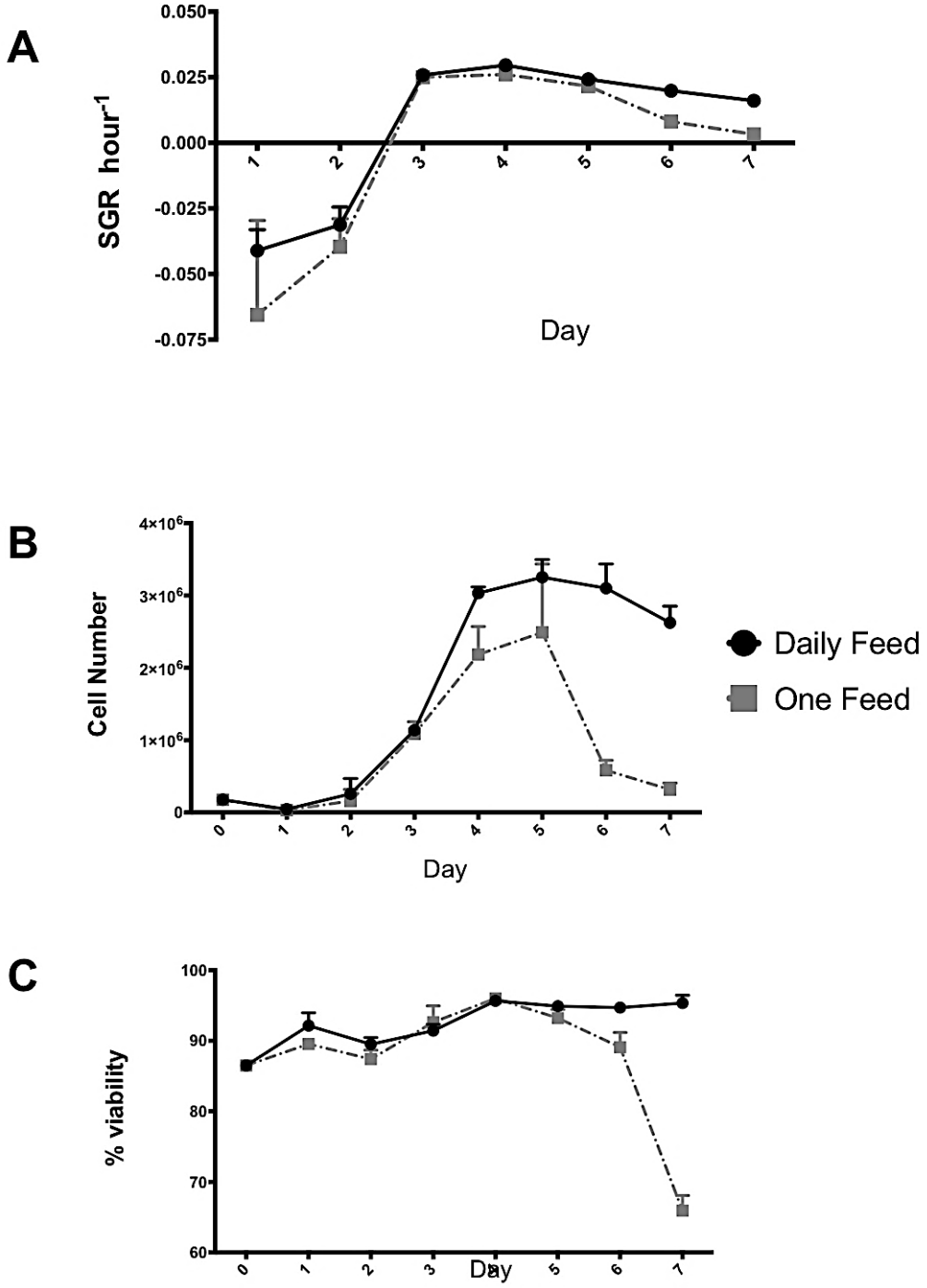


Figure 57. Growth dynamics. (**A**) SGR increased from negative SGR to positive SGR from day 3 onwards peaking at day 4 for the DF condition and day 3 for the OF condition. OF had a greater decrease in SGR over the 7-day passage, SGR differed between the two condition on day 1 ($p=0.0012$). (**B**) Cell number decreased from day 5 onwards for both conditions, similar to the SGR OF had the most significant decrease in cell number and cell viability (**C**). From day 4 onwards there was a significant difference in cell number yield between the two conditions: day 4 ($p<0.0001$); day 5 ($p=0.0002$); day 6 ($p<0.0001$); day 7 ($p<0.0001$). Error bars indicate standard deviation, $n=6$.

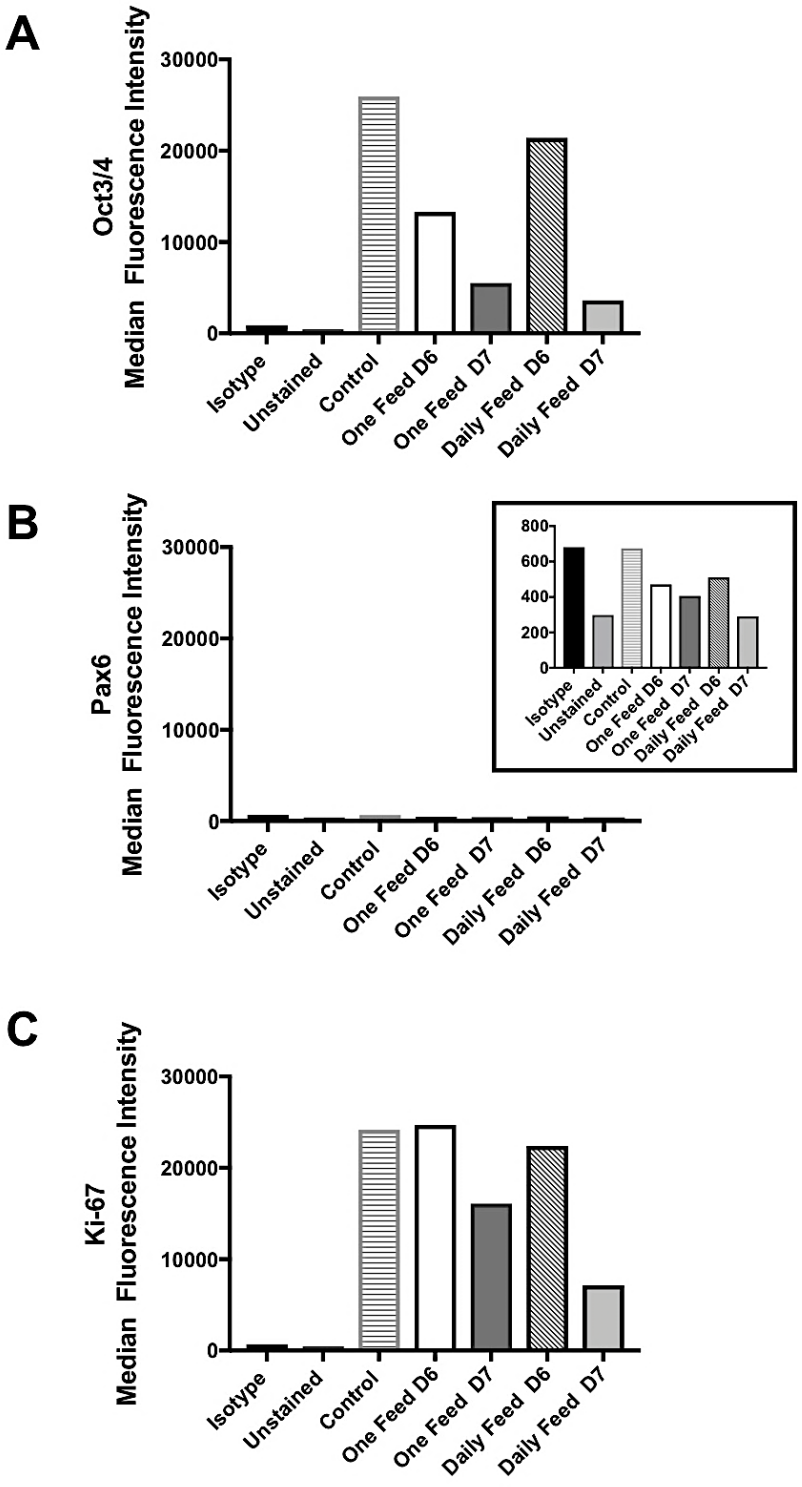


Figure 58. MedFI values for OCT3/4, Ki67 and PAX6. OCT3/4(**A**) and Ki67(**C**) were both highly expressed in both conditions. (**B**)PAX6 expression levels were lower in comparison to OCT3/4 and Ki67, inset shows the very low levels of PAX6, all conditions are under 800 for their MedFI values. OCT3/4 and Ki67 decreased from day 6 to day 7, resulting in lower expression levels overall when compared to the control (day 0) and both conditions at day 7.

5.4. Discussion

The work carried out in this chapter demonstrates that the H9 cells are capable of growing, proliferating and have a normal genetic profile. The banking reproducibility and vial variability study revealed that there was no significant variation in the growth of different vials, however, varying cell numbers were recovered from different vials following thaw. This highlights the importance of cell counting both before cryopreservation and following thaw. Moreover, the use of defined seeding densities instead of split ratios is reinforced, as cell counting and set densities would standardise the seeding procedure following thaw. Therefore, negating the fact that banked vials, regardless of cell counting prior to cryopreservation, would have different cell numbers upon thaw. Inter-passage variation was observed in the expression of OCT3/4 and Ki67 which both decreased from passage to passage (**Figure 46**). It is unclear whether the expression continues to decrease through several passages or if it fluctuates as observed in the longitudinal EC 2102 Ep experiment where the pluripotent markers OCT3/4 and SSEA-1 both fluctuated over 10 passages (**Figure 33E**). It can be inferred that the decrease in Ki67 is due to the higher cell numbers obtained in the following passage after thaw. Therefore, by the harvest point of the second passage the cells have started to slow down in terms of proliferation, which results in decreased expression of the proliferation marker, Ki67. Reduction on OCT3/4 could be attributed to spontaneous cell differentiation, however the lack of differentiation molecules and absence of PAX6 expression suggests that if the cells are in fact differentiating, it is not towards a neural stem cell lineage[122].

The initial expansion experiments have illustrated that there is variation between different passages during H9 pluripotent cell expansion. Typically, post thaw cells have low cell viability and SGR values. Cells seeded at 10,000 cells/cm^2 do not perform as well as those seeded at 20,000 and 30,000 cells/cm^2 as they have lower cell viabilities and take longer to reach confluency as their SGR is lower in comparison. This suggests that a lower cell seeding density influences the growth and quality of cells obtained during pluripotent expansion. These experiments have shown that the cells perform better at a higher seeding density such as 20,000 cells/cm^2 and following a 'recovery' passage subsequent to being thawed from cryopreservation. Both the increase in density and recovery passage resulted in improved proliferation rates, total cell number yields and cell viabilities in comparison to both cells seeded at 10,000 cells/cm^2 out of cryopreservation and even cells seeded at 10,000 cells/cm^2 one passage after cryopreservation. The high resolution growth curve studies demonstrated that one day after seeding the cells are often at a low or negative growth rate which increases significantly from day 2 onwards (**Figure 42A**, **Figure 53A**and **Figure 57A**). Furthermore, in experiment 5.3.5 during passage 1 at day 2 the cells are still at a negative growth rate while at passage 2 on day 2 they are at their peak, highlighting the necessity of a recovery passage. This could be due to lack of cell attachment in the

initial stages of culture caused by inadequate conditioning of the medium to sustain cell attachment and growth.

Establishing that the cells need a recovery passage represents a key element for manufacturing considerations; as resources will need to be adjusted, while taking into account the impact of the recovery passage on overall production timelines and product costs. The growth limitation experiment similarly shows a negative SGR at day 1 and day 2 for cells in their first passage from cryopreservation. It is only from day 3 onwards that the SGR starts to increase, suggesting that the cells either need a long passage length out of cryopreservation, up to five days or they should have at least one passage prior to allow their SGR to stabilise. The length of an optimal culture period for cells out of cryopreservation is shown to be four days as after this point, growth limitation based on both density and nutrient availability (**Figure 57**) is observed. However, the total cell numbers suggest that cell growth starts to decline from day 5 onwards, with nutrient availability factoring in from day 3 onwards as the DF (density inhibition) and OF (nutrient availability) conditions diverge at the this point (**Figure 57B**). When the cells have been subjected to at least one passage, the passage length for cells seeded at 10,000 cells/cm^2, can be reduced to a four-day passage. This is due to the decrease in SGR and cell viability from day 4 onwards, due to the high levels of confluency resulting in cell death. For cells seeded at 20,000 cells/cm^2 the passage length can be decreased to a three-day passage as more cells would be seeded while the SGR is similar to 10,000 cells/cm^2.

Analysis of different feeding regimes demonstrated that route 2, when the cells were only subjected to a medium exchange on day 2, resulted in cells with higher or comparable cell viability, SGR and cell number yield to the control condition which was subjected to daily medium exchange (**Figure 51** and **Figure 53**). Route 2 also exhibited an increase in OCT3/4 and Ki67 between passages, over the 2 passages of the experiment. In general, the control and route 1 behave similarly, while route 2 and route 3 behave similarly; this can be attributed to the time points in which the ROCK*i* is removed. The control and route 1 both have ROCK*i* removed on day 1 when they are subjected to a medium exchange (**Table 24**). In the second run of the experiment it is evident that route 3 is significantly different to the other conditions in terms of SGR, cell number yield and its metabolite profile, typically in an undesired manner i.e. lower SGR, cell viability and higher LDH SMR.

Interestingly between the two runs of the feeding regime experiment (section 5.3.5), the way in which route 3 performed at the end of each passage differed. For instance, route 3 had the lowest cell yield and SGR at the end of passage 1 in the first run but the highest in the second run at the end of passage 1 (**Figure 51A** & **B** and **Figure 53A** & **B**). At passage 2 the opposite was observed as the cell yield and SGR at the end of the passage was one of the highest in the first run while being the lowest in the second run. This drastic change in behaviour and outcomes was not observed for the other routes, which

behaved fairly consistently between the two runs. It can be theorised that the changes observed in route 3 were due to an inconsistency in cell behaviour due to the lack of medium exchange and build-up of waste within the culture vessel. Another potential cause for the variation could be attributed to differences in manipulation of the cells or measurement error, however both are unlikely as the variation is not observed in the other routes which were treated at the same time by the same operator. The observed inter-run variation could also be attributed to the fact that cells in the first run had a pre-passage prior to being seeded into the experiment, while the cells in the second run were seed directly from cryopreservation. However, it is unclear how this would selectively impact the route 3 condition the most.

These results suggest that it is not necessary to subject the cells to daily medium exchanges, however nutrient availability is important to prevent a decline in cell SGR and viability. Ideally the cells should be fed at least once and ideally on day 2 (route 2) as it appears to be an appropriate medium exchange time point. This is also evident in experiment 5.3.6 where the OF and DF conditions both significantly increased their SGRs from day 2 to day 3, therefore medium exchange to replenish nutrient availability as this time point would help to support and sustain cell growth, particularly for a four-day passage (**Figure 57**). Furthermore, day 2 is shown to be have the highest levels of lactate production, as such, a medium exchange to remove the accumulated lactate would be beneficial to the cells.

The rate of metabolite production shows the impact of nutrient availability on cell growth i.e. in general the SMR per cell decreases as more cells proliferate as less nutrients are consumed. In the OF condition the impact of restricted nutrient availability as the cells reach growth limitation is highlighted. This was demonstrated at day 5 by the significant decrease in SGR, cell number yield and the decline in cell viability, which was accompanied by an increase in the rate of production of both lactate and LDH. The increase in LDH production showed that the cells were starting to undergo cell death and enter the decline phase[260,263]. Although lactate production would be expected to decrease with less cells, it actually increases; this can be attributed to having less cells in the same volume of medium from day 1 (OF condition) leading to an accumulation of lactate since no medium exchange was performed. Therefore, the per cell rate of lactate and LDH production significantly increases at the point of the highest decline in cell number (day 5), despite there being less cells and less nutrient metabolism.

The use of 20,000 cells/cm^2 as the standard seeding density was further supported by the ROCK*i* experiment which illustrated that even in the absence of ROCK*i*, cells at 20,000 cells/cm^2 are still able to attach and proliferate. For cells seeded at a density of 10,000 cells/cm^2 the optimal ROCK*i* concentration was determined to be 10 μM as this resulted in the highest Cell Index in the range studied. Thus, cells at 20,000 cells/cm^2 are more suitable for use as the standard culture density as they are less sensitive to ROCK*i* concentration, therefore alleviating growth variation due to ROCK*i*. In addition,

cells at 20,000 cells/cm^2 require a shorter passage period, which reduces the use of resources required to culture the cells, which would be economically beneficial for manufacturing considerations. This highlights the notion that process optimisation work can aid process consistencies such as cell growth and quality, while additionally driving down costs, which is important for CTPs which have putatively high cost of goods. For instance, observations in this chapter (Chapter 6 as well) that highlight that ROCK*i* does not have a significant impact on the growth, quality or phenotype of the H9s, therefore it would be sensible to optimise the process by removing its use. As a result, the costs of goods would lower, as it is an expensive reagent and there would be less complexity in the formulation of the culture medium thereby removing an extra source of variation.

5.5. Conclusions

Cells seeded at 20,000 cells/cm^2 performed better than those seeded at 10,000 cells/cm^2 and regardless of seeding density, the cells performed better after at least one passage. This suggests that cells should be cultured for at least one passage following cryopreservation, before they are used for further experiments and for differentiation; as the input cell material would be stabilised and therefore result in less variation. In addition, directly post thaw the cells have poor growth for the first two days of culture. Differences in the phenotype marker profile were observed, particularly the general trend of decreases in Ki67 from passage to passage and occasional decrease in expression of OCT3/4. At present, it is unclear whether the decreased expression in OCT3/4 and Ki67 has a detrimental impact on further passages and the subsequent differentiation process. Furthermore, experiment 5.2.4 (page - 165 -) resulted in notable expression of PAX6, which is unexpected for cells in their pluripotent state. Results from experiment 5.3.5 (page - 168 -) show that the cells do not have to be subjected to a medium exchange daily, as route 2 with only one medium exchange resulted in cells with desirable characteristics when compared to the control of daily medium exchange. Furthermore, the density and nutrient limitation points for the cells were determined, allowing for an informed three-day passage length to be defined at the optimal seeding density of 20,000 cells/cm^2.

The experiments in this chapter have allowed for parameters for H9 pluripotent expansion to be determined in an informed manner. These parameters have been shown to produce cells with suitable growth characteristics while maintaining the cells' phenotypic profile. Therefore, the critical process parameters that would result in the critical quality attributes can be defined. It is proposed that H9 pluripotent culture prior to differentiation should be cultured under the following conditions: a density of 20,000 cells/cm^2, subjected to one medium exchange on day 2 of a three-day passage and cultured for at least one passage prior to differentiation if the cells are directly out of cryopreservation. These conditions result in an average SGR of 0.018 hour^{-1}± 1.5x10^{-3} over three days and cell viabilities up to 95% ±0.4, while producing cells which highly express pluripotent and proliferation markers, OCT3/4 and Ki-67, respectively. The above provides operators with defined and quantifiable criteria for determining "good-quality" starting cell material which is offers less variation compared to the criteria offered in the prescribed protocol: "*Before the differentiation is started, make sure that the hPSC colonies appear pluripotent by visual criteria (i.e., homogeneous-appearing colonies with clear borders and the absence of obvious differentiating zones). If you are using a hPSC culture system other than the one described here, manual removal of spontaneously differentiated colonies might be required. A desirable confluency of hPSC cultures is 70–90% of the well area at the start of differentiation*"[129]. As the starting material of the differentiation process has been analysed, the following chapter will focus on the differentiation of H9 pluripotent cell into vmDA neuroprogenitors.

Chapter 6:

Process Understanding of H9 Human Embryonic Stem Cell Differentiation into Ventral Mesencephalic Dopaminergic Neuroprogenitors

Chapter 6. Process understanding of H9 human embryonic stem cell differentiation into ventral mesencephalic dopaminergic neuroprogenitors

6.1. Introduction

The focus of this chapter is on the prescribed differentiation protocol of H9 human embryonic stem cells (hESCs) into ventral mesencephalic dopaminergic (vmDA) neuroprogenitor cells. The purpose of the differentiation is to obtain vmDA neuroprogenitors for cell characterisation and ultimately transplantation into patients suffering from Parkinson's disease (PD). The work carried out demonstrates the challenges that are associated with process transfer, process development and reproducible, efficient cell culture protocol development. The work carried out in the previous chapters i.e. protocol standardisation has informed the work in this chapter. For instance, the densities and passage length of the pluripotent cells are based on information accrued in Chapter 5.

6.1.1. The Lund University differentiation protocol

Chapter 2 (2.2.3. *In vitro* differentiation of pluripotent cells into vmDA) discussed the recent advances that have been made with regards to vmDA neuroprogenitor differentiation protocols. This work is concerned with the process development of the Lund University differentiation protocol (**Figure 59**). This protocol is a sixteen-day hESC differentiation process that has had many iterations over the years, the current work is based on the 2017 method, which is the most recent published iteration[129]. This protocol is designed to produce vmDA neuroprogenitors which have been shown to innervate into the target site of the striatum[24]. The cells are cultured on Biolaminin-111 which was chosen as it is naturally expressed in the brain and does not support growth of pluripotent cells, this favours growth of the differentiated cells allowing for more efficient and purer differentiation. This Biolaminin-111 matrix allowed the cells to be cultured without a feeder layer and eradicates the use of a polyornithin/fibronectin/laminin matrix mixture negating the use of embryoid bodies. The removal of feeder layers facilitated translation of the protocol into a xeno-free protocol, making it more adaptable to good manufacturing practice (GMP) compliant manufacturing. Neural induction of the hESCs is achieved through a dual SMAD approach, previously discussed in Chapter 2. The collaborators at Lund University highlighted that CHIR99021 and SHH-C24II concentrations are important for correct vmDA patterning. In the prescribed protocol the cells should have a ventral mesencephalic identity by day 11, which can be assessed by the presence FOXA2, OTX2 and LMX1A, presence of these markers by day 11 should proceed to give the rise to the desired vmDA neuroprogenitor cell population by day 16. LMX1A/B expression is integral to yielding vmDA neuroprogenitors as absence of these homeobox

factors has been shown to result in neurons of a non-dopaminergic fate due to *LMX1A/B* expression being necessary for downstream genes and factors of vmDA neuroprogenitors[92,121].

This protocol comprises of the medium formulations described in Chapter 3. A prescribed cocktail of small molecules consisting of SB431542, noggin, CHIR99021 and SHH-C24II are added when the cells are seeded and during medium exchange for the first seven days, specifically on day 2, 4 and 7 (**Figure 59**). At day 9 the cells are subjected to medium exchange with the FGF8-N2 medium which has FGF8 added whilst SB431542, noggin, CHIR99021 and SHH-C24II are removed. The B27 base medium has FGF8, BDNF and ascorbic acid added to it and is used to re-plate the cells at day 11 and for medium exchange on day 14 (**Figure 59**). The protocol revision used in the present work uses GMP reagents and procedures in an attempt to adapt the protocol for clinical manufacturing.

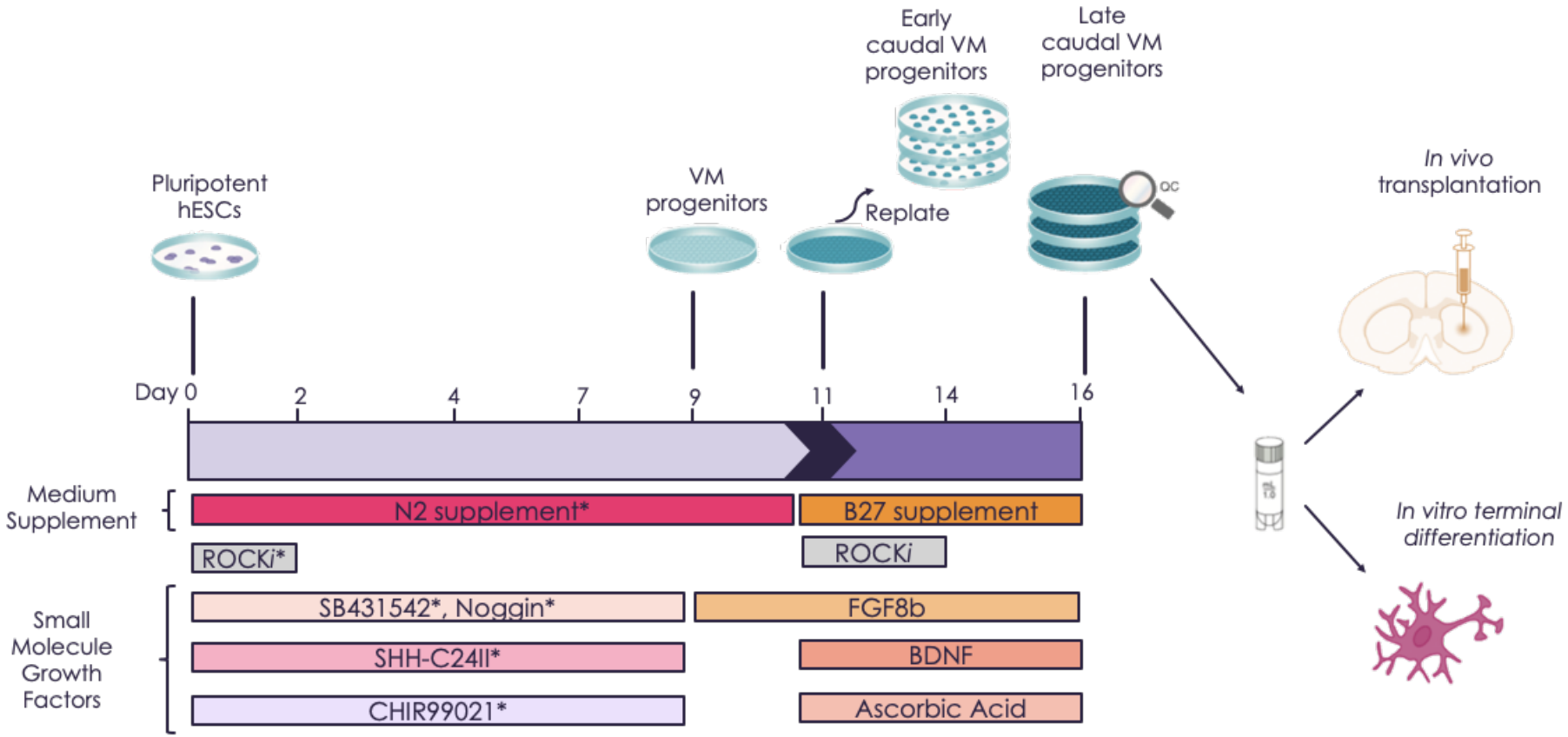


Figure 59. Schematic of the complete differentiation process from hESCs into late ventral mesencephalic dopaminergic progenitors. Medium exchanges are performed on day 2,4,7,9 and 14; on day 11 the cells are harvested and reseeded. ROCK inhibitor is added on day 0 and 11. SB431542 and noggin are used as neurulation factors in the medium, whilst SHH-C24II and CHIR99021 are used to pattern then neural stem cells towards a ventral midbrain linage. The timeline illustrates the time points at which the various differentiation molecules are added into the process. Image adapted from Nolbrant et al, 2017.

6.1.1.1. The small molecules used in the differentiation medium.

This section briefly describes the essential differentiation molecules and media components used in the protocol and their relevance to the differentiation process.

CHIR99021 – is a highly potent and selective, glycogen synthase kinase 3 (GSK-3) inhibitor, inhibiting the GSK pathway allows for activation of the Wnt/β-catenin pathway by allowing the secreted Wnt-protein to bind to the appropriate receptors[126,264]. This pathway is important for the maintenance and function of hESC in terms of their growth and differentiation[265].

SHH-C24II – is part of the Hedgehog protein family, this family is involved in the production of signals that result in the body plan, late stage embryogenesis, creation of tissue boundaries and patterning in bilateral organisms[105]. SHH-C24II in particular is involved in the signalling and regulation of the CNS[91].

SB431542 – is one of the SMAD inhibitors, this targets the inhibition of the SMADs from the Transforming Growth Factor β (TGF-β) family through highly potent and selective phosphorylation blocking of the activing receptor-like kinase receptors (ALK4, 5, and 7)[266].

Noggin – is the second of the SMAD inhibitors, this targets particularly the inhibition of the SMADs from the bone morphogenetic protein (BMP) family[122,125].

FGF8-b – fibroblast growth factor 8b is involved in a range of biological processes including embryogenesis and morphogenesis. FGF8b plays a crucial role in the organisation and maintenance of the midbrain/hindbrain boundary[267].

BDNF – is a neurotrophic factor that belongs to the neurotrophin family which induces survival, development and function of neurons, in particular the differentiation of progenitors into neurons[268].

Ascorbic Acid – is an essential nutrient that is involved in a range of biological processes, particularly as an electron donor for certain enzymes as an enzymatic cofactor. In particular, it is an electron donor for dopamine B-hydroxylase which plays a role in the biosynthesis of norepinephrine from dopamine[269].

N2 supplement – is a commercially available premixed supplement for neural cell culture, the components within the N2 supplement encourage initial commitment and differentiation of stem cell populations towards a neural fate[270]. Neural specific genes are supported in N2 supplemented medium whilst the growth of undifferentiated is inhibited as post-mitotic neurons are favoured[252,270,271].

B-27 supplement – is used towards the later stages of neural differentiation protocols as it supports the growth and attachment of mature neurons, the supplement is particularly high in antioxidants[260,272,273].

RHO Kinase Inhibitor Y27632 (ROCK*i*) – is a rho-associated protein kinase inhibitor that increases cell survival after dissociation[274]. ROCK*i* is added to media used for cell seeding and replating for both the hESCs and cells undergoing the differentiation process.

6.1.1.2. Characterisation strategy

The characterisation strategy employed by Lund uses immunocytochemical (ICC) analysis and gene expression analysis to determine the progenitor cell identity. ICC analysis is carried out using a range of antibodies for vmDA progenitor stage markers including FOXA2, LMX1A and OTX2, for mature neurons derived from the progenitors TH, MAP2, and EN1 are the markers used to identify terminally differentiated neurons, typically at day 44-50[109,129]. Polymerase chain reaction (PCR) and quantitative real-time PCR (qRT-PCR) are used in conjunction with ICC to analyse the genetic profile of the cells to ascertain their identity. The complete set used to profile the cells consists of fifteen genes, including two housekeeping genes (**Table 29**). Although not prescribed in the protocol, a flow cytometry panel was created for characterisation of the cells. The protocol was created by collaborators at Miltenyi Biotec who worked with both Lund University and Loughborough University on various aspects of the overall project. The markers used in the flow cytometry panel are detailed in **Table 30**.

Table 29. Genes used for qRT-PCR analysis by Lund University, table adapted from Nolbrant et al, 2017.

Gene	Full name	Relevant details
ACTB	Actin beta	Housekeeping gene
BARHL1	BarH like homeobox 1	Ventral diencephalon/lateral midbrain VM/ventral
CORIN	Corin, serine peptidase	VM/ventral diencephalon/ventral hindbrain
EN1	Engrailed homeobox 1	Caudal VM
ETV5	ETS variant 5	Caudal midbrain
FOXA2	Forkhead box G1	VM/ventral diencephalon/ventral hindbrain
FOXG1	Forkhead box G1	Forebrain
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Housekeeping gene
HOXA2	Homeobox A2	Hindbrain
LMX1A	LIM homeobox transcription factor 1 alpha	VM/ventral diencephalon
LMX1B	LIM homeobox transcription factor 1 beta	VM/ventral diencephalon
OTX1	Orthodenticle homeobox 1	Midbrain
OTX2	Orthodenticle homeobox 2	Midbrain/forebrain
PAX6	Paired box 6	Dorsal/lateral populations
PAX8	Paired box 8	Caudal midbrain

Table 30. Flow cytometry markers and fluorophores used in the prescribed Miltenyi Biotech characterisation protocol.

Marker-Fluorophore	Marker function/specificity	Desired expression in day 16 vmDA neuroprogenitor cells
FOXA2 -APC	Rostral ventral midbrain/ caudal ventral midbrain/ ventral hindbrain	Positive
IAP-PE	vmDA specific surface marker integrin associated protein	Positive
OTX2-FITC	Midbrain / forebrain	Positive
NKX6.1-APC	Gli transcription factor / generates red nucleus progenitors	Negative
NKX2.1-FITC	Ventral forebrain / rostral ventral midbrain marker	Negative
SOX1-PE	Primitive neurectoderm / lateral floor plate	Negative
OCT3/4-APC	Pluripotency marker	Negative
Ki67-FITC	Proliferation marker	Between 10-60 % expected Positive
PAX6-PE	Dorsal / lateral populations / roof plate	Negative

6.1.2. Process transfer

The aim of the present work was to enable efficient translation of the protocol (**Figure 59**) into a manufacturing process through process understanding and process optimisation activities. In order to optimise the protocol, it was first necessary to understand the protocol, thus site visits and meetings with collaborators at Lund University were organised. This served as a process transfer activity where the researchers from Lund University demonstrated the key aspects of the protocol, such as the seeding procedure, medium composition and cell manipulation at day 0 and medium exchange at day 7.

A cause and effect diagram (Ishikawa diagram) was used to review the overall process and the important factors to consider that could result in a poor manufacturing outcome (**Figure 60**). A poor manufacturing outcome in this context would be cell death, low cell yields, incorrect differentiation and a highly heterogeneous cell population. Eight categories were used to assign causes to, these were *process results; contamination; people; method; measurement; equipment; environment* and *materials*. These categories were also used to carry out a failure modes and effects analysis (FMEA) to identify process steps that would result in failure of an experiment or production run in terms of obtaining vmDA neuroprogenitors. For the FMEA, the *process results, contamination*, *people* and *method* categories were merged into a single category labelled *cell manipulation*. The FMEA was carried out with Lund University personnel, fundamental process steps of the protocol were discussed, several points were raised and ranked in terms of their risk and frequency (**Table 31**). The FMEA provides details of the critical process parameters (CPPs), as determined by experienced users of the protocol, that are essential to the process in order to efficiently obtain the critical quality attributes (CQAs) of the vmDA product. The process controls detailed in **Table 31** were controls that were used by the collaborators at Lund University in their process.

A separate part of the process that also had to be transferred was the flow cytometry protocol provided by Miltenyi Biotec. The protocol was created considering the work being carried out by Lund University in order to provide a characterisation method that can be used for quality control, in terms of identifying and sorting for the desired differentiated cells. This protocol used a three-part panel with panel 1 consisting of pluripotency/neurostem markers; panel 2 consisting of ventral midbrain dopaminergic neuroprogenitors markers and panel 3 consisting of lateral midbrain markers used to identify potential contaminant neuroprogenitors. A summary of the markers is shown in **Table 30**, the protocol utilised pluripotent cells as the control sample for the gating strategy.

The FMEA formed the basis of the differentiation experiments carried out in the present work, which investigated issues of consistency; cell death; cell seeding density; and small molecule concentrations. These failure modes were deemed to have the most significant impact on the success of the

differentiation process. Initial experiments were carried out in order to gain experience in using the prescribed protocol, after which further experiments were designed to understand the protocol and the CPPs. Design of experiments (DoE) were used to carry out multifactorial analysis of the variation caused by factors such as seeding density and small molecule concentration. This was with the aim of executing protocol optimisation to increase the process efficiency whilst producing a translatable, robust protocol for clinical scale manufacturing. The purpose of the protocol would be to highly standardise the process, minimise human-based sources of variation, reduce uncontrolled parameters and increase consistency.

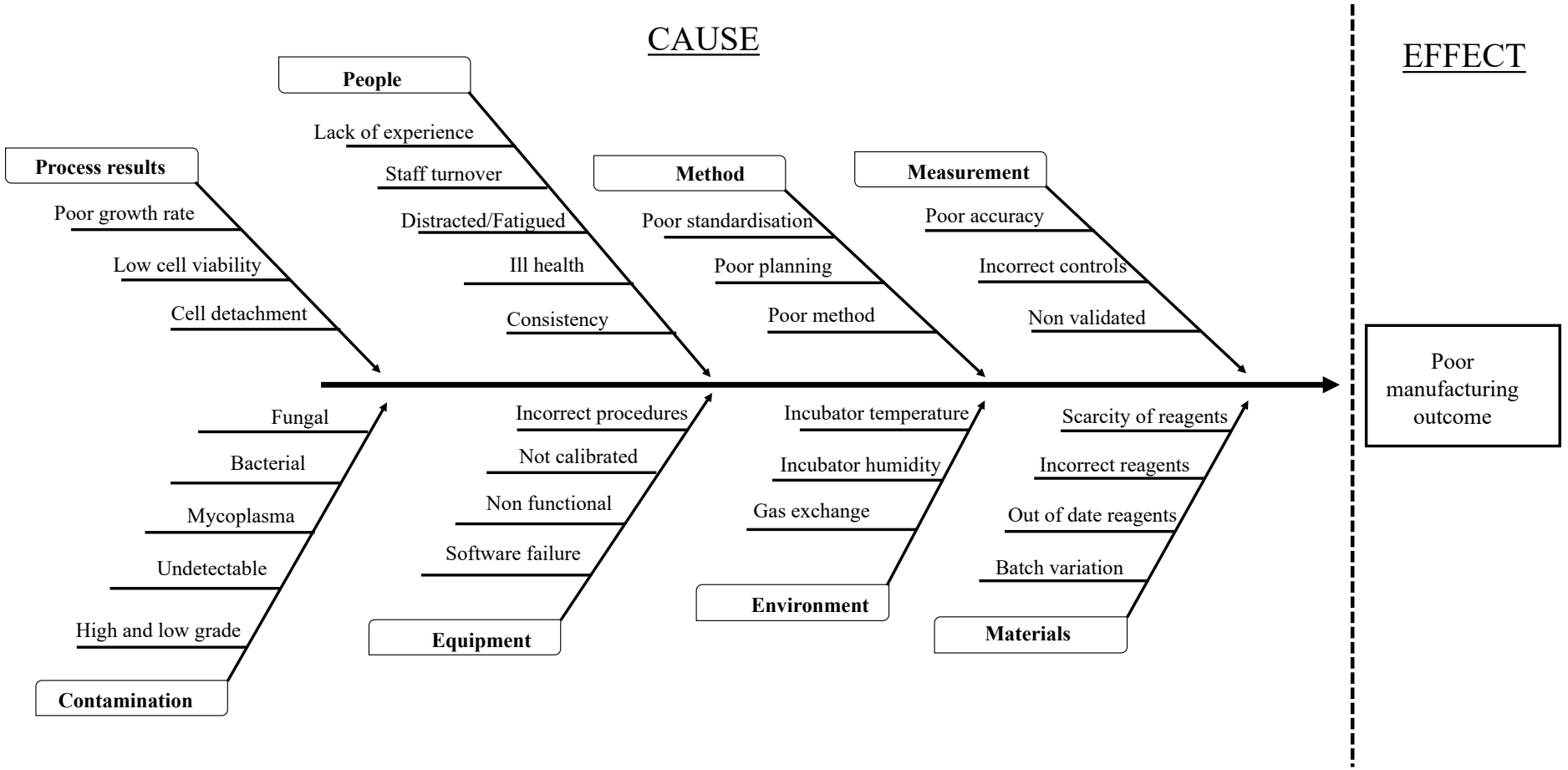


Figure 60. Cause and effect diagram (Ishikawa diagram) detailing potential causes of a poor experimental or manufacturing process for a cell-based product. Eight causal categories were used to determine potential root causes of an undesired effect.

Table 31. Failure mode and effects analysis of the key process steps of the vmDA differentiation protocol, determined by discussion with collaborators at Lund University. The risk priority number was obtained by multiplying the severity ranking by the occurrence rating and by the detection rating (severity x occurrence x detection). The rankings were assigned values from 1 to 10, higher values for the severity and occurrence indicate a failure that would have a severe effect on the process and a high likelihood of occurring, respectively. For the detection rankings, a low value indicates a failure mode that would be easy to detect, while a higher value indicates a failure mode that is difficult to detect or not detectable until the end of the process.

Process Step	Potential Failure Mode	Potential Failure Effect	Severity	Potential Cause	Occurrence	Process Controls	Detection	Risk Priority Number
Cell Manipulation	Contamination	cells would be not used, resulting in batch loss	10	poor aseptic technique	2	personnel training, disinfecting materials used	1	20
	Cell detachment	Reduced yields	5	poor coating effieciency, cell death	6	personnel training	4	120
	poor user consistency and adherence to the protocol	variation resulting in out of specification end product	6	poor training, poorly defined protocol	5	personnel training	8	240
Measurement	Flow cytometer sampler or lasers not working	Loss of product characterisation capability	5	unknown cause	4	efficient equipment maintenance	2	40
	No controls	Potential for incorrect characterisation	5	Poor planning or oversight	2	efficient inventory monitoring	1	10
	no calibrators	Potential for incorrect characterisation	5	Poor planning or oversight	3	efficient inventory monitoring	1	15
	lasers drifting	Skewed result reading	5	Equipment error, no calibration	4	efficient equipment maintenance	4	80
Cell Milieu	Nutrients incorrect supplementation or low concentrations	Poor cell growth	5	Poor or inefficient feeding regime	3	Inspection of the cells	3	45
	CHIR99021 concentration too low or too high	Incorrect or inefficient differentiation	10	poorly defined protocol, inaccurate pipetting	7	use the same pipette	8	560
	Small molecule concentration too low or too high	Incorrect or inefficient differentiation	9	poorly defined protocol, inaccurate pipetting	7	use the same pipette	8	504
	Laminin coating	No cell attachment and cell proliferation	8	poor training, poorly defined protocol	4	personnel training	1	32
	incubator failure	Poor cell growth	10	unknown cause	2	efficient equipment maintenance	1	20
	Cell seeding density too low	Poor Cell growth	7	use of split ratios, poorly defined protocol, inaccurate pipetting	6	personnel training	7	294
Materials	out of date	Incorrect or inefficient differentiation	4	Poor planning or oversight	4	efficient inventory monitoring	1	16
	no stocks	Process cannot be started	10	Supplier failure	6	efficient inventory monitoring	1	60
	spoilt reagents	Incorrect or inefficient differentiation	10	Fridge or freezer failure	4	efficient equipment monitoring	4	160
Equipment	non functional	Process cannot be started	8	unknown cause	3	efficient equipment maintenance	1	24
	Not calibrated	Skewed process or results	6	Poor planning or oversight	3	efficient equipment maintenance	1	18

6.2. Methods

6.2.*1. In Vitro* Cell Culture:

Cells were cultured according to the method described in section 3.2.4.2. H9 Expansion: Standard culture route). The relatively low seeding densities (10,000 – 30,000 cells/cm^2) required to seed the cells for differentiation resulted in low to modest levels of expansion prior to differentiation. Typically, one well of a 6-well tissue culture microtiter plate seeded at 20,000 cells/cm^2 (~177,000 cells per well) can yield an average of 1,600,000 cells per well (180,000 cells/cm^2), affording an almost ten-fold increase in cell number over three days of culture. The resulting pluripotent cells of each well can then be used to seed a further eight to nine well for differentiation, thus highly efficient in terms of cell usage.

6.2.1.1. Differentiation: Run 1 (DR1)

One vial of P8 cells from the H9 bank was thawed and seeded at 20,000 cells/cm^2 onto a Biolaminin-521 coated T25 cm^2 tissue culture flask and cultured for two passages. Following the second passage the cells were seeded at 10,000 cells/cm^2 into three Biolaminin-111 T25 cm^2 tissue culture flasks with N2 differentiation medium containing CHIR99021 at a concentration of 0.9 μM.

6.2.1.2. Differentiation: Run 2 (DR2)

DR2 followed the same culture and experimental design as DR1, however CHIR99021 was added to the N2 differentiation medium at a concentration of 0.7 μM, which was prescribed as the optimal concentration for mesencephalic neuronal differentiation using the H9 cell line[129].

6.2.1.3. Differentiation: Run 3 (DR3)

DR3 followed the same cell culture procedure as DR1 and DR2, the experimental design was adjusted to investigate the effects of different concentrations of CHIR99021 on the survival and differentiation of the H9s. Five concentrations of CHIR99021 (0.6 – 1 μM (**Table 32**) were used in DR3 at a seeding density of 10,000 cells/cm^2 onto Biolaminin-111 coated 6-well tissue culture microtiter plates, each condition was run in triplicate.

Table 32. CHIR99021 concentrations used within experiment DR3. *N*=3 for each condition was used.

	DR3.a	**DR3.b**	**DR3.c**	**DR3.d**	**DR3.e**
CHIR99021 (µM)	0.6	0.7	0.8	0.9	1.0

6.2.1.4. Differentiation: Run 4 & 5 (DR4 and DR5)

Experiments DR4 and DR5 cells were seeded at three densities (10,000; 15,000; 20,000 cells/cm^2) and cultured with media containing varying concentrations of CHIR99021 (0.3, 0.5, and 0.7μM) and the presence and absence of the B27 supplement (**Table 33**). A deviation from the protocol occurred at the replating stage (day 11) of DR4, where some conditions were reseeded at densities lower than the prescribed 800,000 cells/cm^2 due to lower cell yields in those conditions at the day 11 harvest (**Table 34**). Only one condition in DR5 was seeded lower than the recommended density. The cells were seeded on Biolaminin-111 coated 6-well tissue culture microtiter plates, each condition was run in triplicate. Cell counts were performed at day 11 and day 16, while phenotype analysis was carried out at day 16.

Table 33. Experimental design for experiments DR4 and DR5 denoting the concentrations and densities used in the experiments. n=3 for each condition was used.

Density	**(-) B27**	**(+) B27**
10,000 cells/cm^2	0.3μM CHIR99021 0.5μM CHIR99021 0.7μM CHIR99021	0.3μM CHIR99021 0.5μM CHIR99021 0.7μM CHIR99021
15,000 cells/cm^2	0.3μM CHIR99021 0.5μM CHIR99021 0.7μM CHIR99021	0.3μM CHIR99021 0.5μM CHIR99021 0.7μM CHIR99021
20,000 cells/cm^2	0.3μM CHIR99021 0.5μM CHIR99021 0.7μM CHIR99021	0.3μM CHIR99021 0.5μM CHIR99021 0.7μM CHIR99021

Table 34. The reseeding densities of the remaining experimental conditions at day 11. Densities were calculated for the conditions that did not have enough cells to seed at 800,000 cells/cm^2. The replicate column indicates the number of replicates surviving at day 11.

Parent density	Replicate	0.3 CHIR (+ B27)	0.5 CHIR (+ B27)
10,000 cells/cm^2	A	518,000 cells/cm^2	129,000 cells/cm^2
15,000 cells/cm^2	A	800,000 cells/cm^2	124,000 cells/cm^2
	B	681,000 cells/cm^2	
	C	457,000 cells/cm^2	
20,000 cells/cm^2	A	800,000 cells/cm^2	630,000 cells/cm^2
	B	800,000 cells/cm^2	800,000 cells/cm^2
	C	800,000 cells/cm^2	800,000 cells/cm^2

6.2.1.5. Differentiation: Design of Experiment - Definitive Screen 1 (DSD1)

A DoE approach was used to screen the effect of different levels of N2 supplementation and a range of small molecule concentrations. A Definitive Screen Design (DSD) created on Minitab18® (Pennsylvania, USA) was used to produce a matrix for the formulation of different media compositions with different concentrations of the N2 differentiation medium components. A total of thirteen different media formulations were created using low, medium and high concentration levels of each of the small molecules (**Table 35**). To ascertain the effect of these small molecules on the first stage of differentiation process, the FGF8-N2 medium used on day 9 was kept at the same concentration for all the conditions i.e. 100 ng/ml. The experiment was performed up to day 11 which served as the endpoint, sacrificial harvests were performed on day 4, 9 and 11, resulting in three flow cytometry analytical time points and a cell count on day 11. The cells were seeded at 20,000 cells/cm^2 on Biolaminin-111 coated 24-well tissue culture microtiter plates, each condition was run in duplicate.

N.B. One limitation of the work being carried out for GMP manufacturing translation is that experimental replicates had to be constricted, particularly in the DoEs, due to the high costs of the consumables. However, due to inherent methodology of the DoE such as the definitive screen designs employed, the experiments retained statistical power with fewer identical replicates.

Table 35. Definitive Screen Design matrix of the different concentrations used within experiment DSD1. *n*=2 for each condition was used.

Culture condition reference code	N2 supplement (% vol/vol)	Rock (µM)	Noggin (ng/ml)	SB431542 (µM)	SHH-C24II (ng/ml)	CHIR99021 (µM)
M1	1	12.5	125	12.5	600	1
M2	1	7.5	75	7.5	0	0.4
M3	1.5	10	125	7.5	0	1
M4	0.5	10	75	12.5	600	0.4
M5	1.5	12.5	100	12.5	0	0.4
M6	0.5	7.5	100	7.5	600	1
M7	1.5	7.5	125	10	600	0.4
M8	0.5	12.5	75	10	0	1
M9	1.5	7.5	75	12.5	300	1
M10	0.5	12.5	125	7.5	300	0.4
M11	1.5	12.5	75	7.5	600	0.7
M12	0.5	7.5	125	12.5	0	0.7
M13	1	10	100	10	300	0.7

6.2.1.6. Differentiation: Design of Experiments – scale up experiment

A scale up experiment was set up to ascertain the most pragmatic and cost-effective well plate format that would provide a workable number of cells, to carry out both cell growth and cell phenotypic analysis at a higher resolution compared to DSD1(performed in 24-well plate format). One vial of P8 cells from the H9 bank was thawed and seeded at 20,000 cells/cm^2 onto Biolaminin-521 coated T25cm^2 tissue culture flask and cultured for one passage. The cells were seeded at three densities (15,000; 20,000; 25,000 cells/cm^2) and cultured with the M1 medium formulation from experiment DSD1 (page - 201 -). The three densities were seeded onto Biolaminin-111 coated 6-well and 12-well tissue culture microtiter plates, each condition was run in duplicate. Assessment of cell number yield was determined through sacrificial well harvests from each well format and density on day 4, 7 and 9 (**Figure 61**).

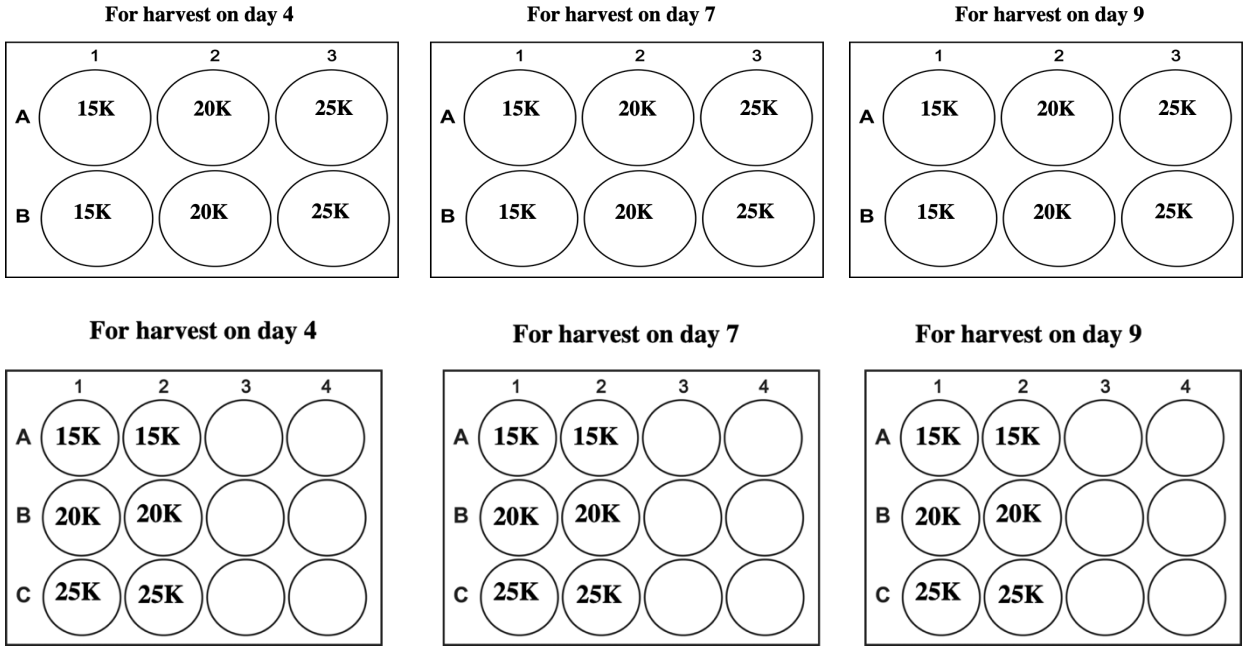


Figure 61. Schematic of the well plate formats used in the experiment to determine a suitable well plate format for a higher resolution DSD. 6-well and 12-well plate formats were used for the three densities 10,000 cells/cm^2 (10K); 20,000 cells/cm^2 (20K) and 30,000 cells/cm^2 (30K). *n*=2 for each condition was used.

6.2.1.7. Differentiation: Design of Experiments – day 11 replating

This experiment looked at the effect of density on day 11 when the cells were replated. In addition, two of the small molecules (FGF8 and ascorbic acid) used in the latter stages of the protocol were included in the experiment design. Cells from the scale up experiment were cultured until day 11 and reseeded into this experiment. Two densities and a midpoint were chosen and combined with a range of concentrations for the small molecules to create a three factor DoE. Nine conditions were obtained with three densities and three concentration levels of the small molecules (**Table 36**). The conditions were seeded onto Biolaminin-111 coated 12-well tissue culture microtiter plates, each condition was run in duplicate and cultured until day 16. Assessment of cell number yield was determined through sacrificial well harvests from each condition on day 14 and day 16.

Table 36. Experimental design matrix of the different concentrations and densities used within the day11 replating experiment. *n*=2 for each condition was used.

Culture condition reference code	Density (cells/ cm²)	FGF-8b (ng/ml)	Ascorbic acid (mM)
C1	400000	50	0.1
C2	800000	50	0.1
C3	400000	150	0.1
C4	800000	150	0.1
C5	400000	50	0.3
C6	800000	50	0.3
C7	400000	150	0.3
C8	800000	150	0.3
C9	400000	50	0.1

6.2.1.8. Differentiation: Design of Experiment - Definitive Screen 2 (DSD2)

The second DSD (DSD2) followed a similar design as DSD1, however FGF8b was also added to the list of small molecules in the screen. In addition, density was added as a factor, three densities were employed (20,000; 25,000; 30,000 cells/cm^2). These factors resulted in an experiment with seventeen different conditions which were seed onto Biolaminin-111 coated 12-well tissue culture microtiter plates, each condition was run in duplicate (**Table 37**). The experiment was run for the full sixteen days, sacrificial harvests were performed on day 4, 9, 11 and 16, resulting in four cell growth and phenotype analytical time points.

Table 37. Definitive Screen Design matrix of the different concentrations used within experiment DSD2. *n*=2 for each condition was used.

Culture condition reference code	Density (cells/ cm²)	N2 supplement (% vol/vol	Noggin (ng/ml)	SB431542 (µM)	SHH-C24II (ng/ml)	CHIR99021 (µM)	FGF-8b (ng/ml)
M1	25,000	1.5	150	15	900	1.5	150
M2	25,000	0.5	50	5	300	0.5	50
M3	30,000	1	150	5	900	0.5	50
M4	20,000	1	50	15	300	1.5	150
M5	30,000	0.5	100	5	900	1.5	150
M6	20,000	1.5	100	15	300	0.5	50
M7	30,000	1.5	150	10	300	1.5	50
M8	20,000	0.5	50	10	900	0.5	150
M9	30,000	0.5	50	15	600	1.5	50
M10	20,000	1.5	150	5	600	0.5	150
M11	30,000	1.5	50	5	300	1	150
M12	20,000	0.5	150	15	900	1	50
M13	30,000	1.5	50	15	900	0.5	100
M14	20,000	0.5	150	5	300	1.5	100
M15	30,000	0.5	150	15	300	0.5	150
M16	20,000	1.5	50	5	900	1.5	50
M17	25,000	1	100	10	600	1	100

The concentration ranges of the small molecules investigated in DSD2 were based on the results and observations from DSD1. Some of the small molecules were kept at the same concentration ranges, while others were changed or removed from the design (i.e. ROCK*i*). The justifications for changing or not changing the ranges and the addition of density are summarised below.

Density: 20,000; 25,000; 30,000 cells/cm^2

Reasoning: Previous experiments with 10,000, 15,000 and 20,000 cells/cm^2 showed that cells seeded at 10,000 cells/cm^2 started to detach and die by day 4 unless B27 supplement was added upon seeding. Therefore, this experiment was designed to look at higher densities, 15,000 cells/cm^2 was excluded as the scale up experiment illustrated that cells at 15,000 cells/cm^2 in a 12-well plate format had poor growth characteristics. As a result, 30,000 cells/cm^2 was added to the experimental design.

N2: No change. 0.5:100; 1:100 and 1.5:100.

Reasoning: N2 was kept the same as it is a complex mixture of compounds which would be difficult to ascertain exactly what mechanisms and which components within it are responsible for the supportive effects observed in DSD1. Therefore, N2 was kept the same and density was added as an extra factor

which might interact significantly with N2 due to its supportive effect i.e. less support for higher densities due to less availability.

SB431542: Changed. 5, 10 and 15 μM

Reasoning: The concentration ranges for SB431542 were broadened to see if there is an effect based on SB431542 as DSD1 did not result in significant interactions based on SB431542. The decision to broaden the ranges was made under the assumption that the observed non-linearity is not valid in the ranges previously employed.

Noggin: Changed. 50, 100 and 150 ng/ml

Reasoning: Similar to SB431542, the concentration ranges for noggin were broadened to determine if a change in the interactions would be observed, in comparison to DSD1.

CHIR99021: Changed. 0.5, 1.0 and 1.5 μM

Reasoning: Previous experiments showed that lower concentrations of CHIR99021 result in cell survival, however the cells were not of the desired phenotype. 1μM has been shown to result in the correct phenotype and result in survival, therefore conditions either side were employed to assess if 1μM is the optimal CHIR99021 concentration.

SHH-C24II: Changed. 300, 600, 900 ng/ml

Reasoning: DSD1 showed that SHH-C24II was highly influential, however the ranges used resulted in a failure mode as SHH-C24II is needed for survival. Higher concentrations were also shown to result in improved phenotypic progression, particularly when coupled with higher concentrations of CHIR99021. For DSD2 the concentration range was shifted upwards, removing the 0 ng/ml condition and adding a higher concentration of 900 ng/ml.

FGF8: 50, 100 and 150 ng/ml

Reasoning: This was the first use of FGF8b as a factor in the design. The concentration ranges used followed the same ranges used in the other small molecules, making it similar to the protocol in terms of ratio between factors i.e. noggin is also at 50, 100 and 150 ng/ml.

ROCK*i*: 10 μM

Reasoning: ROCK*i* did not have any significant influence within the thirteen conditions in DSD1 as there was no linearity/interaction observed. In addition, the cells are only exposed to ROCK*i* for the

first two days of the protocol. Furthermore, the ROCK*i* and cell attachment experiment illustrated that a concentration of 10 μM is optimal for H9 cells. As a result, ROCK*i* was not deemed as a priority factor to be investigated and was therefore it was removed from the design.

6.2.2. Flow Cytometry:

The prescribed Miltenyi Biotec protocol was used to analyse the conditions in experiments DR4 and DR5. This protocol followed the three-part panel design (**Table 38**). The samples were prepared as described in Chapter 3.The pluripotent cells were used as the gating control within both experiments.

Table 38. The three panels used in the Miltenyi Biotec protocol, each panel contains three phenotypic markers used to determine the differentiation outcome and loss of H9 pluripotency. The colour of the text represents the laser that excites the fluorophore.

Panel 1	**Panel 2**	**Panel 3**
Pluripotency	*vmDA subtype*	*Undesired neuronal subtype*
OCT3/4-APC	FOXA2 -APC	NKX6.1-APC
Ki67-FITC	OTX2-FITC	NKX2.1-FITC
PAX6-PE	IAP-PE	SOX1-PE

6.2.2.1. Flow Panel Optimisation for the Definitive Screens

The three-part panel was deemed unsuitable for the high-resolution experiments such as DSD1 and DSD2. Therefore, an in-house single multiplex (six-colour) panel was designed for high frequency flow cytometry analysis, that requires small amounts of sample cells (**Table 39**). This was not achievable with the Miltenyi Biotec protocol as each condition would be required to be put into each of the three panels, which was not practical when working with small culture vessels such as microtiter plates. The relevant isotype controls for each channel were used as gating controls.

Table 39. The in-house six colour flow cytometry panel used for DSD1 and DSD2. The colour of the text represents the laser that excites the fluorophore.

Marker	Fluorophore	Indicates	Expression (in differentiated cells)
Oct 3/4	BV421	Pluripotency	Negative
Otx-2	FITC	DA cell fate	Positive
Pax-6	PE	Neuro stem cell fate	Negative
Sox1	PerCP Cy5.5	Neuroectoderm	Negative
Ki-67	PE Cy7/ Vio770	Proliferation	Transient
Foxa2	APC	DA cell fate	Positive

N.B. DSD1 flow cytometry analysis was performed without OTX2-FITC as part of the panel due to the antibody not being available at the time of analysis.

6.2.3. Minitab definitive screen analysis.

Minitab18® (Pennsylvania, USA), version 18.1 was used to analyse the definitive screen design experiments. Statistical design of experiment analysis was used to analyse the MedFI and Pd response, using quadratic analysis of the model parameter terms. Main effects plots were presented to exhibit the parameters that had an effect on MedFI and Pd at different levels of the respective parameter e.g. the effect of differing CHIR99021 concentrations on FOXA2 expression. Pareto charts presenting the standardised effect of model parameters were also utilised. The charts present the magnitude of the effect (from largest to smallest) of the parameters and therefore the extent to which a factor drives an outcome such as Pd away from the mean in terms of standard deviations. The red reference line depicted on each chart represents the level at which standardised effect becomes significant, calculated using the user defined significance value, which in these instances was 0.15.

6.3. Results

6.3.1. Differentiation: Differentiation Run 1 and 2 – Lund protocol exploration.

In both experiments the cells were observed to be lifting off the culture surface at day 2, leaving very sparse small colonies of cells on the surface (**Figure 62**). As a result, no cell counts, or other characterisation was carried out due to lack of cell material.

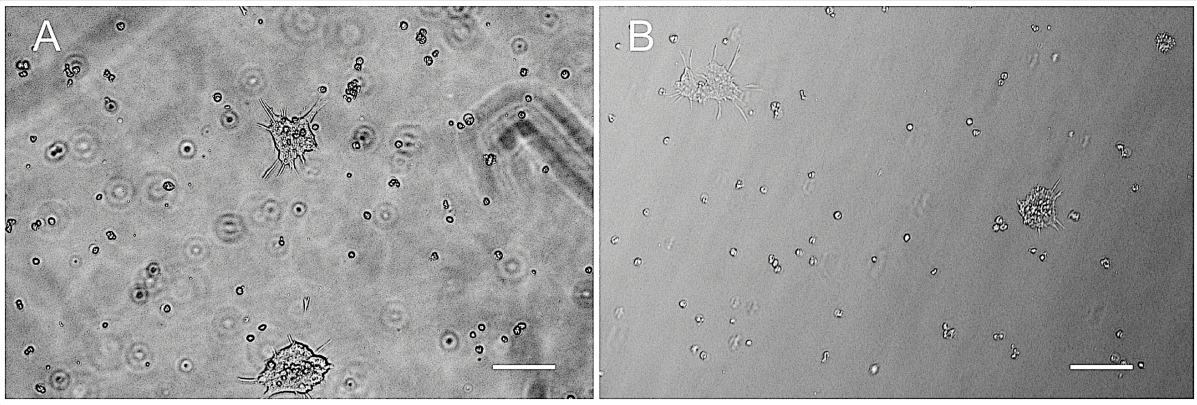


Figure 62. Representative light microscope image of the cells from DR1 (**A**) and DR2 (**B**) showing small sparse colonies and detached cells floating. Scale bar = 400 μm.

6.3.2. Differentiation: Differentiation Run 3 - multivariate analysis of CHIR99021 concentrations

Poor cell attachment was observed on day 1 in the majority of conditions when imagined, using light microscopy. By day 2, most of the cells had attached and small colonies were forming. However, an increase in detached cells and cell debris was also observed. At day 3, most cells had lifted from the surface and those remaining were in the process of detaching (**Figure 63E**). Complete cell detachment was observed at day 4 in the longest surviving condition (DR3.a) which was cultured with 0.6 μM CHIR99021. Overall, cells at all CHIR99021 concentrations lifted from the wells within the first four days of the sixteen-day differentiation process. The two lowest CHIR99021 concentrations (0.6 and 0.7μM) showed the greatest attachment of all the conditions, however this was only sustained until day 4.

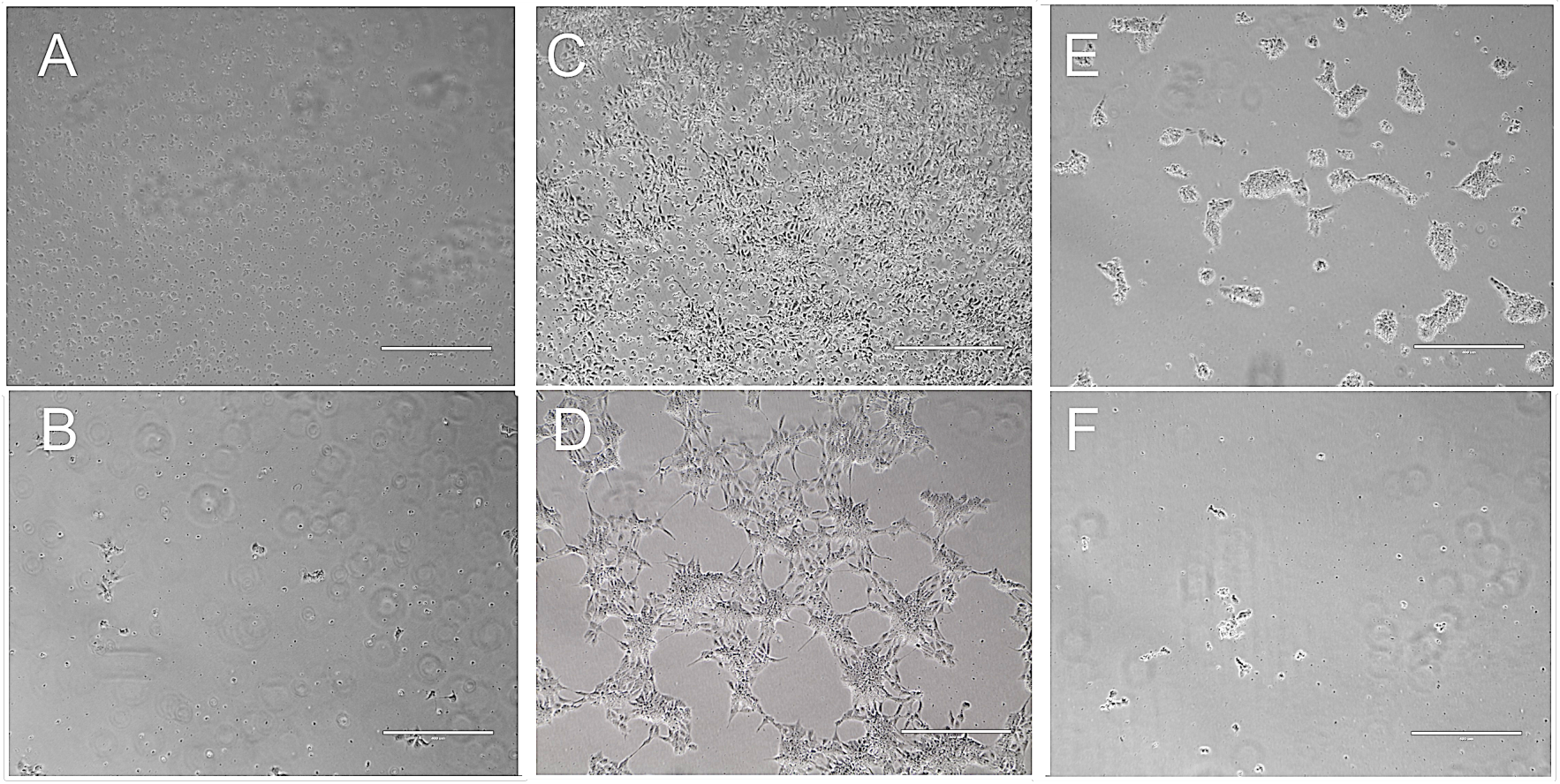


Figure 63. Progressive cell detachment during the early stages of differentiation using medium containing 0.6μM CHIR99021. Light microscope images show cell morphology and the progressive cell detachment. (**A**) Cells at day 1 imaged prior to medium exchange, showing a large number of unattached cells. (**B**) Cells at day 1 following medium exchange, showing very few attached to the culture surface. (**C**) Cells at day 2 imaged prior to medium exchange, showing a lot of cell debris. (**D**) Cells at day 2 imaged following medium exchange, showing colony formation and increase surface coverage. (**E**) Cells at day 3 imaged following medium exchange, poor cell attachment and cell lifting from the surface is observed. (**F**) Cells at day 4 imaged following medium exchange, very few individual cells are observed on the surface and all colonies from day 3 have detached. Scale bar = 400 μm.

6.3.3. Differentiation: Differentiation Run 4 – CHIR99021, Density and B27 multivariate analysis

Six of the eighteen conditions survived the sixteen-day differentiation process. All seeding densities cultured with CHIR99021 at concentrations lower than the recommended 0.7μM were supplemented with B27 from day 0 (**Figure 64**). Cell detachment was observed in all of the other conditions on or before day 7, including the experimental control condition (0.7 μM CHIR99021 at 10,000 cells/cm^2 in absence of B27) which failed by day 2 (**Figure 64**). In all the surviving conditions, confluent sheets of cells were formed by day 9, this was observed earlier in conditions seeded at higher densities (**Figure 65E**). An increase in cell detachment was observed with increasing confluency however, a dense sheet of cells remained on the surface (**Figure 65**). By the replating stage (day 11), due to large amounts of cell death, only half of the surviving conditions had sufficient cell yields to allow for replating at the prescribed density of 800,000 cells/cm^2. The remaining conditions were replated using the total cell number yielded from the cell harvest (**Figure 66**). All conditions survived until day 16 following replating, however the yields of the cell numbers at day 16 were much lower than the cells numbers used on day 11, for some of the conditions (0.3 and 0.5 μM CHIR99021 at 20,000 cells/cm^2, **Figure 67B**).

The SGR data demonstrates that the different conditions were growing at different rates and revealed an increase in SGR between day 11 and day 16 (**Figure 67A**). Phenotypic analysis of the conditions that survived until day 16 (shown in **Figure 64**) revealed that the desired marker expression: OCT3/4$^-$, Ki67$^{-/+}$, PAX6$^-$, FOXA2$^+$, OTX2$^+$, IAP$^+$, NKX2.1$^-$, NKX6.1$^-$ and SOX1$^-$ had not been obtained. Panel 1 illustrated that the cells experienced a decrease in both OCT3/4 and Ki67, however, the cells were still expressing the neural stem/roof plate marker, PAX6 (**Figure 68**.). In addition, there was a lack of dopaminergic cell marker expression (FOXA2, OTX2 and IAP) in panel 2 (**Figure 69**) and the expression of SOX1 in some of the conditions in panel 3 (**Figure 70**). Overall, none of the conditions expressed the correct phenotype regardless of density, CHIR99021 concentration and addition of B27 supplement.

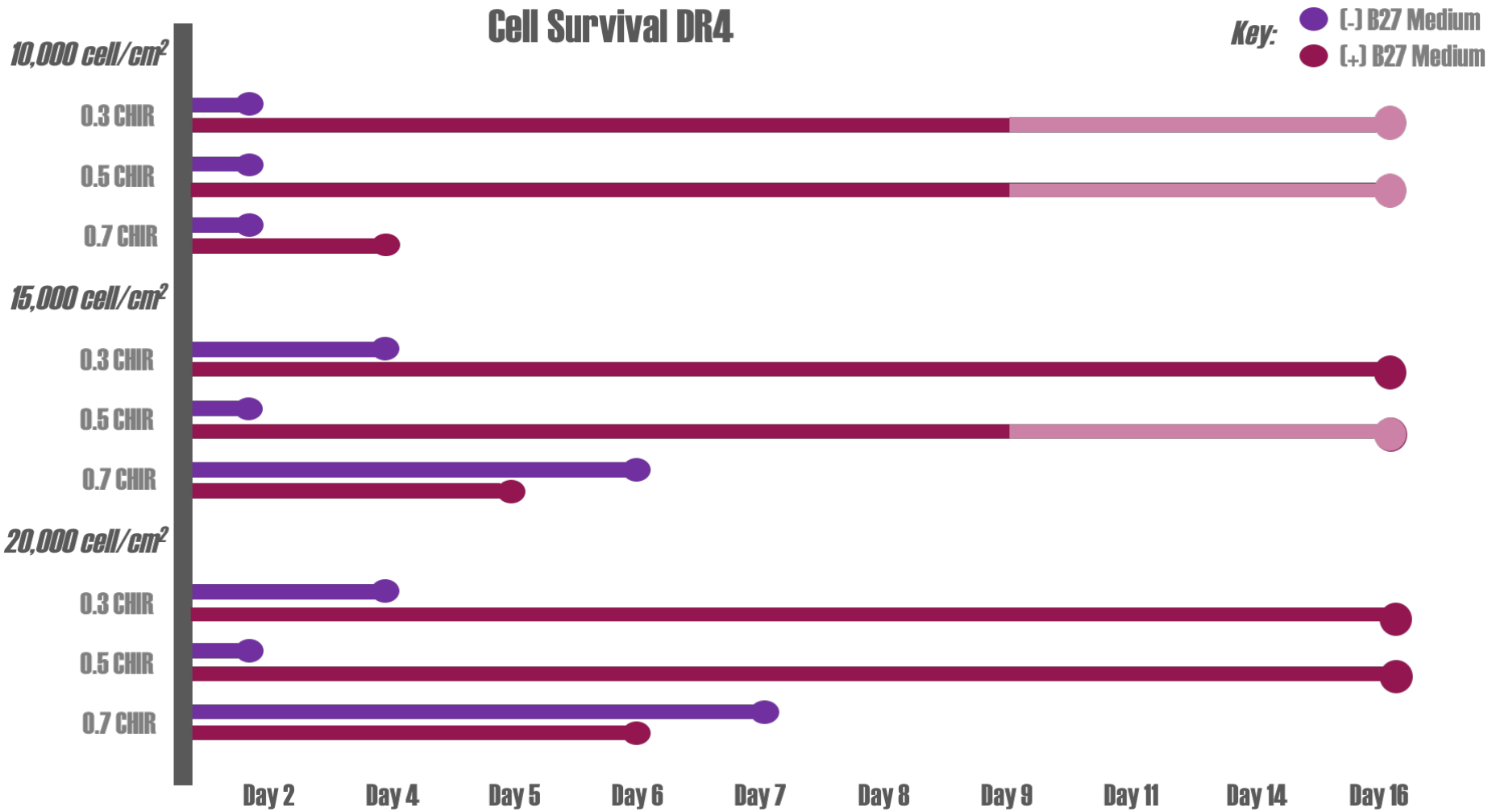


Figure 64: A comparative timeline illustrating cell death time points during the differentiation process. The end points represent the day that the respective condition was terminated due to excess cell death within the culture condition. Three conditions (10k 0.3µM CHIR + B27, 10k 0.5 µM CHIR + B27, and 15k 0.5 µM CHIR + B27) had inconsistent behaviour between their biological repeats, the single surviving wells were carried forward (shown in pink).

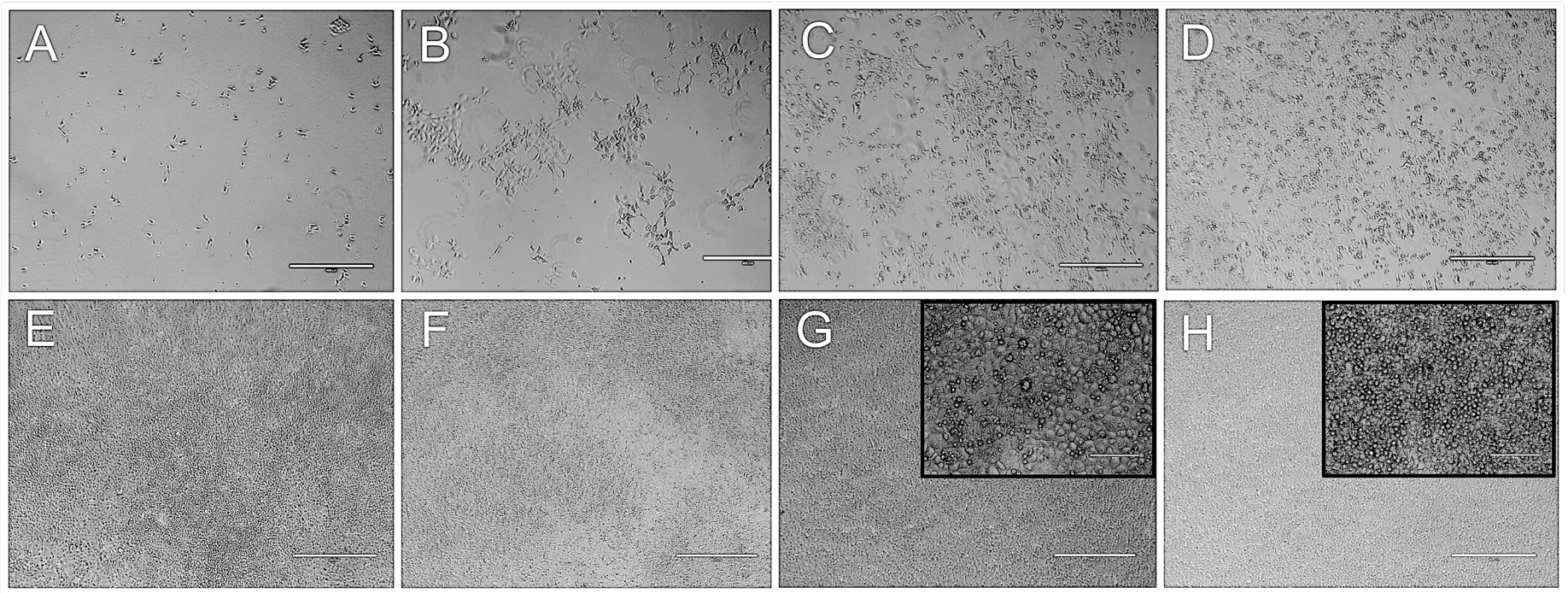


Figure 65. An overview of H9 hESC to vmDA progenitor differentiation. Single cell suspensions were seeded (**A**) which attached start to proliferate and form colonies (**B** and **C**). By day 6 (**D**) the cultures were mostly confluent and increased in confluency to cover the culture surface by day 9 and 11 (**E** and **F**). Following replating at day 11 the cells were fully confluent with no visible culture surface, an increase in cell detachment was observed from day 14 (**G**: inset) and continued to day 16 (**H**: inset). **A**=Day 0; **B**=Day 2; **C**=Day 4; **D**=Day 6; **E**=Day 9; **F**=Day 11; **G**=Day 14; **H**=Day 16. Scale bar = 400 μm, insets scale bar = 100 μm.

Table 40. Condition labels used in the differentiation run 4 and 5 (DR4 and DR5) experiments, detailing both the density and concentration of CHIR99021 used.

Code	Condition
10k 0.3/[0.3]	10,000 cells/cm^2, 0.3 µM CHIR99021
10k 0.5/[0.5]	10,000 cells/cm^2, 0.5 µM CHIR99021
10k 0.7/[0.7]	10,000 cells/cm^2, 0.7 µM CHIR99021
15k 0.3/[0.3]	15,000 cells/cm^2, 0.3 µM CHIR99021
15k 0.5/[0.5]	15,000 cells/cm^2, 0.5 µM CHIR99021
15k 0.7/[0.7]	15,000 cells/cm^2, 0.7 µM CHIR99021
20k 0.3/[0.3]	20,000 cells/cm^2, 0.3 µM CHIR99021
20k 0.5/[0.5]	20,000 cells/cm^2, 0.5 µM CHIR99021
20k 0.7/[0.7]	20,000 cells/cm^2, 0.7 µM CHIR99021

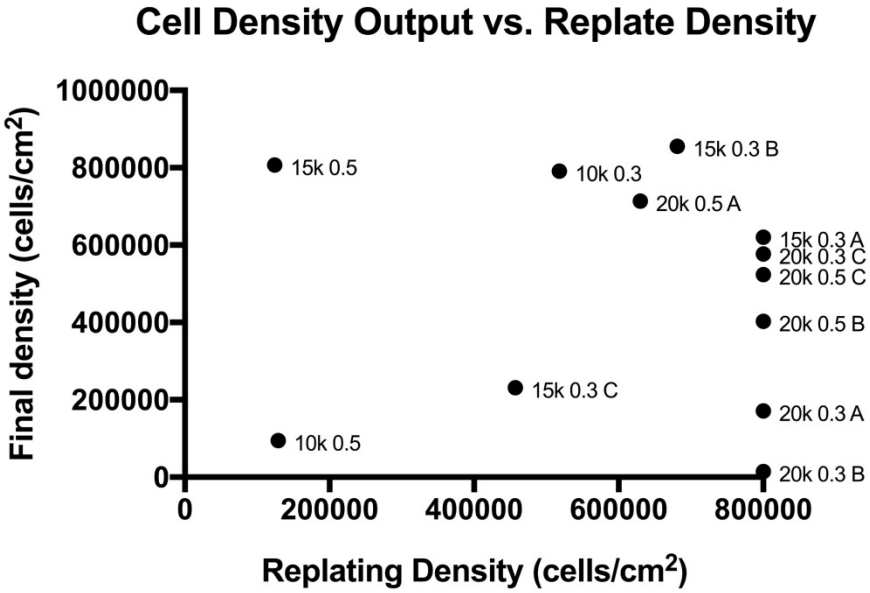


Figure 66. A graph displaying the initial replating density at day 11 plotted against the final density on day 16. On day 11, cells were harvested and reseeded at 800,000 cells/cm^2 where possible. The replating densities were calculated for the conditions seeded below 800,000 cells/cm^2. Conditions replated at 800,000 cells/cm^2 had a range of final cell densities from 14,000 to 833,000 cells/cm^2; this corresponded to 125,000 to 7.4 million total cells per well. Conditions replated at densities <800,000 cells/cm^2 also had a range of final densities with some of them having higher final densities than the conditions at 800,000 cells/cm^2. **N.B**. A, B and C relate to the replicate within each condition.

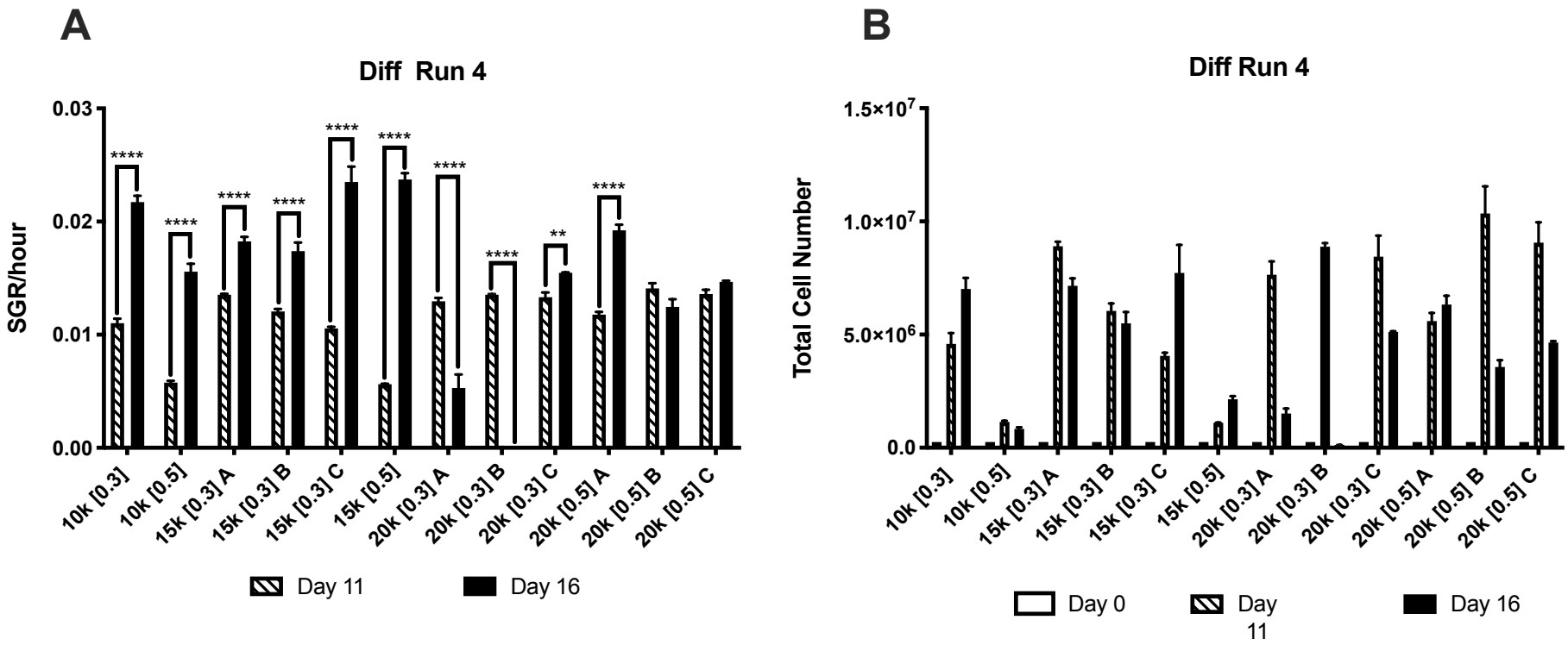


Figure 67. (**A**) Specific growth rate (SGR) and (**B**)total harvested cell numbers from each of the culture conditions that survived in DR4 at both day 11 and 16. The SGR data demonstrated that there were inconsistencies in growth within the conditions, with significant differences in SGR from day 11 to 16 within each condition. The total cell number data demonstrated an increase in cells from day 0 to day 11, followed by a decrease in cell number when the cells were harvested on day 16. In the conditions that did not perform as well such as 10k [0.5] there was little difference in cell number between day 11 and day 16. Conversely, in conditions that initially performed well such as 20k [0.5] the cell number decreases from day 11 to 16 i.e. less than half the cells that were reseeded on day 11 were harvested on day 16. Error bars indicate standard deviation, n=2 for each count. "*" indicates $p<0.05$, "**" indicates $p< 0.01$, "***" indicates $p< 0.001$, and "****" indicates $p<0.0001$.

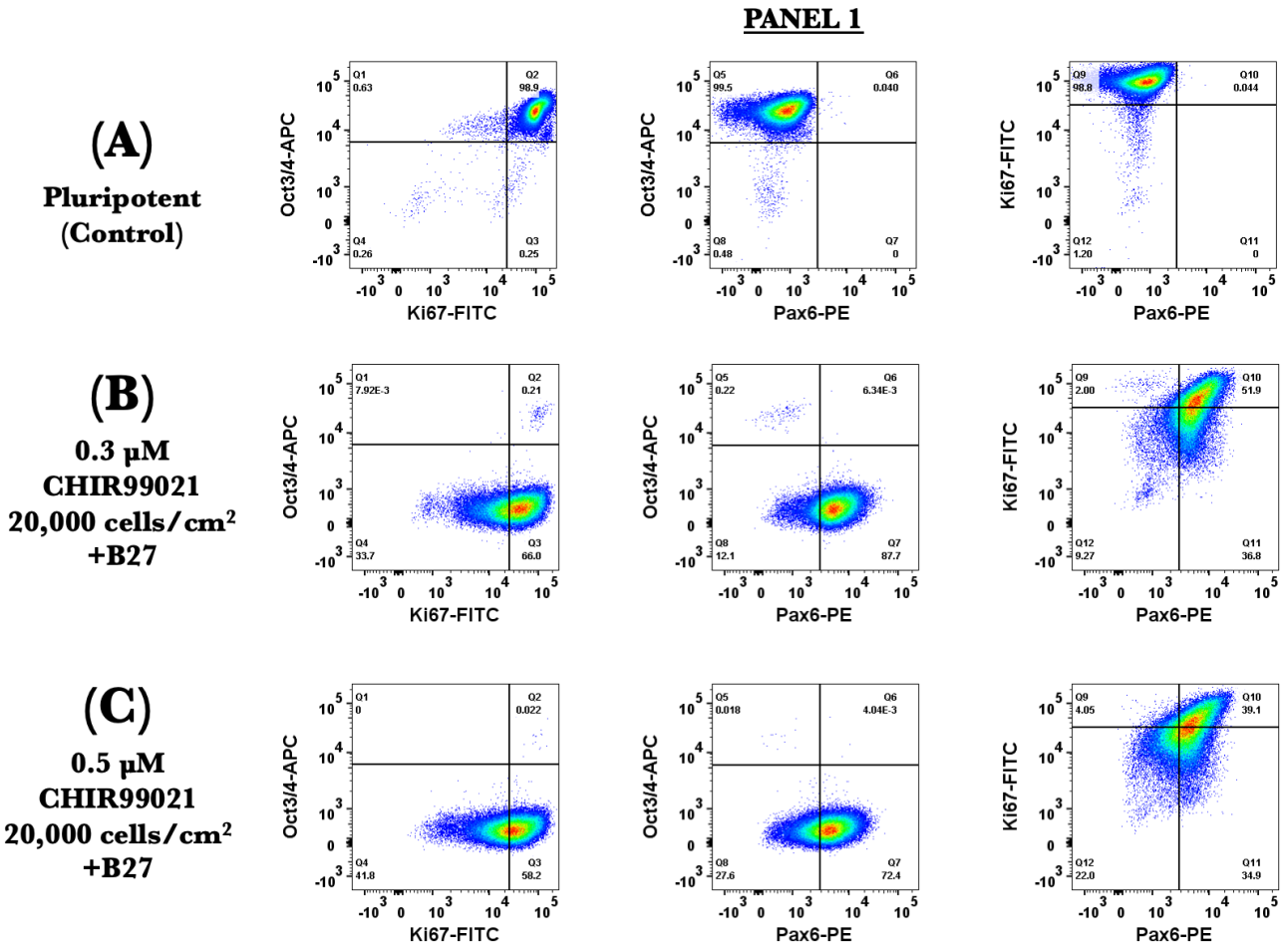


Figure 68. Dot plots of the pluripotent cells, the 0.3 and 0.5 µM CHIR99021 at 20,000 cells/cm^2 with B27 conditions. The key difference between the cell samples is the concentration of CHIR99210. The results show that there was no phenotypic marker profile difference due to CHIR99210 concentration. In the two conditions the seeding density and B27 supplementation remained the same. In comparison to the control, both differentiated sample cells demonstrated a decrease in OCT3/4 and a population shift in terms of reduced proliferation marker Ki67. However, the samples expressed PAX6 at day 16

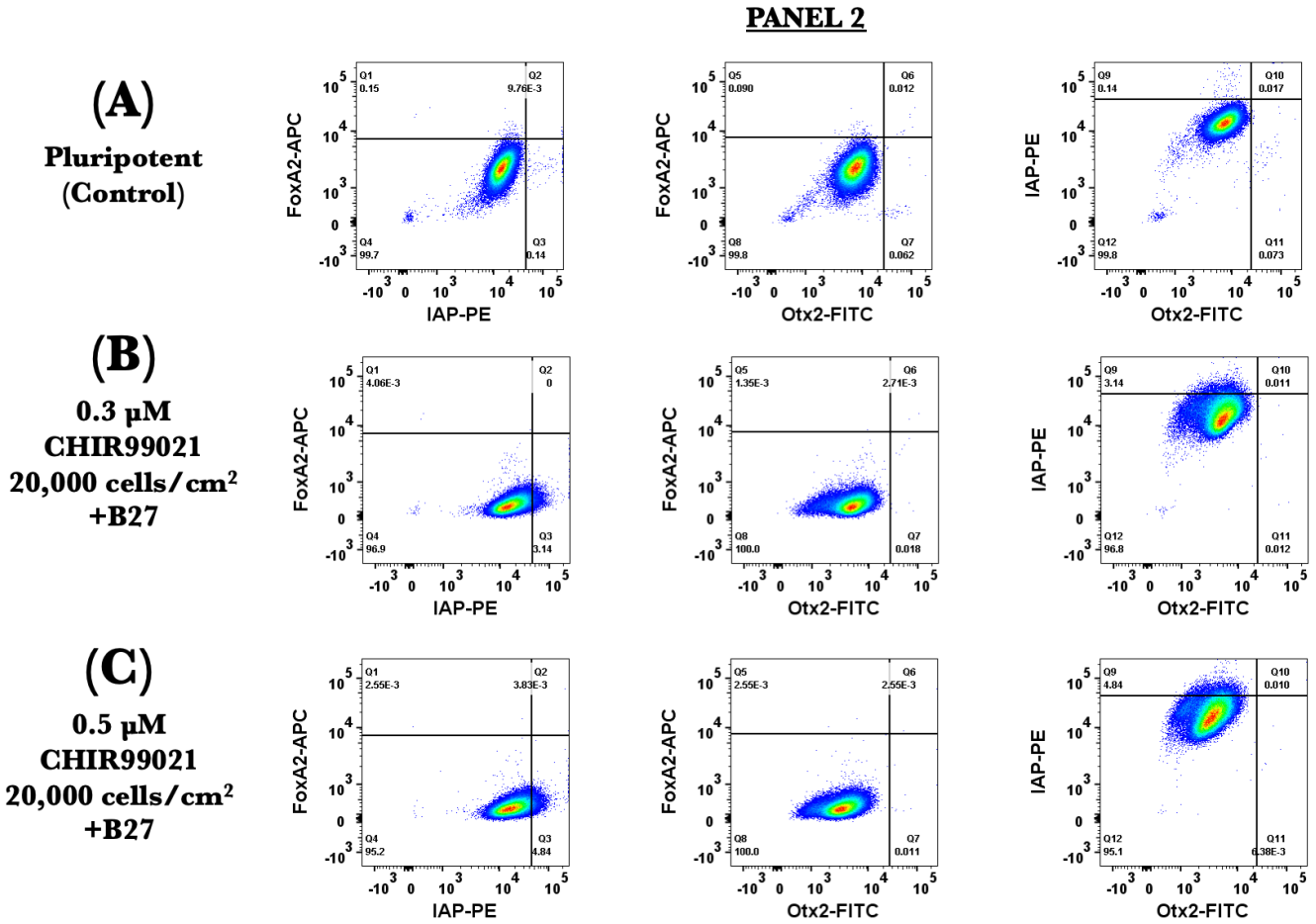


Figure 69. Comparable to panel 1 the cells have differing CHIR99021 concentration (0.3 *vs* 0.5 µM), however the seeding density was equivalent and B27 was supplemented at day 0. In this panel (Miltenyi panel 2) the differentiated cells for both conditions showed little difference in comparison to the pluripotent control cells. The dot plots demonstrate that both of the differentiated conditions are negative for FOXA2 and OTX2 in comparison to the pluripotent cells.

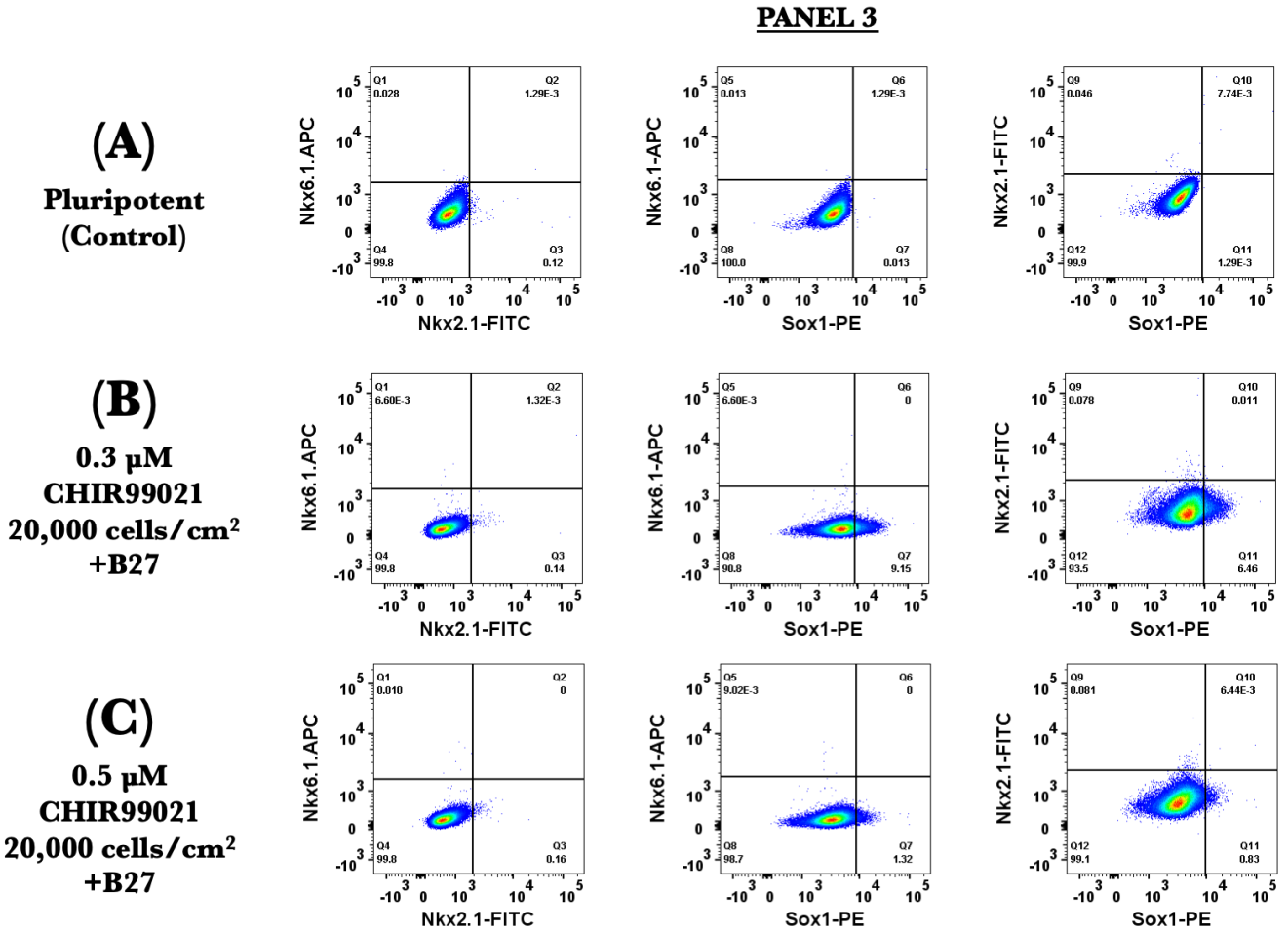


Figure 70. Panel 3 demonstrates the negative expression of NKX6.1 and NKX2.1; however, expression of SOX1 was observed. For the desired cell phenotype all three markers should be negative as these markers are associated with lateral or rostral cell fates which would be considered a contaminant cell type within the cultures.

6.3.4. Differentiation: Differentiation Run 5 - CHIR99021, Density and B27 multivariate analysis

The culture conditions in DR5 resulted in similar observations in comparison to DR4. For instance, the 0.7 μM CHIR99021 conditions in DR5 were the first experimental conditions to be terminated, due to extensive detachment and cell death. However, all conditions in DR5 demonstrated greater consistency regarding the behaviour of the experimental biological replicates, unlike the conditions in DR4. Furthermore, in DR5 the earliest termination point was day 7 (**Figure 71**); much later than the first termination point of day 2 in DR4 (**Figure 64**). SGR and total cell number data also demonstrated less variation between experimental conditions in DR5 compared to DR4 (**Figure 67** vs **Figure 72**). Additionally, in DR5 there was an increase in cell number from day 0 to 11 and also from day 11 to 16 in all conditions, yielding overall better growth and survival compared to DR4 (**Figure 67** vs **Figure 72**).

The flow cytometry data revealed that although the cells were no longer pluripotent due to low levels of OCT3/4, the desired cell phenotype was not obtained from any of the experimental conditions investigated. This was evident from the low expression levels of FOXA2, OTX2 and IAP within panel 2, the dopaminergic neuronal subtype panel of markers. In all conditions the untreated pluripotent cell control had higher MedFI values within panel 2 (**Figure 73**). Three of the conditions (15,000 cells/cm^2 0.3 μM CHIR99021; 20,000 cells/cm^2 0.3 μM CHIR99021+ B27 and 20,000 cells/cm^2 0.5 μM CHIR99021+ B27) had higher MedFI levels of NKX2.1 in comparison to the control, whilst NKX6.1 levels were similarly lower compared to the control in all of the conditions (**Figure 73**, panel 3).

N.B. The codes used in DR4 (**Table 40**) are the same for DR5.

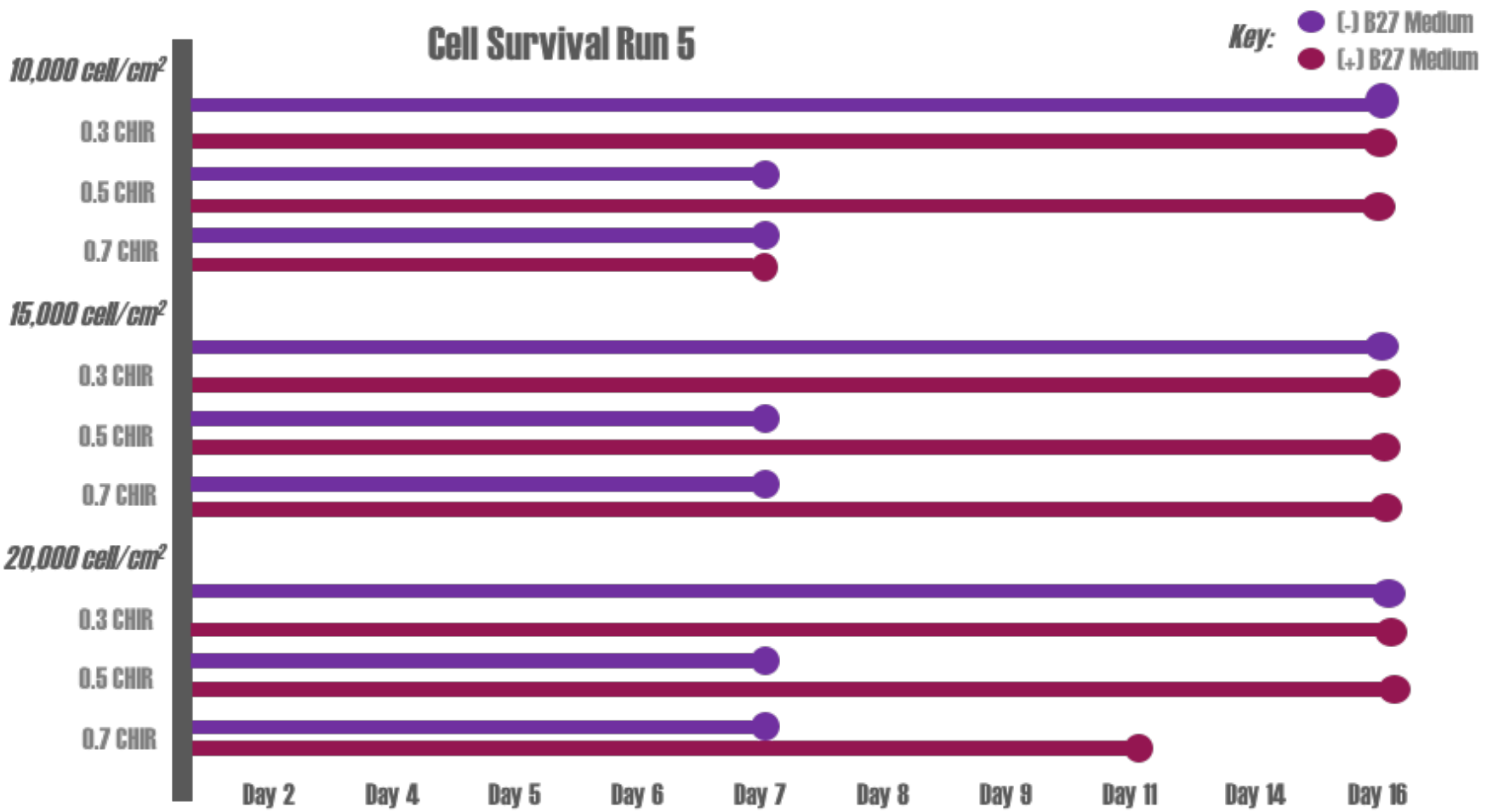


Figure 71. A comparative timeline illustrating point of cell death during differentiation run 5. The end points represent the day that the respective condition was terminated due to excess cell death within the culture condition, if early than day 16. Higher levels of consistency were observed in DR5 in comparison to DR4, all replicates with the conditions behaved the same in DR5, the earliest termination time point was day 7.

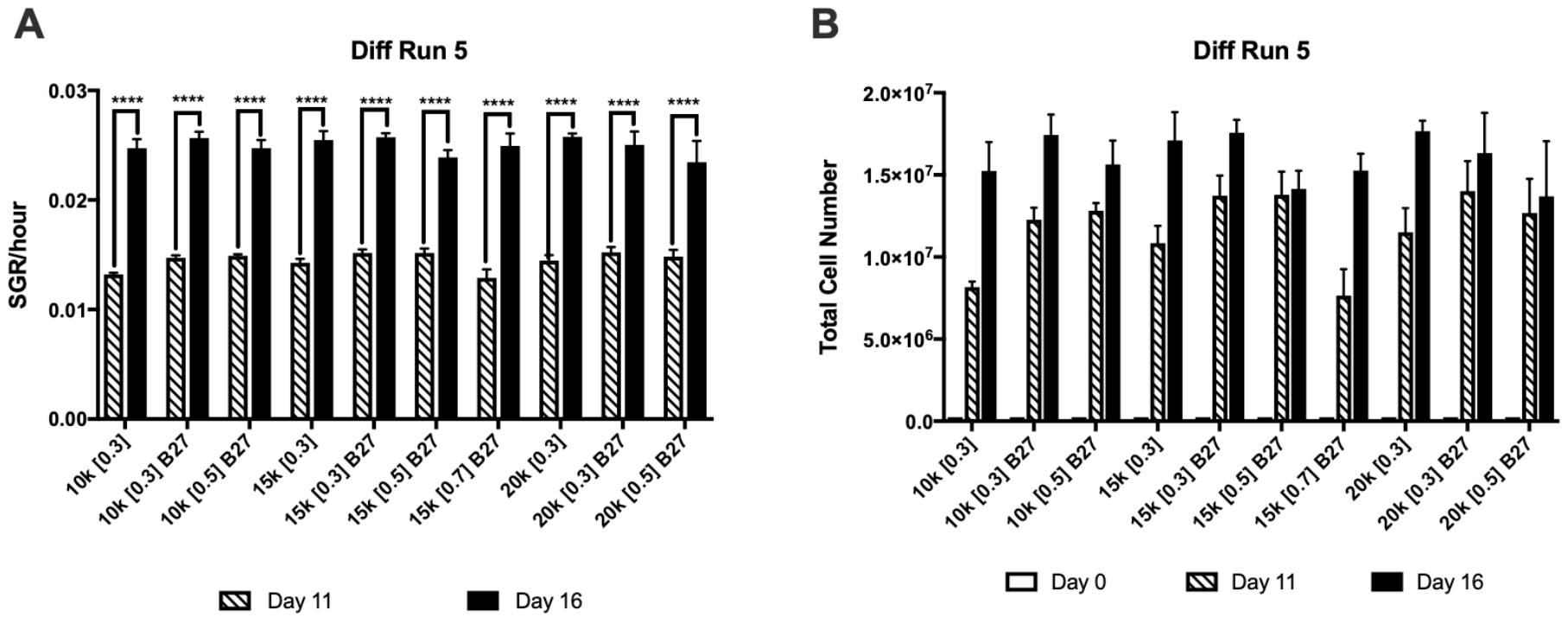


Figure 72. (**A**)Specific growth rate (SGR) and (**B**)total harvested cell numbers from the conditions that survived in DR5 at both day 11 and 16. The SGR data demonstrated a significant difference between day 11 and day 16 in all of the experimental conditions. The cell number data showed an increase in total cell number from day 0 to day 11 and from day 11 to day 16 in all of the conditions. This illustrates a greater consistency and performance in all of the conditions in comparison to DR4. Error bars indicate standard deviation, n=3. "*" indicates $p<0.05$, "**" indicates $p< 0.01$, "***" indicates $p< 0.001$, and "****" indicates $p<0.0001$.

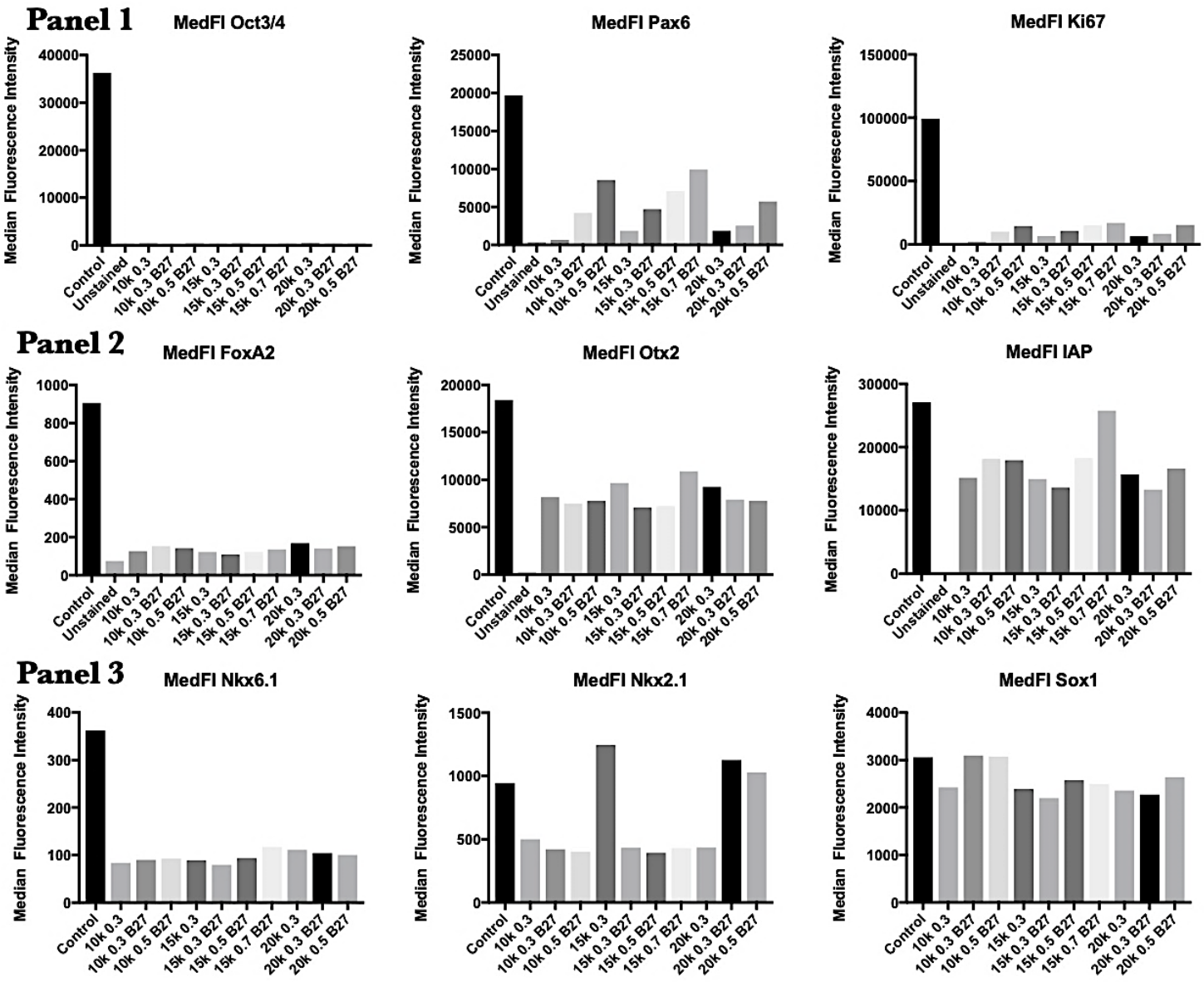

Panel 1
MedFI Oct3/4
MedFI Pax6
MedFI Ki67
Panel 2
MedFI FoxA2
MedFI Otx2
MedFI IAP
Panel 3
MedFI Nkx6.1
MedFI Nkx2.1
MedFI Sox1
Median Fluorescence Intensity
Control
Unstained
10k 0.3
10k 0.3 B27
10k 0.5 B27
15k 0.3
15k 0.3 B27
15k 0.5 B27
15k 0.7 B27
20k 0.3
20k 0.3 B27
20k 0.5 B27

Figure 73. Median fluorescence intensity values of the different markers for all experimental conditions**.** The OCT3/4 MFI data showed that there was negligible expression of OCT3/4 by day 16. There were varying levels of Ki67 expression in the experimental conditions, however they were all lower compared to the untreated pluripotent cell control samples. For the panel 2 markers the MedFI values showed that none of the experimental conditions had higher expression of the putative vmDA markers FOXA2 and OTX2 than the control. Panel 3 showed that all conditions were lower in comparison to the control for NKX6.1 yet some of the conditions had higher NKX.2.1 MedFI value compared to the controls. SOX1 MedFI values showed similar or lower values compared to the controls. The x axis codes are detailed in **Table 40**

6.3.5. Differentiation: Design of Experiment - Definitive Screen 1 (DSD1)

The medium formulations in the definitive screen illustrated that different concentrations of some of the small molecules (N2 supplement, SB431542, SHH-C24II and CHIR99021) had an impact on cell growth, survival and differentiation of the desired vmDA neuroprogenitor cell type (**Figure 78**). In particular, higher concentrations of SHH-C24II and CHIR99021 are required for cell growth and survival (**Figure 78A**); four of the thirteen conditions did not survive until day 11 (M3, M8, M12 and M13), with M12 and M13 being terminated at day 7, M3 and M8 at day 9 due to extensive cell detachment and death (**Figure 74**). In the case of M3, M8 and M12, the concentration of 0 ng/ml SHH-C24II was used (**Table 35**). All other conditions survived until day 11, however there were varying levels of cell yield (~ $5x10^5$ to $2x10^6$) and cell viability (~84 to 98 %) amongst the conditions. The M1and M6 conditions had the highest cell number yield at day 11 (~$2x10^6$ cells), cell viability (97- 98 %) and the highest population doublings (0.54 doublings per day) (**Figure 75**). M2 and M5 had low cell number yields of ~ $5x10^5$ at day 11 and cell viabilities below 90 %. The M2 and M5 conditions also had the highest levels of PAX6 at all time points analysed, whilst the M1 and M6, M9 and M11 experimental conditional had the lowest PAX6 MedFI values and M1 and M6 had high FOXA2 MedFI values that increased incrementally until the day 11 endpoint (**Figure 76** and **Figure 77**).

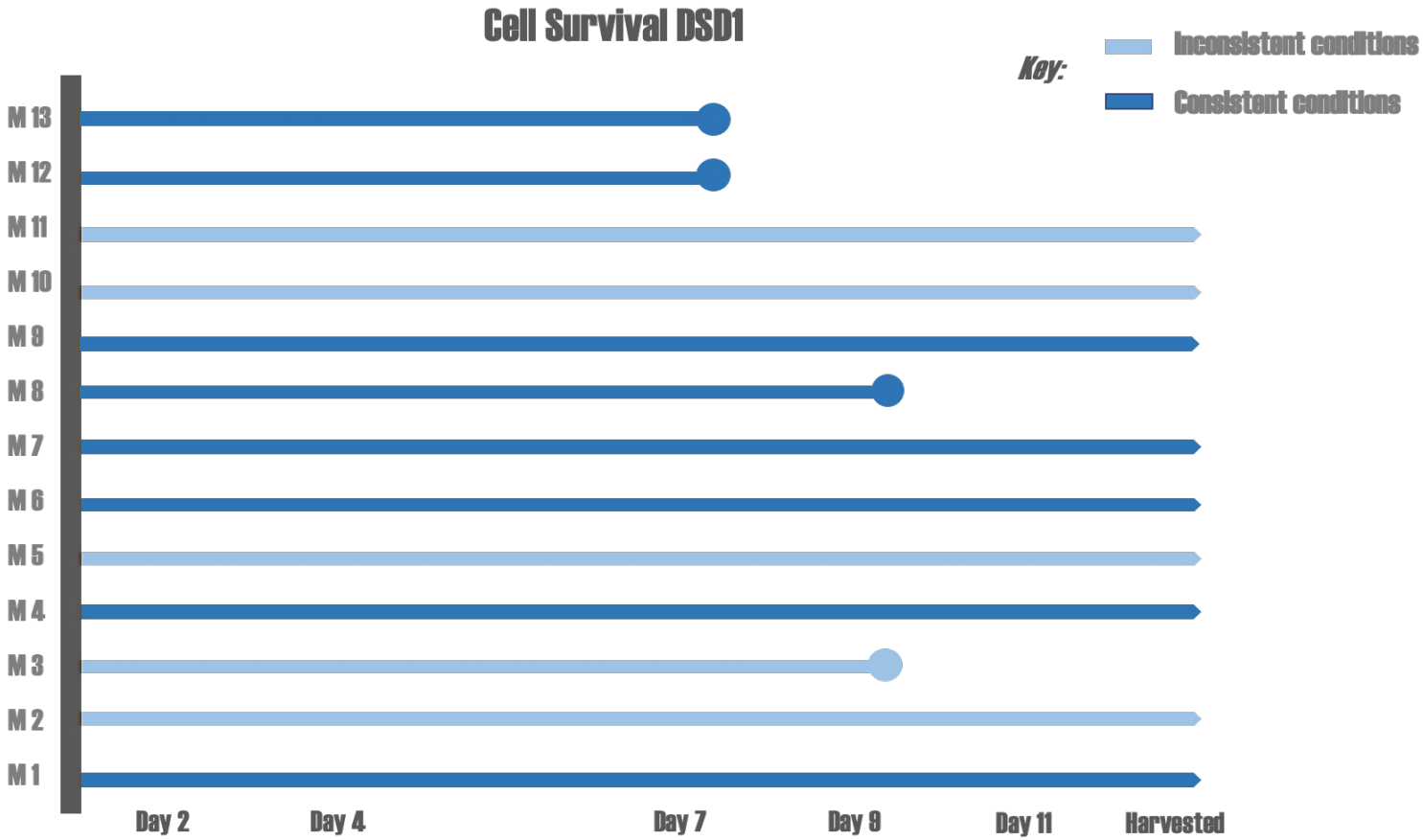


Figure 74.Schematic detailing the termination time points for DSD1. The lighter blue colour represents conditions that were inconsistent throughout the experiment. For instance, some replicates under the M2 conditions were terminated at day 7; however, some were continued until day 11 prior to harvest. The darker blue colour demonstrates the conditions that behaved consistently throughout the experiment both in terms of when they were terminated and when they were harvested i.e. all replicates in M8 were terminated on day 9. M12 and 13 were the first conditions to be terminated due to extensive detachment at cell death by day 7.

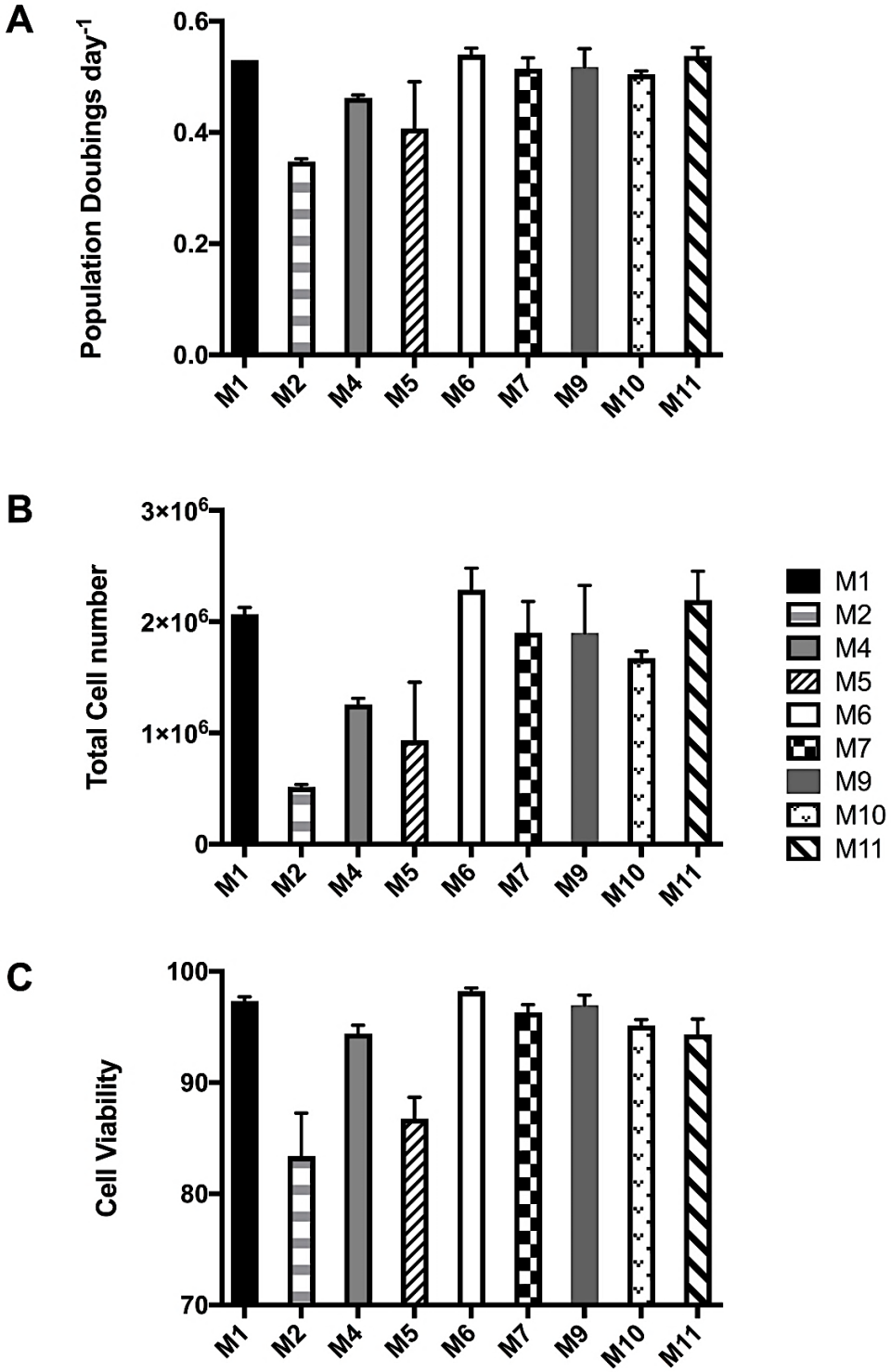


Figure 75. Cell growth and viability data of the different conditions of the definitive screen. For the majority of the culture conditions, the population doubling rate was 0.5 doublings per day, resulting in ~6 population doublings over the 11-day culture period (**A**). The number of cells harvested at day 11 ranged from $5x10^5$ to $2.1x10^6$ with M2 having the lowest cell yield and M6 having the highest (**B**). The cell viabilities ranged from 84 % to 98 %, M2 had the lowest and M6 the highest viability (**C**). Error bars indicate standard deviation, n=4.

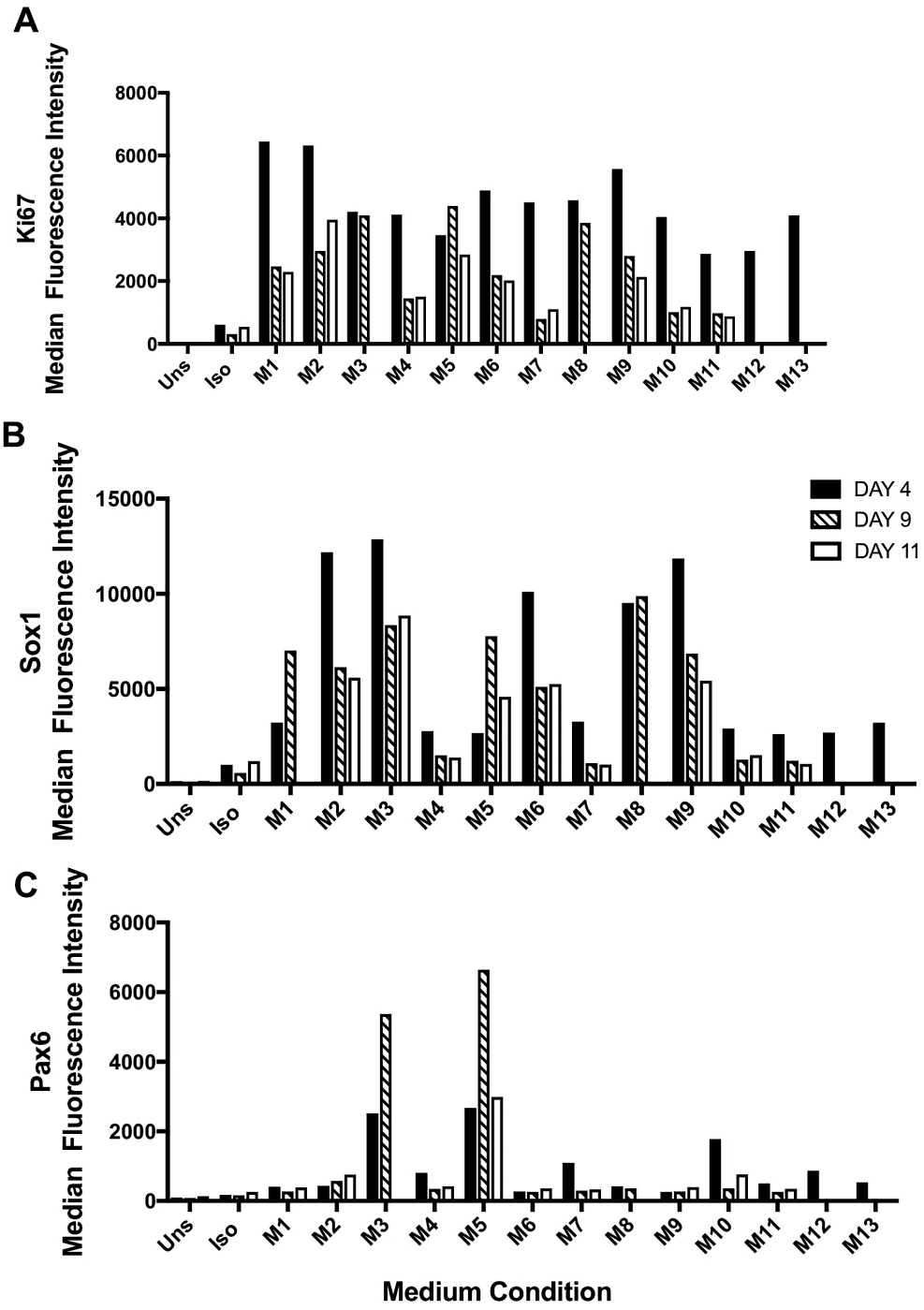


Figure 76. Median fluorescence values. (**A**) Ki67 was observed to decrease from day 4 to 9 in the majority of the conditions. (**B**) SOX1 decreased from day 4 to day 9 and remained at similar levels at day 11. (**C**) PAX6 expression varied amongst the different conditions, initially expressing low fluorescence prior to increasing in some of the experimental conditions (M2, M3 and M5); whilst in other conditions PAX6 expression remained low throughout the eleven-day culture period (M1, M6 andM9), other conditions had a decrease in PAX6 MedFI (M4, M7 and M10). Conditions M2, M8, M12 and M13 were terminated prior to the day 11 time-point, therefore no values were obtained. Uns = unstained; Iso = isotype.

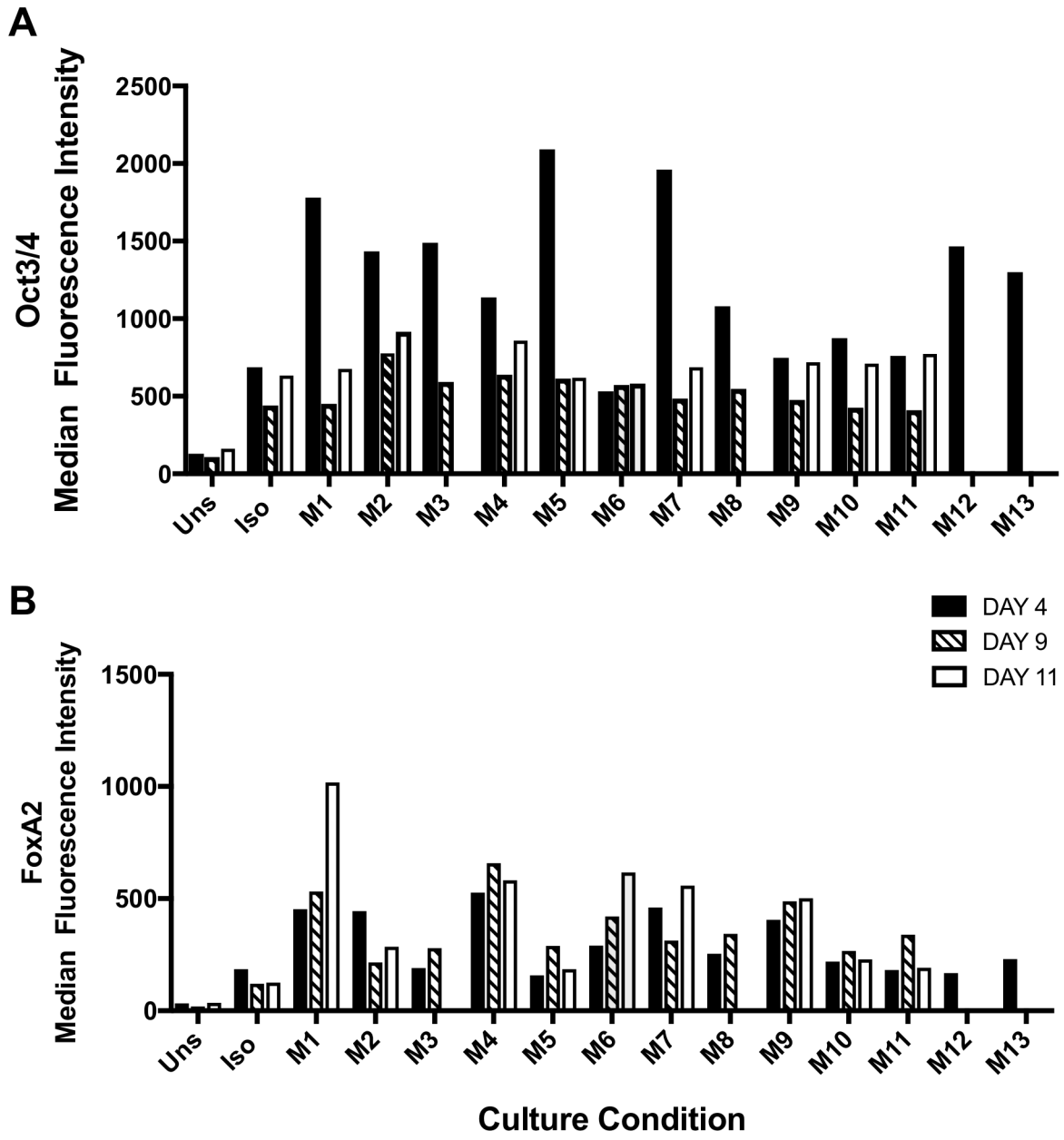


Figure 77. (**A**) OCT3/4 decreased from day 4 to day 9 followed by an increase from day 9 to 11, however this was not to the same level as the MedFI values on day 4. (**B**) FOXA2 increased from day 4 to day 9 in all the conditions except for M3 and M7 where they decreased from day 4 to 9. Some conditions had a decrease in FOXA2 MedFI from day 9 to 11(M4, M5, M10 and M11) while other conditions had an increase in fluorescence (M1, M6 and M7) with M1 having the highest MedFI of all the conditions investigated. Conditions M2, M8, M12 and M13 were terminated prior to day 11, therefore no values were obtained. Uns = unstained; Iso = isotype.

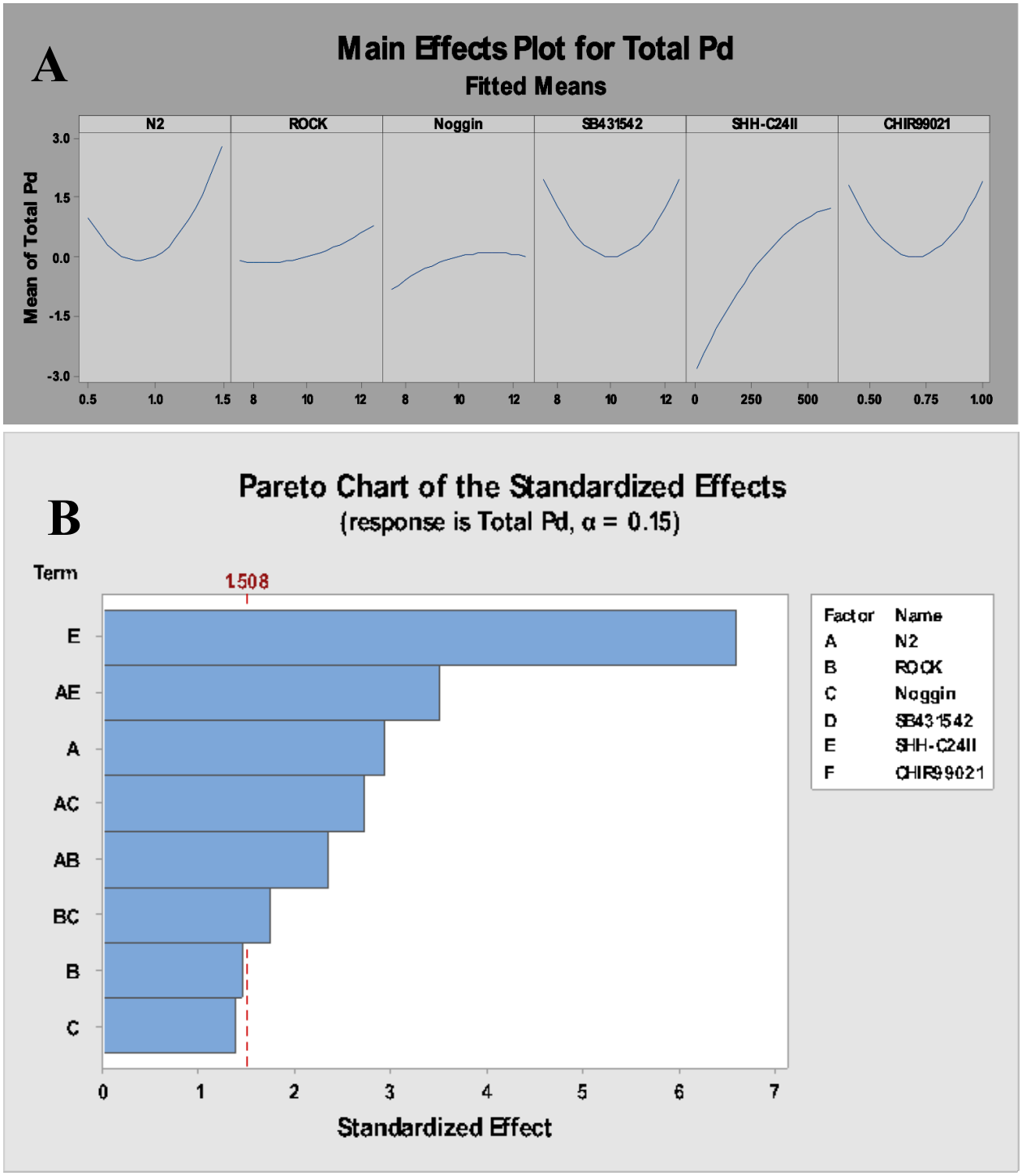


Figure 78. (**A**) Main effects plot of the factors that impacted population doubling rate at the experimental end point day 11. N2 and SHH-C24II had a significant impact on the population doubling rate, higher concentrations of both factors resulted in an increase of doubling rate. (**B**)Pareto chart detailing the level of effect that the different factors had on population doublings at the experimental end point day 11. N2 and SHH-C24II, combined and individually had the largest effect on population doubling rate.

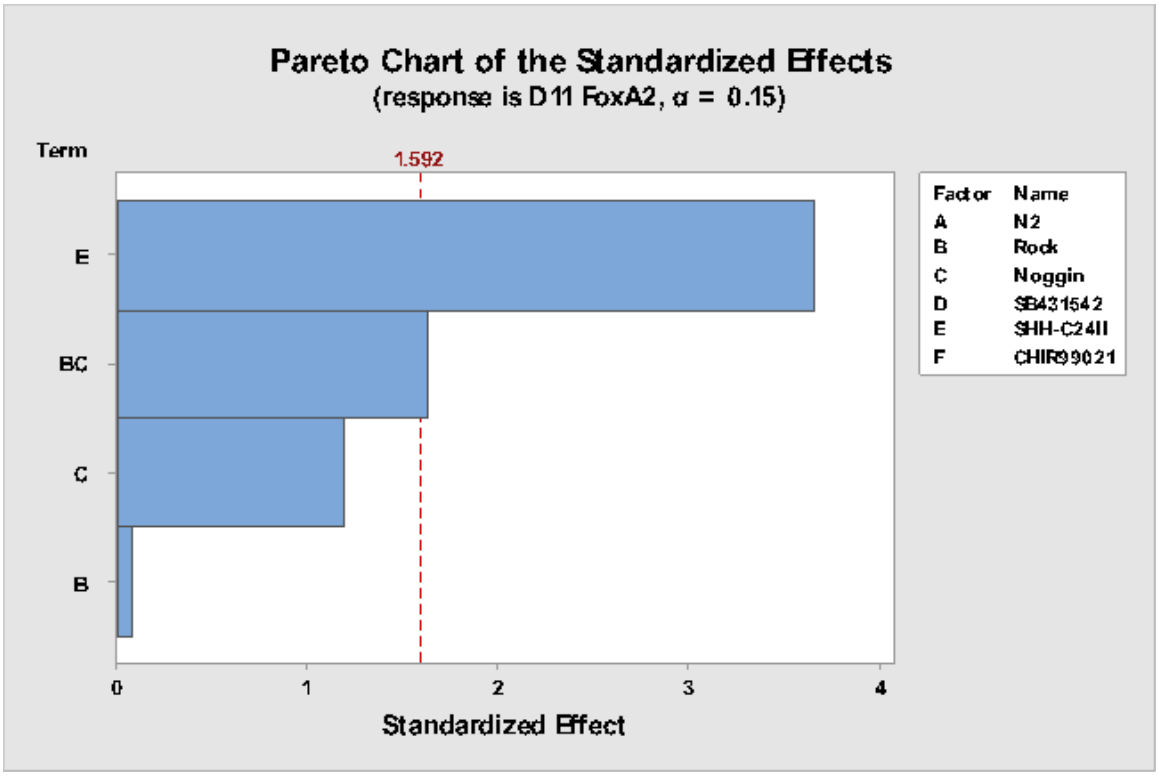


Figure 79. Pareto chart detailing the level of effect the different experimental factors had on FOXA2 expression at the experimental end point day 11. SHH-C24II had the largest impact on FOXA2 expression.

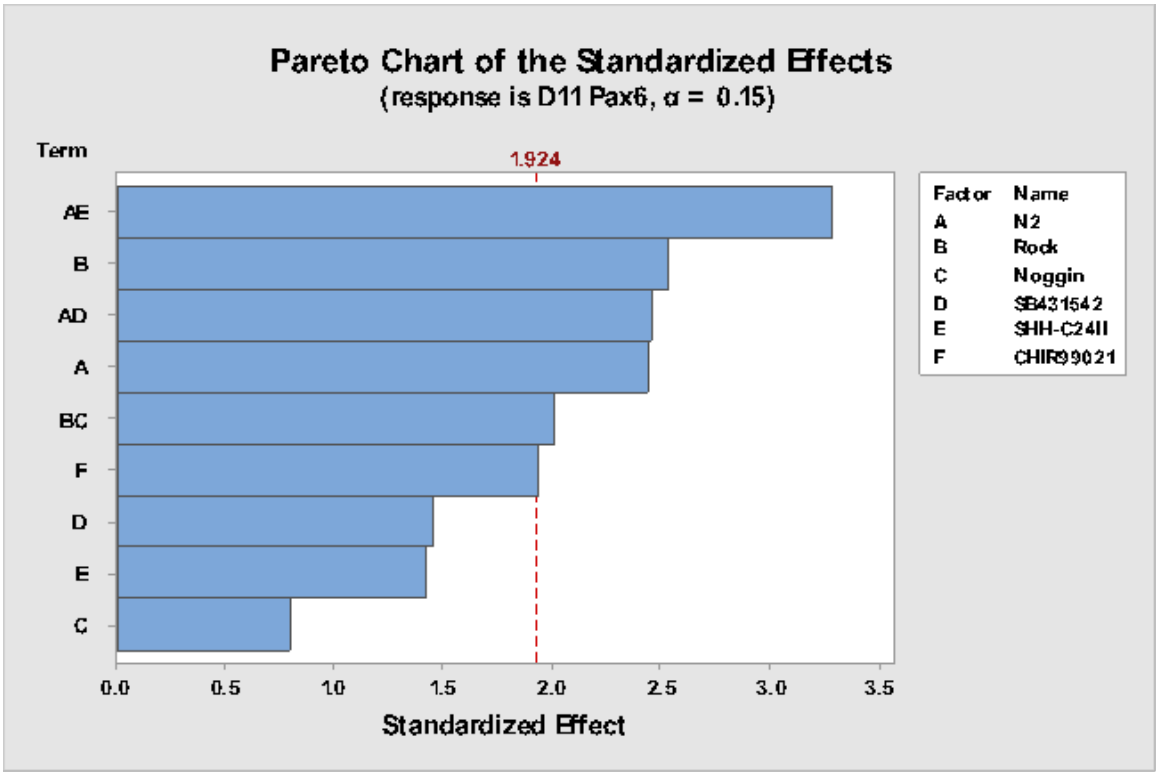


Figure 80. Pareto chart detailing the level of effect the different experimental factors had on PAX6 expression at the experimental end point day 11. N2 supplement by itself and in combination with other factors such as SHH-C24II and noggin influenced PAX6 expression.

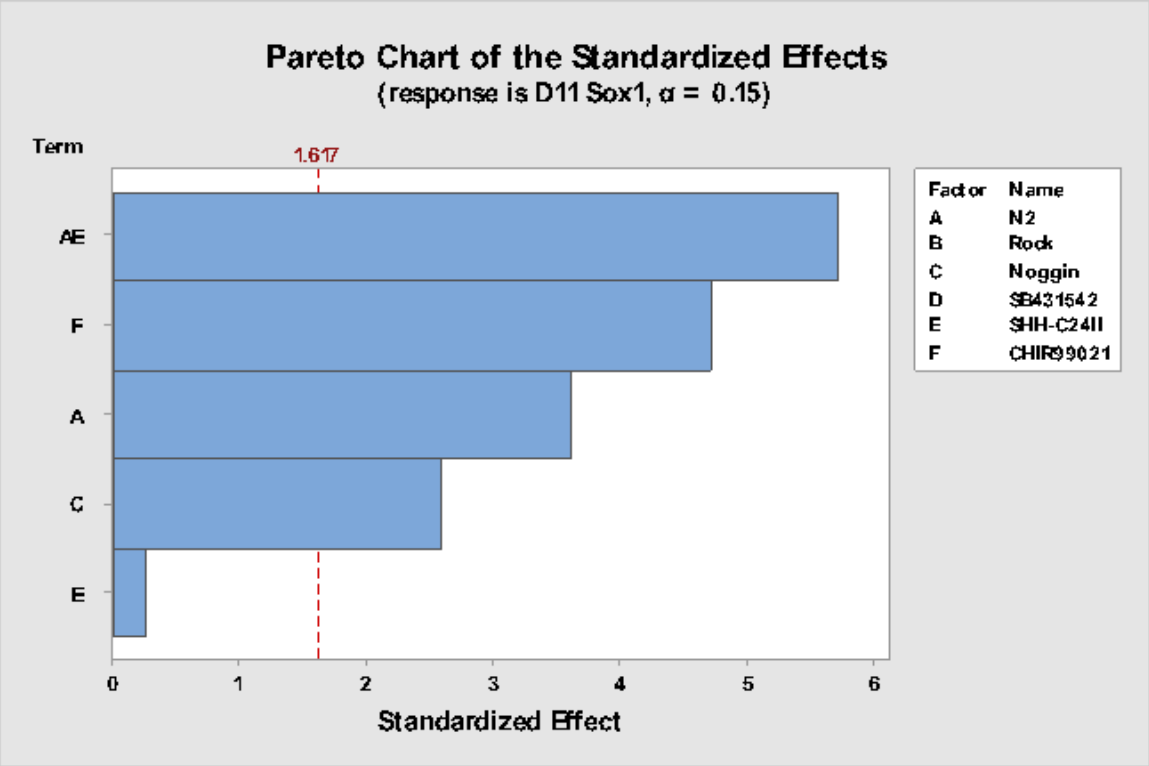
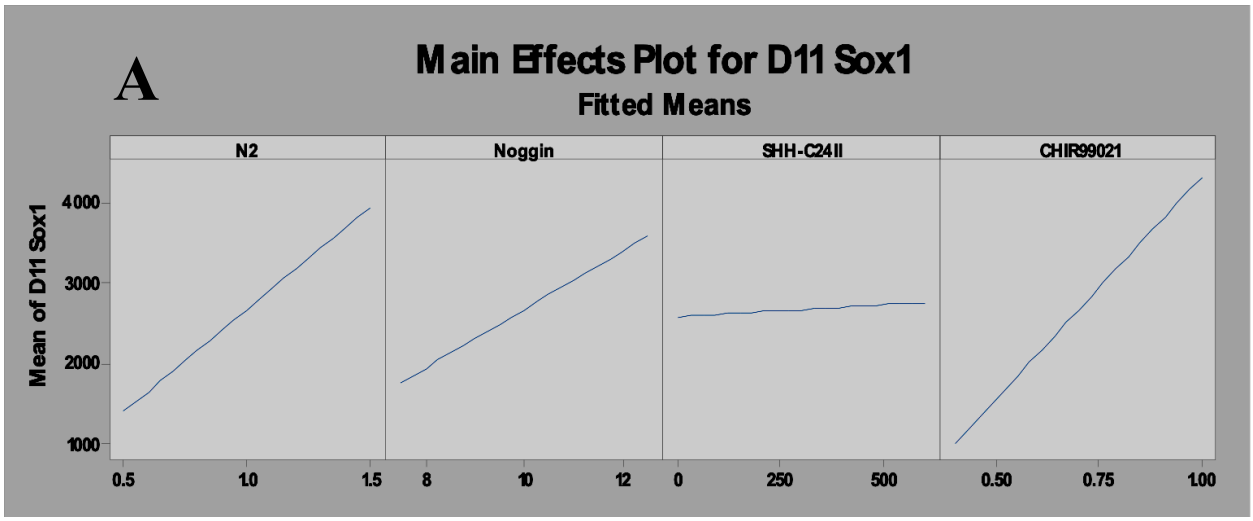
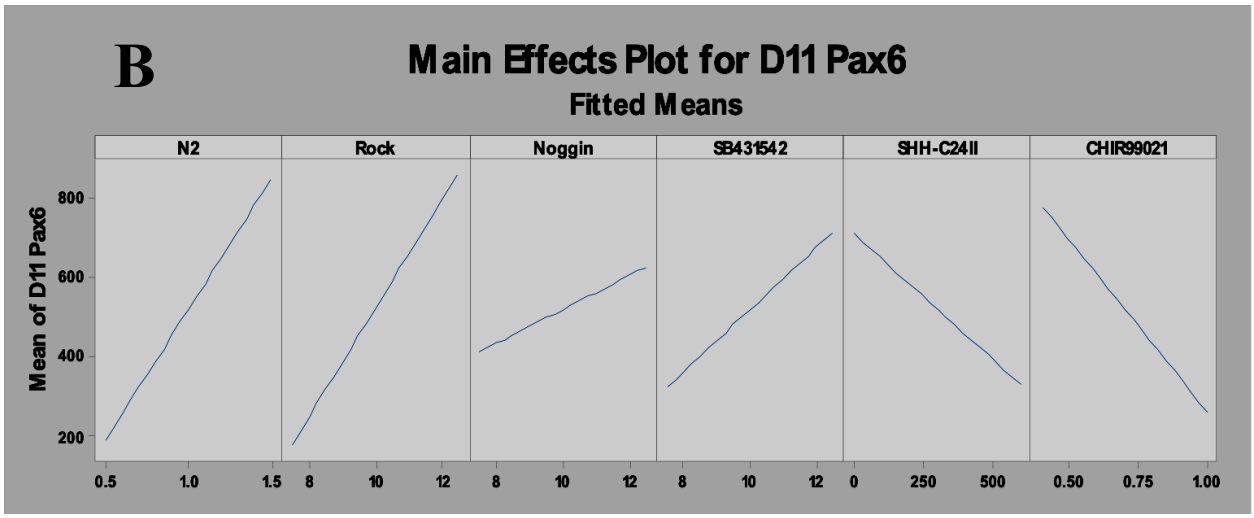


Figure 81. Pareto chart detailing the level of effect the different experimental factors had on SOX1 expression at the experimental end point day 11. N2 supplement, CHIR99021, SHH-C24II and noggin had a significant impact on SOX1 expression.


A
Main Effects Plot for D11 Sox1
Fitted Means
N2
Noggin
SHH-C24II
CHIR99021
Mean of D11 Sox1
4000
3000
2000
1000
0.5
1.0
1.5
8
10
12
0
250
500
0.50
0.75
1.00


B
Main Effects Plot for D11 Pax6
Fitted Means
N2
Rock
Noggin
SB431542
SHH-C24II
CHIR99021
Mean of D11 Pax6
800
600
400
200
0.5
1.0
1.5
8
10
12
8
10
12
8
10
12
0
250
500
0.50
0.75
1.00

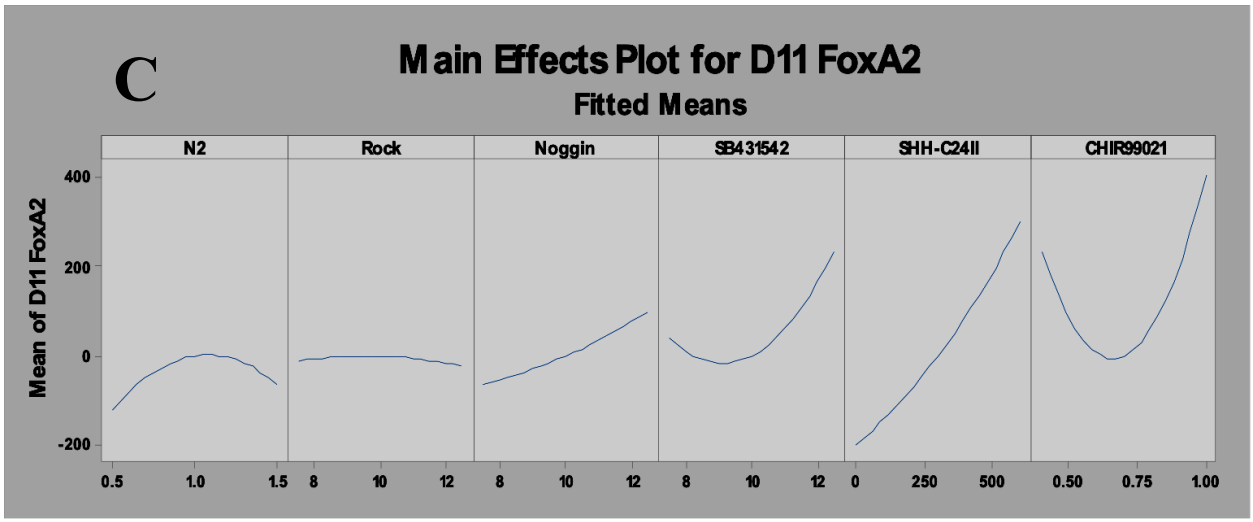

C
Main Effects Plot for D11 FoxA2
Fitted Means
N2
Rock
Noggin
SB431542
SHH-C24II
CHIR99021
Mean of D11 FoxA2
400
200
0
-200
0.5
1.0
1.5
8
10
12
8
10
12
8
10
12
0
250
500
0.50
0.75
1.00

Figure 82. Main effects plot of the factors and their influence on marker expression at the experimental end point day 11. (**A**) N2 supplement, noggin and CHIR99021 exhibited a linear relationship to SOX1 expression, SHH-C24II concentration at the ranges used did not have an impact on SOX 1 expression. (**B**) PAX6 expression had a strong positive linear relationship to N2 supplement and ROCK-inhibitor, SHH-C24II and CHIR99021 had a strong negative correlation to PAX6 expression. (**C**) FOXA2 marker expression main effects, noggin and SHH-C24II had a positive linear relationship with FOXA2 expression, higher levels of both factors resulted in higher levels of FOXA2 MedFI. CHIR99021 had a non-linear relationship to FOXA2 expression, the mid-point concentration afforded the lowest levels of FOXA2 MedFI.

6.3.6. Differentiation: Design of Experiments – scale up experiment.

The SGR of all the conditions decreased from day 4 to day 9 and from day 9 to 11. The decreases were significant for all of the conditions across the days, with the exception of the 6 well plate format at 25,000 cells/cm^2 where no significant decrease from day 9 to day 11 was observed (**Figure 83A**). The cell number yield across the conditions increased overall from day 4 to day 9 and also from day 4 to 11 day. At each time point the SGR increases were significant for the majority of conditions, with the exception of the 12 well plate format at 20,000 cells/cm^2 which had no significant increase in cell number, instead the cell number decreased (**Figure 83B**). This decrease in cell number was also seen in the 12 well plate format at 15,000 cells/cm^2 which had a significant decrease in cell number from 4 to day 9 and also from day 4 to 11. This decreased cell number was also accompanied by a decrease in cell viability in both the 15,000 and 20,000 cells/cm^2 conditions in the 12 well plate format (**Figure 83**C). At day 4 the average cell number yield in the 12-well plate format was ~$1.5x10^6$ cells per well, while at the lower density of 15,000 cells/cm^2 an average of ~$8x10^5$ cells were harvested per well on day 4. The 6-well format plates had yields between $1.5x10^6$ to $2x10^6$ cells by day 4, at day 11 the 6 well plate format had cell yields between $2x10^6$ to $4x10^6$ cells.

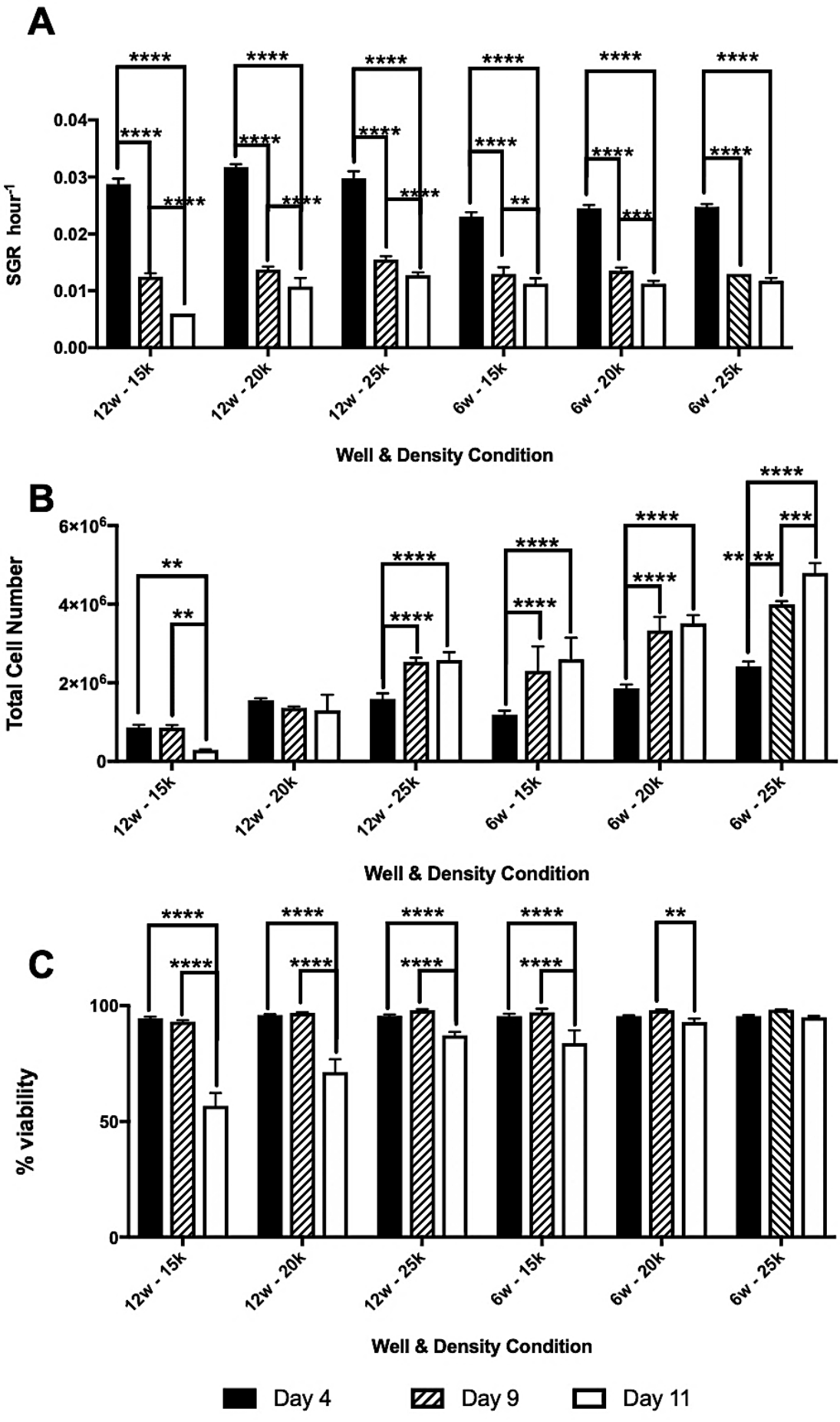

A
SGR hour-1
0.04
0.03
0.02
0.01
0.00
12w - 15k
12w - 20k
12w - 25k
6w - 15k
6w - 20k
6w - 25k
Well & Density Condition
B
Total Cell Number
6×10^6
4×10^6
2×10^6
0
Well & Density Condition
C
% viability
100
50
0
Well & Density Condition
Day 4
Day 9
Day 11

Figure 83. Cell growth dynamics detailing the SGR, total cell number per well and cell viability. (**A**) SGR significantly decreased from day 4 to day 9 and from day 9 to day 11 for all conditions. (**B**) Cell number decreased overall for the 12-well plate format at 15,000 cells/cm^2 and 20,000 cells/cm^2, all other conditions had a significant increase overall in cell number by day 11. (**C**) All of the conditions had a significant decrease in cell viability by day 11, apart from the 6-well plate format seeded at 25,000 cells/cm^2. The 12-well plate format seeded at 15,000 cells/cm^2 and 20,000 cells/cm^2 had the greatest decrease in cell viability from day 9 to day 11, compared to the other conditions. Error bars indicate standard deviation, n=2. "*" indicates $p<0.05$, "**" indicates $p< 0.01$, "***" indicates $p< 0.001$, and "****" indicates $p<0.0001$.

6.3.7. Differentiation: Design of Experiments – day 11 replating

Results indicated that seeding at a lower (400, 000 cells/cm^2) density allowed the cells to proliferate, as these conditions had positive SGR values, whilst those seeded at 800,000 cells/cm^2 had very low or negative SGR values (**Figure 84**). However, SGR values were not significantly different within the conditions in each density i.e. C1, C3, C5 and C7, all at 400, 000 cells/cm^2 did not significantly differ in SGR. Across the two time points the high-density conditions did not have significant increases in cell number, this was also the case for the mid-point condition of 600,000 cells/cm^2 (**Figure 84B**). Differences in cell viability were predominantly observed at day 14 in a range of the conditions, whilst at day 16 the only significant difference was between C1 and C9. Conditions C1 to C6 increased in cell viability from day 14 to day 16, while C7 to C9 had a decrease in cell viability (**Figure 84C**). Images of the cells 2 h after seeding showed that when seeding at a density of 800,000 cells/cm^2 the cells immediately attached and covered the entire available culture surface, leaving a high number of cells unattached.

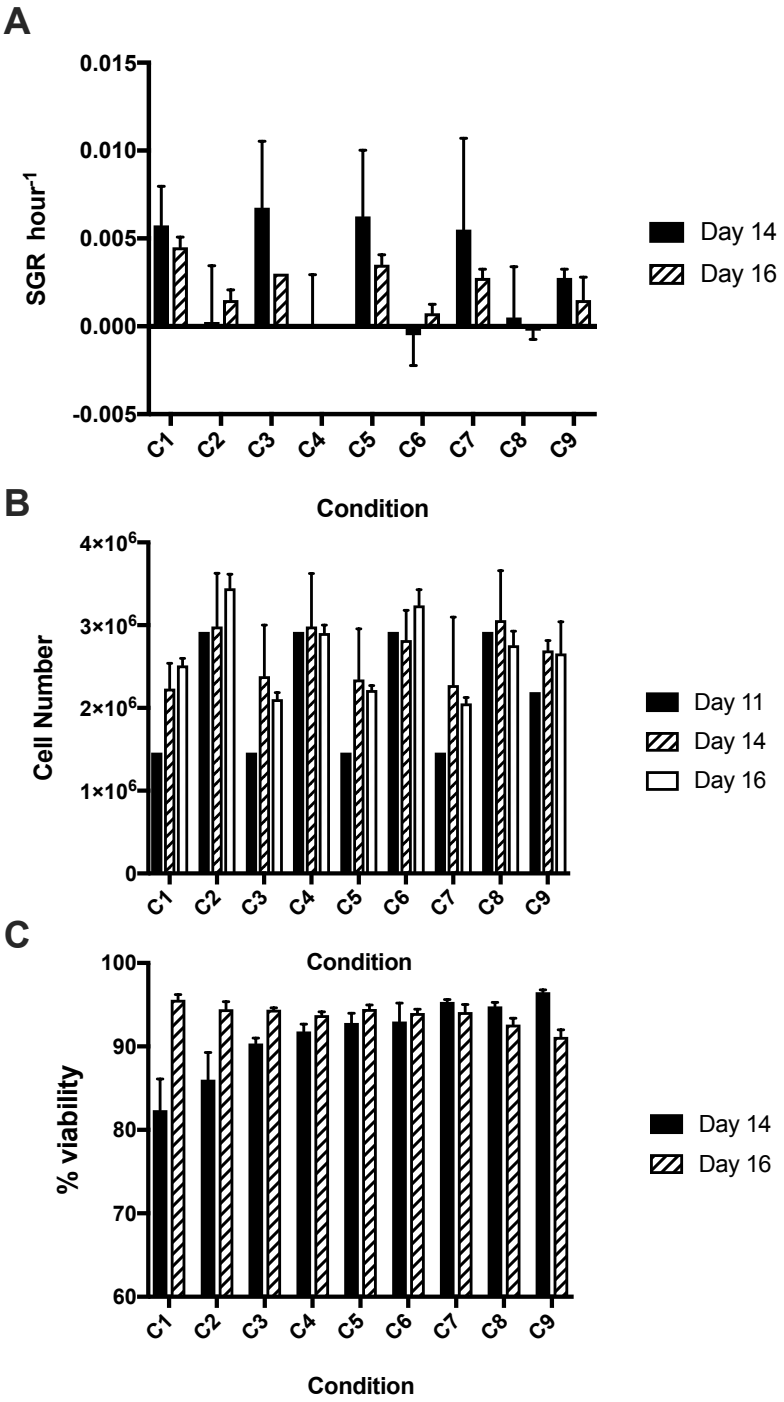


Figure 84. Growth dynamics detailing the SGR, total cell number per condition and cell viability. (**A**) SGR significantly decreased from day 14 to day 16 in all of the conditions p=0.0443 (*). The 400,000 cells/cm^2 density conditions (C1, C3, C5 and C7) had SGR values over 0.005 hour^{-1} whilst the conditions replated at 800,000 cells/cm^2 had very low (<0.001 hour^{-1}) or negative SGR values (**B**) Overall, the cell number increased for all the conditions with average yields between 2x10^6 and 3.5x10^6 per well at day 16; p>0.0001 (****). (**C**) Conditions C1 to C6 had a slight increase in cell viability from day 14 to day 16, while C7, C8 and C9 had a decrease in cell viability. Error bars indicate standard deviation, *n*=2.

6.3.8. Differentiation: Design of Experiment - Definitive Screen 2 (DSD2)

Cells in DSD2 maintained a viability above 85% during the sixteen-day period with all conditions ≥95% by day 16 (**Figure 87**). For the majority of the conditions the cell viability increased from time point to time point or stabilised by day 9, whereby there was a significant increase recorded from day 4. The overall SGR decreased with the majority of the conditions having a negative SGR by day 16 (**Figure 85**). Generally, the cell number increased over the 16 days, however in more than half of the conditions cell number yield decreased from day 11 to day 16 (**Figure 86**). Conversely, the in the remaining conditions there was an incremental increase in cell number yield at each time point, these conditions were: 2, 3, 6, 8, 10,11,13 and 15.

Phenotype analysis revealed a ~six-fold decrease in OCT3/4 MedFI for all of the condition when compared to the pluripotent control sample which resulted in a decrease of OCT3/4 positive cells from 90 % at day 0 to ~ 0.1 % at day16 (**Figure 88A**). On day four all of the conditions were significantly higher in Ki67 MedFI compared to day 9, 11 and 16, with some conditions expressing higher compared to the pluripotent control (**Figure 88B**). The conditions that had the highest FOXA2 MedFI levels were: 1,5,9,11,16, and 17 by day 16, these conditions were largely >60 % positive for FOXA2 (**Figure 89A** and **B**). In addition, these conditions were higher in OTX2 expression at day 16 compared to day 11 whilst being >90 % positive for OTX2 (**Figure 90A** and **B**). Other conditions such as: 3, 4, 6, 7, 8 and 10 had higher FOXA2 MedFI on day 9 or 11 in comparison to day 16. Low PAX6 levels were observed for conditions 1, 5, 9, 16, 17 (**Figure 91A**). Conditions 1, 4, 9, 16, 17 had high SOX1 MedFI expression at day 4 which decreased significantly by day 16 (**Figure 91B**), of these conditions 1, 9, 16, and 17 also had high FOXA2 expression on day 16.

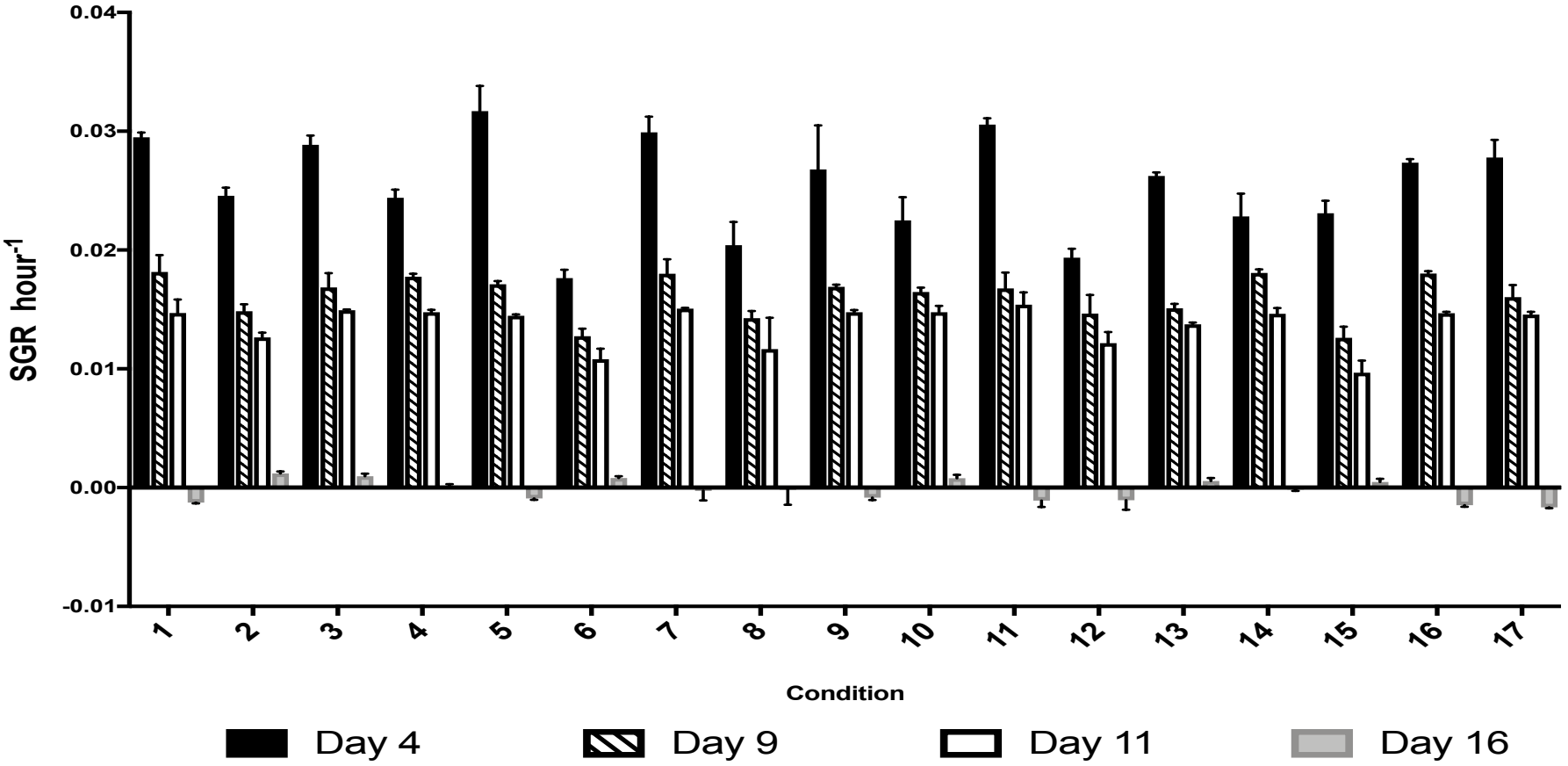


Figure 85. The SGR decreased overall from time point to time point $p<0.0001$ (****), the majority of the conditions had a negative SGR by day 16, when there was the most significant decrease in SGR. Error bars indicate standard deviation, $n=4$.

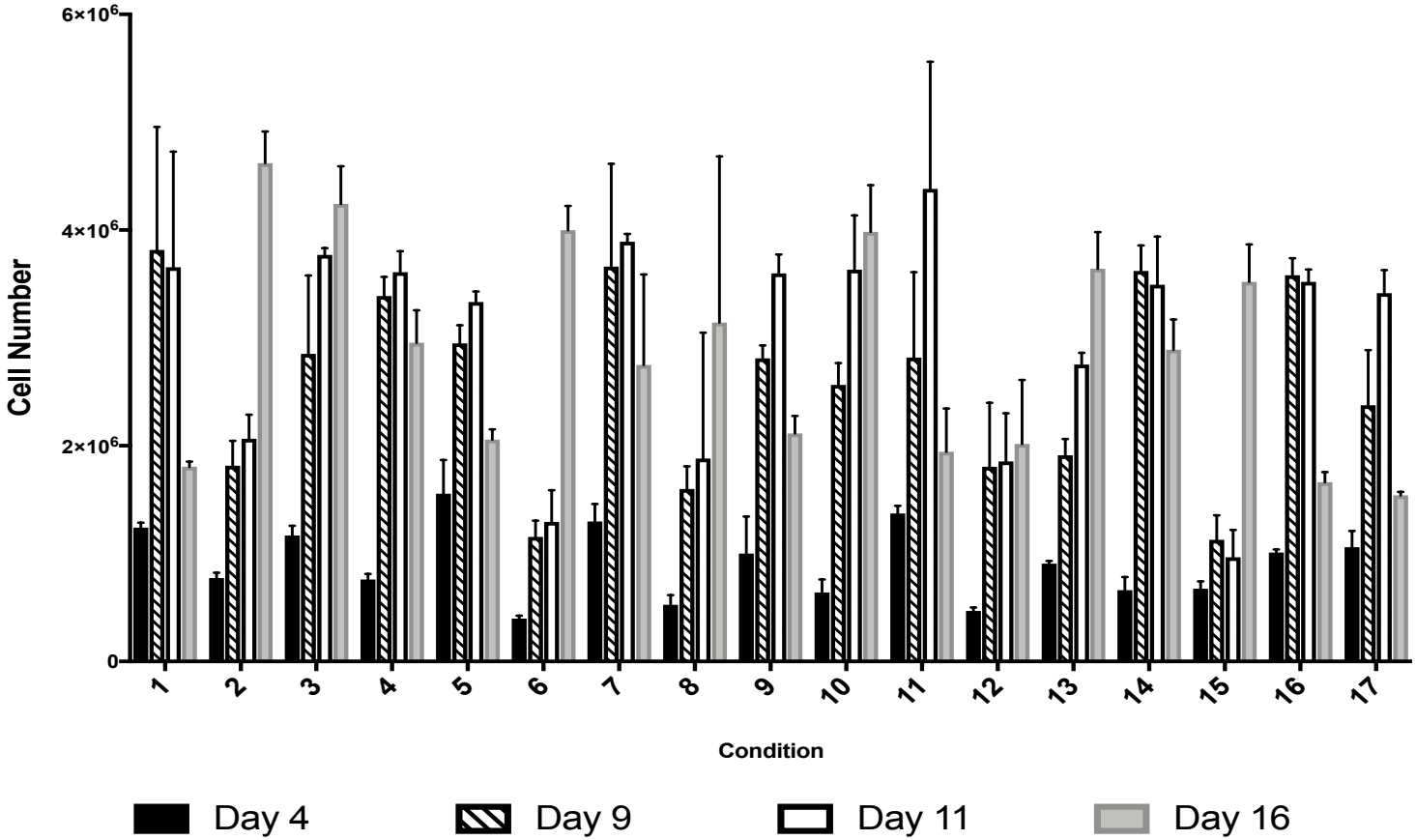


Figure 86. The cell number yields differed amongst the conditions, in particular at day 11 and 16, overall there was an increase in cell yield, however in more than half of the conditions cell number yield decreased from day 11 to 16. Conversely, the other conditions had their highest cell yields on day 16, these conditions were: 2, 3, 6, 8, 10, 13 and 15. Both the time point ($p<0.0001$ (****)) and the culture condition culture ($p<0.0001$ (****)) resulted in significant differences in cell yield. Error bars indicate standard deviation, $n=4$.

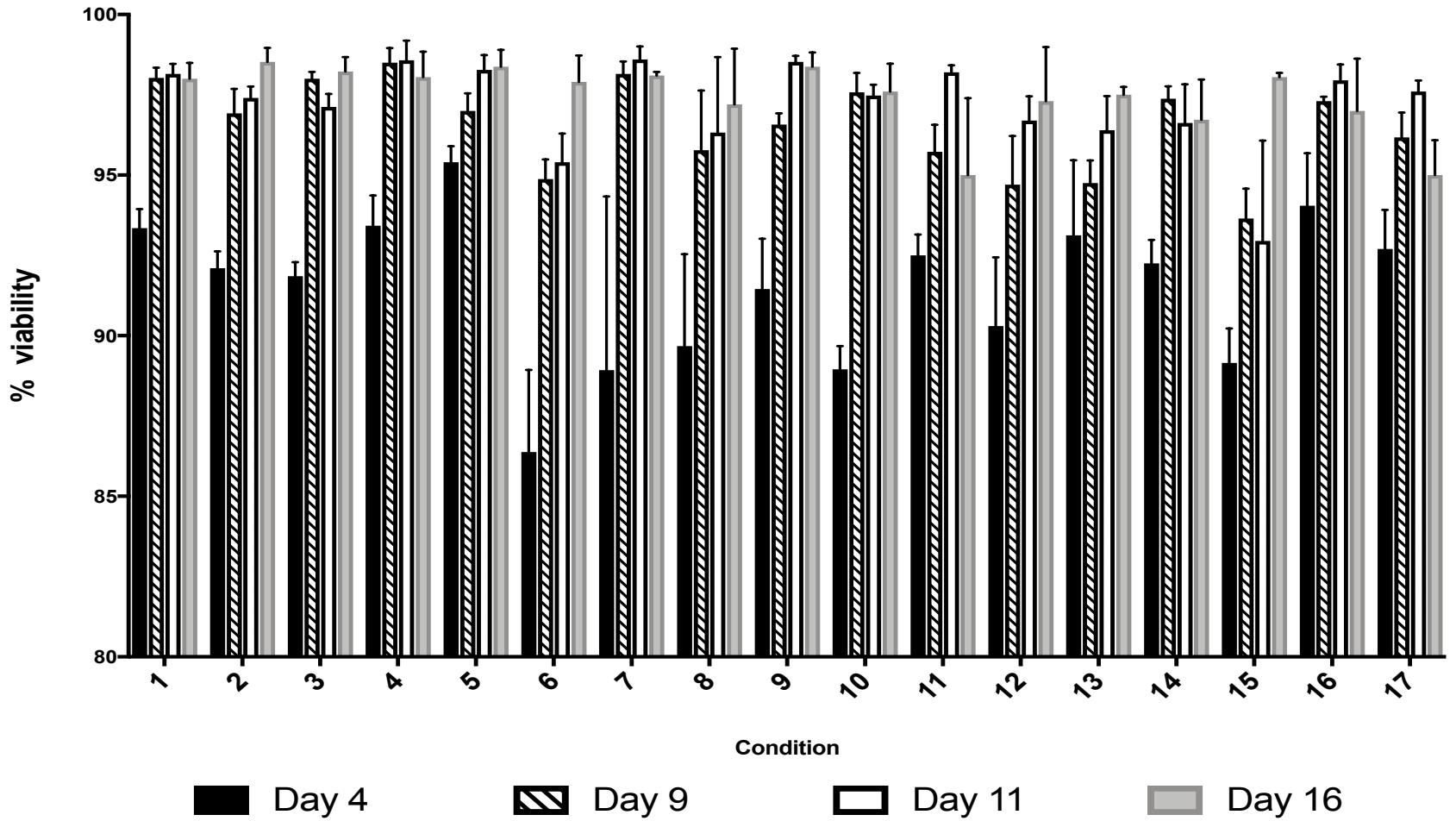


Figure 87. The cell viabilities were high across the 17 different conditions; all conditions were above 85% viability. All conditions except condition 5 and 13, had their most significant increases in cell viability from day 4 to 9; at day 16 the average cell viability was 97.4 ±1.1. Both the time point ($p<0.0001$ (****)) and the condition of culture ($p<0.0001$ (****)) resulted in significant differences in cell yield. Error bars indicate standard deviation, $n=4$.

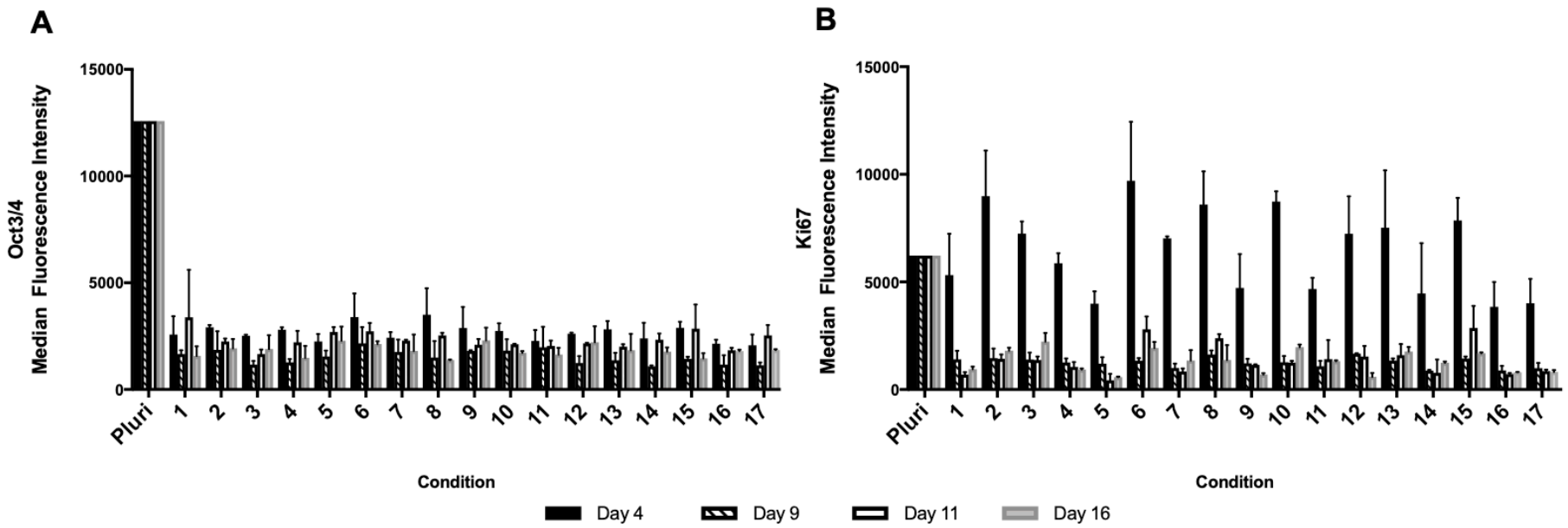


Figure 88. (**A**) OCT3/4 MedFI significantly decreased in expression of OCT3/4 for all conditions when compared to the pluripotent control $p<0.0001$ (****). Overall, the conditions retained a low level of OCT3/4 expression. (**B**) Ki67 MedFI values at day 4 were not significantly different between the pluripotent control and the culture 17 conditions, with the exception of condition 7. However, for all 17 conditions the MedFI at day 4 was significantly higher compared to day 9, 11 and 16, $p <0.0001$ (****). At day 9 all of conditions differed in Ki67 MedFI compared to the control, $p<0.0001$ (****). Error bars indicate standard deviation, n=2.

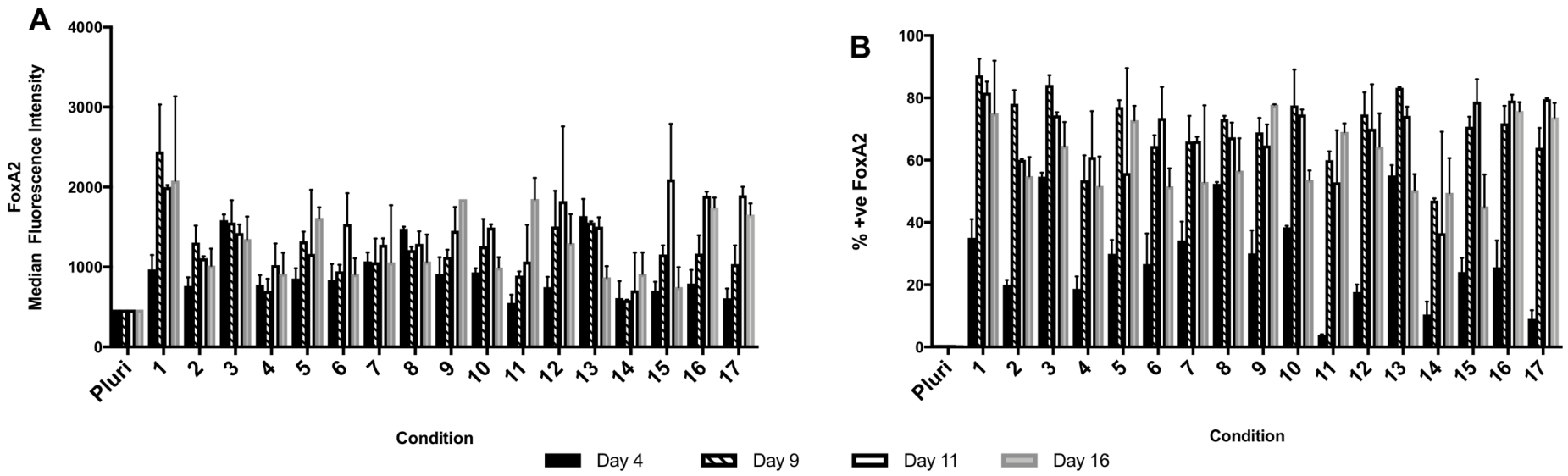


Figure 89. (**A**) FOXA2 MedFI values did not increase significantly over the time points; Except for conditions 1, 9, 11, 16 and 17 at varying time points. Some conditions, such as 12 and 13 had significant decreases in FOXA2 MedFI expression, particularly from day 11 to 16. At day 16 conditions 1 ($p=0.0001$ (***)); 5 ($p=0.0306$ (*)); 9 ($p=0.0025$ (**)); 11 ($p=0.0025$ (**)); 16 ($p=0.0079$ (**)) and 17 ($p=0.204$ (*)) were the only conditions with significantly higher FOXA2 values when compared to the pluripotent controls. (**B**) In general, there was an increase in the percentage of cells that were FOXA2 positive compared to the pluripotent controls. Between day 4 and 9 there was a significant difference in the percentage of FOXA2 positive cells $p<0.0001$ (****). Between day 9 day 11 there was no significant difference whilst between day 11 and 16 there was a lesser degree of significance ($p=0.0224$ (*) compared to day 4 to day 11 $p<0.0001$ (****) and day 4 to 16 $p<0.0001$ (****). Error bars indicate standard deviation, $n=2$.

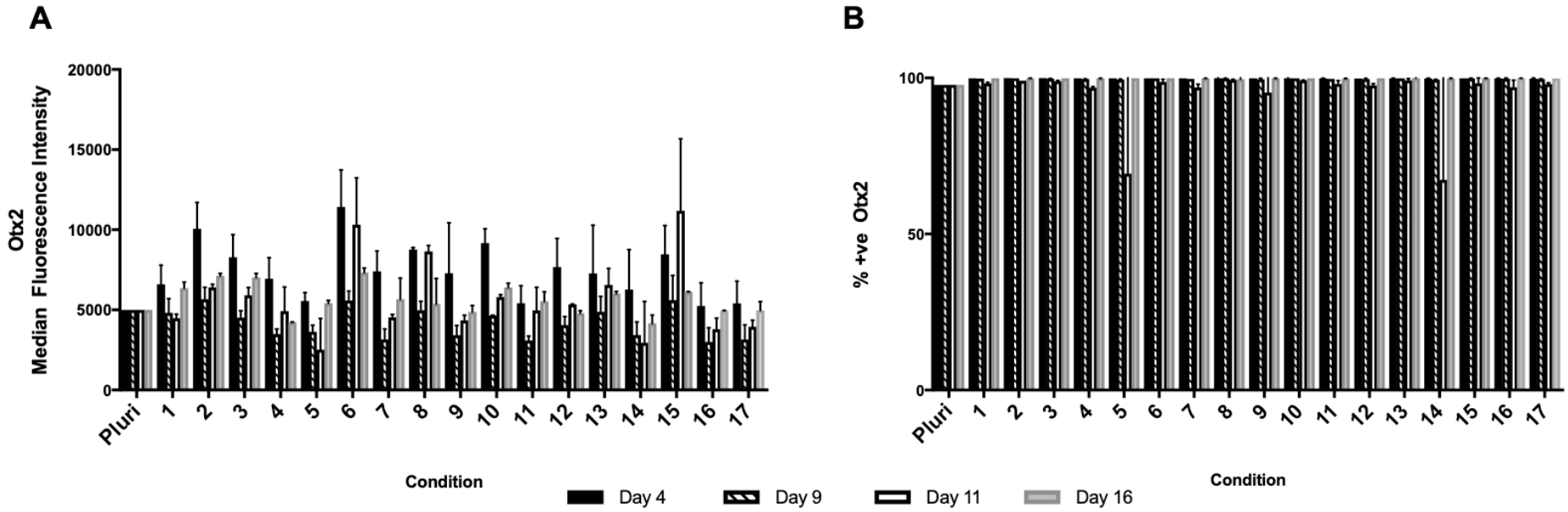


Figure 90. (**A**) Overall, the OTX2 MedFI values did not increase significantly over the time points in comparison to the pluripotent controls, except for condition 2 (p=0.0143 (*)) and 6 p=0.0003 (***)) on day 4, conditions 6 (p=0.0074 (**)) and 15 (p=0.0007 (***)) on day 11. (**B**) The OTX2 percentage positive date illustrates that, with the exception of condition 5 (day 4 to 11 p<0.0001(****); day 9 to 11 p<0.0001(****); day 11 to 16 p<0.0001 (****)) and condition 14 (day 4 to 11 p<0.0001(****); day 9 to 11 p<0.0001(****); day 11 to 16 p<0.0001 (****)) there was no significant difference in the percentage of OTX2 positive cells across the different conditions and across the different time points. Furthermore, the percentage of OTX2 positive cells in the culture condition 17 was not significantly different to the untreated pluripotent control. Error bars indicate standard deviation, *n*=2.

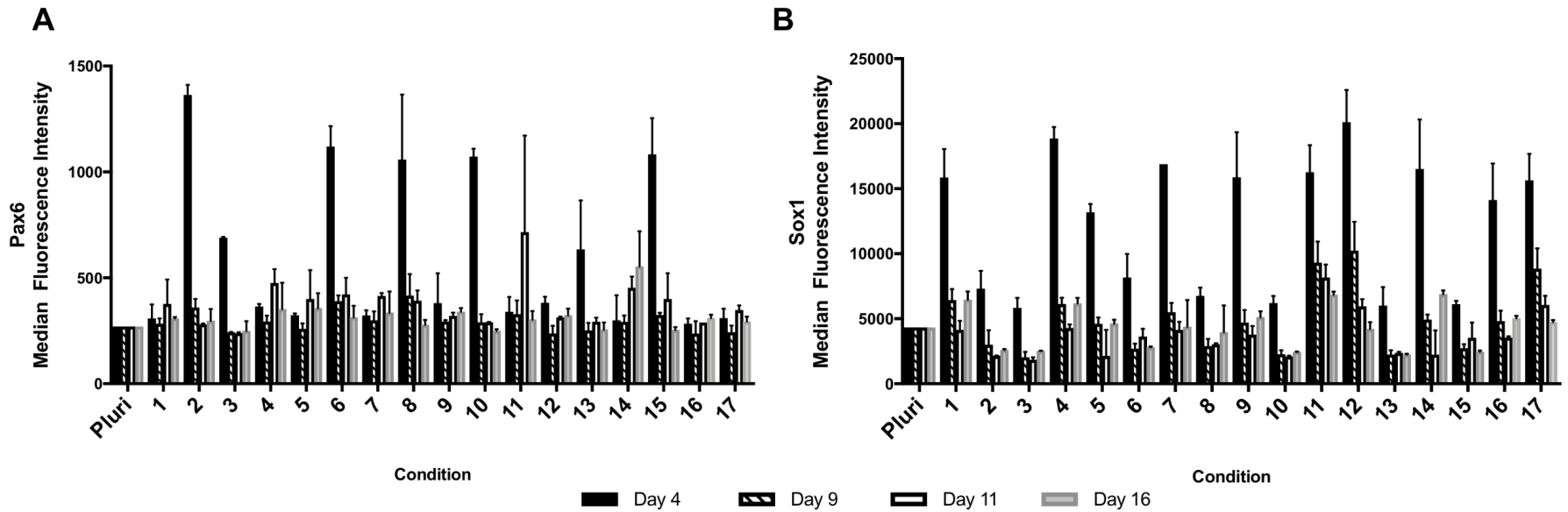
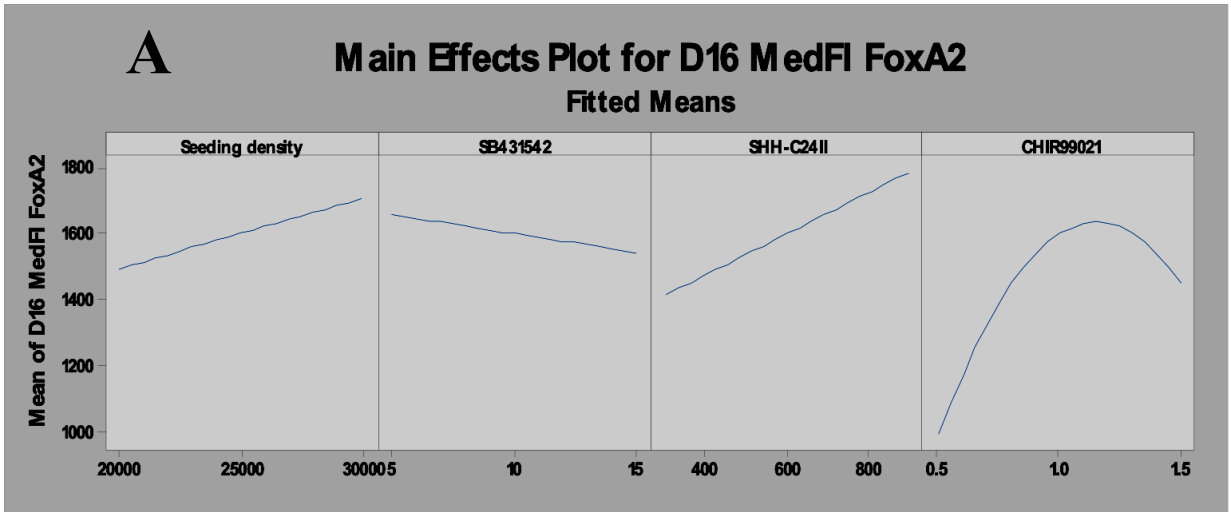
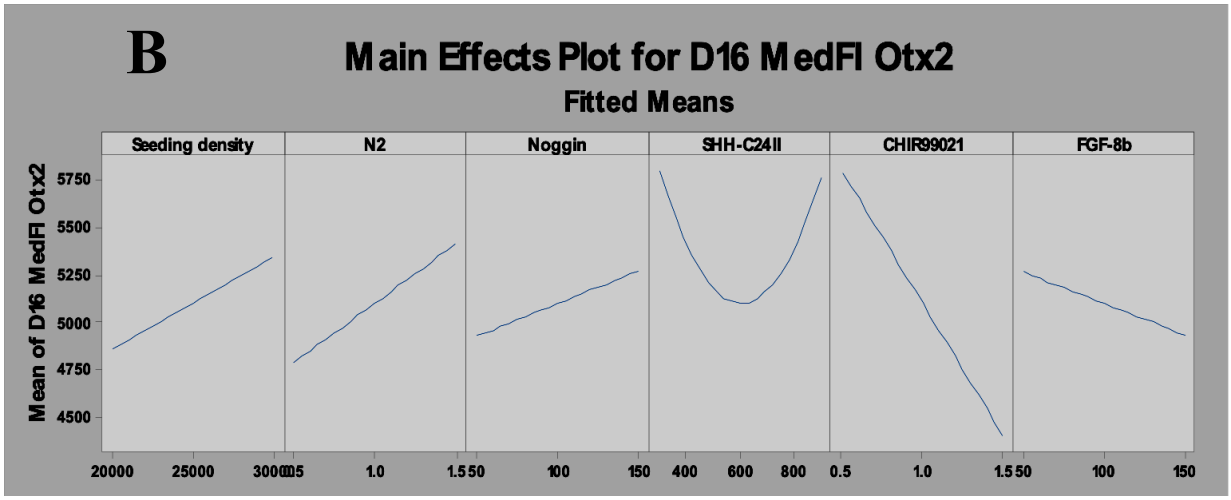


Figure 91. (**A**) Conditions 2, 6, 8, 10 and 15 were significantly different to the pluripotent control on day 4 with higher expression of PAX6. After day 4 the conditions had decreased PAX6 MedFI expression $p<0.0001$(****), which decreased to similar or lower levels compared to the pluripotent controls at subsequent time points. (**B**) SOX1 MedFI expression was highest at day 4 for all conditions; conditions 1, 4, 5, 7, 9, 11, 12, 14, 16 and 17 had the highest SOX1 MedFI expression overall, which were significantly higher in comparison to all other conditions $p<0.0001$(****). Error bars indicate standard deviation, $n=2$.


A
Main Effects Plot for D16 MedFl FoxA2
Fitted Means
Seeding density
SB431542
SHH-C24II
CHIR99021
Mean of D16 MedFl FoxA2
1800
1600
1400
1200
1000
20000
25000
30000
5
10
15
400
600
800
0.5
1.0
1.5


B
Main Effects Plot for D16 MedFl Otx2
Fitted Means
Seeding density
N2
Noggin
SHH-C24II
CHIR99021
FGF-8b
Mean of D16 MedFl Otx2
5750
5500
5250
5000
4750
4500
20000
25000
30000
5
1.0
1.5
50
100
150
400
600
800
0.5
1.0
1.5
50
100
150

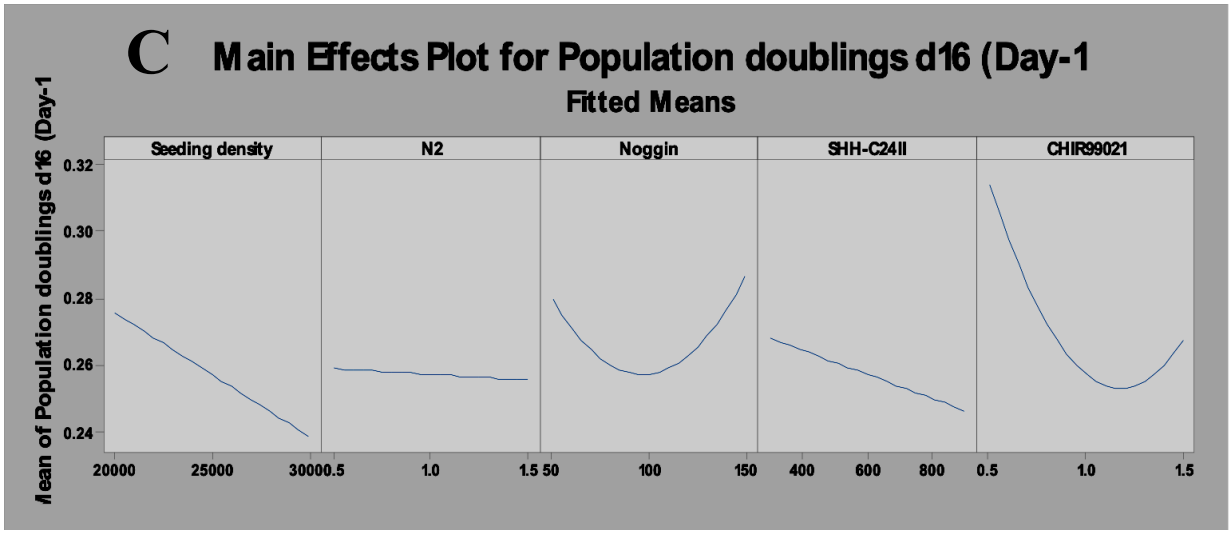

C
Main Effects Plot for Population doublings d16 (Day-1
Fitted Means
Seeding density
N2
Noggin
SHH-C24II
CHIR99021
Mean of Population doublings d16 (Day-1
0.32
0.30
0.28
0.26
0.24
20000
25000
30000
.5
1.0
1.5
50
100
150
400
600
800
0.5
1.0
1.5

Figure 92. Main effects plot of the factors that impacted marker expression and population doublings at the experimental end point day 16. (**A**) FOXA2 marker expression main effects, both seeding density and SHH-C24II have a positive linear relationship with FOXA2 expression, higher levels of both result in higher levels of FOXA2 MedFI. CHIR99021 has a non-linear relationship to FOXA2 expression the mid-point concentration affording the highest levels of FOXA2 MedFI. (**B**) Seeding density, N2 supplement and noggin have a positive linear relationship to OTX2 expression whilst CHIR99021 and FGF-8b have a negative correlation; SHH-C2411 has non-linear relationship where the mid-point resulted in lower OTX2 expression. (**C**) Five of the factors showing differing levels of effect and no effect, in the case of N2 supplement, on the population doubling rate.

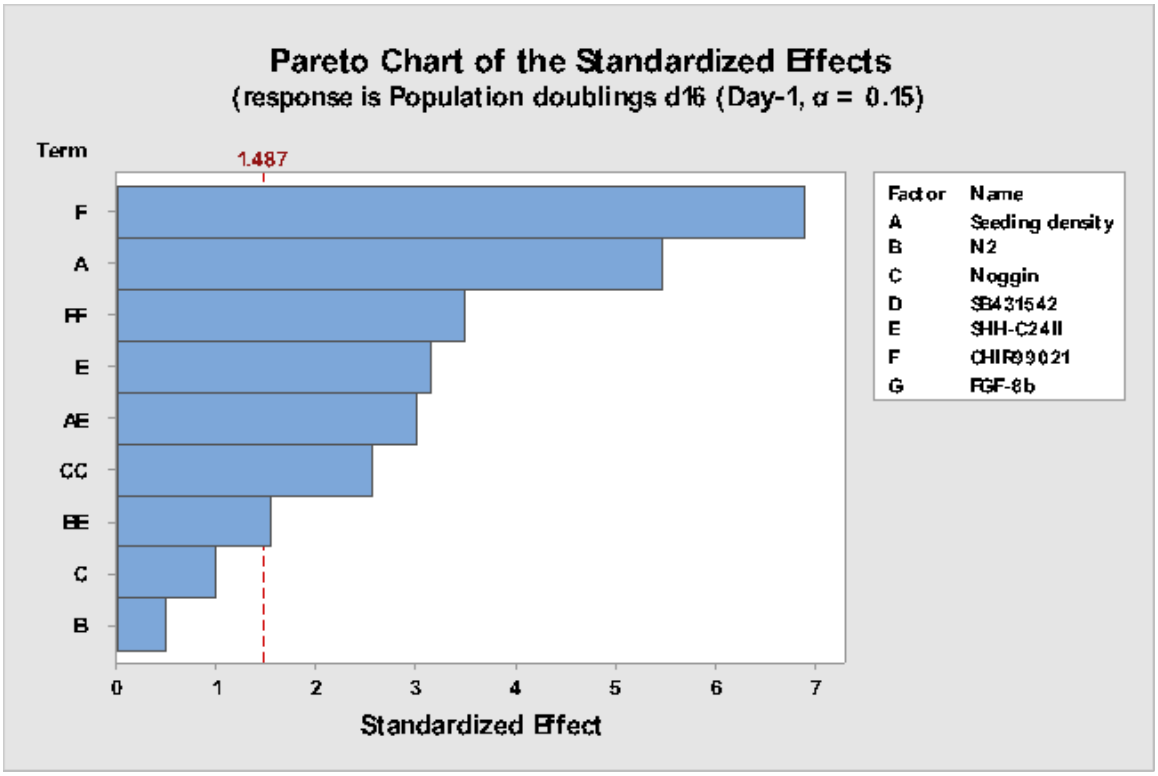


Figure 93. Pareto chart detailing the level of effect the different experimental factors had on population doubling rate at the experimental end point day 16. Seeding density, CHIR99021 and SHH-C24II, individually and when combine with other factors, had the most significant impact on population doubling rate. SB431542 and FGF-8b demonstrated no significant impact on population doubling rate.

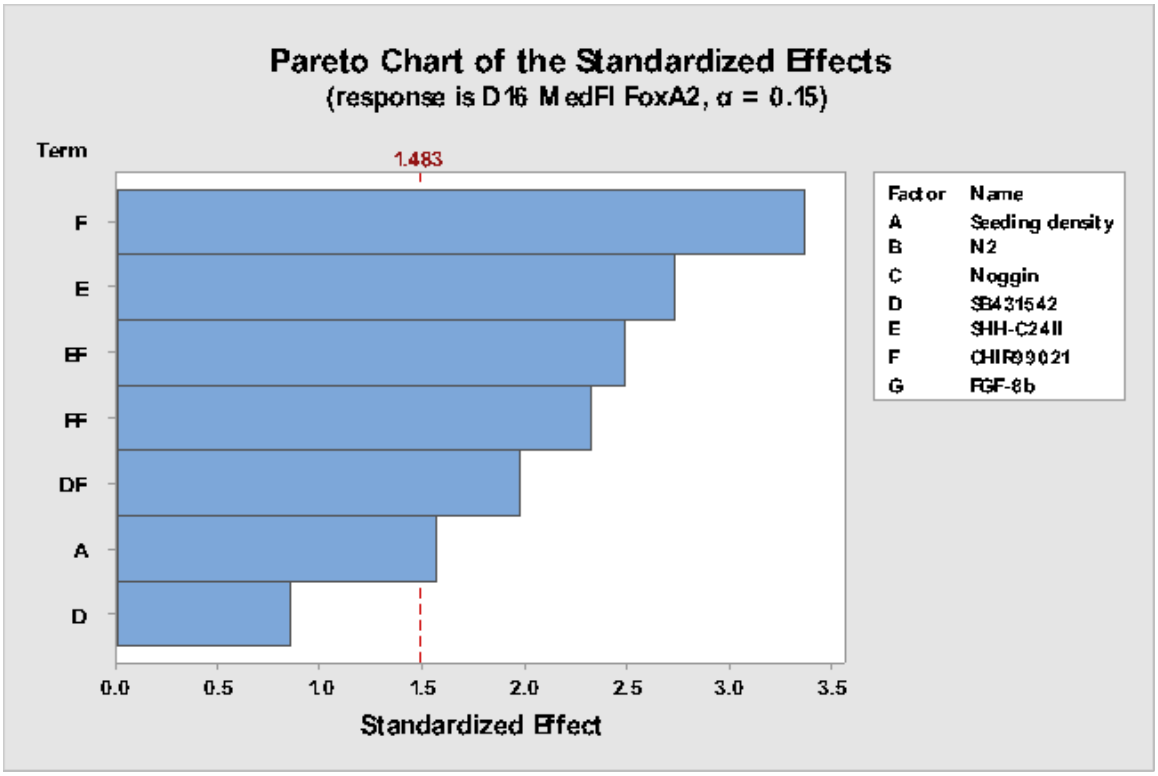


Figure 94. Pareto chart detailing the level of effect the different experimental factors had on FOXA2 expression at the experimental end point day 16. The most significant factor on FOXA2 expression was CHIR99021; SB431542 did not have a significant effect. CHIR99021 and SHH-C24II combined, had a significant effect on FOXA2 MedFI expression.

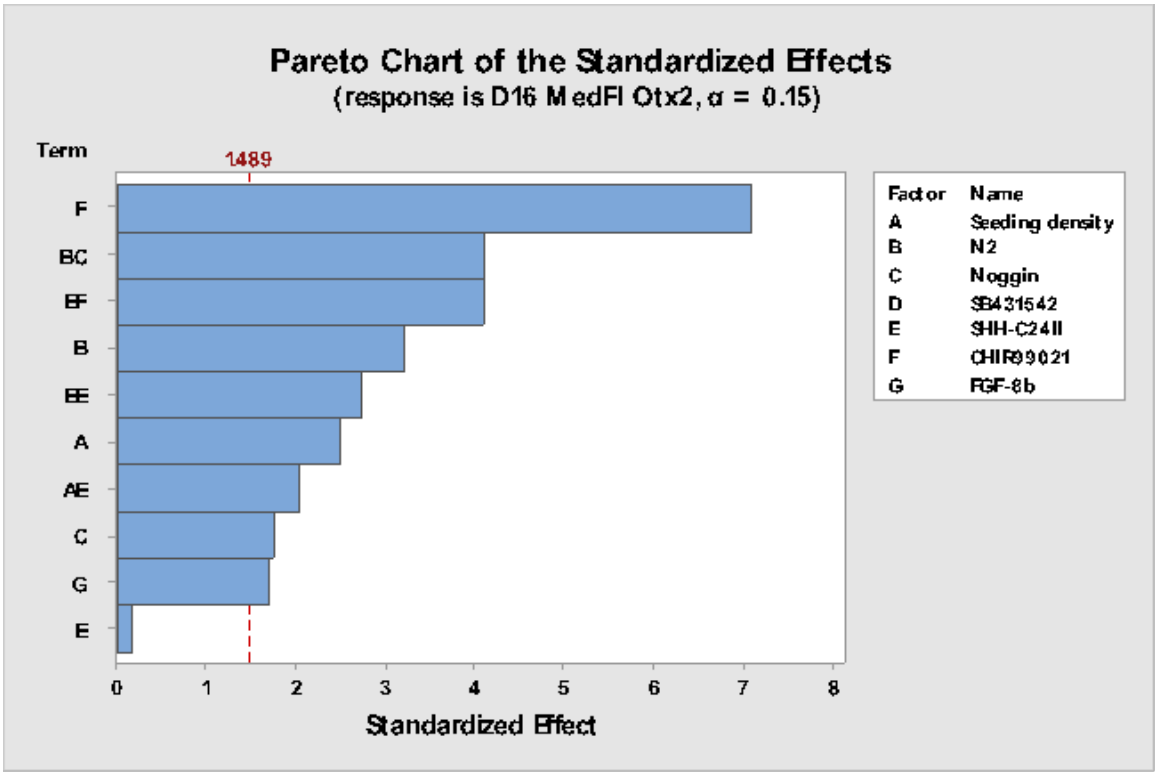


Figure 95. Pareto chart detailing the level of effect the different experimental factors had on OTX2 expression at the experimental end point day 16. The most significant factor on OTX2 expression was CHIR99021; SHH-C24II and SB431542 did not have a significant effect. CHIR99021 and SHH-C24II combined had a significant effect on OTX2 MedFI expression.

6.4. Discussion

This chapter illustrated the challenges that are associated with the translation of a protocol from another institution. In a series of publications[24,124] Lund University presented results that reported the protocol to be capable of yielding the desired vmDA cell type with proven functionality in preclinical studies of rat PD models[85,106]. However, the efforts of five researchers within Loughborough University have not been able to yield cells with the characteristics described in the literature published by the collaborators at Lund University, using their protocol as prescribed[109,129]. As a result, the range of experiments described in this chapter were performed to ascertain the optimal conditions for successful H9 hESC to vmDA differentiation. The following sections discuss the key findings from the present work, specifically discussing parameters including seeding density; small molecule concentration; cell death; cell identity and protocol reproducibility. A summative discussion is presented in order to address how these issues have impacted on protocol optimisation and highlight the key learnings from the current work.

Initial work carried out highlighted the issues concerning process transfer and inter-lab variation concerning differentiation outcomes. A range of experiments were used to ascertain the sources of variation and to investigate conditions that would result in cell survival and appropriate vmDA phenotypic profile. Results from these experiments revealed that the protocol conditions as prescribed did not result in successful differentiation and cell survival without significant changes to the protocol. For example, increased seeding density, changes to the small molecule concentrations and supplementation with B27 from an early stage.

To further investigate the most appropriate conditions needed to obtain the desired cell phenotype a definitive screen DoE was used for six of the different factors used within the initial seven days of the differentiation protocol. These factors were N2 supplement, ROCK*i*, noggin, SB431542, SHH-C24II and CHIR99021. This resulted in an experiment with thirteen different medium compositions using three levels of concentration for each factor, using the protocol conditions as the midpoint. The results of the definitive screen demonstrated that N2 supplement supported a range of different outcomes based on both the measured outcomes of cell phenotype (Day 4, 9 and 11) and cell growth dynamics (cell counts on day 11). ROCK*i* was observed to have a negligible impact on the differentiation process, as it supported cell growth and differentiation in all of the experimental conditions investigated. With regards to noggin, SB431542, SHH-C24II and CHIR99021, the experiments revealed a variety of different outcomes; demonstrating the sensitivities of these factors and their influence on cell survival and phenotype. Both linear and non-linear interactions were observed which had not been previously detected in prior experiments. SHH-C24II in particular was significantly influential as since conditions

void of SHH-C24II completely failed. This did however reduce the power of the experiment due to the failure of the cells cultured under these conditions; furthermore, the low cell numbers resulted in a lack of experimental duplicates as the conditions had to be pooled which reduced the overall resolution of data produced.

As a result of the first definitive screen, information regarding the supportive and non-supportive ranges of the six factors investigated was accrued. The second definitive screen was designed to further probe into the behaviours observed in the first screen. The ranges of the factors were adjusted in the second screen, moving away from protocol conditions as thus far all previous experiments had proven the prescribed protocol parameters and concentrations to be inadequate for obtaining vmDA neurons (typically the protocol conditions were the first ones to fail). The culture vessel size was changed and scaled up to increase cell yields, as low cell numbers reduced the resolution and power of the first definitive screen in terms of growth dynamics. Therefore, more cells in duplicate could be obtained without pooling the samples, such as the case in DSD1. FGF8b and density were added as additional factors, resulting in an investigation to determine the key factors impacting the first nine days of the differentiation process.

6.4.1. Seeding Density

Prior chapters have shown that the densities at which cells are seeded at have an impact on both yield and the cell dynamics within a culture system. Overall, density plays a crucial role in cells survival during the differentiation process. The experiments highlighted a synergistic relationship between seeding density and cell survival, typically higher seeding densities performed better than the prescribed density of 10,000 cells/cm^2. Conditions with cells cultured at the prescribed density of 10,000 cells/cm^2, without B27 supplementation, detached within the first seven days of the protocol, in all the experiments carried out. However, this lower density when supplemented with B27 from day 0 resulted in increased cell survival, for instance as seen in experiment DR5. Moreover, results from Chapter 5 show that expansion cultures seeded at 10,000 cells/cm^2 fail to perform even when cells are in a pluripotent state. Thus, the data suggests that increasing seeding density has a beneficial effect on survival of conditions not supplemented with B27, since conditions seeded at 15,000 or 20,000 cells/cm^2 survived until day 7 as a minimum in DR5 and in the definitive screen experiments (DSD1 and DSD2). Amongst the conditions that survived, initial seeding density contributed not only to how quickly the cultures became confluent, but also whether or not they were replated at 800,000 cells/cm^2 in DR4 and DR5. All but one of the conditions initially seeded at 20,000 cells/cm^2 were replated at 800,000 cells/cm^2, whilst none of the 10,000 cells/cm^2 conditions had sufficient cell yields at day 11 to allow for replating at the recommended density (in DR4).

In DSD2, density was added as an additional factor to the DoE, to investigate its impact on cell feedback/ self-cell influencing factors. Prior to the experiment, the scale up experiment was devised to establish a culture vessel format with a range of densities that would result in a countable number of cells at day 4 (the first harvest time point), whilst also being economically favourable and manageable in terms of scale and manipulation. The results demonstrated that the 12-well plate format had higher SGR values compared to the 6- well plate format. However, in the 12-well format a density of ≥20,000 cells/cm^2 is favourable, since the 15,000 cells/cm^2 condition did not perform well. This was evidenced by lower SGR values compared to the 20,000 and 25,000 cells/cm^2 conditions in the 12- well format group. The lower SGR resulted in lower cell number yields at day 11 for the 15,000 cells/cm^2 condition in comparison to 20,000 and 25,000 cells/cm^2; in addition, the 15,000 cells/cm^2 condition had the lowest cell viabilities at day 11.

The present work illustrates the importance of seeding density and it is an element of the protocol that should be highly defined and standardised (**Figure 93**), as operators and different laboratories have different methods of cell counting[275–277]. This easily introduces variation into the outcomes of the protocol, as shown in the present work, incorrect cell seeding densities can be a failure mode of the process. Here, lower densities have been shown to result in poor cell growth dynamics and poor cell viability.

6.4.2. Replating

At the replating stage of the protocol it was not apparent why the Lund protocol prescribed such a high seeding density; 800,000 cells/cm^2. The data showed that seeding densities up to four-fold less of the protocol replating density are capable of yielding similar cell numbers to conditions replated at 800,000 cells/cm^2 (**Figure 66**). In DR4, some conditions had poor cell attachment and growth, therefore, these conditions were replated using the total harvested cell number, resulting in seeding densities differing from the prescribed 800,000 cells/cm^2 (**Figure 66**). As anticipated, the cells seeded at lower densities initially had lower culture surface coverage, however images collected at day 14 illustrated that resulting cultures looked “cleaner” (**Appendix 5**) with less cell debris in comparison to those seeded at 800,000 cells/cm^2. Images recorded of the cells seeded at the protocol density showed complete coverage within two-hours post replating, with a number of non-adhered and floating cells observed due to the lack of available culture surface (**Figure 96**).

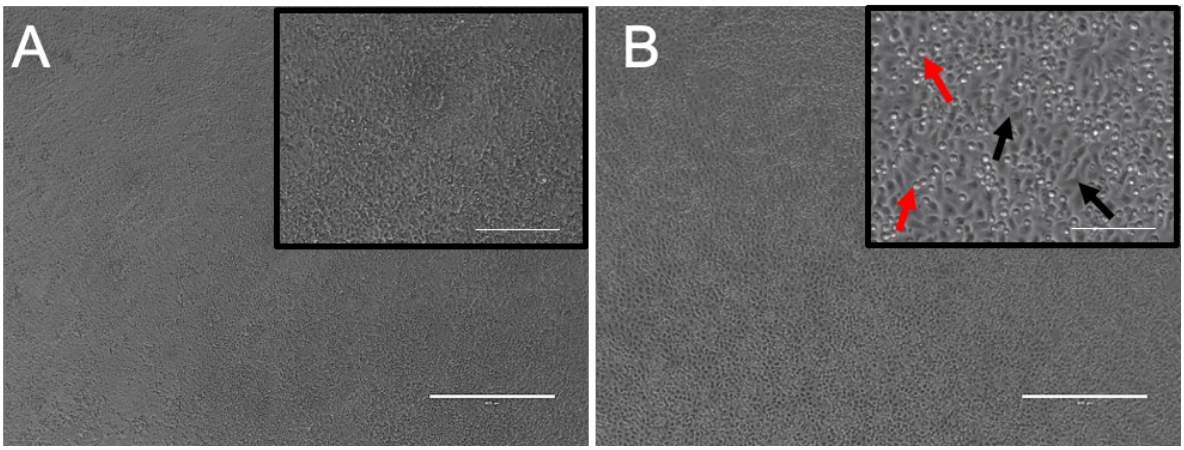


Figure 96. (**A**) Images of cell at day 11 before replating, inset shows zoomed in sections of the well. (**B**) Image of cells at day 11, two hours after replating, the culture surface is fully covered, the inset image shows cells attached (black arrows) and unattached cell (red arrows) due to lack of available culture surface. Scale bar = 400 μm, inset scale bar = 100 μm.

The day 11 replating experiment further explored the effect of seeding density, highlighting a grouping in SGR behaviour (400,000 cells/cm^2 *vs.* 800,000 cells/cm^2), based on replating density. The concentrations of FGF8b and ascorbic acid were represented equally in the two groups, suggesting that the observed behaviour was influenced by the density and not the small molecule concentrations. In addition, SGR values were not significantly different within the density conditions, suggesting that the different concentrations of small molecules do not have an effect on cell proliferation, in the ranges explored. The 400,000 cells/cm^2 conditions had positive SGR values, whilst those seeded at 800,000 cells/cm^2 had very low or negative SGR values. Observations of the cells showed that the cells immediately attached and covered all of the available culture surface when seeded at such high densities (**Figure 96**). This resulted in a high number of cells that remained unattached, attributing to the negative SGR observed in the high-density conditions (C2, C4, C6 and C8). Furthermore, no changes in cell phenotype were observed in conditions replated at densities lower than 800,000 cells/cm^2. This indicated that it is not necessary to replate the cells at such a high density, instead less cells can be used, potentially increasing the efficiency of the protocol.

6.4.3. Small Molecule concentrations

The experiments explored a range of different small molecule concentrations, with some of the small molecules having more of an influence (N2, SHH-C24II and CHIR99021) compared to others (ROCK*i*). DSD1 showed that the influence of ROCK*i* was insignificant since it was supportive of cell attachment for all conditions and the inhibitor is only used to supplement the medium in the first 48 hours of the protocol. DoE analysis of the small molecules demonstrated that ROCK*i* did not have an impact on desired marker expression for FOXA2 (**Figure 79** and **Figure 82C**). Therefore, ROCK*i* was removed from the experimental design used in DSD2.

The effect of CHIR99021 concentration on cell survival initially seemed to be dependent on both seeding density and addition of B27; with the addition of B27, a lower CHIR99021 concentration was desirable, whilst without B27 a higher seeding density was required. Interestingly, the original prescribed protocol concentration (0.7 μM) only survived when other protocol parameters such as the seeding density was increased and/or B27 supplement was added at day 0. The definitive screen results again demonstrated that 0.7 μM CHIR99021 is not a favourable concentration as the M12 and M13 conditions (both at 0.7 μM CHIR99021) were terminated at day 7 due to extensive cell detachment and death; with M13 being the protocol control medium formulation. This further illustrated that 0.7 μM CHIR99021 is not a conducive concentration for successful cell survival and differentiation, even at an increased seeding density, as used in DSD1 this concentration results in lower FOXA2 expression (**Figure 82C**).

The definitive screens highlighted the complex interactions between the different concentrations of supplements and small molecules, such as N2 supplement, CHIR99021 and SHH-C24II, the two vmDA patterning factors. Interactions between N2 supplement and SHH-C24II were observed to have an effect on expression of markers such as PAX6 (**Figure 80**) and SOX1(**Figure 81**) The M2 and M5 conditions in DSD1 had the highest levels of PAX6 expression at all sample points, suggesting incorrect cell lineage fate and/or preservation of a neurostem cell phenotype. This can be attributed to the absence of SHH-C24II and lower concentrations of CHIR99021, which highlights the necessity of SHH-C24II in the patterning and differentiation process. Moreover, M1 and M6, M9 and M11 had the lowest PAX6 MedFI values and M1 and M6 had high FOXA2 MedFI values that increased incrementally until day 11. Both M1 and M6 in DSD1 and conditions 1, 6, 9, 11, 16 and 17, in DSD2, performed well in terms of cell number yield, cell viability, population doubling rate and phenotypic marker expression. A common feature of these conditions was the higher levels of both SHH-C24II and CHIR99021. Although lower concentrations such as 0.3 μM and 0.5 μM CHIR99021 resulted in improved cell

survival and growth (**Figure 92C**), they did not result in the correct cell phenotype (**Figure 92A**). Instead, the work carried out has revealed that higher concentrations than prescribed, such as 1 and 1.5 μM CHIR99021 and 600 and 900 ng/ml SHHC24II, are required to achieve expression of the desired cell phenotype in terms of FOXA2 expression.

However, the results demonstrated that N2 supplement and noggin had a positive linear relationship to OTX2 expression whilst CHIR99021 and FGF-8b had a negative correlation; interestingly SHH-C2411 reveals a non-linear relationship where the mid-point resulted in lower OTX2 expression (**Figure 92B**). This exhibited further complexities in small molecule concentrations, as low concentrations of SHH-C24II (300 ng/ml) afforded higher OTX2 expression but lower FOXA2 expression while lower CHIR99021 concentrations (0.5 μM) resulted in higher OTX2 expression but lower FOXA2 expression (**Figure 92A** and **B**). This highlighted the challenge in determining the optimal concentration combinations to achieve the desired FOXA2 and OTX2 co-expression.

6.4.4. The effects of B27 supplementation on day 0 of the protocol

The data illustrated that the addition of B27 supplement significantly improved cell survival in conditions containing a low concentration of CHIR99021 and those seeded at 10,000 cells/cm^2 (**Figure 64** and **Figure 71**). This highlights the supportive effect of the B27 supplement which was recommended in the troubleshooting section of the Lund protocol[129] . It is unclear exactly under what mechanism the supplementation of B27 aids cell survival, but it can be inferred that the additional nutrients and antioxidants in the B27 formulation provide a beneficial effect in terms of cell survival[171,278]. However, this does lead to successful differentiation as B27 supplemented conditions were not of the desired cell phenotype in any of the conditions in DR4 and DR5. It is worth noting that B27 supplementation may interfere with the initial commitment and differentiation of the cells normally induced by N2 medium[171]. Especially, as B27 supplement is normally used in later stages of differentiation as it favours attachment and growth of mature neuroprogenitors[273], whilst N2 inhibits growth of undifferentiated cells[279].

6.4.5. Cell identity

Cell phenotype was the predominant measure of outcome success as cell survival itself did not guarantee the desired cell phenotype would be obtained. The high-resolution cell phenotyping revealed interesting expression patterns related to some of the markers used in the flow cytometry panels. Indicative markers such as PAX6, which are meant to confirm that the cells have successfully progressed into neural stems cells are essential for determine the trajectory of differentiation. PAX6 is

expected to be transient in neuroprogenitors[25,122], particularly those that are vmDA fated and thus should not express PAX6 at the end of the differentiation process as it is an early neuroectodermal marker[107,280]. Thus, the observed expression of PAX6 at day 16 in some conditions suggested incorrect cell linage fates.

Another observed pattern of PAX6 showed that lower levels of expression on all of the days (the four sample points used) is concomitant with higher FOXA2 expression on day 16 as seen in conditions 1, 5, 9, 16, 17 in DSD2 (**Figure 89** and **Figure 91**). Conversely, conditions with high PAX6 on day 4 did not result in high FOXA2 at any time point analysed. This seems to suggest that the phenotypic expression (PAX6) of the cells day at 4 has an impact on their ability to further differentiate towards the desired vmDA cell lineage, particularly in terms of FOXA2 expression. Conditions 1, 4, 9, 16, 17 had high SOX1 on day 4 which decreased significantly by day 16; of these conditions 1,9,16, and 17 also had high FOXA2 expression on day 16. These conditions, 1, 4, 9, 16 and 17, had high levels of CHIR99021, either 1 μM or 1.5 μM and ~ 70% or above of the cells in these conditions were FOXA2 positive. The best condition was M1(DSD1) and condition 1 (DSD2); cells cultured in DSD2 were able to achieve high FOXA2 expression levels that peaked on day 9, followed by a decline in expression at day 11 and 16. This expression decline was observed in the range of experiments carried out and by collaborators at Miltenyi Biotec (**Appendix 4**). Expression of the vmDA markers, FOXA2 and OTX2, was demonstrated to be highly influenced by SHH-C24II and CHII99021 as these factors individually and/or combined had the most significant effect on vmDA marker expression (**Figure 94** and **Figure 95**).

In terms of other markers, the OCT3/4 MedFI data demonstrated that there was negligible expression of OCT3/4 in the experimental conditions by day 16, which is expected since the cells should no longer be pluripotent. There were varying levels of Ki67 in the experimental conditions, however they were all lower than the pluripotent control sample, which is expected as the cells would still be proliferating, albeit at a slower rate. In DR4 and DR5 when the Miltenyi Biotec flow cytometry protocol was employed, panel 3 revealed that all of the conditions has lower NKX6.1 expression compared to the pluripotent control, while some of the conditions had higher NKX.2.1 MedFI values compared to the control. SOX1 MedFI values showed similar or lower values compared to the control, suggesting that panel 3 does not identify the presence of contaminant/incorrectly differentiated cells. Thus, a more definitive panel of markers should be devised to adequately distinguish between pluripotent cells, desired cells and contaminant cells.

Although the cells did not express the contaminant phenotypic markers including NKX6.1 they were also not expressing high levels of the desired cells phenotype. This suggested that alternative containment markers, such as GBX2 known to be present in midbrain-hindbrain boundary should be

explored[94,267]. The use of these markers might capture the cells that were expressing high level of PAX6 on day 4 and low levels of FOXA2 on day 16. It was apparent that cells with lower levels of PAX6 expression were obtained from conditions with higher concentrations of CHIR99021, highlighting that the GSK inhibitory effect of CHIR99021 interacts with the mechanisms that governs FOXA2 (**Figure 92A**) and PAX6 expression[25,127,264,265]. Further studies concerning the use of PAX6 are necessary to understand its influence on the differentiation trajectory of the cells and its relationship to the small molecules. For instance, systematic alteration of N2 supplement concentration and the neurulation factors, noggin and SB431542, which were shown to significantly impact PAX6 expression (**Figure 81** and **Figure 82B**). In addition, daily sacrificial analysis of the cells would provide greater resolution of differentiation trajectory under the culture conditions.

6.4.6. Flow cytometry results and protocol alteration:

Flow cytometry analysis made it apparent that the Miltenyi Biotec protocol panel setup (**Table 38**) had limitations, particularly for use in high frequency phenotyping; as it is inefficient from a cell resource perspective. The Miltenyi Biotec protocol requires each condition/sample to be divided into three separate aliquots so that each aliquot is analysed by one of the three panels. This is because simultaneous analysis of the all nine markers is not possible due to each panel using the same three fluorophores APC, FITC and PE. Hence a single multiplex panel was formulated, which facilitated more efficient use of the cell samples, particularly in the DoEs whereby the samples were limited in terms of cell number. An alteration to the gating strategy was made by forgoing the use of the pluripotent cell sample as the control. Instead, an isotype control strategy was employed to better distinguish between positive and negative populations within the panel. This change in control was due to high levels of fluorescence observed in the pluripotent cells in panel 2, where the cells were in the upper decade on the log scale for the dopaminergic markers (**Figure 69A**), which is unexpected since the cells had not been exposed to differentiation molecules. As a consequence, the gates for panel 2 were skewed towards the higher decades on the scale (**Figure 69B** and **C**), making it difficult to ascertain if the fluorescence signal was due to the effects of the differentiation or not.

In the majority of the experiments performed, the putative vmDA markers FOXA2 and OTX2 (panel 2 markers of the Miltenyi Biotec protocol) expression was low in terms of MedFI. This suggested that either the markers themselves are not highly expressed in the cells obtained, therefore resulting in low levels of fluorescence, or that the current markers are not adequately definitive for identifying the product. The latter can be deduced to be the case, as the percentage positive data of OTX2 expression showed that ≥98% of the cells were positive for OTX2 expression. This was regardless of the cells being in a pluripotent state or having undergone differentiation. Furthermore, the percentage of cells

expressing OTX2 remained high across the 16-day process as evidenced by the data from the four sampling points at day 4, 9, 11 and 16.

6.4.7. Discussion summary of the key experimental findings.

Overall, the protocol in its prescribed form failed in terms of successful vmDA differentiation. The data highlighted the inadequacy of the culture parameters outlined in the differentiation protocol provided[129]. Despite the completion of process transfer activities (discussed in section 6.1.2. Process transfer) and a strict adherence to the protocol, all efforts to differentiate the H9 cells using the protocol were unsuccessful. Furthermore, issues concerning protocol reproducibility were raised since the experimental control conditions also failed to survive. Alterations to various aspects had to be employed, in an attempt to get the cells to survive past day 4. As aforementioned the FMEA looked into the critical process steps that were deemed to have a potential significant effect on the process. The experiments carried out in the present work aimed to dissect, interpret, analyse and attempt to control these process failure modes. In the adapted FMEA, **Table 41** control actions provided by the present work are detailed. The majority of the controls were linked to the cell manipulation process and cell milieu steps, this highlighted the high variability that is observed due to these steps. This illustrated how poorly defined protocols can hamper the outputs of the process, due in part, to inadequate process understanding and control. Consequently, initial attempts were unsuccessful with regards to yielding vmDA neurons, moreover, initial attempts did not succeed in maintaining cell survival past day 4. The suggested controls and troubleshooting provided by Lund University were also incapable of successfully yielding vmDAs, and despite the cells surviving until day 7 when suggested changes were employed, the cells failed to survive the entirety of the 16-day differentiation process. In addition, the controls themselves, such as the earlier addition of B27 at day 0, are vague and offer limited understanding to the root cause of the variability/failure observed.

Table 41. Adjusted FMEA of **Table 31**. Detailing the process optimisation steps taken to improve or understand the corresponding failure mode. Recommended actions to improve the protocol are given as a result of the experiments and observations of the present work.

Process Step	Potential failure mode	Prescribed Process controls	Risk Priority Number	Process Optimisation steps	Recommended Actions
Cell manipulation	Contamination	personnel training, disinfecting materials used	20	n/a	Frequent aseptic technique competency testing, laboratory maintenance, routine testing
	Cell detachment	personnel training B27 supplementation	120	Highly defined cell culture protocol to increase standardisation and consistency	Standardised coating procedures
	poor user consistency and adherence to the protocol	personnel training	240	Highly defined cell culture protocol to increase standardisation and consistency	Use of defined protocol with minimal operator decision making.
Measurement	Flow cytometer sampler or lasers not working	efficient equipment maintenance	40	n/a	efficient equipment maintenance
	No controls	efficient inventory monitoring	10	n/a	efficient inventory monitoring
	no calibrators	efficient inventory monitoring	15	n/a	efficient inventory monitoring
	lasers drifitng	efficient equipment maintenance	80	n/a	efficient equipment maintenance
Cell Milieu	Nutrients incorrect supplementation or low concentrations	Inspection of the cells	45	Carried out experiments to understand the metabolic profile of the cells	Use of defined protocol with minimal operator decision making. Monitoring of nutrient consumption, online if possible to allow for informed feeding strategy
	CHIR99021 concentration too low or too high	use the same pipette	560	Carried out a series of experiments to determine the optimal CHIR99021 concentration in a systematic manner user DoE	Employ a DoE to determine optimum concentration for cell line of choice. Use regularly calibrated pipettes
	Small molecule concentration too low or too high	use the same pipette	504	Carried out a series of experiments to determine the optimal concentrations in a systematic manner user DoE	Employ a DoE to determine optimum concentration for cell line of choice. Use regularly calibrated pipettes
	Laminin coating	personnel training	32	Highly defined cell culture protocol to increase standardisation and consistency	Standardised coating procedures
	incubator failure	efficient equipment maintenance	20	n/a	efficient equipment maintenance
	Cell seeding density too low	personnel training	294	Highly defined cell culture protocol to increase standardisation and consistency. Experimentation to examine the effect of different seeding densities at various stages of the process and their impact of cell growth and differentiation success.	Use of defined protocol with minimal operator decision making and automated cell counting. Personnel training
Materials	out of date	efficient inventory monitoring	16	n/a	efficient inventory monitoring
	no stocks	efficient inventory monitoring	60	n/a	efficient inventory monitoring
	spoilt reagents	efficient equipment monitoring	160	n/a	efficient inventory monitoring
Equipment	non functional	efficient equipment maintenance	24	n/a	efficient equipment maintenance
	Not calibrated	efficient equipment maintenance	18	n/a	efficient equipment maintenance

The initial experiments DR1- 3 illustrated that culturing the cells under the Lund prescribed protocol conditions such as seeding at 10,000 cells/cm^2 did not result in successful cell differentiation. A multivariate experimental design (DR4 and DR5) and definitive screens (DSD1 and DSD2) were employed to the survey different culture conditions and media formulations. This approach demonstrated the complexity of the differentiation process whilst also highlighting the difficulties that arise with attempting to control such multifaceted culturing conditions. In DR4 and DR5 the more favourable culturing conditions during differentiation were identified to be those where the medium contained lower concentrations of CHIR99021 (0.3 and 0.5 μM) and the addition of B27 supplement at day 0 (1:100 concentration). In conjunction with these, a higher seeding density (15,000 and 20,000 cells/cm^2) was also more favourable for cell survival, this resulted in subsequent experiments employing higher seeding densities. Alterations to CHIR99021 and SHH-C24II concentrations have been shown to be key components in improving the outcomes of the differentiation process. As previously mentioned lower concentrations (of CHIR99021 in particular) have been shown to increase cell survival, however they did not yield vmDA cell types but were more likely to form diencephalic cells due to a lack of GSK inhibition[87,94,129]. The definitive screen experiments showed that a range of different medium formulation can support vmDA differentiation, typically these formulations are high in CHIR99021 (1 and 1.5 5 μM) and SHH-C24II (600 and 900 ng/ml). This resulted in a shift away from the concentrations prescribed (0.7 μM and 300 ng/ml, CHIR99021 and SHH-C24II, respectively) in the Lund protocol in order to achieve cell survival post day 7 as a minimum whilst maintaining increased levels of FOXA2. This highlighted that small molecule concentrations are a key CPP that should be controlled in order to achieve the desired CQAs.

The lack of survival in the control conditions may be attributed to common process transfer issues, such as pipette calibration discrepancies between institutions. Pipette inaccuracy and cell count inaccuracy may be responsible for the prescribed, yet sub-optimal differentiation conditions, visualised as the purple cross in **Figure 97**. If variability in equipment is assumed, this can be inferred to be the cause for the necessary alterations to both the seeding density and the small molecule concentration. Discrepancies in the actual concentrations and cell numbers reported in the protocol would result in the observed inconsistency when executed at different laboratory sites, as the actual concentrations and cell numbers being employed would differ. For instance, the perceived optimal conditions for H9 differentiation is 0.7 μM CHIR99201 at 10,000 cells/cm^2, would actually be 1.0 μM CHIR99201 at 20,000 cells/cm^2 due to the pipette and counting procedures being offset; i.e. non-calibrated pipettes and the variability of manual cell counting. The data in the present work does show that upward shifts in cell seeding density and shifts either side of the protocol for the small molecule concentrations resulted in improved outcomes, in particular, upward shifts (depicted as the blue box in, **Figure 97**). The data showed improved survival with better phenotype outcomes when cells were seeded at higher

densities and altered small molecule concentrations were utilised, predominantly with higher concentrations of CHIR99021 and SHH-C24II.

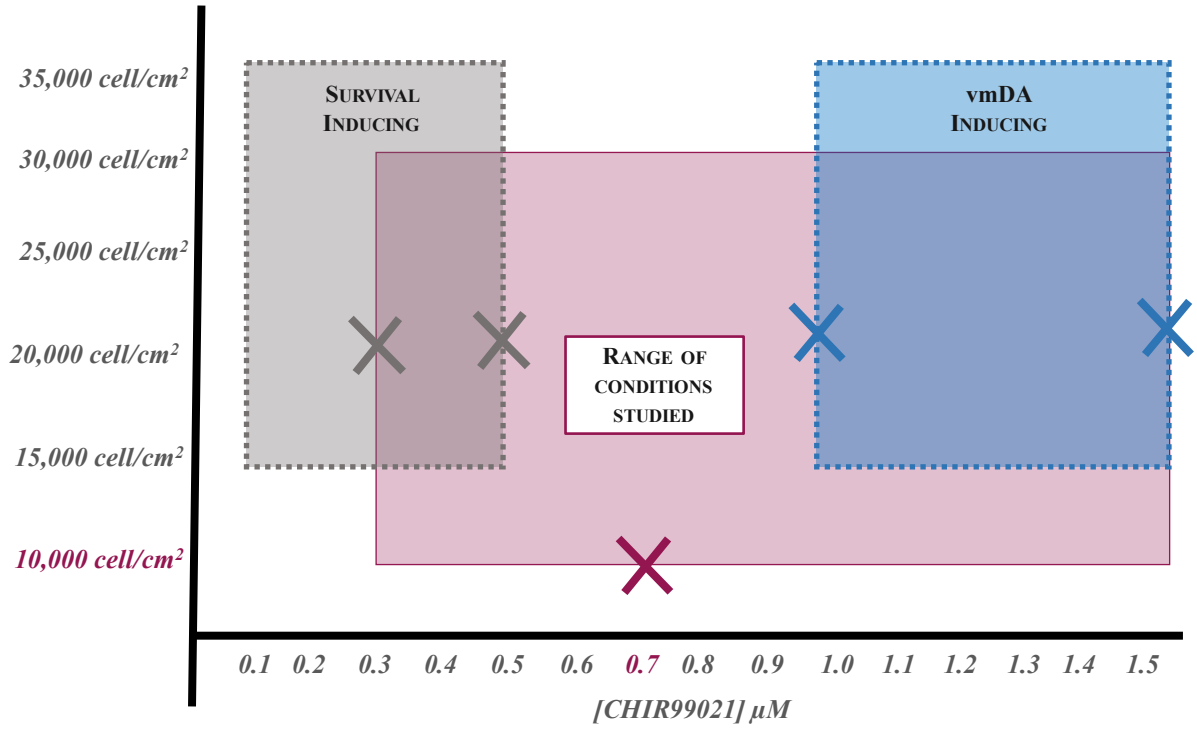


Figure 97. Process Transfer diagram, showing the range of densities and CHIR99021 concentrations explored. Central purple area shows the space studied, parameters in purple text represent the protocol conditions and the perforated boxes show the range of conditions that resulted in cell survival and/or vmDA differentiation. The grey box and crosses represent the parameter conditions that were observed to be optimal for cell survival given the results from DR4 and DR5. The blue box and crosses represent parameters from DSD1 and DSD2 that induced vmDA cell phenotype and cell survival. 20,000 cells/cm^2 is a common feature in the best performing conditions, while the prescribed Lund protocol density, 10,000 cells/cm^2 and central CHIR99021 concentrations (0.6-0.9 µM) do not perform well in the range of conditions explored.

6.5. Conclusions

It is evident that there were process transfer challenges that required a significant amount of process optimisation to be performed. Regarding seeding density, higher densities 15,000 to 30,000 cells/cm^2 were shown to be conducive to cell survival and growth. However, lower seeding densities, 150,000 – 600,000 cells/cm^2, at the replating stage have the potential to increase the efficiency of the protocol by 50% as a density of 400,000 cells/cm^2 was shown to have higher SGRs; therefore, 50% less cells can be used to replate at day 11 whilst still obtaining a similar yield at day 16. From a product manufacturing standpoint, this would fully exploit the cells and double the product yield that can be obtained at day 16; this ultimately increases the resultant batch sizes from the same volume of input cell material. This is highly desirable in a manufacturing context as production cost could be reduced and overall production efficiency would be increased. High resolution phenotypic tracking has provided insight into the phenotype trajectory of the cells, i.e. levels of PAX6 at day 4 had an impact on cell lineage fate. Process knowledge such as this can be used to develop assays to ensure that the process is achieving the CQAs throughout. For instance, determination of PAX6 levels early in the process can be used as go/no-go criteria for proceeding with a manufacturing run, which can result in cost and resource savings if a batch going towards the wrong lineage is halted prior to the end of the process.

Adjustments to the initial Lund protocol has resulted in the development of culture conditions capable of surviving the differentiation process however, the validity of the vmDA identity has not yet been adequately achieved to allow for translation into a manufacturing process. Moreover, further characterisation and process understanding are required in an effort to build a portfolio of cell markers that can be used to definitively identity and potentially quantify optimal ranges of the CQAs for a manufacturing process. This information would be valuable for any further efforts centred around identity and potency assay development. In addition, further investigations are required to ascertain the explicit optimal conditions for H9 differentiation, since current ranges that are either side of the protocol (0.7 μM), in terms of CHIR99021 concentration, have resulted in cell survival with low expression yields of the desired vmDA cell type (range explored 0.3-0.6 μM and 0.8-1.5 μM). Further experimentation with the most promising candidates from the DoE such as M1 and condition 1 (DSD1 and DSD2, respectively), would facilitate validation of a design space that produce a robust and reproducible protocol, suitable for translation into a manufacturing process.

6.6. Chapter Bridge

The work detailed in this chapter demonstrates the challenges in translating a research protocol into a robust manufacturing process, whilst highlighting the value of informed-DoE based protocol conception. Early consideration of process control and multivariate process understanding would allow for the product development timeline to progress with ease and greater efficiency, by negating the laborious process optimisation that has been the focus of this work. Early consideration of the whole product development journey is the key takeaway from this body of work and this chapter in particular, as such the following chapters will explore how early economic considerations can have a beneficial impact for developers as they are more informed about their product. Specifically, if developers have an understanding of resource utilisation, cost of goods and the ability to make informed data-based process changes that do not have a detrimental impact on the budget; they are likely to progress effectively through the product development pathway.

The next chapter will explore the key challenges in CTP adoption and reimbursement. These challenges are pertinent to the current CTP climate, which is favourable due to the onset of some of the first cell and gene therapies such as the CAR-T therapies by Gilead (Yescarta) and Novartis (Kymriah) being approved[281]. However, CTPs such as Yescarta and Kymriah have very high price tags (£272,000 and £ 282,000 respectively) and thus there are adoption and reimbursement challenges that need to be addressed, to ensure sustainable and efficient adoption of CTPs[281–283]. The following chapter will explore health technology assessment, reimbursement, adoption and implementation of CTPs in the context of the UK and Canadian healthcare systems, with a focus on the former.

Chapter 7:

Exploration of the Challenges in the Adoption of Regenerative Medicine Therapies

Chapter 7. Exploration of the Challenges in the Adoption of Regenerative Medicine Therapies

7.1. Introduction

The focus of this chapter is on the exploration and evaluation of key challenges in the adoption and reimbursement of CTPs. Scientific success in terms of positive clinical trial data for safety and efficacy is a milestone achievement in the development process of therapeutic products, however, this alone does not guarantee that a product will be commercially successful. Instead, commercial success is determined by a product's ability to demonstrate value through both clinical effectiveness and financial cost-effectiveness within a health care setting. The objective of this chapter is to determine the post-regulatory challenges associated with economic evaluation, adoption, reimbursement and commercial success of CTPs. In Chapter 2 the basic principles of health economics, health technology assessment (HTA) and product commercialisation considerations were discussed, and these themes provide context to the challenges being addressed in this chapter. The challenges will be explored in the context of the UK and Canadian healthcare systems, with a focus on the former. These two healthcare systems share commonalties in some aspects of their provision to healthcare as they are both publicly funded systems. In addition, the work carried out in this chapter has been in collaboration with institutions both in the UK and Canada.

N.B. Due to the specialist nature of this topic area, it is recommended that the glossary from Chapter 2 is reviewed to ensure that the correct meaning of the terms are understood in the context of the present work.

7.1.1. Healthcare in the UK

The Department of Health and Social Care (DHSC) oversees the development of polices and guidelines that aim to provide the best quality healthcare to patients. Healthcare in the UK is provided publicly by the National Health Service (NHS) which was established in 1948[284]. There are currently four versions of the NHS, one for each of the countries in the UK: NHS England, NHS Scotland, NHS Wales and the Health and Social Care in Northern Ireland. NHS England is the largest of the 4 systems as it caters to a population of over 54 million people and manages over 85% of the ~ £147 billion budget which equated to £124 billion in 2019 [284]. Unless otherwise stated, NHS England will be the service discussed in this thesis and will be referred to herein as the "NHS". The NHS is funded through general taxation, national insurance contributions and fee-for-service income, although the latter represents a small proportion of the total budget income[284]. Residents of the UK are entitled to healthcare that is free at the point of use, with the exception of specific care such as dental treatment and ophthalmic care[285]. Under the DHSC there are three executive non-departmental public bodies of interest in the area of health economics and reimbursement, which are NHS England, NHS Improvement and The National Institute for Health and Care Excellence (NICE).

7.1.1.1. NHS England and NHS Improvement.

Under the Health and Social Care Act 2012, NHS England and NHS Improvement are jointly responsible for pricing of healthcare resources[286,287]. NHS England is responsible for budgeting, amongst other responsibilities such as the organisation, operation and commissioning of the English NHS through allocating funds to the Clinical Commissioning Groups (CCGs). NHS Improvement sets the rules for determining the tariff for the NHS services and ensures that procurement, choice and completion of healthcare services are performed with the patients' best interest in mind. NHS Improvement specifies the "currencies, national prices, the method for determining those prices, the local pricing and payment rules, the methods for determining local modifications and related guidance that make up the national tariff payment system"[287]. The work carried out by these two agencies is multifaceted since the healthcare needs of over 50 million people must be considered; therefore, it is necessary to take account of high cost interventions that have significant impacts on the budget. These costs are separated so that the representative reimbursements costs are not skewed due to high cost drugs, chemotherapy, devices and therapies such as the recently approved CAR-T therapies for cancer treatment. As part of the mandate of providing high quality care to patients, NHS England and NHS Improvement have mechanisms to ensure that the reimbursement system is dynamic and current, such as facilitating the appraisal of new drugs and innovative technologies[287]. This enables patients to have access to the best available care and for the healthcare budget to be used in the most efficient and effective way to ensure equity of healthcare. In general, it is the responsibility of NHS England to

specify which healthcare interventions and services should be reimbursed using a national tariff price, once these services have been identified it is the duty of NHS Improvement to set the price.

7.1.1.2. The National Institute for Health and Care Excellence and its role in health technology assessment

The National Institute for Health and Care Excellence (NICE) is an independent non-governmental institution that is funded by the DHSC[288]. The institution was initially established in 1999 in order to standardise care and health outcomes within the NHS, as variations in health outcomes up to this point had become prevalent[289]. For example, patients with the same clinical condition were receiving different types and standards of care depending on where they were in the country, leading to the phenomena of the 'postcode lottery'[288]. As a result, there were differences in health outcomes and quality of life for patients due to their geographical location. The role of NICE is to provide guidance in matters of health and social care to the NHS and ensure that appropriate services of a high quality are made available without variation amongst patients, thus, abolishing the notion of the health postcode lottery. NICE also helps to resolve uncertainty regarding the best interventions to use that provide the best value for money within the NHS by appraising the available options. Additionally, the institute also provides clinical guidance to set the national standard on how people with certain conditions should be treated, to ensure the best care is provided.

NICE has core principles that it operates on, these are (i) *Scientific rigour*, which ensures the best available evidence is assessed in order to develop guidelines and recommendations[289]. (ii) *inclusiveness* and (iii) *transparency* are also important since NICE is a publicly funded entity. Therefore, the relevant stakeholders are involved in the development of a recommendation or guidance, typically this includes the relevant healthcare professionals and patient advocacy groups. In order to ensure transparency, the recommendations and guidance carried out by NICE is available and free to access on their website[290].(iv) *Independence* is a founding principle of NICE, therefore it is operated at arm's length of the government as a public body. As such the public has the right to challenge NICE's guidance and recommendations or even take it to court. (v) *Timeliness* and (vi) *being current* are also import aspects of NICE so that the healthcare being provided is up to date, meaning that they regularly review their guidelines and support their implementation. Ultimately NICE should be reasonable and accountable for its guidance decisions[290].

There are four main guidelines that NICE provides guidance over which are; NICE guidelines, interventional procedure guidance, technology appraisals guidance and the medical technologies and diagnostics guidance[290]. Of importance to this is work are the latter two guidance, since the technology appraisals guidance assesses the clinical and cost-effectiveness of health technologies such as medical

devices, treatment procedures, diagnostic tools and pharmaceutical products in addition to biopharmaceuticals. Medical technologies and diagnostics guidance helps with the timely adoption of clinically effective and cost-effective technologies within the NHS.

In terms of appraisals, assessing the value of new interventions is a key activity carried out by NICE[289,291]. This activity is challenging as "value" is subjective and expressed differently for different stakeholders i.e. for patients value often refers to the perceived health benefits and improvements achieved from an intervention; whereas for payers value would refer to cost-effective interventions that provide patient benefits and cost savings. In such a case NICE carries out assessments to determine if the new intervention provides more benefits than the current standard of care within the NHS[290,291]. The use of cost-effectiveness and quality adjusted life years (QALYs) in these assessments provides a standardised and comparable assessment of the value of a new intervention. Value is not considered at an individual level but for the averaged population since budgets do not currently allow for value to be based on each individual within the NHS; opportunity costs are also an important element of the value as funds have to be redirected to fund new interventions[291–293]. NICE also considers value for the developers as they understand that developers have to make a return on investment for the products they provide to the NHS, which is the main buyer of healthcare services and products in the UK. Following a recommendation from NICE it is the responsibility of NHS England to commission the recommended technology, this involves implementation and reimbursement decisions on how the technology will be adopted and paid for through the NHS' payment system.

7.1.1.3. The NHS payment and reimbursement system.

The payment system uses different models to account for the complex challenge of budget control, resource allocation and healthcare quality improvement. Two of the main payment models include block payments and capitation[294,295](**Table 42**). Block payments are used for making payments for hospital services through an agreed fixed sum payable in instalments for a broad set of specific services, independent of the number of patients that may use the service. Capitation payment is a model used for providing payments for general practitioner (GP) services, again for an agreed fixed sum, however in the capitation model the sum is based on the size of the population served by the GPs[296]. Capitation is designed to allow healthcare providers to offer availability and access to comprehensive services.

Table 42. Summary for the four main payment models used in the UK healthcare system, adapted from Wright et al (2017)[297].

Model	Description
Block budgets	Provider payments for all services provided are bundled together, with a lump sum paid to providers at intervals, independent of the level of activity
Capitation	Providers receive bundled payments on a per patient basis for the services the patient receives
Case-based payments	Providers receive a prospective fixed sum for an episode – rather than single instance of care
Fee-for-service	Providers are paid retrospectively per unit of activity undertaken

In 2003 a new case-based payment model was introduced[287,298], which was termed payment by results (PbR), in PbR providers of care are reimbursed on based on the number of patients seen and their respective diagnosis and treatment. Currently, PbR is now referred to as the national tariff payment system (NTPS) which is updated annually. Under this system there are different tariffs for different service types and each tariff covers the full cost of the activity e.g. the consultant fees, nursing, bed space, meals, consumables, equipment used and most drugs over a treatment spell[287]. The tariffs are set against different currencies which are applied to different categories of services such as in-patients, outpatients and emergency services[287]. For inpatients the currency used is called Health Resource Groups (HRGs – section 7.1.1.3.a.), for outpatients the currency is based on attendance and the procedures carried out; emergency service currencies are based on attendance and critical care days. In simpler terms, currencies are the unit of healthcare for which a payment is made, for example an elective day case major hip procedure with no complex complication is a currency[287,296,299]. Currencies can take several forms covering different time periods from an outpatient attendance, a stay in hospital or a year of care for a long-term chronic condition. Tariffs are the set prices paid for each currency, for example, the elective day case major hip procedure with no complex complication from admission to patient discharge was priced at £5,431in 2018[287].

There are four key elements to the tariff system which include; the treatment, coding, grouping and the tariff itself. Treatment refers to the procedure and care provided for all patient settings (inpatient, outpatient and emergency services). The care provided during treatment is then coded by clinical coders to reflect the currencies used in the system. These codes provide information regarding the diagnosis, interventions and procedures that the patient receives. In addition, data such as the age of the patient

and the length of their stay in the care setting are included in the coding. Grouping is the process that applies an HRG based on the codes and data provided regarding the patient's treatment (explained in section 7.1.1.3.a.). Once the grouping has been carried out, a tariff can be applied based on the HRG and type of admission, payments from the commissioner to the provider are then made based on the calculated tariff (**Figure 98**). Providers are paid for the number and complexity of the patients they see; each provider is paid the same for providing the same services so there is no competition due to national pricing.

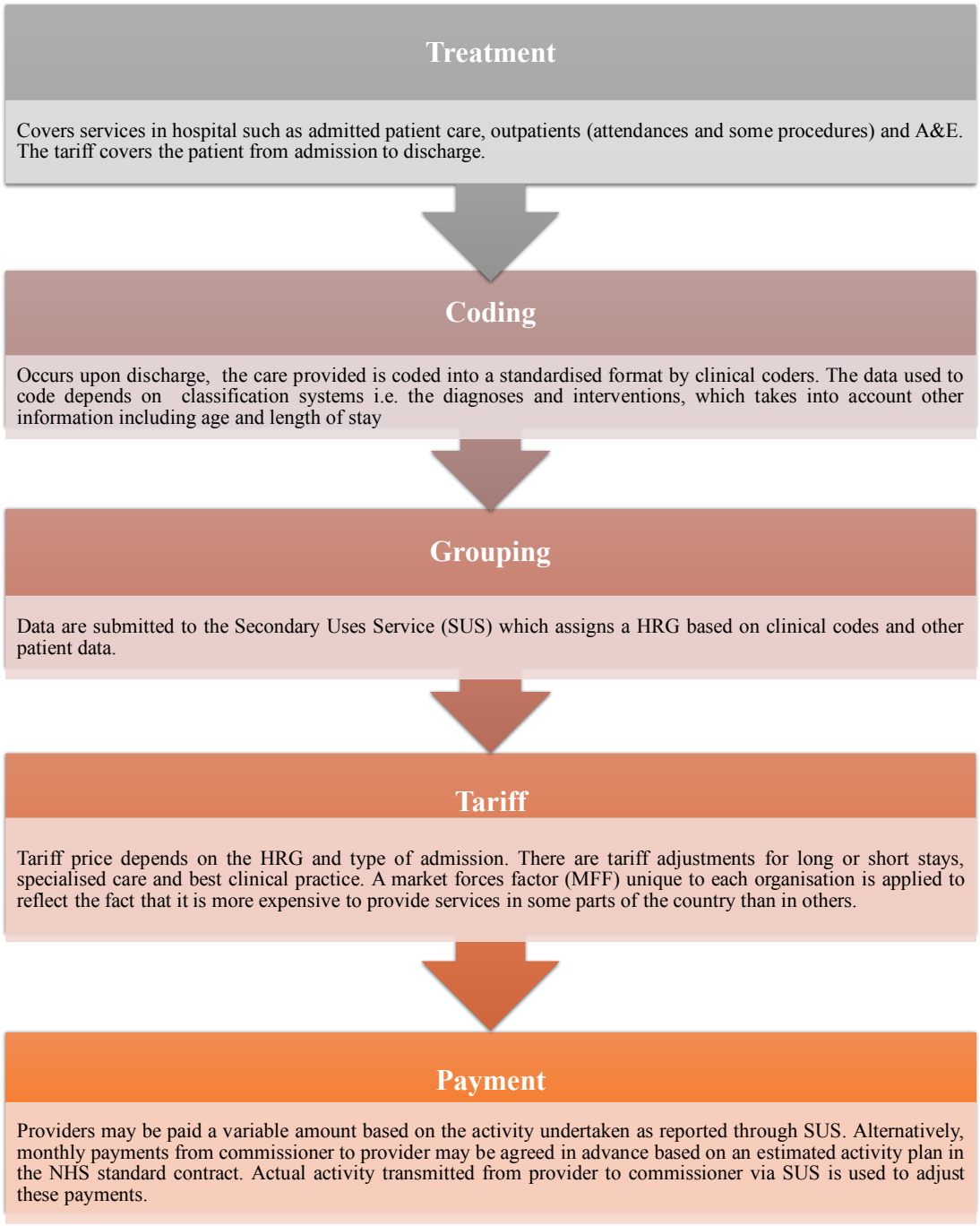


Figure 98. The process of healthcare provision, from treatment to payment under the PbR national tariff payment system.

7.1.1.3.a. Health Resource Group coding:

A Healthcare Resource Groups (HRG) code is derived from a combination of age, gender, diagnosis, procedures, complications and length of stay[295,300]. HRGs were first introduced in 1991, and since then there have been several revisions with the latest set being HRG4+ updated in 2017[301]. HRGs are updated annually to ensure that the payments to service providers are appropriate for the work being carried out, updates are based on consultation from expert working groups which include practitioners and policy makers. The composition of the HRG is based on clinical detail whereby treatments which consume a similar amount of resources are grouped together. This is based around HRG chapters, there are currently 22 chapters which are broadly based on anatomical areas i.e. HRG E refers to treatments related to cardiac disorders. These chapters are further defined to take into account treatments and complexities, resulting in over 1,600 HRG codes[302]. Upon discharge clinical coders apply the HRGs to code the diagnosis and interventions applied to the patient on a local hospital level using a patient administration system (PAS). The data is then sent to the national secondary uses service (SUS) which group clinical codes into HRGs and calculate the payment to be made based on the most applicable HRG, currency and tariff[287,303,304]. An example of two case studies of the HRG system and payment process is shown in **Appendix 6**.

7.1.2. Healthcare in Canada

Similar to the UK, healthcare in Canada is publicly funded and it available to all Canadian citizens and permanent residents within the thirteen provinces and Canadian territories. The Canadian healthcare system, Medicare, is a product of the Medical Care Act of 1966, which was later encompassed into the Canada Health Care Act of 1984[305,306]. In comparison to the UK healthcare system, Canada has a more decentralised approach to the administration and delivery of healthcare. The Health Care Act sets the standards which each of the thirteen provinces and territories must meet in order to be reimbursed for their expenditure under the Canada Health Transfer[305,306]. In brief, the Health Care Act aims to ensure that health care is provided under five core principles: (1) under public administration thus on a not for profit basis; (2) comprehensively in terms of medically necessary services; (3) universally to all Canadian residents; (4) portability in cases where residents are temporarily out of their province and finally; (5) with ease of accessibility and uniformity regardless of one's ability to pay[305,306]. While the Canada Health Care Act provides federal level guidance on the provision of health, spending decisions are made at a provincial and territorial level by each of the thirteen provinces and territories.

In Canada, ~70% of total health care spending is by public means and the services provided are free at the point of use[307]. The remaining 30% is spent privately through out-of-pocket payments and private insurance on services such as optometry and dentistry[307,308]. Additional health insurance covers the cost

of services not covered under Medicare. Most hospitals in Canada are publicly funded and are set to operate on a fixed budget as a means of controlling costs[309–311]. However, GPs are private and typically operate on a fee-for-service basis under the coverage of the province or territory they are situated in[309,312,313].

Medication costs are also a private expense however; prices are negotiated at a federal level by the Patent Medicine Prices Review Board (PMPRB) for Canada's federal, provincial and territorial drugs plan[314]. Drugs plans are managed by the provincial and territorial governments, whose responsibility it is to determine what prescription drugs are publicly funded and the associated eligibility criteria. Not all citizens and residents are eligible for coverage through public drugs plans, typically coverage is given to "those most in need, based on age, income, and medical condition."[177] Pricing and reimbursement decisions regarding medication in Canada occur at three levels; federally by the PMPRB, by the individual provincial and territorial drug plans, and by the Common Drug Review[315,316].

7.1.2.1. Canadian Agency for Drugs and Technologies in Health

Adoption and reimbursement in Canada is multistage process, initially the new product must be approved by Health Canada for safety and efficacy measures. Once approved the maximum ceiling price for the new drug is set by the PMPRB and this value is submitted for HTA for clinical and/or cost-effectiveness evaluation. The Canadian Agency for Drugs and Technologies in Health (CADTH) is a national agency similar to NICE in the UK, in that it provides healthcare decision-makers information, advice, evidence and recommendations related to the use of medication and medical devices[317]. CADTH has a range of programs and services, of particular interest to the present work are their common drug review (CDR) and health technology management programs.

7.1.2.1.a Common Drug Review

The CDR is a process that most drugs must go through prior to entering the public system. The CDR provides recommendations concerning reimbursement and funding decisions for the provincial and territorial public drug plans, with the exception of Quebec[317]. Publicly funded drug plans in the provinces and territories must work within their budgets to provide equity and value for patients, thus the CDR assists the drug plans in decision-making with regards to which drugs they should pay for[316]. The CDR receives and reviews submissions by systematically evaluating the clinical evidence provided by the developer/manufacturer, followed by pharmacoeconomical analysis to determine the cost-effectiveness and economic impact of the drug.[315,318]. To do this, the CDR must bring together medical experts and patients to frame the investigation by comparing the benefits and side effects of the product in relation to existing treatment options. In addition, the cost effectiveness of the drug is compared to

existing options, the CDR can also include the societal impact of a new treatment as part of their analysis. The CDR process produces summarised evidence for the Canadian Drug expert committee (CDEC), the CDEC uses this information to provide a non-binding recommendation to the drug plans[319,320].

The CDEC's recommendations cover the optimal use of the drug and the level of funding that the drug might receive. The committee is usually comprised of a chair, public members, and experts in areas such as health economics and drug utilisation[320]. The committee deliberates on aspects such as the available clinical studies, comparators, therapeutic advantages, cost and cost-effectiveness. The deliberations result in a non-binding recommendation from the committee, detailing whether the drug should be publicly funded or not. It is then up to the individual drug plans to make a decision whether to fund the drug or not and how the drug will be funded based on the CDEC recommendation.

7.1.2.1.b. Health Technology Assessments

The health technology management programs provide assessments concerning the "clinical and/or economic evidence on health technologies"[321]. In Canada health technologies relate to "prescription drugs, diagnostic tests, and surgical, medical, or dental devices and procedures — but not broad health system issues, such as information technology, program delivery, staffing, and finance."[321] . HTAs are performed by ad hoc multidisciplinary teams created to suit each project being assessed. Similar to NICE, HTA assessments by CADTH are published and made freely available to anyone.

The HTA function of CADTH has a national approach, such that HTAs are "normally reserved for topics of pan-Canadian interest"[316,321]. The assessments provide evidence for health policy, purchasing and clinical practice decision-making. These analyses evaluate both the clinical and cost-effectiveness of a treatment, taking into account its budget impact as well as any potential ethical, legal and social impacts of the treatment[321,322]. CADTH also offer a Technology Review, which is used for reviews that do not require a comprehensive HTA i.e. a review with an economic component only.

CADTH HTAs undergo a rigorous prioritisation process, whereby each technology must be assessed for appropriateness under CADTH's mandate before it can progress further[322]. The criteria include: duplication of effort i.e. determining if similar reviews are being carried out elsewhere; need i.e. does a decision need to be made and is the impact of the decision substantial to patient care; and stage of diffusion i.e. is the technology currently or imminently available[323]. These criteria are considered to ensure that efficient use of assessment resources are used and to determine the importance of the assessment for the benefit of patient care for technologies relevant to Canada. Once a technology is

deemed appropriate it is prioritised based on its clinical, budget and population impact as well as the jurisdictional interest and its effect on health equity[323]. Details of the scores and categories used for both the appropriateness and prioritisation assessment can be found on CADTH's website[324].

7.1.2.2. Patent Medicine Prices Review Board

The Patent Medicine Prices Review Board (PMPRB) is a federal agency established in 1987 to ensure that Canadian consumers are not charged excessive prices for patented medicines[314,325]. The PMPRB only has jurisdiction over 'factory-gate' prices set by the manufacturers and not on the final prices set by the wholesalers and pharmacies that sell to the end consumer[314,325]. Furthermore, the PMPRB's role is currently limited to patented medicines, thus the price of generic medications is not regulated by the PMPRB.

As part of the review process a scientific and economic analysis is carried out to determine how appropriate the price is[314]. The scientific analysis considers the level of therapeutic improvement of the new drug; the levels range from breakthrough improvement to no improvement. The economic analysis determines the appropriateness of the price based on: the price of the drug in the relevant market; the price of similar drugs in the same therapeutic class; and the price of the drug and similar drugs in other comparator countries, typically the UK, USA and some western European countries[177,325]. The individual drug plans are then responsible for deciding at a provincial and territorial level the reimbursement conditions for the drug. The final price paid by the public drug plans is negotiated by the provinces, territories and the manufacturer through the pan-Canadian Pharmaceutical Alliance (pCPA).

In addition to its regulatory role the PMPRB is also responsible for the reporting of pharmaceutics pricing trends in the industry. The reporting is carried out annually and reported back to Parliament. The annual report covers aspects such as: sales of patented drugs, price trends, international drug prices *vs.* Canadian prices, patent drug utilisation and Canada's drug expenditure in a global context[314]. In addition, the PMPRB also reports on research and development expenditure within Canada related to patented pharmaceuticals[314].

7.1.3. Challenges in CTP adoption and reimbursement.

Even with the understanding of healthcare systems and their reimbursement mechanisms covered above, CTPs still face the challenge of understanding how to navigate through the various adoption and reimbursement pathways, that have for so many years been aimed at pharmaceutical drugs and medical devices. Some of these challenges were previously discussed in section 2.4.6. Considerations for developers.

These considerations highlight some of the key issues that have slowed CTP adoption. For instance, a prevalent issue is understanding the evidence requirements for "curative" CTPs to generate data that adequately supports adoption and reimbursement. The notion of a curative CTP would be desired by payers, particularly for chronic diseases such as PD and diabetes, however due to the lack of long-term clinical data these claims are yet to be affirmed. Thus, appropriate evidence generation is a challenge that CTPs face in order to satisfy both regulators and payers that their products will afford the clinical and cost benefits proposed[326,327]. Therefore, appropriate data generation to support decision making for CTPs is a challenge that still needs to be addressed. In addition, overcoming barriers to adoption such as hospital infrastructure, payment structures and how to offset costs are key challenges for CTPs.

The small number of CTPs currently available typically target small populations (typically less than 5,000 patients), thus they are given special or conditional designations as these therapies are often considered as a "last chance" therapy option for patients[26]. However, if CTPs are to ever become a mode of mainstream treatment, ad hoc evaluations and approvals will not be an efficient mode of adoption and sustainably growing the cell therapy industry (CTI). Instead, the aforementioned challenges need to be addressed in a holistic manner, meaning there should be clear understanding of the adoption pathways for CTPs, if they are indeed any different to drugs, biologics and medical devices in terms of evidence generation, economic modelling and payment structures.

There is currently scarce evidence of policies and pathways for CTPs, although many countries have now begun to form regenerative medicine committees and expert groups for areas such as HTA[326–328]. However, areas such as payment mechanism, pricing, code generation (for payment) and reimbursement schemes still remain largely elusive. Bodies such as NHS England and NHS improvement currently do not have defined information on how payment and pricing will or will not differ for CTPs. Currently, CTPs such as Kymriah and Yescarta that have been approved are being reimbursed under specific funding, which in both cases is the Cancer Drug Fund[329].

Should an onset of non-cancer CTPs be successful for market authorisation then it is not clear how these would be funded. Other important considerations include:

- Tariff/Code structures - will they be different for each nuanced treatment or will they be blanketed?
- Will there be budget impact adjustments to facilitate the costs of CTPs?
- Will managed risk agreement structures be employed?
- Are outcomes-based pricing/payment schemes going to be used for CTPs?
- Would there be changes in the willingness to pay for these therapies due to their long time and potential curative benefits which could offset costs?

The current lack of clear and defined understanding of the reimbursement and adoption pathways for CTPs in the literature and amongst some expert organisations, lead the author seek other avenues to understand what the key challenges in the adoption of CTPs were. As a result, the author founded a working group to address some of the key challenges that CTPs must overcome in order to successfully traverse the reimbursement and adoption pathway. The working group, **Ch**allenges in the **A**doption of **R**egenerative Medicine **T**herapies (CHART), was set up in collaboration with Medicine by Design, a regenerative medicine initiative at the University of Toronto, the Centre for Commercialization of Regenerative Medicine (CCRM) and the Toronto Health Economics and Technology Assessment Collaborative (THETA). The working group setup a workshop, inviting selected UK and Canadian experts to discuss these adoption challenges.

7.2. Methodology

In order to address the question of what the key challenges in the adoption of CTPs are, a quasi-action research methodology was utilised using the CHART workshop as a vehicle for obtaining the data to answer the question. An action research approach allowed active participation in the research. The action research methodology used followed an approach similar to that described by Bogdan and Biklen (2007), where by "systematic collection of information that is designed to bring about social change"[330]. In the case of the present work the goal was not to bring about social change, but to provide recommendations on what the key challenges that need to be addressed are, for CTP adoption to be better facilitated. There are various frameworks that exist for the purposes of action research[330–332], in the present work a five-step process was employed (**Figure 99**).

Figure 99. Schematic of the action research methodology framework employed, detailing the five-step process used to investigate the challenges in the adoption of CTPs.

The first step of selecting the focus was inspired by the limited literature and lack of general consensus concerning whether there are any key differences in the HTA methodology for evaluating CTPs and what the payment and reimbursement structure for these putatively expensive therapies would be. Overall the focus was on the adoption challenges of CTPs after the point of regulatory approval. In terms of clarifying the theories currently around CTP payment mechanisms and adoption, the literature provides some key perspectives. This mainly involves the use of managed entry agreements (MEAs) to facilitate payment of expensive therapies[333,334]. Most theories concerning adoption of CTPs are from manufacturing companies in the industry, mainly looking to ensure that they products can be adopted on the market. However, organisations such as the Cell and Gene Therapy Catapult have made efforts on a system level, with the creation of their manufacturing hub and the advent of the Advanced Therapy Treatment Centres (ATTCs).

Step three of the process involved identifying the research questions; six theme areas were initially identified, which were: *evidence of clinical effectiveness, health economics, social values towards regenerative medicine, health technology assessment, payment system mechanisms* and *adoption & implementation*. To facilitate meaningful inquiry into the themes, more detailed questions where formulated, these are presented in the agenda under each theme/session (**Appendix 7**). Collecting the data to answer the question(s) was the fourth step in the process and this was achieved by realisation of the actual workshop in Toronto, Canada. The data collected was valid as the participants of the workshop were peers in the CTP, regenerative medicine, HTA and health economics areas therefore they were appropriate sources of information. Reliability of the data being collected was important to consider, the speakers and discussants in the workshop were experts who actively conduct the assessments, and actively perform research and work in the relevant areas that were discussed. Therefore, there is confidence in the reliability of the data collected as it was obtained credible sources who represent many of the varying aspects of the adoption pathway. In addition, the use of speakers and discussants from both the UK and Canada assisted in triangulation of the validity and reliability of the data as various speakers and discussants were asked to contribute to each session. This provided multiple sources of data for each session reducing the level of potential bias in the qualitative data being obtained.

Data analysis was carried out following collection, for the purposes of the present work the discussion points from the workshop were sorted and sifted to identify the key trends and overall conclusions from each theme. This was performed by recapping the session recordings and identifying the crucial points from each session and where possible, finding published data to support the points. This led to step five of the process that is the reporting of the results/data, the report can be found in the following discussion section of this chapter. In addition, a white paper was commissioned as a result of the workshop discussions, providing a medium to demonstrate to policy makers to the issues that were identified and

need to be addressed to facilitate CTP adoption.

In relation to the topic of bias and subjectivity it is important to note that each of the different parties participating in the workshop had varying intentions and expected outcomes for attending the workshop. For companies such as Takeda, Gilead and BlueRock, the workshop provided an opportunity to learn about and ask questions regarding the HTA process and the potential adoption and implementation pathways that are being considered for CTPs. For the HTA agencies, the workshop provided an opportunity for them to share their experience with CTPs to date whilst also allowing them to publicise the programmes they offer that can help developers through the pathway, such as early scientific advice. The academics within the workshop shared their current findings and the methodologies that they have been working on with regards to CTP HTA and considerations such as social value systems. The clinicians present were able to use the workshop as an opportunity to share their experiences with CTPs, in particular, CAR-T therapies and the current challenges they have encountered in the trials that have been run. It is important to acknowledge the subjective nature of the discussion points that arose, since these are based on the work of parties presenting their work and experiences, albeit in the context of the CTI. However, the majority of speakers present at the workshop were based from institutions that have the notion of producing unbiased, fact-based information; therefore, the work that they presented was adequately objective. In addition, due to the international audience present, the conference speakers were encouraged to provide factual based findings which in most cases had also undergone peer review.

7.2.1 Workshop organisation

The author was involved in all aspects of the workshop and took lead on aspects such as: formulating the agenda, inviting speakers and discussants/panellists, facilitating the running of the workshop on the days it ran and presenting at the workshop.

The purpose of the workshop that was to facilitate discussion to identify the challenges faced in CTP adoption. Thus, the workshop acted as a method of primary qualitative research by means of expert opinion and formulated discussions. Expert opinion was sought from invited attendees specialising in regenerative medicine and other relevant areas with regards to HTA, adoption, reimbursement and health economics. The attendees were asked to be speakers or discussants/panellist on a specific area(s) considered to be hindering the adoption of CTPs, including evidence generation, economic evaluations and implementation challenges. The invited attendees were approached through collaborations, previous networking engagements and recommendations of the author and other members of the organising committee. An agenda was devised by the author and the organising committee to facilitate

targeted discussion during the workshop.

The experts invited where from institutions that were deemed to be highly involved and influential in the topics set out in the agenda (**Appendix 7** and **Appendix 8**). Representatives from NICE and CADTH were invited to discuss the HTA process and to establish if there are nuances to the process when CTPs are being assessed. Representatives from the Office of Health Economics (OHE) and the Centre for Health Economics – University of York (CHE) were invited to discuss the methodologies involved in economic evaluation and health economic analyses. The Oxford Academic Health Science Network (Oxford AHSN) provided insight into the challenge of adopting CTPs and the steps being taken to facilitate adoption. It was imperative to also engage with CTP industry and clinicians to ensure the discussion points were relevant to the challenges currently being faced. Therefore, Hitachi Chemical Advanced Therapeutics Solutions, LLC (HCATS) was invited to the workshop as they have broad understanding of CTPs at different phases of development. Thus, facilitating discussion of the issues around evidence generation and adoption for the developer perspective, while being agnostic as they have a range of client products. In addition, representatives from Gilead and Takeda were invited to provide insight into their experiences with market access for CTPs. Clinicians and academics in health policy and bioethics were invited to speak on issues of social values towards CTPs.

7.3. Discussion

The workshop started with an introduction (Session 1 in the agenda, **Appendix 7**) highlighting the context in which the workshop was conceived, which is discussed in section 7.2.1. (page - 283 -) It was acknowledged that while there are many aspects involved in the adoption of CTPs, reimbursement and adoption were the main focus of the workshop. A definition of "advanced therapy medicinal products" (ATMPs) was provided to ensure a commonality of understanding within the workshop. ATMPs, are synonymous to the CTPs that have been discussed throughout the present work, however ATMPs include gene therapies as well as cell therapies. CAR-T therapies such as Yescarta and Kymriah were frequently used as examples to provide context as CAR- T therapies are currently the flagship therapy of the cell and gene therapy industry[281,283].

The following sections provide an overview of the presentations and outcomes of the workshop sessions and discussion panels. Prior to the workshop, presenters and discussion panellists were provided with questions to consider, these are presented at the start of the session summaries below and also in the workshop agenda, (**Appendix 7**). Where applicable references have been provided to support the discussion points, footnotes have been used to highlight discussion points specifically from the workshop. The presentation slides of the workshop presenters have been provided in the appendix, where permission has been granted for use in the present work.

7.3.1. Evidence of clinical effectiveness: Session 2

The purpose of this session was to explore the key challenges in providing evidence of clinical effectiveness of CTPs. The presentations were provided by a Senior Scientific Adviser from NICE and a haematologist from the Princess Margaret Cancer Centre, Toronto, with experience of administering CAR-T therapies. The discussion panel was comprised of the NICE Scientific Adviser, the Princess Margaret Cancer Centre haematologist, the dean of Health Sciences at Simon Fraser University and a biomedical engineer from HCATS.

Posed questions:

- ATMPs (specifically CTPs) – does their promise of cure/long term effect make them different?
- How to satisfy both payers and regulators by producing adequate data?
- What is the evidence supporting the benefits and safety of ATMPs?
- How do we ascertain the long – term clinical benefits for ATMPs?
- What is the structure of the data/evidence?
- What is the strength of the evidence?

7.3.1.1. Session Summary

Benefit to the patient was highlighted to be the key question for consideration when contemplating clinical effectives for the purposes of HTA and cost-effectiveness analysis. Demonstrating and understanding how well an intervention works compared to established modes of practice is crucial to the evidence data provided to decision makers and payers. The issues of evidence and demonstration of long-term clinical benefits is paramount as the cost of CTPs is high, thus they must present value. However, currently there is uncertainty regarding the long-term benefits and potential adverse effects of CTPs as the data currently available is immature and scarce, thus their potential curative effect cannot be substantiated[173,335]. Typically, CTPs are developed by academic institutions or start-up companies with limited funding and lack of experience with HTA process; this can result in inadequate evidence generation for the purposes of satisfying the HTA process. Another point that was highlighted was the lack of randomised clinical trials (RCTs) and small sample sizes associated with CTPs as single-arm trials that currently routine practice for CTPs[335,336]. Furthermore, current trials can only provide surrogate or intermediate data as the final clinical outcomes are unknown. In addition, due to the short length of trials (6 – 36 month follow up period) and the resultant data, how well clinical effectiveness

is maintained and the potential for future adverse effects is yet to be established. Therefore, current data is subject to extrapolation, follow-up and accruing observational and real-world data[i].

In the context of CAR-T therapies, evidence such as objective response rate (ORR); ongoing response (OR); complete response (CR); progression free survival (PFS); overall survival (OS); adverse effects such as cytopenia and Eastern Cooperative Oncology Group (ECOG) scores are required to provide robust data that can be assessed against comparators[337,338](**Table 43**). Furthermore, the data should be obtained from randomised trials with larger sample sizes, to offer sufficient statistical power in each arm of the trials. This would adequately inform the HTA process and provide value to the decision makers and payers. Extended research post-market authorisation would be a mode of providing additional and real-world data. However, there are issues with adequately capturing the data in a valuable way[ii].

Table 43. Definitions of the typical outcome measurements used in the oncology area for CAR-T trials.

Outcome Measure	Definition
Complete response (CR)	Signifies the absence of all detectable cancer post treatment, this is the best result that can be reported in cancer prognosis
Eastern Cooperative Oncology Group (ECOG) score	A performance scale used to assess disease progression (in oncology) and assess the daily living abilities a patient
Objective response rate (ORR)	The percentage of patients whose cancer shrinks or disappears after treatment
Overall survival (OS)	The length of time from either the date of diagnosis or the start of treatment for a disease, such as cancer, that patients diagnosed with the disease are still alive
Progression free survival (PFS)	The length of time during and after the treatment of a disease, that a patient lives with the disease but it does not get worse

[i] CHART workshop presentation/discussion point
[ii] CHART workshop presentation/discussion point

7.3.2. Health economics: Session 3

Health economics are an important element of the reimbursement and adoption process; therefore, a session was dedicated to discussing cost-effectiveness evaluations and any methodological challenges that are related to evaluating CTPs. The presentation was provided by a member of the Centre for Health Economics (CHE), University of York. The panel included the research fellow from CHE, a senior principal economist from the Office of Health Economics, an assistant professor from the University of Waterloo (Canada) school of pharmacy and the senior scientific advisor from NICE. As in the previous session CAR-T therapies were referenced as a case study to provide context.

Posed questions:

- What is different about reimbursement strategies for ATMPs?
- How to evaluate the cost effectiveness of ATMPs?
- How affordable are ATMPs/can they be affordable?

7.3.2.1. Session Summary

Evidence, price and affordability were highlighted as some of the key challenges in economic evaluations. Evidence challenges encompass issues such as the appropriateness of surrogate endpoints used to inform evaluations when endpoint trial data is not available; evidence generalisability; assurance of sustained health outcomes and quantification of bias in non-randomised studies. These evidence challenges result in a high level of uncertainty as the input data for evaluation models would not be robust. For example, surrogate endpoints with an unclear relationship to overall survival and health related quality of life, offer no benefit to the HTA and decision-making processes.

The challenge of price and affordability associated with CTPs can be attributed to the complexity of production and administration of CTPs. High cost of goods figures results in higher prices being demanded by the manufacturers, which ultimately increases the price for the payer. Furthermore, the complexity of introducing or adjusting infrastructure and acquiring accreditation to administer CTPs presents additional costs to the payer that have a significant budget impact. Consideration of these challenges and the uncertainty they result in prompted assessment of the appropriateness of current methodologies in HTA for evaluating CTPs. Two studies were commissioned to assess how fit for purpose the existing HTA and reimbursement process are for CTPs[328,339]. The reports by Hettle *et al* (2017) and Crabb *et al* (2016) provide a detailed summary of the assessment and appraisals carried out. In brief, the assessment found that the methodology and decision framework used for NICE technology appraisals is applicable to CTPs[328,339]. However, there are elements to CTP evaluation that present

challenges to the HTA process, for instance, appropriate extrapolation. Extrapolation is a key driver of cost-effectiveness and therefore results in pronounced decision uncertainty. Solutions to dealing with uncertainty were contemplated, a leasing method of payment was considered to be a method for reducing decision uncertainty[328]. This highlighted the need for practical, workable payment methodologies for CTPs that deal with uncertainty and reduce budget impact at a point were mature evidence is limited, for instance, MEAs[328,334]. In the period after the reports were released, two CAR-T products have been evaluated and recommended for use, both under a MEA using the NHS Cancer Drug Fund[340,341]. This mode of commissioning has thus far provided a reimbursement mechanism for CAR-T therapies; however, such bespoke commercial and payment arrangements do not provide a sustainable long-term strategy. Furthermore, as the advent CTPs continues non-oncology-based therapies will also need to be reimbursed and the Cancer Drug Fund would not be applicable to them, therefore, modes of routine and sustainable commissioning are needed[iii].

The session presentation highlighted some of the key issues experienced thus far regarding CAR-T appraisals. For example, defining the target populations for marketing authorisation that is broader than the populations used in the trials; choosing appropriate comparators/ standard of care; extrapolation of survival data; cost and price; implementation; administration; and management of potential adverse effects[iv]. While these are CAR-T-specific challenges that have been identified, these are likely to be the challenges faced by CTPs in general. Choosing the target population is an activity that needs to be carefully considered and appropriate controls should be used to ensure robust trial data is obtained that provides evidence of clinical effect. The costs that are used within HTA have a significant impact on the cost-effectiveness of a health technology. Therefore, the costs that are included in an evaluation should be justified and representative of the economic impact of the therapy. Costs associated with adverse effects and use of agents such as immunosuppressants should be included as they are likely to have an impact of the budget of the payer.

The presentation illustrated the limitation of trials compared to real world evidence in the case of CAR- T appraisals. Patient selection was deemed to be important as data for ECOG 0-1 patients was reasonably consistent in comparison to ECOG 2 or greater patients. Furthermore, there were key differences in the rate of immunosuppressant use (three times higher); double the use of incentive care and higher readmission rates in the real world compared to the trials.

[iii] CHART workshop presentation/discussion point
[iv] CHART workshop presentation/discussion point

7.3.3. Social values toward regenerative medicine: Session 4

Both clinical evidence of effectiveness and health economics are crucial pillars of the reimbursement and adoption pathway, however with new technologies such as CTPs, it is important to also consider a societal perspective, as ultimately the therapies and technologies are being assessed for the members of society that need them. Therefore, session 4 was included in the agenda to explore the role of social values in the context of CTPs such as CAR-T therapies. The presentation was provided by the director of the University of Toronto's Joint Centre for Bioethics. The discussion panels included the Joint Centre for Bioethics director, the dean of Health Sciences at Simon Fraser University and a professor from the Institute of Health Policy, Management and Evaluation, University of Toronto.

Posed questions:

- What is the public/patient perception of ATMPs?
- Can an appropriate case study example be used?
- Should patient preferences be considered?

7.3.3.1. Session Summary

Regenerative medicine/CTPs have long offered the promise of personalised medicine that has higher efficacy compared to traditional "gold standard" therapies whilst also having the potential to be curative. This promise would provide a paradigm shift in the administration of health care, from a model that is 'good for most' to one that focuses on the individual patient and emphasises pragmatic issues regarding healthcare; rather than system level health policy for the general public[v]. Such a shift in paradigm for public healthcare systems such as the NHS and those in Canada is almost unfathomable to healthcare providers due to the complexities of administration and budget restraints. This raises the question as to whether CTPs can ever truly be used in the mainstream and deliver on their promise.

Responsible innovation offers a framework in which a collective approach towards future innovation can be fostered[342]. This approach can be used to address social needs and challenges by engaging stakeholders with a unified set of values in order to provide solutions in a sustainable way[343]. Responsible innovation in health can help address issues such as health equity, frugality and inclusiveness, which are all key consideration for HTA in publicly funded healthcare systems[342].

[v] CHART workshop presentation/discussion point

The inclusivity of the societal perspective is required in order for HTA processes to be transparent. Many HTA committees also include a lay member, for instance the NICE technology appraisal committee and CADTH's CDEC. In addition to this, policy makers should have public engagement that facilitates broad sampling of perspectives and elicitation of social values[326,344]. A societal voice would arguably result in increased public awareness and understanding of the value and potential of new therapies. This would improve trust between the public and the private institutions due to increased dialogue, particularly around the issues of effectiveness, so that there is reduced risk of over promising with new therapies. Furthermore, this would mitigate the risk of hype and the potential for public disappointment, as there would be greater awareness from the public that is appropriately informed, as opposed to the current reliance on sensationalised media reporting. Availability of information is important, and it should be appropriately disseminated and transparent, which is currently not that case i.e. less than 45% of clinical trials publish their results[vi]. Increased information regarding therapy efficacy would permit greater understanding of the how therapies work. This may also potentially reduce the occurrence of health and stem cell tourism, since the information provided allows potential patients to be better informed. Rather than the current reliance on claims made by the media and some unaccredited clinics which is often not supported by published scientific evidence[vii].

[vi] CHART workshop presentation/discussion point
[vii] CHART workshop presentation/discussion point

7.3.4. Health technology assessment overview: session 5

The previous sessions investigated some of the key considerations that are involved in the HTA process. This session combined those elements and provided an overview of the HTA process from both a Canadian and UK perspective. Presentations were given by the Senior Scientific Adviser from NICE and the Pharmaceutical Reviews Senior Advisor from CADTH. The discussion panel was comprised of both the advisors from NICE and CADTH, the Assistant Deputy Minister and Executive Officer of Ontario's Public Drug Programs, Government of Ontario and the vice president of market access and external affairs from Takeda, Canada. Akin to the previous sessions CAR-T therapies were referenced as a case study to provide context to the HTA process.

Posed questions:

- What is the data and how is it packaged into a HTA process?
- The specifics of an evaluation, what is the HTA journey like?
- What is the decision-making process/formula?
- HTA and clinical processes - do they differ?

7.3.4.1. Session Summary

From NICE's perspective the two key questions to consider during the HTA process concern benefit and costs. In terms of benefits, the question is how well the technology works in comparison to established practice in the health service. The cost question considers how much the technology under assessment costs as a means of treatment/intervention, in comparison to the established practice in the health service. The appraisals are formally referred by the DHSC to NICE in order to carry out a review of the clinical and economic evidence provided by health technology developer/manufacturer. The review process leads to a recommendation on the use of new and existing technologies for use within the NHS (in England). Value is at the core of the HTA process and considers elements such as: clinical effectiveness; cost-effectiveness; end of life extension; innovation; degree of need; equity and non-health objectives (**Figure 100**). NICE employs a standard cost-effectiveness threshold (£20,000 - £30,000 per QALY) which is stipulated by the voluntary scheme for branded medicines pricing and access agreement[345,346]. However, there is a certain range of flexibility if other elements of the value core are deemed to carry weight, for instance the threshold can be pushed as high as £50,000 per QALY if the technology addresses a non-health objective of the NHS or it is life extending at an end of life stage. The end of life criteria for technologies includes the ability to demonstrate that the technology is: suitable for patients with a short life expectancy (less than 24 months); capable of offering an extension

of life that is greater than three months, and that the estimates of life extension are robust, plausible and objective.

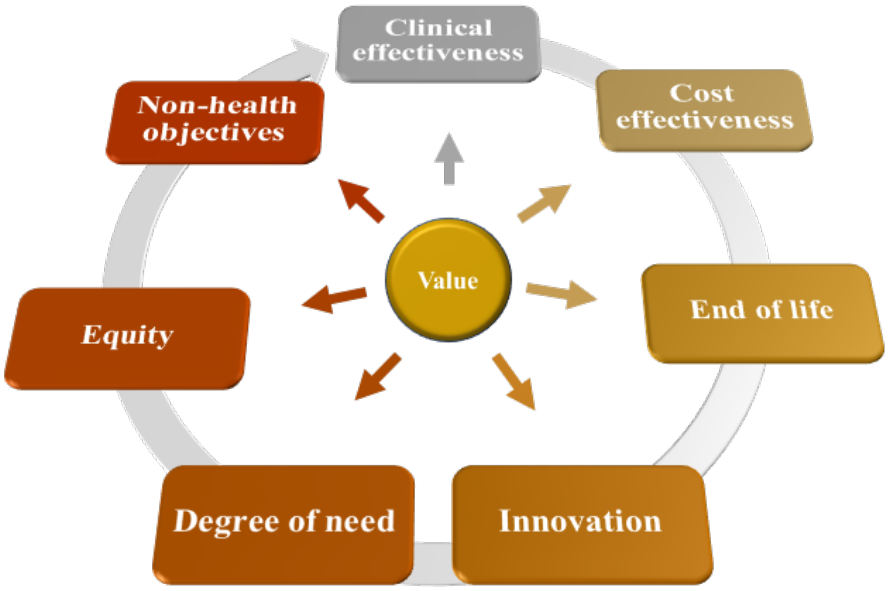


Figure 100. The seven elements that NICE uses to determine value of a health technology during technology appraisals. Adapted with permission from NICE.

In addition to clinical and cost-effectiveness, NICE also considers affordability; while a technology might be cost-effective it does not necessarily mean it can be afforded by the healthcare system. For instance, a technology might be cost effective in the long-term, however budget restraints might mean that there are no funds in the budget to pay for implementing and adopting the technology. Therefore, NICE performs budget impact tests to ascertain a balance between value and affordability. Currently, the budget impact threshold is set at £20 million per year in the first three years of the adoption of any new technology[344]. If a technology exceeds the threshold, negotiations for access arrangements can be considered in order to facilitate the adoption of cost–effective, high budget impact technologies.

Managing uncertainty and risk is crucial for CTP appraisals; post-recommendation data collection is one way one mitigating risk when the clinical evidence is immature, as it generates evidence that can be used to reassess the effects and value of a technology in the mid and long-term. From a financial perspective, commercial negotiations can help reduce the financial risk and burden imposed on the payer by novel technologies, by lowering prices or entering risk sharing agreements. In the case of CAR-T therapies commercial negotiations were carried out after they were recommended for use through the Cancer Drug Fund. One of the conditions imposed in the agreement for reimbursement

requires that further data collection post-recommendation (currently five years of data collection) is obtained to avoid withdrawal from listing on the Cancer Drug Fund. The data collected in the managed access period includes PFS, immunoglobulin usage and OS[viii]. This mechanism provides access to the patients while allowing for uncertainty to be reduced as additional data is collected.

NICE has been active for 20 years now and in that time many of its processes have been updated to keep up with healthcare developments. For instance, the advent of regenerative medicines resulted in a study of the appropriateness of NICE's HTA methods for assessing regenerative medicine technologies. The study used a hypothetical example product based on early clinical data for related real products (CAR-T therapy) supplemented with hypothetical evidence. In addition, as of April 2019, NICE has started cost-recovery activities for technology appraisals and highly specialised technology appraisals. Another development is the expansion of NICE's scope by reviewing all new drugs approved by drug licensing agencies in addition to new uses of already approved drugs by 2020[ix]. NICE has also been encouraging manufacturers to engage with the HTA process earlier in their product development timelines, in particular, prior to clinical development plans being finalised. NICE's scientific advice service offers manufacturers the ability to obtain guidance on how to develop and generate appropriate evidence, consider cost-effectiveness and obtain an understanding of the HTA process from the perspective of decision makers. Furthermore, to streamline the adoption process NICE encourages manufacturers to plan for the regulatory and HTA pathway simultaneously.

The Canadian/CADTH perspective to HTA is fairly similar to NICE, an overview is provided in section 7.1.2.1. Unlike NICE, the HTA recommendations carried out by CADTH are non-binding and it is up to the individual provinces and territories to make a decision as to whether or not a new technology is adopted. CADTH broadly has two streams for HTA: one for pharmaceuticals, and one for clinical interventions/devices. In both cases CADTH assesses whether the technology should be adopted[x]. Choosing which stream a technology is evaluated under depends on the mechanism of adoption, if the technology should be funded and placed on the drugs formulary list it will undergo HTA for a pharmaceutical. If the mechanism of adoption requires funding and a complex implementation system, HTA for a clinical intervention is used. In both cases, value, as with NICE, is the key consideration, however the deliberative work used differs between the two pathways within CADTH. The deliberative pathway evidence package for clinical interventions has additional elements such as legal, ethical and implementation considerations (**Table 44**).

[viii] CHART workshop presentation/discussion point
[ix] CHART workshop presentation/discussion point
[x] CHART workshop presentation/discussion point

Table 44. A comparison of the elements evaluated in CADTH's HTA process under the drugs and clinical intervention deliberative frameworks. These elements are used to determine the value of a technology and whether or not it should be adopted. Adapted with permission from CADTH.

Considerations	Drugs	Clinical Intervention
Relevance and unmet need	✓	✓
Benefits	✓	✓
Harms	✓	✓
Patient perspective	✓	✓
Economic impact	✓	✓
Implementation	✗	✓
Legal	✗	✓
Ethical	✗	✓
Environmental impact	✗	✓

In the case of CADTH's recent CAR-T assessment a clinical intervention deliberative framework was utilised that analysed clinical effectiveness cost-effectiveness, ethical issues, implementation considerations, patient perspective and care giver perspective. CADTH uses a Health Technology Expert Review Panel (HTERP) comprised of seven core members: a chair; public member; ethicist; health economist; three health care practitioners; there may also be up to five expert members included in the panel. The HTERP recommended Norvatis' Kymriah CAR-T therapy for adoption on the condition that the initial price was reduced. The HTERP also recommended the consideration of an interprovincial agreement to ensure equity of health provision and the development of clear eligibility criteria. Furthermore, similar to NICE, the recommendation called for the collection of outcomes data post recommendation in order to generate real-world evidence. A pan-Canadian standardised approach with defined outcomes was proposed as means of obtaining data for future reassessments to assess long-term effectiveness, safety and cost-effectiveness of the health technology.

While there are differences in the manner in which NICE and CADTH perform their HTAs there is a lot of commonality in the results of their work, which is to provide recommendations (albeit non-binding in Canada) for the adoption of health technologies. Although agencies assess for the value of the technology using independent frameworks, there are similarities in the way in which effectiveness is assessed, in particular, clinical effectiveness. As a result, NICE and CADTH now collaborate to offer parallel comprehensive scientific advice, which streamlines the process for technologies aiming to apply for recommendation in both countries. Those who participate in the service can obtain practical advice and clarification about the similarities and divergence between the two markets.

7.3.5. Payment system mechanisms: session 6

In the previous sessions the way in which healthcare interventions are paid for was highlighted as a crucial issue. Despite the MEAs offering an ad hoc solution, this is not sustainable in the long-term. This session did not have a formal speaker to provide a presentation, however there was a decision panel that led the discourse comprised of the research fellow from CHE, the senior principal economist from the Office of Health Economics and the vice president of market access and external affairs from Takeda Canada. A round table approach was used in this session and all attendees were invited to provide their perspectives on CTP payment mechanisms.

Posed questions:

- Do we need novel systems or adjustment of codes for payment?
- What are the funding mechanisms/risk sharing agreements?
- Are there any novel payment mechanisms?

7.3.5.1. Session Summary

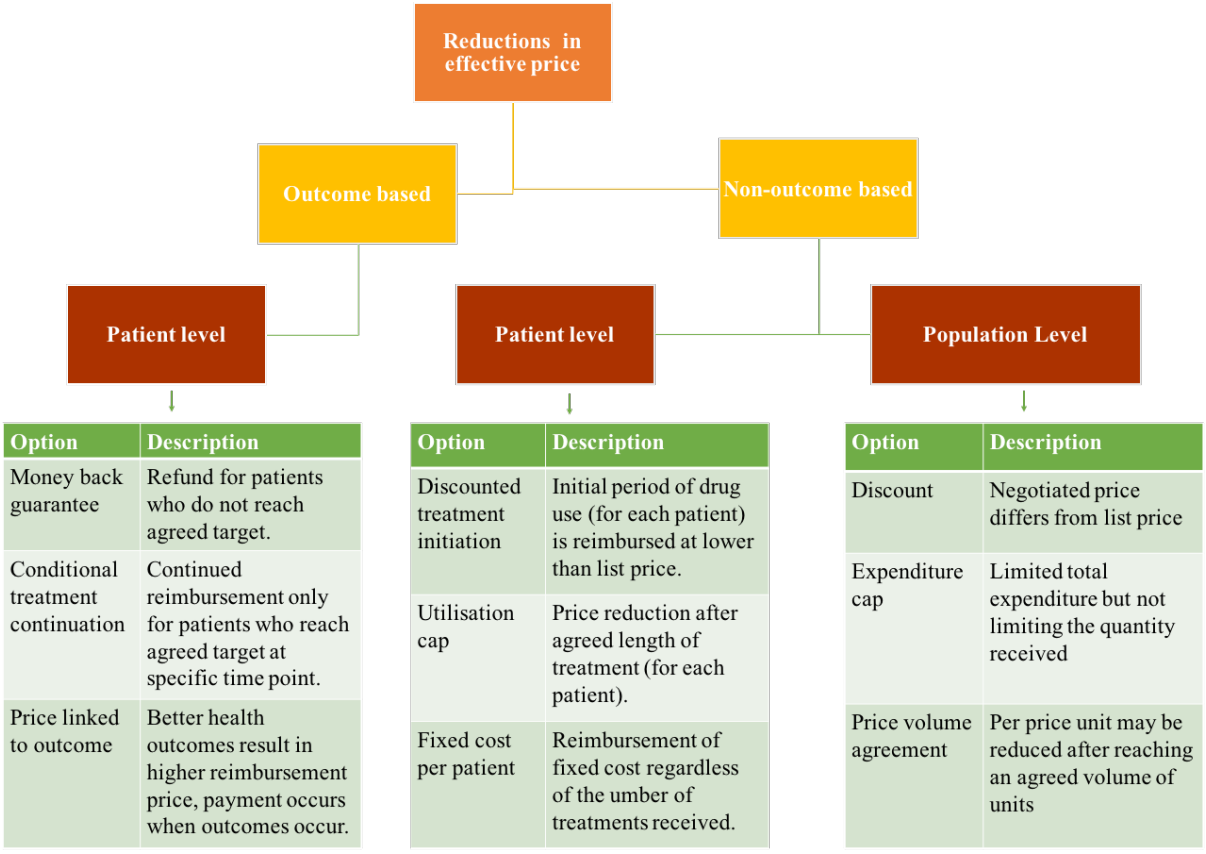
There is a lot of progress being made in terms improving payment systems and reimbursement in healthcare. In the UK efforts towards new payment models, integrated care systems, developing sustainable payment systems and assessing the future of Payment by Results are areas being addressed by organisations such as NHS Improvement and NHS England[xi]. However, paying for CTPs still presents a major challenge for public health care systems since both the UK and Canada have budget constraints. Highly priced health technologies such as CTPs require different solutions in comparison to traditional small molecule therapeutics, as the high up-front costs and uncertainty in health outcomes results in a lot of risk for the payer. Therefore, mechanisms such as MEAs are necessary to mitigate the financial risk of novel, immature and expensive technologies[347].

There is yet to be a fully defined approach to paying for disruptive and expensive technologies. Mechanisms such as the Cancer Drug Fund are only useful for oncological therapies such as CAR-T therapies, for other disease indications robust and sustainable modes of payment that do not significantly offset budgets are required. Even in the case of the Cancer Drug Fund, it is unsustainable to expect future CAR-T therapies such as those treating solid tumours to also be recommended within

[xi] Westminster Health Forum Seminar - Priorities for improving payment systems and reimbursement in healthcare. 3rd May 2018.

this funding pot, as the funding source is finite. Therefore, long term strategies are required in order to be able to routinely commission and pay for CTPs.

The aforementioned MEAs offer a means of providing reductions for the effective pricing of new technologies. The agreements can be outcome based or non-outcome based, and are typically associated with evidence generation post-recommendation[334,347]. The purpose of the agreements is to provide technologies where there are no other alternatives for patients while facilitating evidence development to reduce the payer's financial risk due to the uncertainty associated with novel technologies. **Figure 101** outlines MEA options that can be used for reimbursement.



Option	Description
Money back guarantee	Refund for patients who do not reach agreed target.
Conditional treatment continuation	Continued reimbursement only for patients who reach agreed target at specific time point.
Price linked to outcome	Better health outcomes result in higher reimbursement price, payment occurs when outcomes occur.

Option	Description
Discounted treatment initiation	Initial period of drug use (for each patient) is reimbursed at lower than list price.
Utilisation cap	Price reduction after agreed length of treatment (for each patient).
Fixed cost per patient	Reimbursement of fixed cost regardless of the umber of treatments received.

Option	Description
Discount	Negotiated price differs from list price
Expenditure cap	Limited total expenditure but not limiting the quantity received
Price volume agreement	Per price unit may be reduced after reaching an agreed volume of units

Figure 101. Taxonomy depiction of the price reduction schemes that can be used for both outcome and non-outcome based managed entry reimbursement agreements. Adapted from Grimm *et al* (2016).

Other financing schemes have been suggested to address the issues of high up-front costs and affordability associated with CTPs[334]. Amortisation, offers a financing route in which the high up-front costs of treatment are spread over time to reflect the time period over which benefits are realised[335]. "Pay for performance" is a mechanism in which the total price paid is linked to a measure of performance (e.g. Objective Response Rate). This requires monitoring of performance measures and the payment mechanisms to ensure that the appropriate reimbursement is obtained. Leasing is another

option that can be used, this is a method that combines the concepts of amortisation and pay for performance i.e. payments are made over a set time period for as long as the measure of performance is met[335]. Other innovative financing schemes are also being considered, for instance methods that aim to utilise the expertise of financial market solutions. This has the potential to better manage financial risk as the finance industry is well established and skilful in managing high financial risk[xii].

[xii] CHART workshop presentation/discussion point

7.3.6. Adoption and implementation of ATMPs: Session 7

Implementation was a subject area that was mentioned repeatedly in the previous sessions. Following approval and reimbursement recommendation, defining how a technology is implemented is the final hurdle before it can effectively reach patients. This session was devised to explore how CTPs might be implemented in a manner that is efficient and sustainable. The presentation for this session was provided by the Head of Clinical Innovation Adoption from the Oxford Academic Health Science Network (AHSN). The discussion panel included the Head of Clinical Innovation Adoption, a haematologist from the Princess Margaret Cancer Centre, the Senior Director of Market Access and Government Relations from Gilead Sciences and the director of Health Solutions from MaRS Partnerships.

Posed questions:

- Do sessions 1-6 help address the adoption challenges (were any tangible action points raised?)
- What were the key challenges identified and were there any solutions put forward?
- Do we need changes in infrastructure? (ATTC – discussion point?)
- How do we facilitate effective adoption of ATMPs?
- What are the barriers to adoption?
- Where are the evidence gaps?
- Policy aspects?

7.3.6.1. Session Summary

The pathway to market access is established, however the pathway to adoption and implementation of CTPs is less clear. Implementation of CTPs requires specialised infrastructure, accreditation and personnel training that is not currently routinely available. Adoption and implementation require concerted efforts from multiple stakeholders such as health care commissioners, policy makers, clinicians and service providers. In the UK the NHS is a 'single payer' system which presents potential advantages compared to highly privatised and fragmented healthcare systems (e.g. USA healthcare system) for adoption and implementation. This potential advantage is due to the centralised commissioning, coordination, standardisation, risk and data management that is facilitated by having a single (predominantly) system for healthcare provision.

The Consolidated Framework for Implementation Research (CFIR) was used to provide context into the key considerations related to the challenges of adoption and implementation of healthcare technologies[348]. Three elements of the framework (inner setting, outer setting and individual characteristics) were highlighted in a UK context concerning the adoption and implementation of

CAR- T therapies within the NHS. [xiii] The inner setting highlights the institutional barriers to adoption that have been faced by CAR-T centres in the UK. Accreditation resulted in highly resource intensive commitments in order to adjust policies, pathways and guidelines within the respective centres allowing for them to be deemed qualified to administer the therapies. Contracting associated with the CAR-T manufacturers was variable between the different hospital centres and it was highlighted that negotiations should be centralised to aid in the interpretation of contracts. Governance of the process and the key stakeholders involved in the implementation is highly sought after to ensure that all requirements of the multifaceted CTPs pathways are adequately managed. [xiv] The capacity to carry out the activities required to implement CAR-T therapies is a crucial element, the appropriate equipment and staff needs to be available as well as ward and intensive treatment unit capacity. In addition, staff training presented a challenge as releasing personnel for training time was not always achievable[xv]. This illustrated that generic training in CTPs is needed as it is not feasible or sustainable to release staff for training for each individual CTP prior to adoption. Patient management is important in to ensuring accessibility to eligible patients; this requires a national level effort to identify capacity and carryout referrals as appropriate.

The outer setting to implementation and adoption of CTP is emergent, efforts by the Cell and Gene Therapy Catapult to drive adoption has resulted in innovation hubs that focus on different areas of the CTP realisation pathway. The Advanced Therapy Treatment Centres (ATTCs) develop models based on best practises in specific areas; processes are then shared amongst the network to improve efficiency amongst the system[349]. The ATTCs have been established to carry out the initial exploration of the infrastructural and process requirement challenges (**Figure 102**), the learnings from the centres will then be rolled out to other centres in the UK which will provide standardised approaches to the implementation and adoption of CTPs.

[xiii] CHART workshop presentation/discussion point
[xiv] CHART workshop presentation/discussion point
[xv] CHART workshop presentation/discussion point

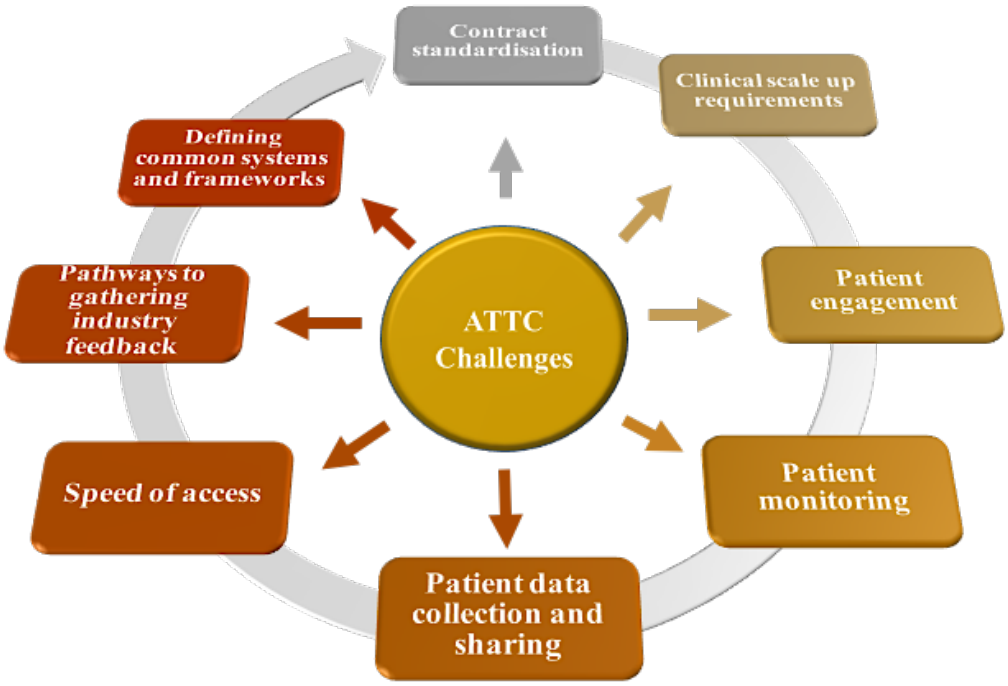


Figure 102. Adoption challenges being explored under the ATTC model. Adapted from the Cell and Gene Therapy Catapult.

Under the CFIR, individual characteristics to consider include; patients; clinicians; managers and policy makers. Crucially patient education is important as patients are the recipients of the therapies; thus generating and maintaining positive public and patient perception of CTPs is important for social and ethical considerations of adoption. Equally as important is the support of clinical leaders as they are the stewards of successful adoption and patient experience. Therefore, clinicians should be involved in implementation and adoption activities so that there is clarity about their role and responsibilities. Commissioning managers, at a higher organisational level, are key to the implementation process as technologies that are adopted should be in line with the strategic priorities of the healthcare commissioner group. It is up to commissioning managers to provide compelling business cases and assess appropriate levels of risk and benefit associated with adopting technologies, especially those that are resource intensive such as CTPs[xvi]. The policy level is fundamentally overarching as without the political will to support the CTP industry efforts such as the ATTCs and their aim to foster implementation would not be possible. In addition, policy makers can work closely with organisations to ensure that training is adequate for future workforces that are skilled in the implementation of CTPs, instead of relying on unstainable ad hoc staff training for each new CTP.

[xvi] CHART workshop presentation/discussion point

7.3.7. Workshop summary.

The workshop highlighted several areas that need to be addressed in order for the adoption of CTPs to be better facilitated. In addition, the workshop presentations and discussions illustrated that while CTPs maybe novel in their mechanism of action, the way in which they are assessed for value is not novel and does not need to be bespoke. Ultimately, at the core of reimbursement and adoption is value; payers look at what value is being provided by a technology. There is no need to change the way in which health economic evaluations are carried out as essentially it is still a matter of assessing clinical benefits, risks and costs.

Fundamentally, conventional HTA frameworks can be applied to CTPs; the challenge is ensuring that both value and risk are adequately captured by the methods, given the current commonness of immature CTP evidence. The CTI needs to address the uncertainties associated with the evidence currently being provided in the early onset of these CTPs applying for approval and funding. The challenge of evidence is exacerbated by schemes that allow for early regulatory approval, while this is beneficial for patient access, it presents a hindrance to HTA as the trials utilised for regulatory approval can only provide short-term follow-up data. The way in which evidence is generated for CTPs needs to be addressed in order to combat the issue of uncertainty, and randomised clinical trials with greater sample sizes, should be employed[336,350,351]. The value of real-world evidence is undisputed for emergent therapies, however, there should be consensus on how the data is standardised, captured and utilised for reassessments and analysis of long-term effects.

This raises the question as to whether to apply pragmatic decisions based on available evidence or to delay decisions until further data becomes available. A solution that is gaining prominence in light of this question is the use of MEAs as a means of mitigating the long-term evidence risk associated with CTPs. MEAs have proven to be essential to the initial adoption of CTPs, however this requires engagement between multiple stakeholders to ensure appropriate sharing of risk. Of the MEAs currently being employed outcome-based payment methods offer a solution for CTPs that facilitate risk mitigation for the payer. In addition, outcome-based payment methods, encourage developers to provide high quality products to ensure they are reimbursed and avoid the penalty charges that are associated with not meeting defined clinical outcomes. It is important to take account of the anticipated benefits of the technology and the value of the uncertainty around these health outcome estimates in an objective manner when using MEAs. An objective perspective is necessary to ensure that only those technologies that have the potential to be effective are adopted. Thus, avoiding the adoption of expensive therapies with potentially limited benefits, albeit in a risk mitigated manner. Furthermore, there should be appropriate incentives employed for all stakeholders to ensure future evidence generation. The incentives help to provide accountability for the stakeholders i.e. the payer should ensure targets are

met to avoid paying with limited evidence of value and the providers should ensure the data is obtained in order to sustain reimbursement. Additionally, even under MEAs it is essential to consider the potential costs of implementation reversal associated with CTPs, should reversal be necessary[xvii].

Funding pots such as the Cancer Drug Fund have thus far facilitated the commissioning and adoption of novel CTPs, albeit only within the oncology space. Thus, a vital point of scrutiny with regards to CTP adoption is sustainable affordability, it is important to question whether the healthcare system can sustainably bear the costs of implementing these technologies. This will become a prominent issue as more and more therapies for different disease indications are approved at a regulatory level; demand for access to these therapies will be inevitable. However, as to whether CTPs can be routinely commissioned remains to be seen. Therefore, although HTA and payment mechanism are two separate entities, they are both crucial to the feasibility of adoption, thus there is value in these activities being carried out simultaneously, so as to avoid recommended therapies that cannot be paid for. This is a lesson that should have been learned by cases such as Glybera (where the product obtained regulatory approval but was deemed too expensive to be adopted and reimbursed[221]). This emphasises the notion that a therapy may be available but if it cannot be paid for, it cannot be used to treat the patients that need it[221].

People hold a vital role in the adoption process, ultimately people are the stewards to the adoption of therapies, from patients, to clinicians, administrators and the manufacturers themselves. Therefore, it is important to have a representation of all stakeholders during the adoption pathway as the perspective of all parties is crucial; i.e. if patients and clinicians do not want the technologies being offered, it serves little purpose to adopt them. The patients and clinicians should be advocates for new technologies, this requires them to be well informed to enable them to appropriately and critically assess the value of these technologies, from their perspectives. Furthermore, it is a question of social values when it comes to addressing how much risk should be taken to achieve potential benefits, especially with novel technologies where benefits and risk are not fully known. In addition, a societal perspective is important in public healthcare systems as equity is a major consideration, thus the opportunity costs posed to the public as a consequence of CTP adoption would be significant, particularly in times where budget constraints are ubiquitous. Moreover, in terms of equity, how to prioritise access and the justifications for it are significant social challenges that should be addressed. Public discourse about these social challenges should be encouraged, as well as discourse regarding the prices of therapies to ascertain whether or not they are justified to be so high form a societal perspective[xviii]. Akin to this, discourse, is the need to appropriately disseminate information to the public so that media hype and overpromising is avoided.

[xvii] CHART workshop presentation/discussion point
[xviii] CHART workshop presentation/discussion point

Currently, most issues and lessons learned regarding the adoption of CTPs have been the result of CAR-T therapies. It is important to note that some of the lessons may or may not be applicable to other disease areas and CTPs. However, the CFIR issues raised above are likely to be a universal challenge faced by all CTPs/ATMPs, for instance intensive treatment unit capacity, appropriate personnel training, robust supply chain management and cold storage logistics. This highlights the importance of an organised infrastructure in the successful implementation and adoption of CTPs. This is likely to require investment of capital in order for hospitals to be accredited and suitable for routine administration of a wide range of CTPs. This process will require coordination and collaboration to ensure sustainability and standardisation of procedures to guarantee patient equity of access and quality. Furthermore, for patients to benefit from future CTPs, changes are required in terms of infrastructure and other areas such as data collection and workforce training. Fundamentally, such drastic changes will require support from the 'top down', as well as the political will to advance the industry and foster adoption[xix]. In the UK, institutions such as the Cell and Gene Therapy Catapult are examples of the support mechanisms and infrastructures that is needed. Furthermore, the ATTCs offer niche innovation hubs that should help to facilitate more efficient adoption and implementation approaches.

Innovation in policy frameworks and business models is necessary to successfully and efficiently translate research from the bench to beside for the benefit of patients. Such innovation equally needs to be as informed by empirically grounded evidence. A system-level view is essential to avoid unintended consequences of targeted reforms. This not only requires policy level engagement, but also engagement from all stakeholders of the supply chain. In the UK, NHS England's commercial deals with manufacturers allowed for early and rapid introduction of CAR-T therapies compared to the rest of Europe, highlighting the influence and power of political will in facilitating the adoption of technologies[175]. Thus, the main next steps that were outlined at the end of the workshop included: policy maker engagement, interacting with other relevant stakeholders, addressing the issues of evidence generation and understanding the future payment system mechanisms.

[xix] CHART workshop presentation/discussion point

7.4. Conclusions

The adoption of CTPs is a multifaceted challenge that requires policy makers, academics, clinicians, patients and manufacturers to work together on all stages of the pathway in order to progress the CTI as a whole. HTA helps the decision-making process for the payer, since a fundamental issue with CTPs is the uncertainty of their long-term benefits. Thus, payers cannot be guaranteed the true value of what they are paying for, due to the immaturity of the evidence currently available. Expenditure of public budgets, in the case of both Canada and the UK, on such technologies requires both opportunity cost and equity to be strongly considered. Therefore, HTA for CTPs should be capable of providing robust analyses to aid the payer in their decision-making process. Global efforts in HTA have great value, the collaboration be between NICE and CADTH illustrates this; and this may be a mode that facilitates more streamlined assessments, especially in terms of clinical effectiveness, since it is inevitable that cost analysis will vary from country to country. However, such collaborations provide opportunities to learn from other existing systems, which is crucial for having adaptive and innovative internal systems for HTA and adoption.

Data presents a considerable challenge within the CTI, in order to generate strong evidence clinical trial designs should aptly capture the necessary data to satisfy the needs of both the regulators and the payer. This should include safety, efficacy, clinical effectiveness, cost-effectiveness and the other value elements of the respective HTA agency. It is never too early to start thinking about how to generate evidence. Both NICE and CADTH offer scientific advice programmes on HTA process preparation that encourage developers to engage with the HTA process and its requirements as early as possible, to ensure that their data is as robust as possible when it comes to their final submission.

In terms of adoption, it is mainly the implementation of CTPs that requires restructuring and adaptation to ensure that these technologies are provided efficiently and effectively. Efforts such as the UK's ATTCs and personnel training are steps towards the right direction to adequately deliver CTPs. In addition, collaborative efforts between different treatment centres are required to effectively and efficiently implement CTPs, which presents a significant challenge in a jurisdictional structure such as the Canadian healthcare system. The NHS is well placed for outcomes-based payment mechanism as it is a single payer system, and all patients within the system have an identifier. Unlike in the USA/private payer systems where the patient is likely switch between different healthcare providers. Therefore, this organisational feature should be exploited in order to provide improved patient management whilst also providing real-world evidence of the long-term clinical effect of technologies post-recommendation.

The topical and prominent example of CAR-T therapies has made HTA agencies evaluate how to assess CTPs, the studies carried out have shown that while HTA processes are applicable to CTPs, the

combination of great uncertainty coupled with the high potential for substantial patient benefits means that innovative payment methods are needed in order to provide access, while mitigating risk. How sustainable payment systems will be established currently remains unclear and this is an area that requires more work and understanding to ensure that more CTPs are approved and reimbursed in the near future. Siloed funds such as the Cancer Drug Fund are not a realistic solution for all therapies, thus arrangements for routine CTP commissioning is necessary which will undoubtedly require budgets allocations to be adjusted as appropriate. In addition, alternative ways of financing and paying for expensive therapies such as CTPs should be evaluated. Other industries such as the aviation and financial investment industries have the potential to provide alternative payment methods for these presently expensive therapies. In the interim, risk sharing offers a viable method that is set to be common practice until the true value and long-term benefits/effects of CTPs are established.

7.5. Chapter bridge

This chapter has explored the key challenges in the adoption of CTPs, the valuable experience and expertise of the workshop participants illustrated that there are areas that need to be addressed. In addition it was demonstrated that CTPs are desirable and there is work and research being carried out to facilitate their adoption. The majority of the learnings from this chapter have been in the context of CAR-T therapies due to their current prominence. The following chapter returns focus back to the dopaminergic neuroprogenitor candidate therapy, the price headroom and potential cost-effectiveness of the candidate therapy were explored using validated assumptions. Early stage economic evaluation tools were also used to determine the potential economic impact and return on investment. In addition, the key points of consideration from this chapter, in terms of the different themes, were evaluated against the candidate therapy to ascertain how it would fair in areas such as social values, HTA and implementation.

Chapter 8:

Exploration of the Pricing, Cost-Effectiveness and Adoption Potential of the Candidate Therapy

Chapter 8. Exploration of the pricing, cost-effectiveness and adoption potential of the candidate therapy

8.1. Introduction

The work carried out in this chapter aimed to provide insight into the potential cost-effectiveness and commercial viability of a cell therapy product (CTP) based treatment for Parkinson's Disease (PD). Furthermore, the challenges and workshop discussion points from the previous chapter were explored in the context of the candidate therapy to ascertain how it would perform, if it was to progress through development and apply for market authorisation, adoption and reimbursement.

This chapter aims to demonstrate the use of the headroom method discussed in Chapter 2 (section 2.4.4. Headroom method) and an in-house cost of goods (CoGs) model as a means of determining the commercial viability of the candidate therapy, herein after referred to as the fictional product, "DopaCell", for the purposes of the analysis. As aforementioned in chapter 2 the headroom analysis method is a tool that can be used throughout the product development timeline as an iterative means of assessing commercial viability and as a go/no-go decision-making tool during product development. At an early stage, the headroom analysis can be used to determine if further resources should be provided into a product's development or not. In latter development stages, prior to efficacy data being obtained, headroom assessments can be used to assume the cost-effectiveness, adoption and gross profit potential of a product.

8.1.1. The economic impact of PD

PD is a progressive and debilitating disease that, unfortunately, currently has no cure and existing modes of treatment simply manage the symptoms[67,225,352]. Therefore, the quality of life of PD patients inevitably worsens over the course of the disease, with medication decreasing in efficacy with time and the costs incurred increasing[41,70,353]. These outlays include but are not limited to: costs of higher medication doses, home modifications, adult care, personal care, loss of income (due to early retirement for either the patient and/or care giver), hospital visits and other lifestyle changes[69,354,355]. These costs impact different stakeholders: some costs are incurred by the healthcare payer i.e. the NHS, some are incurred by insurance companies (particularly in healthcare systems such as the USA) and some costs are incurred directly by the patients themselves[214,356,357].

The literature illustrates that the economic impact of PD is significant. In the UK, PD has a prevalence rate of 140,000 at a cost of ~ £2 billion annually , this prevalence rate is set to increase to ~160,000 and

200,000 per annum by 2025 and 2035 respectively[358,359] (**Figure 103**). PD patients have a higher rate of non-elective hospital admissions for treatment of motor decline, urinary tract infections, pneumonia and hip fractures[204]. In a study commissioned by NHS England, it was reported that PD-related admissions amounted to £777 million over a four-year period between (2009 to 2013)[204]. Moreover, PD patients had higher rates of emergency admissions and higher costs due to longer hospital stays compared to a similar population without PD[204]. A study conducted in the USA reports that the national economic healthcare burden of PD in 2010 was ~ $14 billion, 55 % higher than a similar population without PD[358]. The disease burden suffered by the patients, the cost of PD to healthcare systems and the increasing elderly population highlights the need for improved treatment strategies that do more than merely manage the symptoms of PD. Thus, it is of paramount importance to formulate treatment options that significantly improve the quality of life of patients, address the underlying disease pathology and consequently reduce the economic impact of PD for both payers and patients.

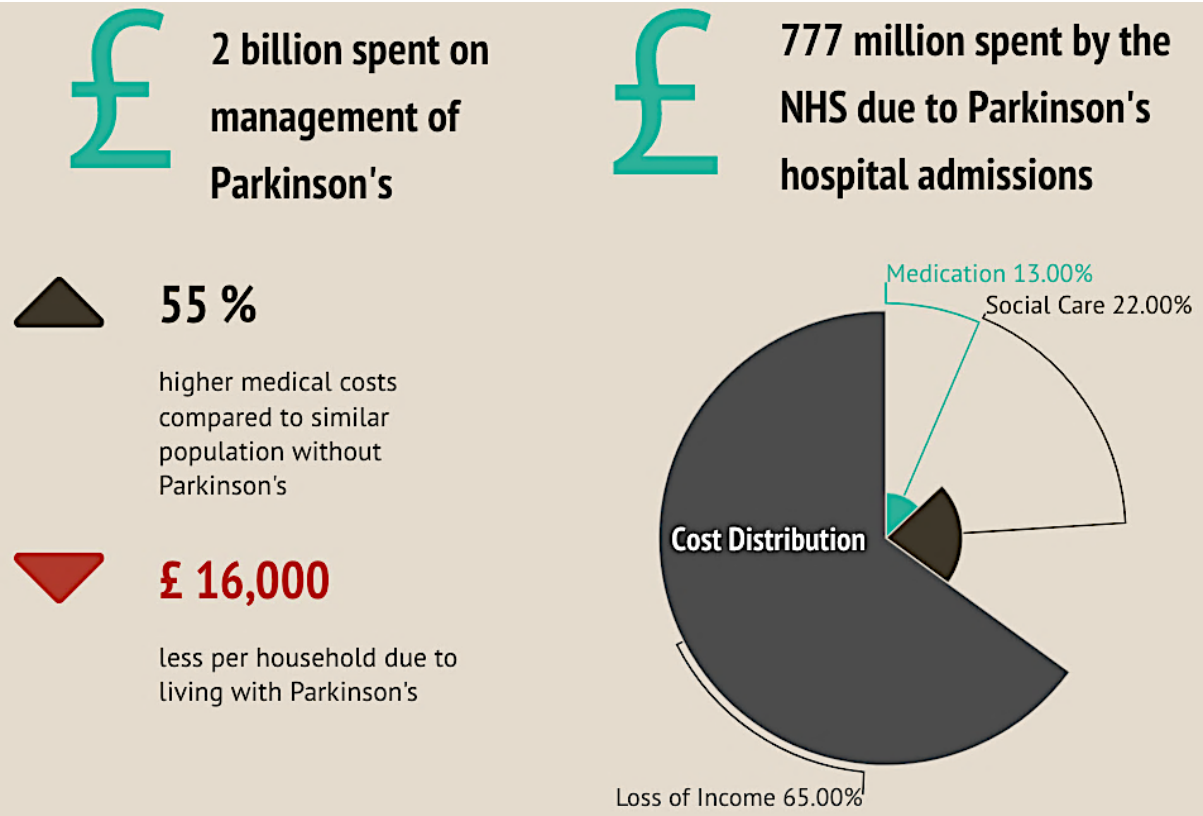


Figure 103. Summary of the economic burden of Parkinson's disease. Data obtained from the literature [204,358,359].

8.1.2. Health economic evaluations for PD treatment strategies

Economic evaluations of healthcare products and interventions are common practice nowadays due to the growing budget constraints that health care systems face. Health Technology Assessment (HTA) agencies such as the National Institute for Health and Care Excellence (NICE) and the Canadian Agency

for Drugs and Technologies in Heath (CADTH) have to assess the costs and benefits of new technologies to determine their value and cost-effectiveness. Approximately 30 model-based economic evaluations specifically assessing PD interventions have been carried out and reported in the literature over the last two decades, with the majority of these occurring in the last decade[360,361]. Folse *et al* (2018) and Becerra *et al* (2016) provide a comprehensive overview of PD economic analysis and cost-effectiveness studies[360,361]. The majority of evaluations for PD have been related to medication therapy and deep brain stimulation (DBS); however, evaluations for hypothetical cell and gene therapy interventions have also been performed[344,362–366] (**Figure 104**). Markov models and decision trees are used to model the costs and disease progression and are typically set over a five to ten-year time horizon. The assessments reported in the literature use Hoehn & Yahr (H&Y) stages and the Unified Parkinson's Disease Rating Scale (UPDRS) to measure health outcomes, patients at H&Y stages II to V with 'off' time >25% are usually chosen [360,361] (**Table 45**). This population is more likely to benefit from significant changes in quality of life from treatment due to the symptoms they experience such as pronounced rigidity and tremors. In addition, most models look at motor symptom changes as the means of evaluating treatment effect[360,361], since motor symptoms are most common for PD patients. PD economic evaluations have been carried out in a wide range of countries, mainly developed countries (e.g. the UK, Germany and the USA) that have a larger geriatric population, thus PD is more prominent in comparison to lesser developed countries i.e. those in sub-Saharan Africa[70,360] (**Figure 104**). In many cases, alternatives to therapies dependent upon oral medication such as Levodopa (L-DOPA) are found to be cost-effective, for instance DBS[356,363,364]. This is due to the lack of long-term benefits offered by medication therapies which are reliant upon mechanisms that try and increase the amount of dopamine available.

N.B. A brief summary of the PD scales used. The Hoehn & Yahr (H&Y) stages describe the level of motor function symptoms as the disease progresses, the typical symptoms of each of the five stages are shown in **Figure 3**. The Unified Parkinson's Disease Rating Scale (UPDRS) is a questionnaire based method of assessing both the motor and non-motor symptoms of PD, including mental functioning, mood, social interaction levels and the ability to carry out daily activities[367–369].

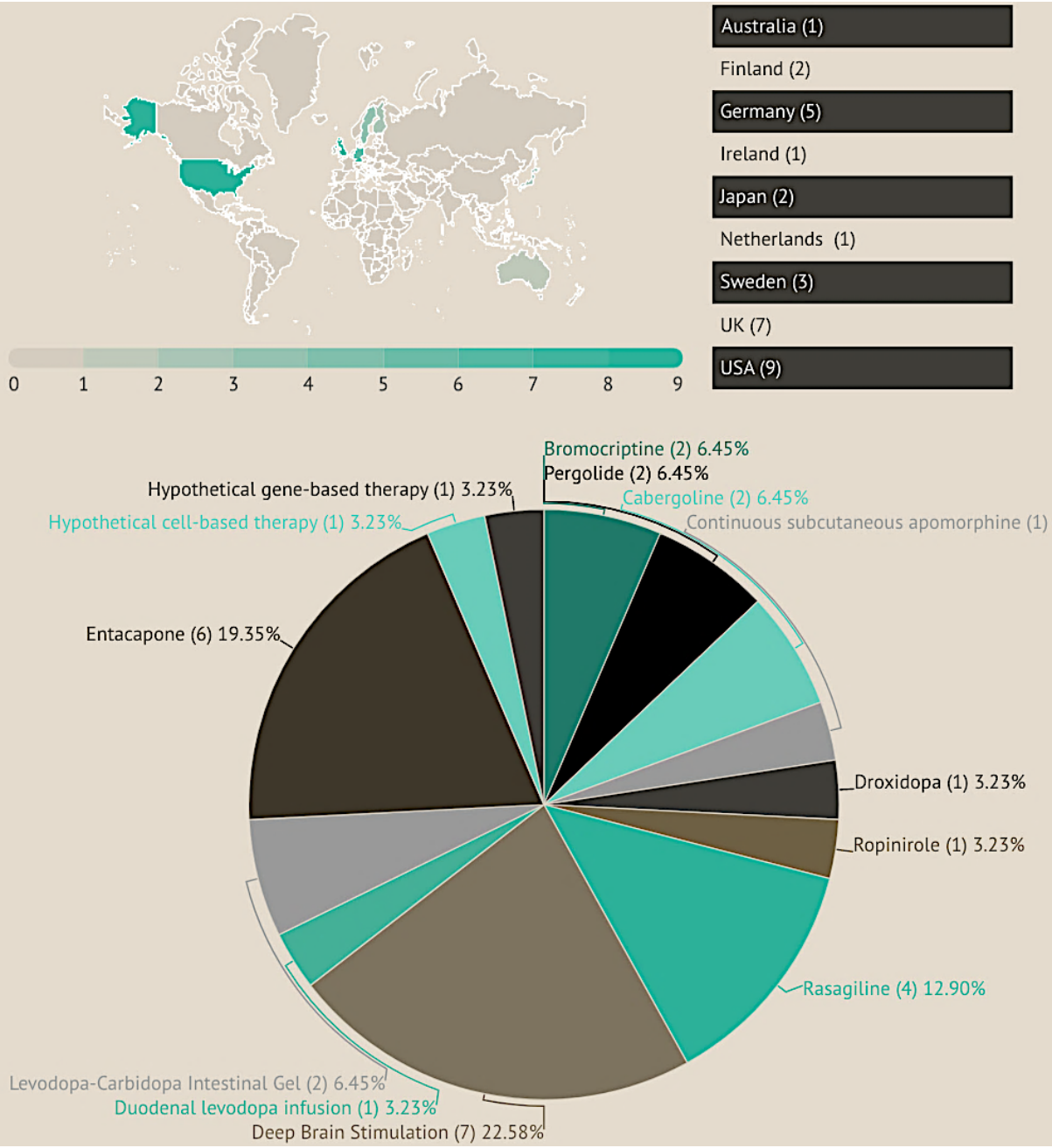


Figure 104. Summary data of economic evaluations for the treatment of PD, detailing the interventions evaluated and the countries that have carried out evaluations. Numbers in brackets indicate the amount of studies that have been carried for the respective intervention and the amount of studies carried out in the respective country. Information summarised from literature searches and studies by Folse et *al* (2018), Nagpal *et* al (2019) and Becerra *et al* (2017).

Table 45. Summary of economic evaluations for the treatment of PD, detailing the measured efficacy outcomes and the PD characteristics of the populations that been evaluated. Information summarised from literature searches and studies by Folse et *al* (2018), Nagpal *et* al (2019) and Becerra *et al* (2017). PD = Parkinson's disease; H&Y= Hoehn & Yahr; QoL = quality of life; UPDRS = Unified Parkinson's Disease Rating Scale.

Outcome Measures	Populations
H&Y stages changes	OFF time > 25%
Motor function analysis	Full range of H&Y stages
OFF time state	Pre PD medication
UPDRS score	Early PD
QoL score	Advanced PD

To date, there have been very few studies conducted to economically evaluate cell and/or gene therapies for the treatment of PD, with only two studies identified by the author[344,365]. The study by Hjelmgren *et al* (2016) performed a predictive evaluation of dopamine cell replacement, for health outcome data they utilised clinical data from patients that had undergone neural transplantation using foetal derived ventral mesencephalic tissue[365]. Robust cost data was not included in the study as the authors did not provide cost of good estimations for the therapy due to the tissue being obtained from elective abortions. The study did however conclude that dopamine cell replacement therapy could result in long-term cost offsets and QALY gains for the patients[327].

More recently Jorgensen *et al*, used a Markov model to determine the cost-effectiveness and budget impact of a potential gene therapy for PD. Their model used a lifetime time horizon of 20 years, both costs and QALYs were discounted at a rate of 3.5% (the standard rate used by NICE) the analysis was carried out in the context of the UK and USA markets[344]. The study concluded that a PD gene therapy would provide substantial value, however the two markets would result in a difference in price potentials due to differences in willingness to pay and budget impact thresholds. Both of these hypothetical cell and gene therapy evaluations show the potential health gains and cost savings that can be obtained by disease modifying treatments for PD such as cell and gene therapies[344,365]. Akin to the study by Jorgensen *et al*, the present work will analyse the potential cost-effectiveness, price potential and budget impact of DopaCell in the context of a UK market.

8.1.3. CTPs as cost-effectiveness case study for PD

The adoption of CTPs requires them to be highly efficacious and cost-effective as there are other therapeutic strategies such as DBS, L-DOPA, dopamine agonists and enzyme inhibitors available for PD patients, some of which (DBS) can be used for patients at the advanced stages of the disease[370]. Therefore, to be deemed as cost effective, CTPs must prove to have additional benefits in order to be cost-effective and receive positive health technology assessments (HTA) appraisals. For example, they should exhibit "(i) long-lasting, major improvement (60–70%) of mobility and suppression of dyskinesias and/or (ii) improvement of symptoms resistant to other treatments or modification of disease progression"[370].

PD is an ideal case study for exploring the reimbursement potential of a CTP. Firstly, since PD is an incurable and progressive disease with currently no disease modifying treatments available, thus there is an effectiveness gap a CTP such as DopaCell could cover. Secondly, PD affects a larger population in comparison to other disease such as the cancers that are treated by CAR-T therapies, therefore it is a good case study to explore the use of a CTP in a large population and its economic impact. Third, studies have been carried to determine transition times from one H&Y stage to the next and the costs associated with the different stages of the disease[195]. Therefore, using assumptions of DopaCell's efficacy, its potential effectiveness and QALY impact can be obtained in an informed manner for use in the headroom assessment. Finally, due to the allogenic nature of DopaCell, it has the potential to be an off-the-shelf product with lower associated production and logistics costs in comparison to CAR-T therapies; this could be favoured with regards to regulatory and reimbursement evaluation.

8.1.4. CTP benefits and drawback for PD

The potential benefits of a CTP for the treatment of PD are discussed in Chapter 2 section 2.1.6.3. Here, a brief summary of the potential benefits is given. The use of a CTP is potentially advantageous in comparison to current PD therapeutic strategies as a CTP can provide sustained/prolonged neurorestoration. Foetal tissue-based trials, which have a similar mode of action to DopaCell[24], have demonstrated clinical improvements in patients for ≥18 years[71,75,371]. This has been evidenced by a restoration of normal striatal dopaminergic function, measured by fluorodopa (F-DOPA) uptake and imaged using positron emission tomography scanning[371]. This improvement has been observed three to five years postoperatively, resulting in some patients being able to discontinue their use of anti-Parkinsonian medication for over 15 years. Other patients benefited from decreased medication dosage such as L-DOPA by 46 – 65 % per day post-operatively[81]; consequently, this could result in reduction of polypharmacy, a strategy associated with controlling PD medication side effects. Results from PD utility scales have also shown significant improvements, for instance average H&Y improvements from

H&Y 3.71 down to H&Y 2.5 have been reported[78]. This change in H&Y stages is significant enough to result in amelioration of patient quality of life aspects such as reduced time on the 'off' state, decreased sleep disturbance and less pronounced motor symptoms, permitting reinstatement of one's driving license[78].

Although current cell-based trials for PD treatments have shown potential benefits, it must be noted that this mode of treatment does still face some drawbacks and challenges. For instance, graft induced dyskinesia (GID) has been observed in some trials, causing concern regarding the efficacy of cell-based treatments[84]. Furthermore, two double-blinded trials conducted in the 1990s showed no significant therapeutic benefit as a result of the procedures[62,84,372]. This triggered a decade long halt in CTP trials for PD, as the benefits of such an emergent and costly treatment strategy was brought to question. In addition, due to the allogenic nature of the foetal based therapies, immunosuppressants are a requisite of the therapy. The duration of use varies dependant on the chosen regime; the reported length of use ranges from 6 to 48 months postoperatively, with 6 – 18 months being the most common duration[373]. Furthermore, although the transplanted foetal grafts have demonstrated clinical benefit for up to eighteen years, the pathogenesis of the disease persists, evident from post-mortem analysis of transplanted patients showing the presence of Lewis bodies in the grafts[13,71,84].

Nonetheless, since the specific aetiology of the disease is not known, therapeutic targets to prevent disease pathogenesis and progression are not yet a reality. Therefore, even though it is currently not possible to stop the spread of the disease to the grafted cells, a dopaminergic CTP such as DopaCell offers a viable therapeutic option because "(i) disease propagation is slow, (ii) the majority of grafted neurons are unaffected after a decade, and (iii) patients experience long-term improvement"[373]. The neurorestoration offered by a PD CTP improves quality of life in terms such as reduced OFF-time; which is significantly important to PD patients; "patients with PD would likely seek treatment that would minimize the amount of 'off-time' experienced per day"[366]. This highlights the requirement and significance of a treatment strategy that provides such desired patient benefits.

8.1.5. Proposed CTP patient criteria

The ideal stage of intervention with a CTP for PD is not unknown, similar to DBS; although DBS is typically reserved for patients with advanced PD with life expectancy exceeding five years. In addition, commissioning criteria for DBS states that DBS candidates should "have symptoms of motor complications severe enough to significantly compromise function and quality of life"[374]. Furthermore, "strategies such as adding a catechol-O-methyl transferase (COMT) inhibiter, adding a monoamine oxidase inhibitor (MAOI), switching to long acting agonists or adding amantadine for dyskinesia should have been tried and failed or be considered unsuitable"[374] prior to treatment with DBS. In addition, "All

options for best medical therapy will have been considered, tried or exhausted by a movement disorder consultant neurologist working with a functional neurosurgery team"[374]. Additionally, the DBS in movement disorders policy states that DBS "should not be performed in patients with clinically significant cognitive decline (dementia), marked postural instability, 'on' freezing or 'on' falls."[374] These criteria form a sound basis for candidate selection for trials/treatment of PD with a CTP.

Foetal tissue based and other cell-based PD trials have previously selected patients under the age of 65 at H&Y stages III – V, with idiopathic PD for ten to fifteen years and no cognitive impairment upon assessment[362,375–377]. Patients at H&Y stage III are considered to be in the severe/advanced phase of PD where they spend an increased amount of time in the 'off' state regardless of medication (**Figure 3** and **Figure 4**). Patients fitting this prolife, in addition to the above DBS criteria would be ideal candidates as the CTP has the potential to offer improvement in quality of life, possibly improving H&Y back to H&Y II.

Treating patients prior to prolonged disease progression could result in prolonged states of higher quality of life, provided that the disease is not permitted to rapidly develop to advanced and debilitating stages[354,378]. For instance, CTPs have allowed for patients to cease use of PD medication post-operatively due to improved symptoms, prolonging higher quality of life[84,373,379,380]. Furthermore, CTPs could potentially delay the onset of advanced PD whilst inhibiting the incurrence of the substantial costs associated with disease progression[381]. This is pertinent, as worldwide increases in life expectancy, especially in westernised countries, will inevitably result in higher populations of patients with advanced PD. In addition, as PD is a disease of the elderly population, delaying the onset of advanced symptoms for ten to twenty years would significantly improve quality of life in the latter stages of patients' lives and prove to be cost-effective for a healthcare resource intensive population. Therefore, delaying the costlier, later stages of PD is important, since this forms part of the valid argument for the use of a disease modifying treatment such as a CTP[378]. Despite CTPs such as the DopaCell not being curative, they have the potential to offer better patient benefits and increased cost-effectiveness for a range of stakeholders.

8.2. Methodology and analysis

The purpose of the work carried in this chapter is to evaluate the commercial viability and potential cost-effectiveness of the candidate therapy (DopaCell). Using the headroom method, the author aimed to answer the following questions:

- What is the price potential of a human embryonic stem cell (hESC) based therapy for PD?
- Would a hESC-based therapy for PD be cost-effective?
- Could a hESC-based therapy lower the economic burden of PD?

8.2.1. Information required

In order to attempt to answer the questions posed above, essential information including intervention costs and health utility data was required, to populate the evaluation models used to carry out the analysis. These information categories will be discussed in further detail in the following sections.

8.2.1.1. Health Utilities

Health utility values are used to quantify the quality of life levels of patients and are integral to cost-effectiveness analysis. Due to the lack of clinical trial data for DopaCell, the outcomes from the literature of the foetal mesencephalic tissue transplants has been used as an informed estimate; of the health gains that could potentially be afforded by DopaCell. Health utility data for the comparators (generic medication therapy and (DBS) was also accrued from the literature.

8.2.1.2. Costs

Costs are equally as integral as health utilities to cost-effectiveness analyses. Understanding the components that make up the total costs used in economic evaluations is important, thus it is fundamental to qualify and quantify the resources used and validate the bearer of the costs. This helps to distinguish between direct and indirect costs (**Table 46**). Arguably, economic evaluations that consider indirect costs better reflect the cost-effectiveness of an intervention as they provide a holistic view of disease and the treatment option costs.

Table 46. The cost inputs that have been considered for cost-effectiveness analysis.

Direct cost	Indirect costs
• Hospitalisation costs • Procedure cost • Product costs	• Loss of productivity costs • Lifestyle change costs • Side effects costs • Societal costs • Care costs

8.2.2. The information used in the model and analysis:

8.2.2.1. Health Utilities:

The analysis assumes treatment for patients with a baseline health utility of 0.42 and stage III of the H &Y scale with high levels of 'off' time i.e. 50 % or more [382]. The comparator used in the present work is generic anti-Parkinsonian medication which can provide a health utility of 0.55 (**Table 48**) and general DBS surgery, providing a health utility of 0.66 (**Table 47**). The DopaCell health-related quality of life data assumes that the patient is able to return to a perfect health state as a result of the therapy i.e. normal headroom assumption.

Adjustments to the headroom were made to account for the fact that perfect health is not an achievable result from use of the therapy. Instead, treatment results in improvements to H&Y stage II with less than 50 % 'off' time were utilised, which corresponds to a health utility value of 0.72. This resulted in assessment of DopaCell as a perfect therapy (classic headroom) and in an attuned manner that is a clinically realistic and provides significant improvement of a whole stage in the H&Y scale (DopaCell adjusted). Literature analysis shows that this is an achievable level of improvement as average reductions of 1.2 H&Y stages have been reported[78]. The adjusted headroom health utility value is based on long term outcome analysis that has shown:

1. Up to a 40 % decrease in motor symptoms[35]
2. 40 % decrease in 'off' time observed in some patients[35]
3. Significant quality of life improvements being obtained e.g. patients regaining their driver's license due to improvements, ability to sleep with less disturbances, par-taking in hobbies *etc*.[78]
4. Striatal F-DOPA returning to normal levels, resulting in cessation of medication[383]
5. Decreases in L-DOPA dosages between 46 – 65 % per day post-operatively[81]

Table 47. DBS heath utility data obtained from the literature and the averaged value used for the present work.

Baseline	Increment	Health Utility	Details	Reference
0.29	0.47	0.76	Base line used was 0.29 in the study	Yianni *et al* (2013)
0.42	0.224	0.644	-	Moon *et al* (2017)
0.42	0.17	0.59	70% reduction in motor complications. Quality of life improvement of 30% over medication	DBS policy (2013)
0.42	0.3	0.72	Utility represents transition from H&Y III 50 -75 % OFF time to H&Y II OFF time 0- 25%	Eggington *et al* (2014)
0.42	0.169	0.589	-	Pietzch *et al* (2016)
0.394	**0.2666**	**0.6606**		**Average**

Table 48. Heath utility data for medication therapy obtained from the literature and the averaged value used for the present work.

Study Baseline	Increment	Study Health Utility	Health Utility from model baseline	Details	Reference
0.564	0.08	0.644	0.5	Assumption: drugs carry on working in the best way for period analysed therefor the level of health utility is sustained, however increased doses would be necessary thus an increase in cost incurred	Moon *et al* (2017)
0.53	0.06	0.59	0.48		DBS policy (2013)
0.418	0.242	0.66	0.662		Eggington *et al* (2014)
0.42	0.15	0.57	0.57		Pietzch *et al* (2016)
0.483	**0.133**	**0.616**	**0.553**		**Average**

8.2.2.2. Costs:

The sensitivity analysis ranges used in the present work were obtained from a combination of typical ranges in the literature that are representative of the assumptions being made in the present work. In general, the value of 25 % above and below the baseline was utilised for the sensitivity analysis. However, for some factors, such as miscellaneous costs e.g. the use of immunosuppressants and loss of productivity, the sensitivity ranges were different and ranged from 0 – 100 %. The use of immunosuppressants ranged from no use of immunosuppressants i.e. 100% below the baseline and 50% above the baseline representing a 50 % longer period of immunosuppression. The miscellaneous costs for medication therapy ranged between 50 – 100%, representing further indirect costs other than care and hospital costs, 50% above the baseline representing additional indirect costs such as household modification and lifestyle changes; while no changes and no addition costs were represented by 100% below the baseline. For loss of productivity the range incorporated non-inclusion of loss of productivity such as loss of income representing 100% below the baseline and 25% above the baseline presenting a modest literature-based increase to indirect costs

8.2.2.2.a. DopaCell and DopaCell adjusted

The total cost of DopaCell to the payer is comprised of manufacturing costs with a 50% mark-up, surgery costs, hospitals costs, pre-operation costs and miscellaneous costs. The manufacturing cost is based on an in-house CoGs model that was employed for the present study. The administration of DopaCell would involve an invasive surgical approach which makes up 17.5 % of the total costs, furthermore the patients have to undergo pre-operative assessments and hospital stays which also increases the cost. Immunosuppressants (miscellaneous costs) also represent a substantial outlay contributor accounting for 25% of the total costs when administered for the first twelve months, post-operatively. The amounts used to determine cost are shown in **Table 49** and are based on taking an average of reported costs from the literature (**Appendix 21**).

Table 49. DopaCell costs, the costs are the same for both DopaCell and DopaCell adjusted. The CTP variable cost represents the development/manufacturing costs as determined by the in-house CoGs model with a 50% mark-up. All other variables were sourced from current literature.

Variable	Amount (£)	Details	References
CTP	15,000	Development cost of £10,000 with a 50% mark up as the price to the payer	Parmar (2018) Eggington *et al* (2014) Pietzch *et al* (2016)
Surgery	7,000	Stereotactic procedure, theatre costs and personnel costs	
Hospital	1,700	Neurosurgery follow up costs	
Pre-operative	712	Pre-operative stay and assessments	
Miscellaneous	8,000	Immunosuppressants at a cost of £8000 per year	
Total	**£32,412**		

Sensitivity analysis was performed on the DopaCell cost input variables mentioned above, to determine which input had the most significant impact on the price/cost of DopaCell to the payer. The baseline used the values reported in **Table 49**. The ranges used in the sensitivity analysis were 25% above and below the baseline for all the variables, except for miscellaneous costs. For the miscellaneous costs the range included no use of immunosuppressants i.e. 100% below the baseline and 50% above the baseline (**Figure 105**).

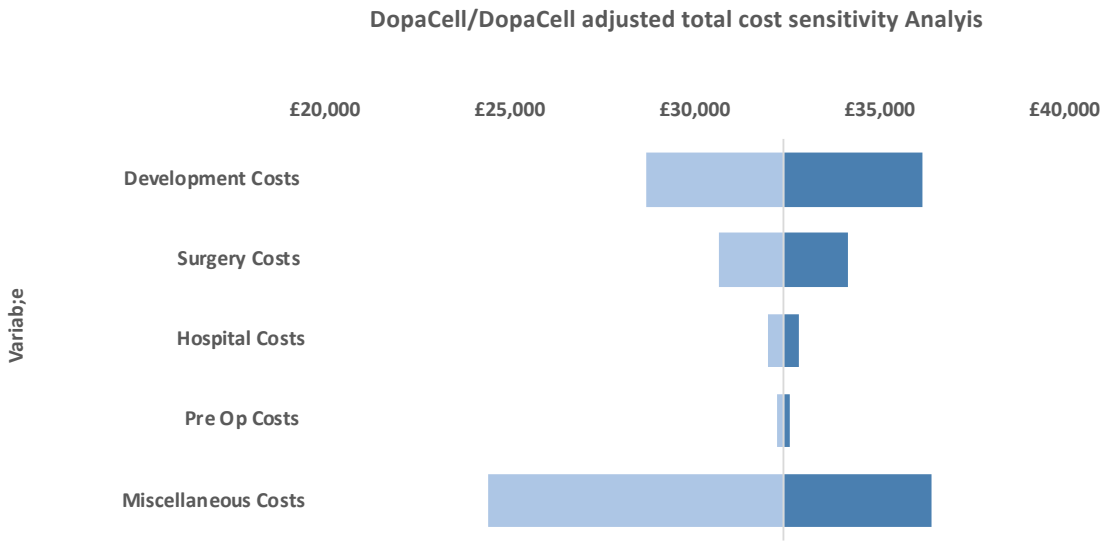


Figure 105. Tornado plot showing the influence of each variable on the final total cost of DopaCell to the payer in the first year. Miscellaneous costs which represent the use of immunosuppressants are shown to be the most influential variable on final total cost. The lighter colour (left hand side) demonstrates the level of cost decrease from the baseline (centre line) due to the respective variable. The darker colour (right hand side) demonstrates the level of cost increase from the baseline due to the respective variable.

8.2.2.2.b. DBS

The cost of DBS included device costs, surgery costs, hospital costs, pre-operative costs and miscellaneous costs. The device cost is based on an average of reported device costs in the literature, similarly the costs of the other inputs are based on the literature averages (**Appendix 15**). The values used to obtain the final cost are show in **Table 50**. The ranges used in the sensitivity analysis were 25% above and below the baseline for all the variables **Figure 106**.

Table 50. Input variables for the total cost of DBS in the first year, the values reported are averages from data sourced from the literature.

Variable	Amount (£)	Details	References
Device	11,928	Includes DBS device, extensions, leads and patient programmer	Moon *et al* (2017)
Surgery	7,000	Implantation procedure, theatre costs and personnel costs	Yianni *et al* (2013)
Hospital	1,776	Neurosurgery follow up costs	DBS Policy (2013)
Pre-operative	709	Pre-operative stay and assessments	Eggington *et al* (2014)
Miscellaneous	1,572	Battery changes ever 3-5 years	Pietzch *et al* (2016)
Total	**£22,986**		

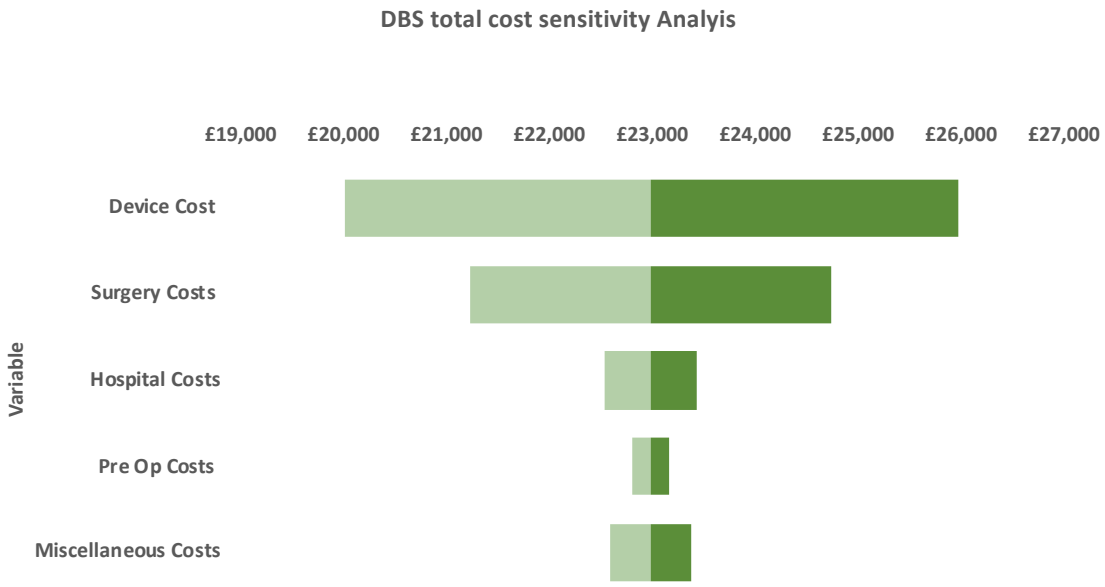


Figure 106. Tornado plot showing the influence of each variable on the final total cost of DBS to the payer in the first year. Device costs are shown to be the most influential variable on final total cost. The lighter colour (left hand side) demonstrates the level of cost decrease from the baseline (centre line) due to the respective variable. The darker colour (right hand side) demonstrates the level of cost increase from the baseline due to the respective variable.

8.2.2.2.c. Medication

The input outlay components for the medication therapy comparator include the cost of the medication, this is based on generic anti-Parkinsonian medication. As medication-based therapies simply treat PD symptoms and do not have a disease modifying effect, additional indirect costs are incurred due to dampened responses to the medication and worsening of symptoms as the disease progresses. Therefore, the total cost to the payer used in this analysis includes indirect cost such as care costs, hospital visit costs and loss of productivity costs (**Table 51**). The addition of indirect costs better illustrates the actual cost of medication therapy and living with PD. The 2017 Parkinson's UK 'The cost of Parkinson's' report highlights the importance of these indirect costs as they have a significant financial impact on PD patients and their families[355,357]. The values used to determine the cost are shown in **Table 51** and are based on an average of reported costs from the literature (**Appendix 18**).

Table 51. Input variables for the total cost of medication therapy in the first year, the values reported are averages from data sourced from the literature.

Variable	Amount (£)	Details	References
Drugs	2,654	Typically costs increase as the disease progresses due to increased dose requirements	Parkinson's UK (2017)
Care	5,221	Personal care and assistance is require due to movement disorder	Weir *et al* (2018)
Hospital	2,157	Require for catchup and stays after fall etc. that increase as the disease progresses	DBS Policy (2013)
Loss of Productivity	11,069	Loss of income due to early retirement	Eggington *et al* (2014)
Miscellaneous	1,000	Modification to household, dietary intake	Pietzch *et al* (2016)
Total	**£22,103**		

The ranges used in the sensitivity analysis were 25% above and below the baseline for all of the variables included, except for loss of productivity and miscellaneous costs. For loss of productivity the range incorporated non-inclusion of loss of productivity such as loss of income representing 100% below the baseline and 25% above the baseline presenting increased value to indirect costs (**Figure 107**). For the miscellaneous costs the range included no further indirect costs other than care, hospital and loss of productivity costs i.e. 100% below the baseline and 50% above the baseline representing additional indirect costs such as household modification and lifestyle changes.

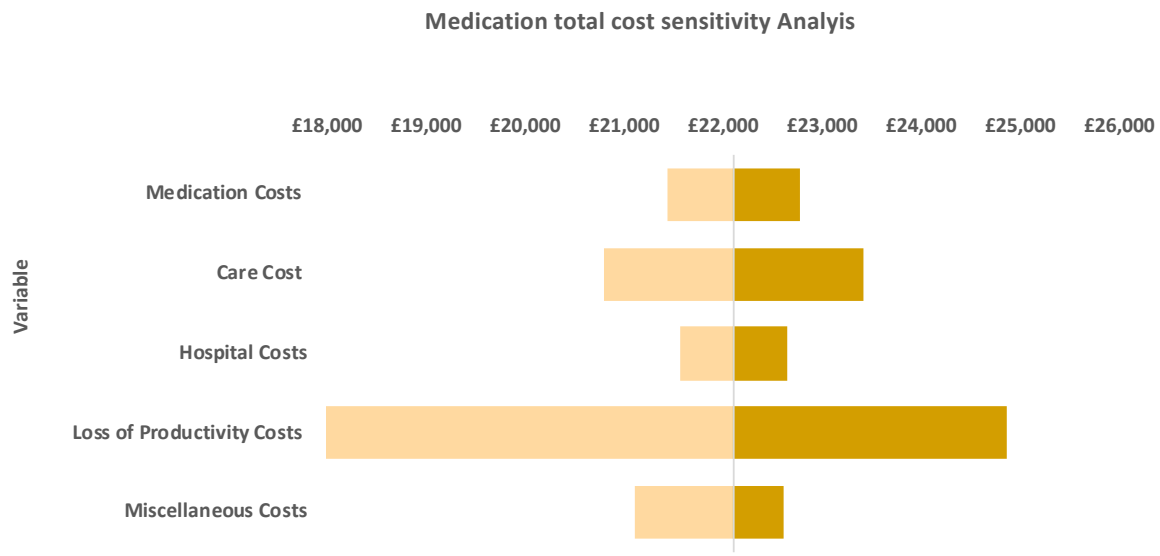


Figure 107. Tornado plot showing the influence of each variable on the final total cost of medication therapy. Loss of productivity costs are shown to be the most influential variable on final total cost. The lighter colour (left hand side) demonstrates the level of cost decrease from the baseline (centre line) due to the respective variable. The darker colour (right hand side) demonstrates the level of cost increase from the baseline due to the respective variable.

8.2.2.3. Cost-effectiveness analysis assumptions

Below are the assumptions used for determined the cost-effectiveness of the CTP:

1. Outpatient hospital visit costs for ten years and medication costs for the first five years are included in the ten-year cost savings analysis for DopaCell/DopaCell adjusted.
2. The patients stop taking medication after five years as a result of DopaCell/DopaCell adjusted.
3. Patients have two years of immunosuppressant use as a result of DopaCell/DopaCell adjusted.
4. Two device battery changes are included in the ten-year cost savings analysis for DBS as well as outpatient costs and medication costs.
5. Medication costs for DBS decrease from year 6 onwards.
6. No loss of productivity is experienced with the use of both DBS and DopaCell/DopaCell adjusted.
7. 3.5% discount rate on the medication and hospital costs associated with DopaCell/DopaCell adjusted and DBS.
8. Medication therapy costs increase by 25% every two years over the ten-year period as the disease progresses.
9. A £30,000 per QALY willingness to pay threshold is assumed.

The assumptions utilised in the analysis are based on typical trends and assumptions observed in the literature, for instance, the timeframe for hospital visits, DBS device battery changes and use of immunosuppressants. Standard practice by HTA agencies such as NICE i.e. the 3.5% discount rate and £30,000 per QALY threshold was used. The medication therapy cost increase by 25% every two years is based on studies that have demonstrated that patient medication costs increase as the disease progresses by 6 – 50 % per year depending on disease severity. The median value of 25 % was used for the analysis.

8.2.3. Headroom assessment

The headroom method makes assumptions regarding a therapy's effectiveness, in the absence of clinical data, this tool is particularly valuable for product developers as it provides financial information from the supplier/developer perspective. Typically, the headroom assumes maximum effectiveness to ascertain cost-effectiveness, under the assumption that; if an intervention is not cost-effective at a maximum effect, then it is unlikely to be cost-effective under any other circumstance. This is especially the case when the intervention is more expensive than the comparator, which is typically the case for CTPs.

To obtain the health utility data the average health utility from medication therapy (0.55) was used to determine the difference in health-related quality compared to the DopaCell/DopaCell adjusted. The difference was assumed over a ten-year period of fixed health utility due to use of the DopaCell/DopaCell adjusted, DopaCell assumes maximum health gains i.e. restoration to 1 QALY (**Table 52**). On the other hand, DopaCell adjusted uses the health utility value obtained from the health gains that have been observed from the foetal tissue trials thus far and relating these outcomes to the H&Y scale and the corresponding health utility (0.72). The difference in QALY (ΔQALY) was calculated as shown in **Equation 10**.

Table 52. QALY calculation input variables for both the DopaCell/DopaCell adjusted.

Variable	Equation symbol	DopaCell	DopaCell adjusted
Health Related Quality of Life (New Therapy)	$HRQoL_{ND}$	1.00	0.72
Health Related Quality of Life (Current Therapy)	$HRQoL_{CT}$	0.55	0.55
Difference in HRQoL	ΔHRQoL	0.45	0.17
Time in health State (Year(s))	t	10	10
QALY	**ΔQALY**	**4.47**	**1.67**

$$\Delta QALY = (HRQoL_{ND} - HRQoL_{CT}) \times t = \Delta HRQoL \times t$$

Equation 10

The ΔQALY was translated into monetary terms by multiplying it with the willingness to pay (WTP) threshold of £30,000 for one additional QALY (**Equation 11**). This determined the potential maximum price to the payer, thus the headroom price point of the CTP given the respective level of incremental health benefit. The greater the ΔQALY the higher the headroom per patient as shown in **Table 53**.

Table 53. Headroom calculation input variables.

Variable	Equation symbol	DopaCell	DopaCell adjusted
QALY	ΔQALY	4.47	1.67
Willingness to Pay	WTP	£30,000	£30,000
Headroom per person	**$maxCost_{PP}$**	**£134,100**	**£50,100**

$$maxCost_{PP} = \Delta QALY \times WTP$$

Equation 11

Obtaining the headroom allows for other predictive outputs such as the potential income that can be acquired by the developer given the size of the eligible population of patients. Here an estimate of 100 patients per year was used based on treating ~ 1% of the ideal candidate population described in section 8.1.4. The maximum income was obtained by multiplying the headroom per person with the number of potential patients (**Table 54**) as shown by **Equation 12**.

Table 54. Market informed headroom input variables.

Variable	Equation symbol	DopaCell	DopaCell adjusted
Headroom per person	$maxCost_{PP}$	£134,100	£50,100
Number of potential patients	N (per year)	100	100
Maximum revenue	**maxRevenue (per year)**	**£13,410,000**	**£5,010,000**

$$maxRevenue = max\Delta Cost_{PP} \times N$$

Equation 12

8.2.3.1. Headroom based financial appraisals

Information on the potential income can provide developers with early hypothetical financial appraisals that can be used to estimate the gross profit of their product. These estimates can be made using the headroom and information on potential sales volumes and the known or estimated development/ manufacturing costs. The potential gross profit and profit margin can be obtained as well (**Table 55**). The gross profit was calculated by subtracting the manufacturing costs from the maximum income and multiplying by the years of sales with N patients being treated per year (**Equation 13**).

Table 55. Gross profit input variables for the first year, based on the treatment of 100 patients.

Variable	Equation symbol	DopaCell	DopaCell adjusted
Maximum income	maxIncome (per year)	£13,410,000	£5,010,000
Years of sales with N patients (total sales volume)	V	1	1
Manufacturing costs	C_{DD}	£1,000,000	£1,000,000
Gross Profit (per product)	-	£124,100	£40,100
Gross Profit (%)	-	93 %	80 %
Annual Gross Profit	**AGP**	**£12,410,000**	**£4,010,000**

$$AGP = (maxRevenue - C_{DD}) \times V$$

Equation 13

Budget impact is a key consideration for developers in the UK market as this can limit the potential sales volumes due to caps on the total expenditure per year a single product/intervention can have, as set by the NHS. This value is currently set at approximately £20 million, therefore using the headroom price points obtained in the present work the number of patients that can be treated per year are: 149 and 399 for the DopaCell and DopaCell adjusted, respectively.

8.2.4. Cost-effectiveness analysis

The sections above reported the potential benefits and costs for the patients and developers regarding use of DopaCell/DopaCell adjusted compared to the standard of care medication therapy. The following sections focus on the potential cost-effectiveness of DopaCell/DopaCell adjusted compared to medication therapy and DBS. The analyses performed here aim to provide information for the payer to aid in adoption and reimbursement decision making. Similar to the analysis in sections 8.2.2 and 8.2.3 the results in this section are over a ten-year time horizon period.

8.2.4.1. Cost and savings of the interventions

The cost of each intervention over the ten-year period is shown below, although DopaCell and DopaCell adjusted have different effectiveness levels there is no difference in the cost, thus only the DopaCell costs are shown. DopaCell expenses in the first year are based on the values shown in **Table 49**, with the addition of £2,655 for cost of medication, totalling up to £35,067. In the second-year outlays include the second year of immunosuppressants at £8,000, hospital visit costs at £500 and the cost of medication again, totalling up to £11,155. The third to fifth year expenses comprise of medication costs and hospital costs discounted at 3.5%. From year six onwards only the hospital visit costs are incurred as patients would have stopped taking anti-Parkinsonian medication and immunosuppressants, resulting in a significant decrease in costs (**Figure 108**).

DBS costs include the outlays detailed in **Table 50** with the addition of £2,655 for the cost of medication, totalling up to £25,641 in the first year. Second to fourth year expenses include medication and outpatient hospital visit costs. At year five, in addition to the fourth-year expenses, £2,000 is added to account for the cost of DBS device battery replacement. From year six onwards there is a decrease in costs as the outlay expense of medication decreases to £1,336. At year ten the cost of another battery replacement is added to the outpatient costs, resulting in a slight cost increase (**Figure 108**).

The medication costs over the ten years are based on the total detailed in **Table 51**, expenditure then increases by 25% every two years over the ten-year period used in the analysis (**Figure 108**). The 25 % increase rate was chosen as it is in line with reported increases in PD cost as the disease progresses[70,354,358]. The indirect costs represent the highest cost proportion as the disease progresses. This is due to costs such as personal care and hospitalisation increasing considerably, as the patients lose their independence due to increased ‘off’ time and worsening of symptoms.

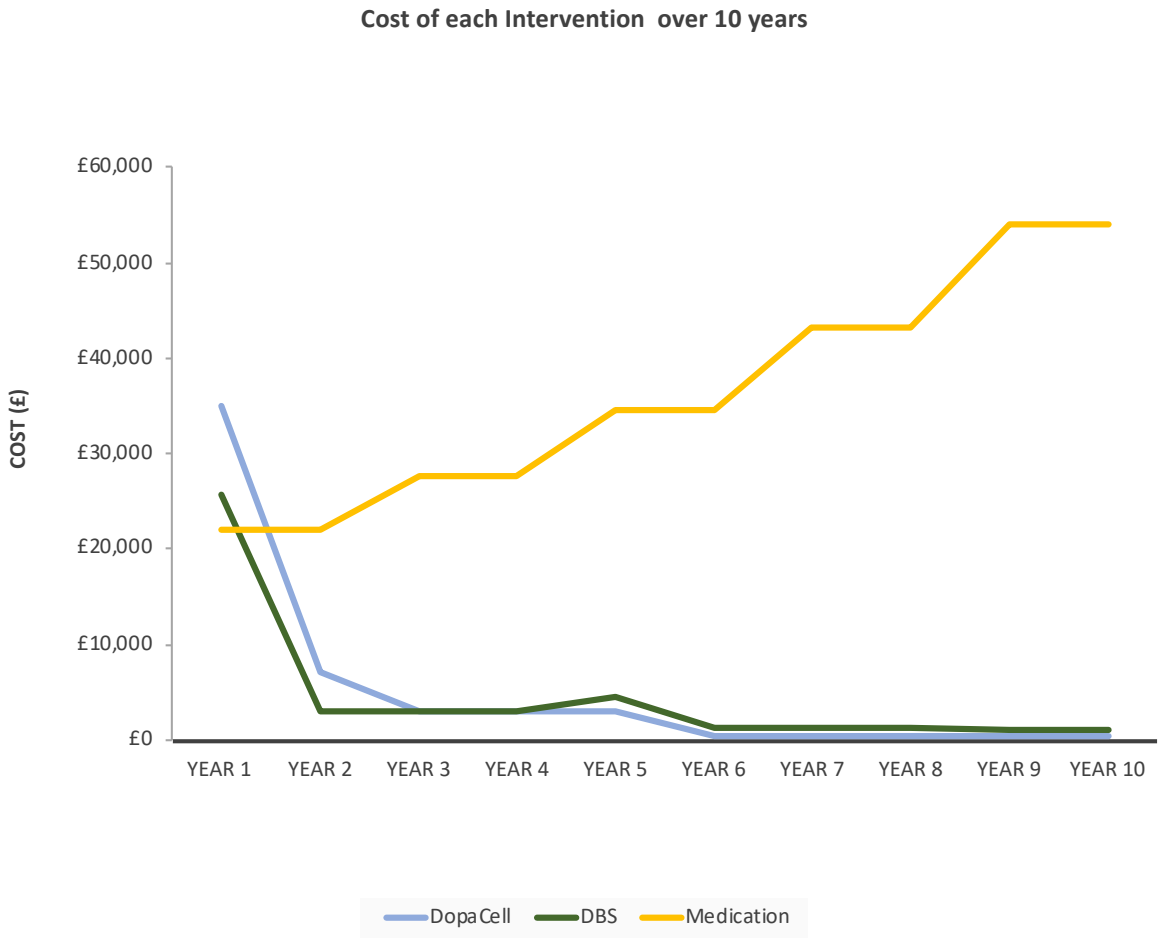


Figure 108. A comparison of the costs of the interventions over a ten-year period demonstrated that both DopaCell and DBS have significant decreases in costs at year two and at year five. Medication costs are shown to increase over the ten-year period, amounting to £53,962 per year, by year ten.

The cost savings were simply determined by taking away the expenditure of the alternative intervention (DopaCell and DBS) from the medication therapy costs for each year (**Figure 109**).

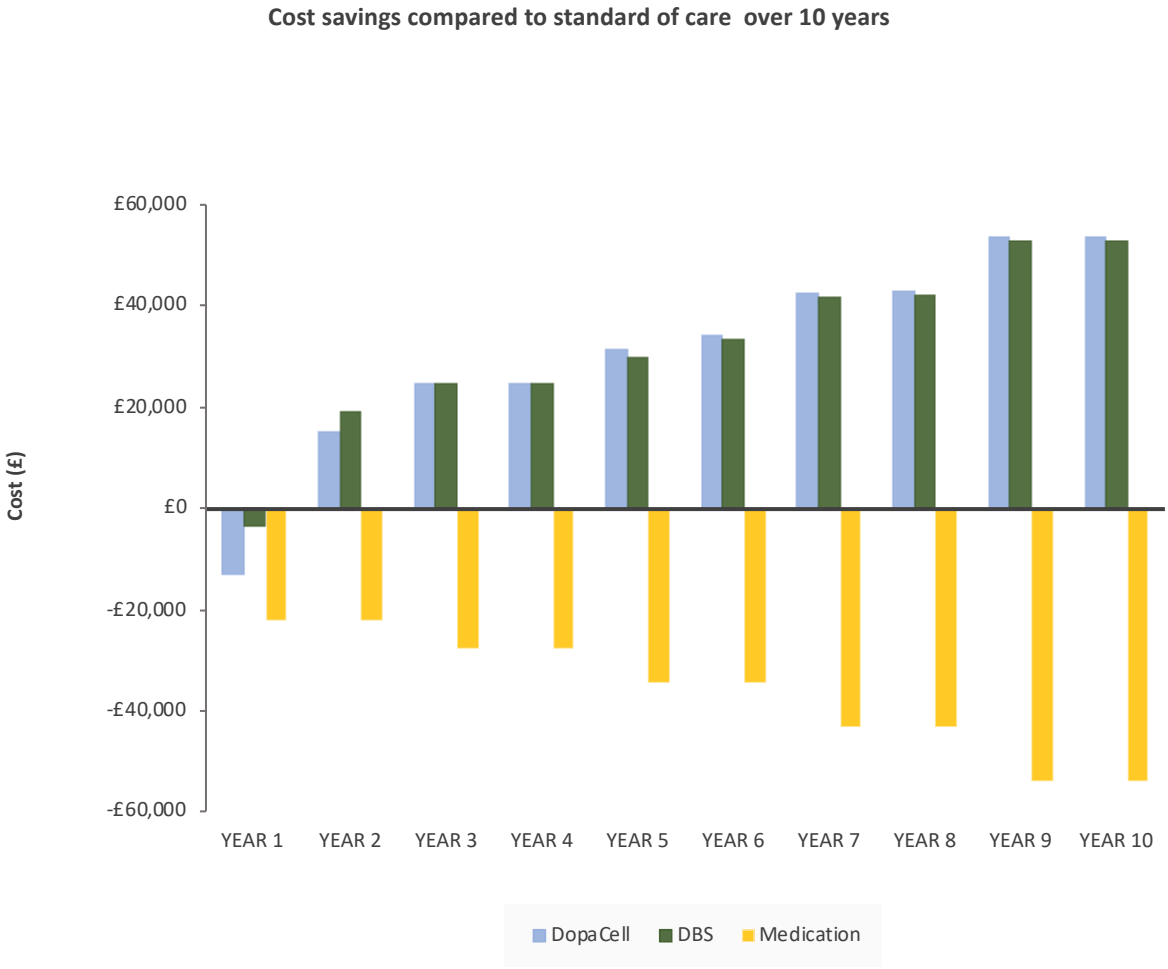


Figure 109. Cost savings for both DopaCell and DBS are observed from year 2 onwards, DopaCell results in higher savings compared to DBS from year five onwards. The cumulative cost savings over the ten-year period are £304,951 and £317,270 for DopaCell/DopaCell adjusted and DBS, respectively. Bars above £0 indicate cost savings.

8.2.4.2. Cost per QALY comparisons

The cost per QALY for each year was calculated by dividing the cost of the respective year by the health utility of the given treatment option, the results were plotted to show the cost per QALY trends over a ten-year period (**Figure 110**).

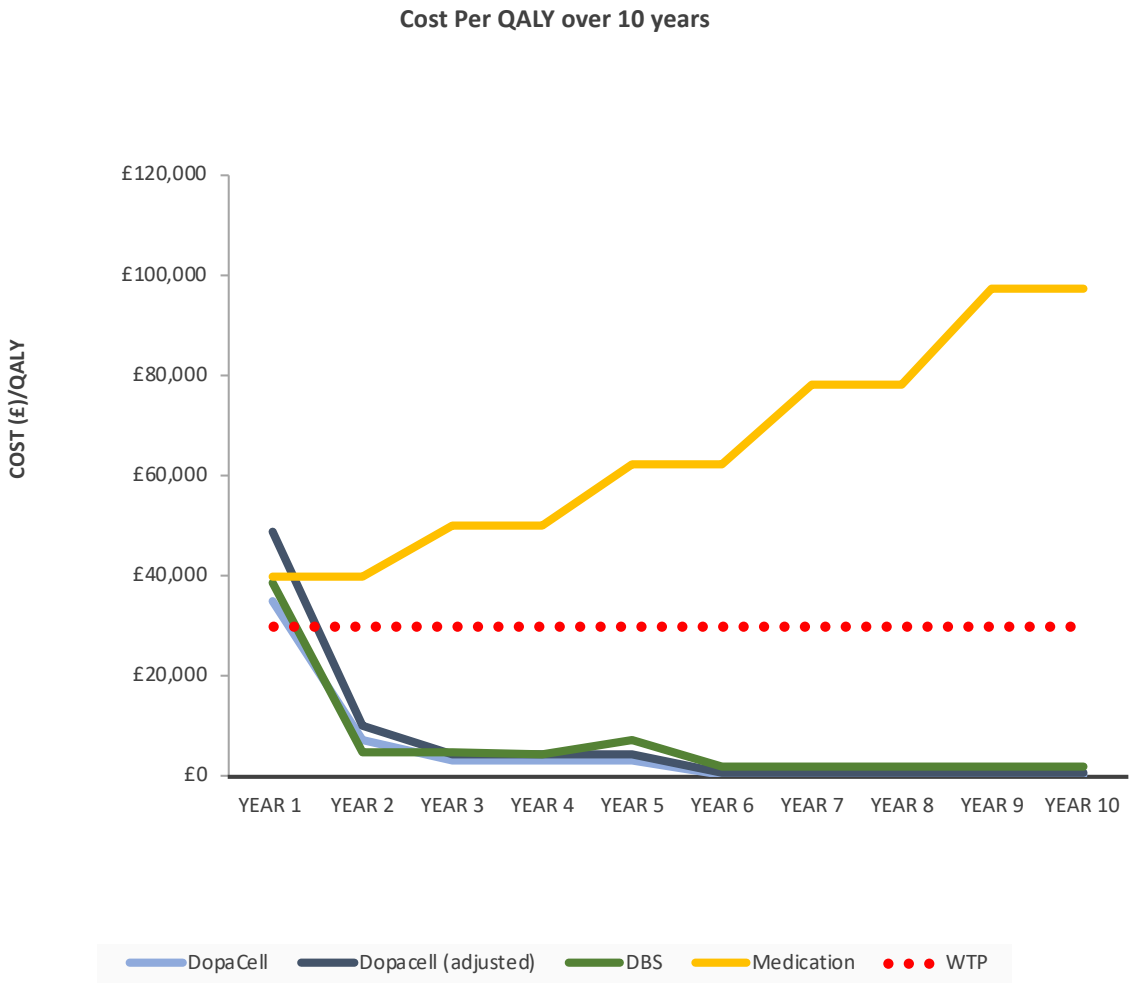


Figure 110. The cost per QALY data shows that from year two onwards the DopaCell/DopaCell adjusted and DBS all fall under the £30,000 per QALY cost-effectiveness threshold (red dotted line). Medication therapy costs per QALY increase over the ten-year period.

The average cost per QALY was determined by dividing the cumulative yearly costs by the total QALYs over the ten-year period (**Figure 111**). In order to determine if the CTP would remain cost-effective at higher prices the model was run with a range of sale prices from £15,000 up to £300,000 (**Figure 112**).

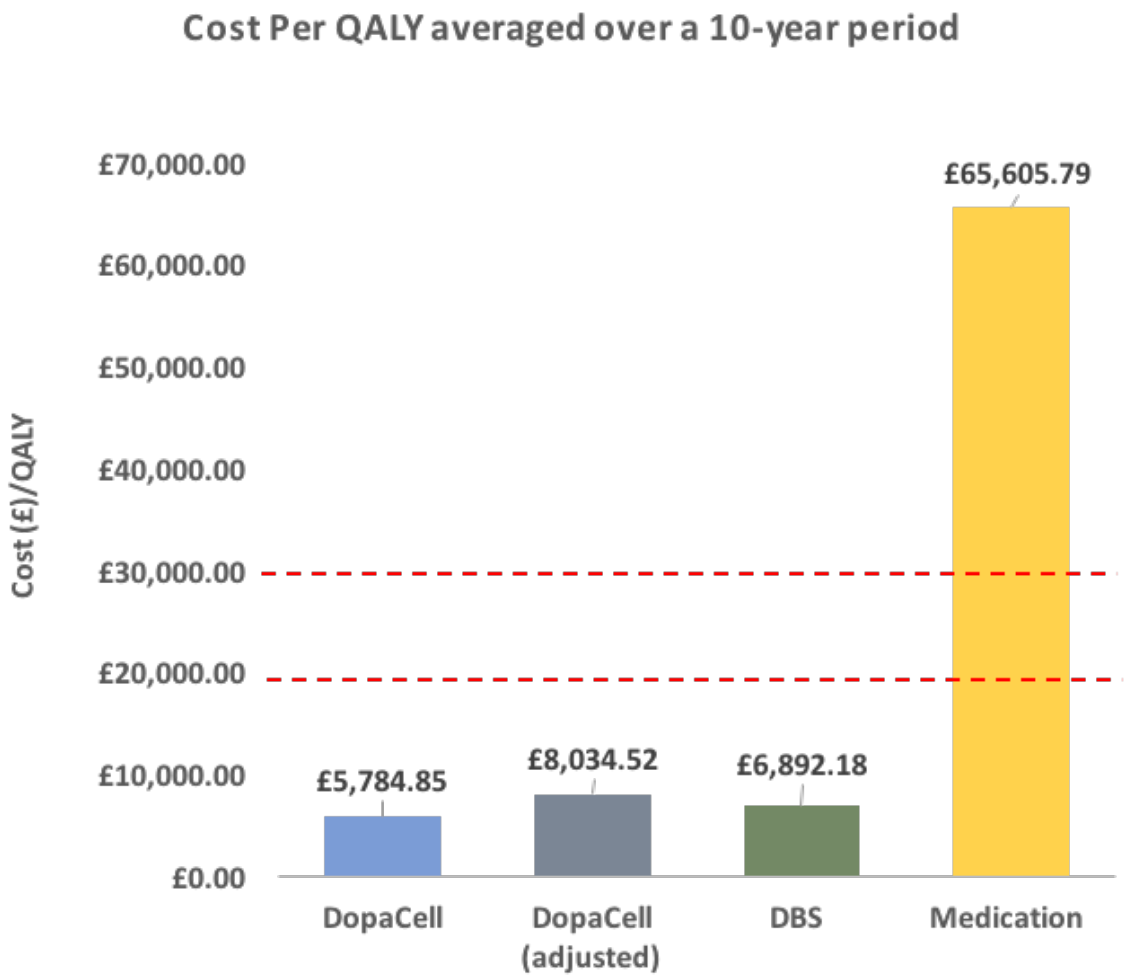


Figure 111. Cost-effectiveness ratios (CER) of the CTP under two health utility assumptions (DopaCell and DopaCell adjusted) with the use of immunosuppressants, DBS and medication over a ten-year period. DopaCell had the lowest cost per QALY, and medication therapy had the highest cost per QALY. Red lines represent the lower and upper willingness to pay thresholds, £20,000 and £30,000 per QALY (indicated by red dotted lines). All of the alternative interventions are below the two thresholds while medication therapy is notably above the upper threshold.

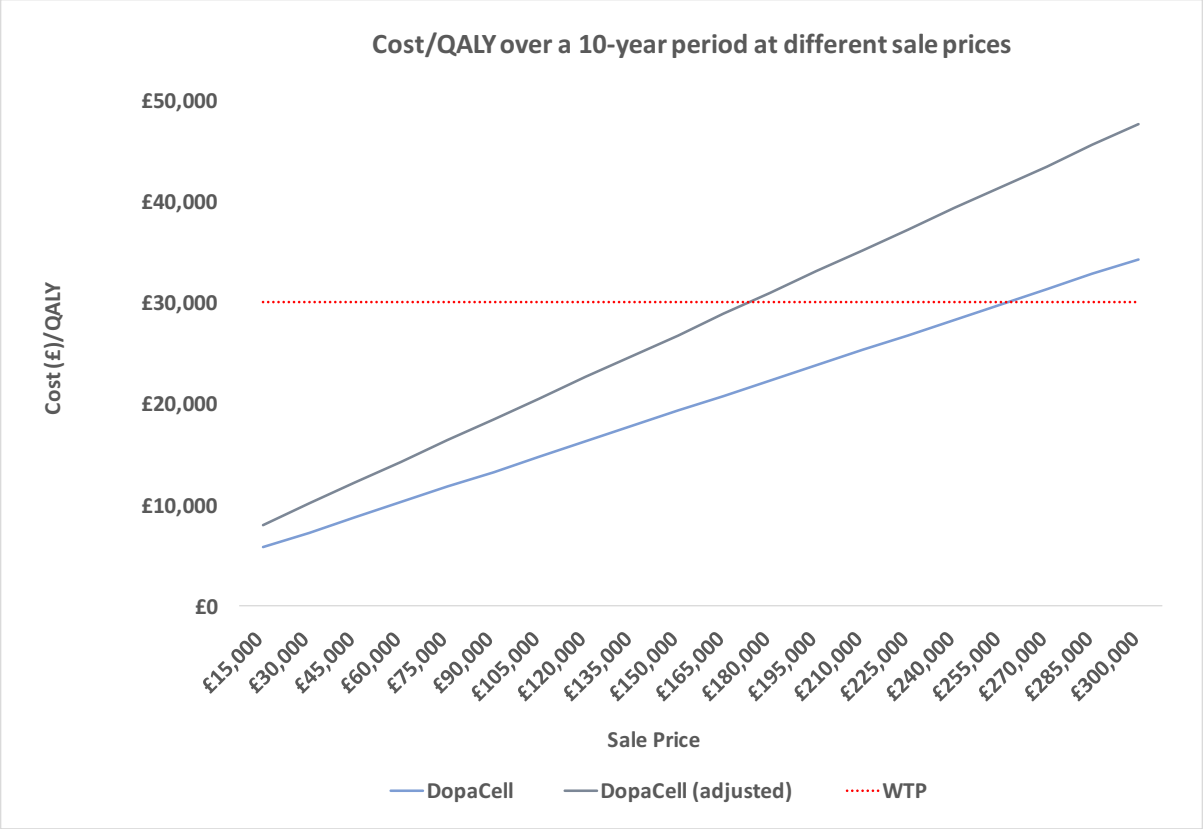


Figure 112. Comparison of costs per QALY for DopaCell and DopaCell (adjusted) over a ten-year period. Data shows the cost per QALY as a result of different sale prices. DopaCell crossed over the £30,000 WTP upper threshold at a sale price of £255,000 (cost/QALY = £31,284) while DopaCell (adjusted) crosses the threshold at a lower sale price of £180,000 (cost/QALY = £30,951).

Over a ten-year period, the alternative treatments are both cheaper and more effective than the standard of care. Thus, it is not necessary to determine the incremental cost-effectiveness as the standard of care (Meds) is dominated by DopaCell, DopaCell adjusted and DBS, which means it is the least cost-effective option in the analysis. (**Figure 113**).

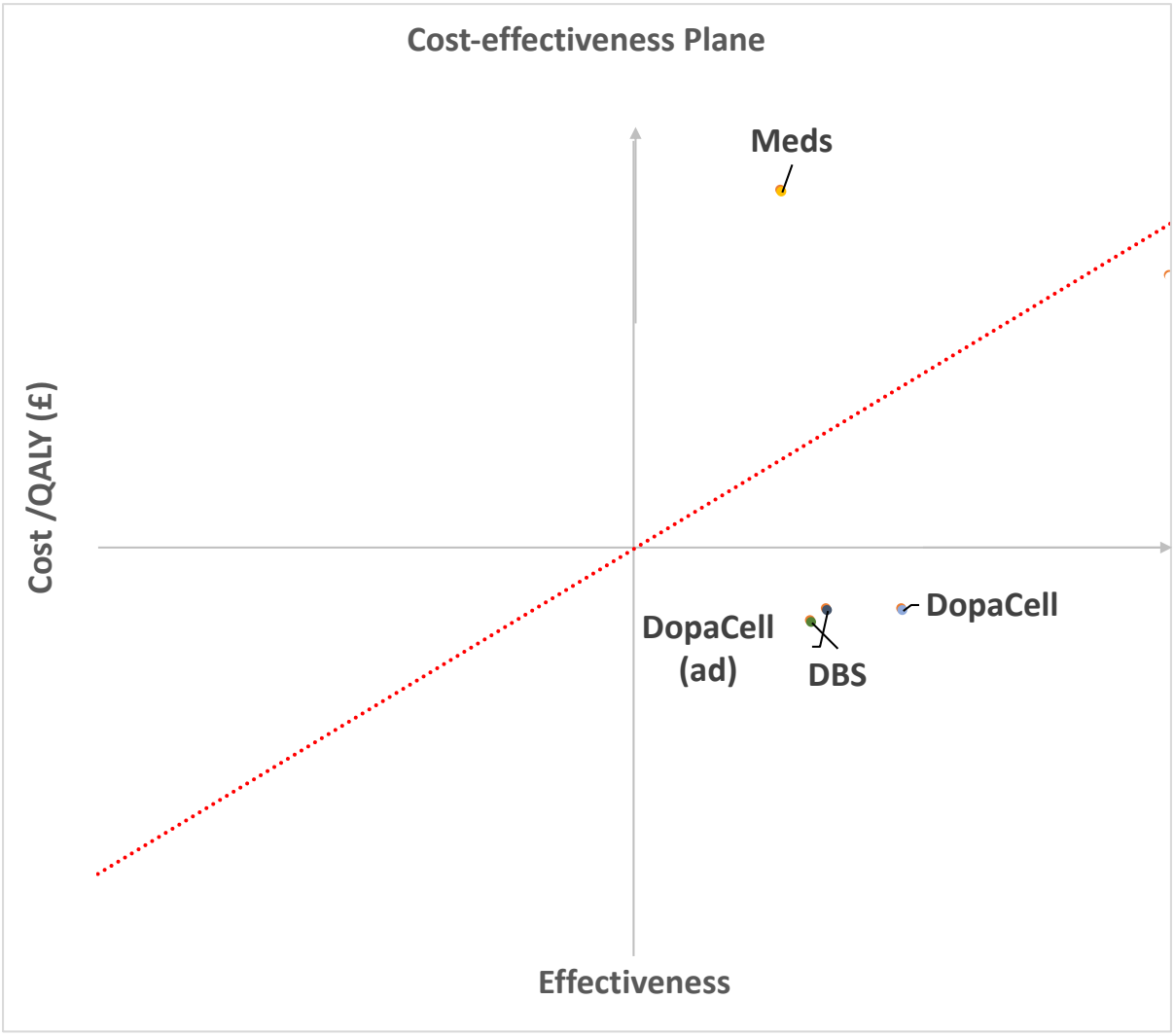


Figure 113. Cost-effectiveness plane shows that DopaCell, DopaCell adjusted (DopaCell ad) and DBS treatment options are all more cost-effective than medication therapy (Meds) over the ten-year period shown by their position in the bottom right hand quadrant. Therefore, medication therapy is dominated by all the other treatment options analysed. Medication therapy is positioned in the top right-hand quadrant and above the WTP threshold of £30,000 (represented by the red line).

8.3. Discussion

The analysis performed above demonstrates that, under the informed assumptions made, a CTP such as DopaCell/DopaCell adjusted would be a cost-effective mode of treatment for PD. In addition, the headroom shows that DopaCell/DopaCell adjusted would be a commercially viable product for the treatment of PD. Both DopaCell and DopaCell adjusted with their differing levels of QALY gains would allow developers to recover the associated development/manufacturing costs and whilst providing an adequate gross profit. Furthermore, the CTP can be priced considerably higher than the £15,000 used in the base conditions of the analysis model. Analysis of different sale prices revealed that DopaCell and DopaCell (adjusted) can be sold at prices up ~£255,000 and ~£180,000 respectively while remaining at under £30,000 per QALY over a ten-year period. Both of these figures are substantially higher than the headroom prices (£134,100 and £50,100 for DopaCell and DopaCell adjusted, respectively), therefore the developer, under the assumptions in the present work, would be able to have abundant gross profits and manufacturing cost recovery. A key driver for the high price potential and maximum headroom is the large effectiveness gap observed in current PD treatment. Although the headroom method operates on the basis of pessimistic assumptions for the standard of care and optimistic assumptions for the new intervention; in the case of PD medication therapy as the standard of care, the afforded health outcomes are already minimal. The health utility scores for medication therapy are low and offer minimal improvement followed by a decrease in effectiveness in the long term. Consequently, the headroom potential for a therapy like DopaCell is high as the effectiveness gap that needs to be covered is large, thus even marginal improvements are considerably more valuable than the current standard of care.

The use of a CTP e.g. DopaCell/DopaCell adjusted provides a cost-effective alternative for the treatment of PD as it offers QALY gains that cannot be provided by current modes of treatment such as medication therapy and DBS. Moreover, the quality of life gains reported in foetal tissues transplant trials show that patients can regain their livelihood and independence[80,373]. This would negate loss of productivity expenses, which have been identified as the biggest contributor to medication therapy costs. The CTP's potential ability to reduce and/or stop the use of anti-Parkinsonian medication allows it to be cost saving, while reducing patient polypharmacy and the side effects associated with PD medication therapy. This is significant in the economic impact of PD as the direct annual costs associated with comorbidity, symptom management and medical treatment have been reported to increase from; ~ £3,000 during the initial stages of the disease to; ~£18,000 at the latter stages of the disease[357]. These costs do not include indirect expense such as hospitalisation costs, lifestyle change costs and personal care costs, which are also reported to increase as the disease progresses[70,358].

Conversely, whilst the expenditure for DopaCell/DopaCell adjusted is high in the first year (~£35,000) it decreases to an average of £1,400 after the patients stop taking immunosuppressants from year 3 onwards with costs dropping to below £500 by year 10 (**Figure 108**). The cost per QALY results show DopaCell is the cheapest option. DopaCell adjusted, which represents a more realistic QALY gain level, is more expensive in comparison to both DBS and medication therapy, however this is only for the first two years, after which it is more cost-effective than both DBS and medication therapy. The expenditure reduction observed for DopaCell/DopaCell adjusted is ascribed to a decrease in medication costs, lower incidence of PD complications, fewer hospitalisations and no loss of productivity being experienced by patients. Thus, highlighting the value of treating PD before it progresses to the advanced stages where it becomes costlier[378]. Under the assumptions made in the present work, the use of a CTP for PD would provide significant cost savings from as early as year 2, even with the added cost of immunosuppressants.

The cost-effectiveness comparison shows that the DopaCell/DopaCell adjusted dominates the current standard of care, suggesting it would likely result in a favourable NICE appraisal decision. Furthermore, as the QALY gains and improvements on quality of life are greater than those provided by DBS, DopaCell/DopaCell adjusted could still be recommended for adoption when appraised against DBS. Recommendation would be likely in the idealist case of DopaCell and in the case of DopaCell adjusted with ICERs of £3,924 and £22,422 respectively, when analysed against DBS for cost-effectiveness over a ten-year period.

As demonstrated by the sensitivity analysis, some variables have a significant influence on the cost of the treatment mode. For medication therapy, loss of productivity was the most influential cost variable, highlighting the importance of including indirect outlays in cost-effectiveness analysis. This is apt for chronic diseases such as PD that have a high societal burden on top of the direct medical expenses. Therefore, including indirect expenses better represents the true cost of the disease, especially when analysed over a long period, such as the ten-year period used in the present work. Sensitivity analysis results of DopaCell/DopaCell adjusted and DBS outlays also showed their respective major cost influencers, which were miscellaneous (immunosuppressants) and device expenses, respectively (**Figure 105** and **Figure 106**). For DopaCell/DopaCell adjusted, a point to consider is as to whether the expense of the immunosuppressants should be included in the cost-effectiveness analysis; as without them the DopaCell/DopaCell adjusted would be ~ 25% cheaper with the same QALY gains, making it even more cost-effective. From a payer's perspective, it would be assumed that they would require all expenditures associated with the use of the therapy to be included, in order to fully evaluate the true cost of adopting DopaCell/DopaCell adjusted as a mode of treatment. Thus, shorter lengths of immunosuppressant use would result in higher cost-effectiveness/savings for DopaCell/DopaCell adjusted.

Due to the relatively low costs of the DopaCell/DopaCell adjusted assumed in the analyses, it could be eligible for reimbursement under a range of different payment mechanisms. Managed entry agreements (MEAs) might not be necessary as the costs are relatively low and the budget impact is not high given the population assumed in the analysis. As CTPs in general are still an emergent mode of treatment, an annuities-based payment structure could be used as means of risk mitigation by the payer[328,334]. If DopaCell/DopaCell adjusted is likened to DBS in terms of resource use, then the procedure employed to administer the DopaCell/DopaCell adjusted could be funded by the NHS Commissioning Board and reimbursed using the appropriate healthcare resource group[374] (section 7.1.1.3.a.). Thus, there is scope for a pre-existing reimbursement funding pathway for the CTP to follow, which is different from the Cancer Drug Fund currently being used for CAR-T cell and gene based therapies[329].

8.3.1. Limitations and considerations about the analysis

As with any model based on assumptions, there are certain limitations that cannot be overcome but should be noted. A major limitation of the analyses carried out is that the DopaCell data assumes full recovery and QALY gains which is not likely to be achieved. However, the purpose of this overestimation of efficacy is to ascertain if the DopaCell could ever a be cost-effective treatment option. Therefore, full gains are presumed on the basis that if the product is not cost-effective at full QALY gains, it is unlikely to ever be cost-effective. DopaCell adjusted aims to address this limitation by providing a scenario when the CTP provides more realistic QALY gains. These gains are informed by available data from foetal tissues transplant trials, which have a similar mode of action to the DopaCell/DopaCell adjusted, however the cell source is hESC (for DopaCell/DopaCell adjusted).

Another limitation of the analysis is the use of a static healthy utility value (0 % discounting rate) for all modes of treatment. In particular, for medication therapy this overestimates its costs-effectiveness as it is putative that health utility decreases overtime, with the use of medication as the patient experiences reduced response and efficacy. Thus, the cost per QALY would increase over the ten-year period as the cost increases and the healthy utility decreases. Lastly, the use of immunosuppressants until year two could be considered an overestimation as the typical length of use is 6-12 months. Thus, DopaCell/DopaCell adjusted would become cost-effective at an earlier time point, if shorter time lengths of immunosuppressant use are employed.

8.3.2. Adoption of the DopaCell/DopaCell adjusted under the context of the CHART workshop

The following section aims to evaluate the adoption potential of DopaCell/DopaCell adjusted by employing the key points of consideration from the six adoption challenge themes from Chapter 7.

Evidence of clinical effectiveness – DopaCell/DopaCell adjusted would face similar challenges to the CAR-T therapies in providing long-term evidence of clinical effectiveness. Using the preclinical work that has been carried out to demonstrate that hESCs are comparable to the foetal tissue cells, it could be inferred that the *in vivo* efficacy would translate for hESCs in human subjects. Currently only surrogate data from the foetal tissue clinical trials can be used as a measure of the efficacy of DopaCell/DopaCell adjusted. Thus far, some patients have shown notable clinical improvements and engrafted cells have been shown to remain intact for up to 24-years. However, these results have been obtained from clinical trials of small sample sizes (7 – 50 participants)[84,384]. In addition, the lack of randomised clinical trials (RCTs) would not be deemed favourable by regulators and payers, thus RCTs would need to be employed to ensure robust evidence generation. Progression free survival (PFS) would need to be demonstrated, however the length of adequate PFS would need to be established as PD is a disease of the elderly, thus the PFS could be shorter than other illnesses i.e. paediatric and cancer illnesses.

Health economics – health economic evaluation of DopaCell/DopaCell adjusted would not be different to how other health technologies are evaluated. The NICE and CHE reports have demonstrated that the current methods of evaluation were applicable [328,339]. The main challenge from an evaluation perspective is the uncertainty associated with a novel therapy, particularly if there is limited evidence of long-term clinical effectiveness.

Social value towards regenerative medicine – as a form of treatment DopaCell/DopaCell adjusted is likely to be looked upon favourably as there is currently no mode of treatment that would offer the potential neuro restorative effects of DopaCell/DopaCell adjusted. PD patients and charities are advocates for CTP based treatments, this is evident from the encouragement and funding of research in the area stem cell therapies for PD. In addition, there are commercial companies such as BlueRock Therapeutics that have funded with vast amounts of money (USD 225 million) to progress CTPs treatments[385,386]. For instance, their first product in their pipeline is a H9 hESC derived dopaminergic neuro progenitor product, similar to DopaCell/DopaCell adjusted, which will be commencing first-in-human clinical trials in 2019[84,387].

Health Technology Assessment – similar to the health economics, there would be no difference in methodology employed for DopaCell/DopaCell adjusted in terms of the HTA process. The requirements to demonstrate value would still be the same in the case of HTA through NICE or

CADTH. Under the CADTH deliberative framework, DopaCell/DopaCell adjusted would be evaluated as a clinical intervention therefore additional elements such as implementation would need to be considered, in addition to the core measures of cost and clinical effectiveness.

Briefly, using NICE's factors for value (**Figure 100**) (*clinical effectiveness, cost effectiveness, end of life, innovation, degree of need, equity* and *non-health objectives*) DopaCell/DopaCell adjusted would be cost effective as demonstrated by the analysis performed above. Assuming similar results to the foetal trials, DopaCell/DopaCell adjusted would result in clinical effectiveness and potentially extend patient end of life, particularly in terms of quality of life. Due to the difference in mode of action and the potential to be neurorestorative, DopaCell/DopaCell adjusted would likely provide value in terms of innovation. In addition, DopaCell/DopaCell adjusted could meet a high degree of need in a disease area were symptom management is typically the best that can be achieved. Both equity and non-health objectives are difficult to determine as they are subjective to the internal priorities and organisational strategies of healthcare providers such as NHS England and its clinical commissioning groups. Overall, under the cost and evidence assumptions made, DopaCell/DopaCell adjusted would be likely to undergo a favourable HTA through NICE. In addition, DopaCell/DopaCell adjusted is cost effective at a range of different price ranges and even at the realistic health utility level of DopaCell adjusted. This is in comparison to the CTPs such as Kymriah and Yescarta which have very high price tags of up to £282,000[388].

Payment system mechanisms – as highlighted in Chapter 7, sustainable methods of payments for CTPs are yet to be established. In the case of DopaCell/DopaCell adjusted how it would be commissioned would depend on the price that is offered to the payer. The prices shown in the present work have ranged from £15,000 to a realistic £180,000 per patient. Thus, depending on the position of the price on this spectrum, routine commissioning could be possible, potentially even without the use of MEAs assuming a lower price and sufficient evidence generation. Under the assumption that DopaCell/DopaCell adjusted is similar to DBS in terms of resource utilisation, it could be funded *via* payment for the appropriate Adult Neurosurgery and Neurosciences Clinical Reference Groups used by the NHS Commissioning Board. However, if a higher price tag is chosen (i.e. higher than the ~£23,000 price of DBS), MEAs and commercial agreements would be required, how these commercial agreements would work is unknown as the agreements are confidential, as in the cases of Yescarta and Kymriah[175].

Adoption and implementation – many of the lessons from the CAR-T therapies examples in Chapter 7 would need to be considered for DopaCell/DopaCell adjusted, particularly in terms of surgical capacity and personnel training. However, DopaCell/DopaCell adjusted is arguably a well-placed CTP for adoption, the themes explored above demonstrated that it can be assessed and would potentially have a favourable recommendation on the condition that sufficient and robust evidence could be generated.

DopaCell/DopaCell adjusted's similarity to DBS in terms of being a surgical intervention would suggest that it could be easily implemented using the current care pathway employed by DBS. Issues such as personnel training and infrastructure would still need to be considered, particularly in terms of the manipulation and supply chain logistics associated with the cells themselves, as they differ significantly to the DBS device considerations. Therefore, implementation would need to be focused on appropriate technologies for handling the cells to ensure they are thawed and formulated appropriately without impacting their quality, efficacy and safety profile. The allogenic nature of DopaCell/DopaCell adjusted presents the opportunity for a cryopreserved off-the-shelf product, which would make supply chain logistics much simpler and increase access to centres further away from manufacturing sites. This could also reduce costs and reduce waiting times for product access, which is presently a challenge for CAR-T therapies due to their complex autologous manufacturing process.

8.4. Conclusions

The analysis carried out has demonstrated the potential value and cost-effectiveness of a CTP for the treatment of PD. In comparison to the available treatment strategies, a CTP for PD could offer improved efficacy, clinical improvements, cost savings and a neurorestorative mode of action. This would benefit patients in terms of their health-related quality of life and reduce the economic burden associated with the treatment and management of PD. The headroom assessment and cost-effectiveness analysis provided information to answer the questions previously posed in section 8.2 (page - 317 -).

- What is the price potential of a hESC-based therapy for PD?

  The potential price would vary on the level of efficacy achieved by the therapy, the headroom analysis performed here shows that the price could be between £50,000 – £134,000 per patient. This price is significantly lower compared to other cell and gene therapy products that have been recently approved such as Yescarta and Kymriah at £282,000 (Kymriah)[388]. However, the price setting results demonstrated that prices of between £180,000 to £255, 000 could be used while remaining cost-effective.

- Would a hESC-based therapy for PD be cost-effective?

  Yes – the results demonstrate that a CTP such as DopaCell/DopaCell adjusted would be a cost-effective treatment option for PD. Even with the use of immunosuppressants DopaCell/DopaCell adjusted dominates medication therapy, which is the current standard of care. In addition, the CTP is more cost-effective than DBS in the long-term and provides greater cost savings. This is rare for CTPs as they are often costlier than the standard of care, thus a CTP for PD would be likely to receive a favourable recommendation from NICE given the assumptions made, particularly as both DopaCell/DopaCell adjusted are below the £30,000 WTP threshold. In addition, sale price analysis shows that the CTP can be priced at up to £255,000 per patient and still be cost-effective over the ten-year period analysed.

- Could a hESC-based therapy lower the economic burden of PD?

  Yes – a CTP mode of treatment for PD would provide clinical and quality of life improvements that would reduce the majority of costs associated with PD. This is particularly the case as PD is a chronic disease, thus in the long-term significant cost savings would be realised by retarding the progression to the advanced and costlier stages of the disease.

Similar to the aforementioned cost-effectiveness analyses (8.1.2. Health economic evaluations for PD treatment strategies), the DBS and DopaCell analysis carried out in the present work has demonstrated that both interventions would be cost-effective over the use of medication therapy. Medication therapy such as L-DOPA has been in use before health economic evaluation became a prominent exercise. Thus, it can be inferred that if medication therapies such as L-DOPA were subjected to an economic evaluation in the present day, there is a high probability they would not be considered for adoption as they offer minimal health benefits and result in incremental costs in the long term. As a result, they would not be cost-effective, due to their high costs and inability to be disease modifying or to significantly delay the progression of the disease and worsening of symptoms.

The climate for cell and gene therapies is currently emergent however, for the first time since the advent of the area there is continuing and improved understanding of the adoption and reimbursement pathway of these therapies. This is due to the precedence being set by products such as Yescarta and Kymriah[175,283,328,388]. Whilst a large number of assumptions have had to be made in the present work, it is likely that a therapy such as DopaCell would be adopted based on the adoption considerations addressed in Chapter 7. A CTP such as DopaCell/DopaCell adjusted is well placed for evaluation and adoption, particularly if it can afford health benefits and cost savings over a prolonged period, which are unattainable using current standard of care approaches. The true reality of these potential benefits will only be realised once clinical trials using hESCs have been performed, however foetal tissue trials offer hope as patient health improvements have been achieved and sustained over prolonged periods for some patients.

Chapter 9:

Thesis Conclusions

Chapter 9. Thesis conclusions

Chapter 2

The present work used a multidisciplinary approach to investigate the translational requirements for successful product realisation of a dopaminergic cell therapy product (CTP). The scope for the use of CTPs for Parkinson's disease (PD) was discussed and highlighted the limited benefits of current modes of treatment that only manage the symptoms of PD. A CTP mode of treatment was shown to have the potential to offer quality of life improvements that are unparalleled by therapeutic strategies such as medication therapy. The use of CTPs does not stop the progression of the disease, thus is it not a cure, however the engraftment of ventral mesencephalic dopaminergic (vmDA) neuroprogenitors results in restoration of native dopaminergic circuitry. This is a neurorestorative mode of action that significantly retards the progression of the disease and can potentially delay the onset of PD motor symptoms. This is a powerful mode of action, especially as the addition of ten to twenty years of improved quality of life and reduced medication intake would be invaluable for PD elderly patients.

Progress has been made in deriving cells for engraftment and a range of different cell sources have been explored in the literature. The present work has focused on the methods that mimic embryogenesis, the resultant protocols have been through many iterations over the last two decades (**Figure 8**). Key markers such as FOXA2 and cell characteristics such as axon projection have been identified as critical quality attributes (CQAs) in the preclinical studies that have been employed. However, the challenge remains in being able to produce the cells to meet the CQAs in a highly reproducible, standardised, scalable and cost-effective manufacturing process, while satisfying regulators in terms of product safety and efficacy.

Section 2.3. Manufacturing considerations explored some of the key manufacturing process considerations concerning the production of CTPs. Assay generation to ensure product purity and appropriate product identification were some of the key considerations. In addition, potency and safety were highlighted as an integral elements of CTP development, specifically when applying for regulatory approval. In particular, potency forms the basis of process understanding and closely involves concepts such as Quality by Design (QbD) and Design of Experiments (DoE). The role of effective and efficient supply chains was also addressed and crucially the role of technology in the development of CTPs. Fundamentally, it is necessary to consider all of these elements at an early stage of the product development timeline, as means of having a proactive stance and to mitigate risk. With complex products such as CTPs, information that is well characterised and well understood facilitates in depth process control and understanding that allows developers to make informed changes based on a defined design space. Ultimately this can help to increase the chances of commercial and regulatory success.

Commercial success is not guarantee even if a robust scientific program has been used that achieves regulatory approval. A product's commercial success is determined by its ability to enter the market, to reach patients, to benefit patients and finally to be reimbursed. Reimbursement is a necessary goal to achieve as the development costs incurred need to be recovered by the developers in order to fund new research and provide gross profits. Health economics and economic evaluations are the concepts that must be addressed in order to determine the commercial viability and cost-effectiveness of a healthcare product. The basic concepts of health economic decision modelling and the headroom methods were discussed in Chapter 2 (section 2.4) to provide understanding of the essential considerations around the topic of health technology adoption. Issues such as evidence generation, (clinical trial designs), technology appropriateness and budgets concerns were touched upon to highlight the considerations for developers as payers determine the cost effectiveness of their products. For payers new technologies need to provide patient benefits at a cost that satisfies their budgets. On the other hand, developers want their new technologies to be adopted and reimbursed as much as possible. Thus, a difficult decision must be made to satisfy both parties while benefitting the patients.

Chapter 4

Hypothesis: Standardised cell culture procedures result in less variation in growth and cell characteristics.

For successful product manufacture, the standardisation of processes and quality is desired, for CTPs this is not only desired but imperative as the products will end up being administered to patients. The work in this chapter highlighted the challenges associated with biological and protocol variation that has hampered the robust standardisation of CTPs. The use of the EC 2102Ep cell line illustrated that even when a putative reference cell-line is used, there is innate biological variation that cannot be controlled. This was highlighted by the use of data driven, robust and standardised culture conditions that provided an insight into some of the behaviours and characteristics of the cells. Importantly, the feeding regimes that the cells are exposed demonstrated a significant effect on SMR and phenotypic marker expression. Initially, seeding density was proposed to a major source of culture variation, however the experiments illustrated that while density has a role in the observed variation, it is actually a complex interaction between density and feeding regime that impacts cell growth, gene expression and cell phenotype. The longitudinal experiment highlighted the value of understanding the effects of long-term passaging as this in itself resulted in cell characteristic changes. For allogenic therapies that might require hESCs pre-culture processes, the effect of the pre-culture period will need to be understood so that the cells are guaranteed to have the same CQA upon initiation of all differentiation process.

The variation observed in this chapter demonstrated the issues that need to be addressed by the cell therapy industry (CTI) in order for products to be developed in a well standardised manner for better translation. Increased standardisation and process control allow for CQAs to be achieved, which is important for products that will be introduced to a patient's body. This will become more of a challenge as the industry moves towards allogenic therapies as it will be imperative to ensure batch to batch variation is kept as low as possible and product potency is guaranteed in every release. Modes of standardisation that are employed will need to ensure that they have process monitoring capabilities to ensure that the product is within its CQA tolerance range.

To further aid in variation reduction, human operators will need to be removed from processing cells as much as possible, systems such as the Miltenyi prodigy are potentially well placed to assist in the removal of operator variation. Automation along with well-defined and standardised cell-culture protocols should result in less variation in growth and cell characteristics. However, biological variation will still remain a challenge to address, nonetheless the removal of operator variation results in the de-compounding of both sources of variation. This is integral for reproducibility and successful use of cell material in cell therapy manufacturing.

Chapter 5

Hypothesis: Variation of measured cell outputs can be reduced by characterising input cell material and standardised procedures.

The importance of standardisation was highlighted in Chapter 4; standardisation is also crucial to the culture of input cell material for further processes. Critical material attributes (CMAs) of inputs such as pluripotent cells are important to the manufacturing process, the pluripotent cells that are used in the differentiation should be of the best quality. However, there is limited guidance as to what the best quality should be, thus the work in this chapter focused on creating a profile of characteristics that could be used to benchmark good quality input cells. This was important as the growth dynamics and characteristics of the cells prior to differentiation process should be understood and robust CQAs should be established. The culture process of the pluripotent cells should also be standardised to ensure that input cell material is consistent prior to differentiation, as inconsistency in the input cell material would inevitably result in variation of the end product. This is typically the case in autologous therapies such as CAR-T therapies where patient cell variation is common, resulting in differences in cell quality and cell yields. For allogenic therapies such as the candidate therapy, this can be easily mitigated by standardisation of input CMAs to ensure product quality consistency.

The range of experiments carried out established optimal culture parameters such as passage length, seeding density and feeding regime that would result in consistent output pluripotent cells for the differentiation. The reduction of passage length and feeding frequency that was demonstrated is desirable in product manufacturing as it uses less resources and thus reduces costs. Additionally, in a manual cell culture process this would result in less operator intervention, which would mitigate the risk of variation.

Chapter 6

Hypothesis: Multi-parameter analysis and high-resolution phenotype analysis can provide information on differentiation trajectory, critical process parameters and critical quality attributes.

Chapter 6 illustrated that CHIR99021 is a major driving force for the differentiation process. In addition, SHH-C24II, by itself and in combination with CHIR99021, is important for obtaining cells with the putative FOXA2 dopaminergic marker. PAX6 exhibited interesting trends, particularly at the earlier stages of the differentiation process as its express or lack of, can potentially be used to determine the phenotypic trajectory of the cells. This predictive capability can potentially be used as an early screening assay to ensure that the cells are progressing towards the desired CQAs, particularly in terms of the determinant phenotypic cell markers. Density and nutrient supplementation were also key factors for successful cell survival and differentiation, the DoE results illustrated the relationship and complex interactions of the various factors involved in the differentiation process. These complex interactions need to be well characterised and linked to both the critical process parameters (CPP) and CQAs to ensure that the drivers of successful differentiation are identified and controlled.

The appropriateness of characterisation tools and cell markers was an important element highlighted in Chapter 6. The flow cytometry panels utilised, both the Miltenyi Biotec and in-house protocol, illustrated that the distinguishing vmDA markers FOXA2 and OTX2 were not exclusive to the desired cell vmDA cell type. Both markers were found in pluripotent cells and in cells differentiated under a range of culture conditions, suggesting that the markers do not adequately discriminate/select for the desired cell type. This presents a fundamental issue to address, as only homogenous cell populations should be introduced to the patients, thus it is important to have discriminatory characteristics that can be used to sort the cells. Moreover, as cell identification and sorting information/assays form part of the technical dossier for the product, it is important they are robust and validated as early as possible.

It is evident from the work carried out that cell differentiation is a very complex procedure that requires the consideration of various different processes and obtaining the correct cell phenotype is integral to a successful differentiation. In combination with the consideration of process standardisation, being able

to track and understand the trajectory of the cells in the differentiation process offers an invaluable ability to validate CQAs and their impact throughout the process. This facilitates data driven process control, which can allow for proactive decision making based on the behaviour of the cells at an early stage. For instance, a decision to terminate or progress with a differentiation process can be made using early signifiers to predict whether or not the cells are on the correct trajectory. This capability would be enabled by integrated process analytic technologies that allow for in-process sampling and monitoring. This ability is one that the CTI is striving for as it permits for enhanced data driven go/no-go process decision making and process control.

The lack of adequate process transfer experienced in the present work offers a valuable lesson about the importance of robust process transfer activities. In addition, the value of validating processes before they are transferred to other parties is highlighted as this would allow for more streamlined process development activities. The complexity of the differentiation process and the multiple parameters involved means that it is imperative to remove any sources of ambiguity that could lead to process variation and ultimately product variation. Results from Chapter 6 revealed that the protocol conditions as prescribed in the published literature did not result in successful differentiation and cell survival. Significant changes to the protocol had to be made by the author and others within the research group at Loughborough University, in order for the cells to survive and differentiate towards the desired vmDA phenotype. Moreover, this highlights the necessity of a vigorous process transfer procedure in order to ensure that results can be recapitulated by different researchers in different institutions, which is imperative for process development and protocol translation.

Therefore, standardisation is yet again highlighted as a key factor to consider, in addition the use of a DoE approach demonstrates the value of experimental designs that facilitate robust data collection which provides in depth knowledge of the CPP and CQA interaction. Employment of such experimental designs at an early stage of protocol and product development is encouraged as it permits for well-informed product development in a validated manner. Furthermore, aspects such as the equipment used, staff training procedures and supply chain should be validated through comparability studies, to ensure that the resultant protocols are inherently robust enough to produce the desired end product. This will greatly assist future activities such as process transfer, protocol translation and scale up, which are vital activities for successful process development and product realisation of CTPs.

Chapter 7

Hypothesis: Cell therapy products are more complex than pharmaceutical therapies, therefore they should be evaluated differently for HTA, reimbursement and adoption.

Chapter 7 illustrated that there are challenges that needs to be tackled regarding CTP adoption, however the majority of these challenges are not unique to CTPs. The current methods of evaluation are applicable to CTPs, the biggest challenge for these novel therapies is providing evidence that reduces the uncertainty of their long-term effects. The generation of evidence of clinical effectiveness needs to be improved by ensuring trial designs employ robust controls and increase their sample sizes while collecting data in the relevant setting. In addition, trials should conduct comparisons with established practice and evaluate relevant outcomes based on their relationship to health-related quality of life and mortality. The use of modelling approaches based on clinical plausibility and presently available data can help to deal the uncertainty of evidence at the time of submission. Furthermore, incentives for real world evidence capture should be established to ensure that data is gathered and can be used to further evaluate the therapies for their long-term clinical effectiveness and adverse effects. The post-appraisals evidence of CAR-T therapies, albeit within a short period of time, has shown that patient selection is important; in addition, both rates of immunosuppressant use and readmission were higher than in the clinical trial setting. This highlights the value of data obtained in real world context, which can only add to the depth of knowledge and evidence about the use of a technology.

The implementation of CTPs is an area that will require concerted efforts from multiple stakeholders to ensure that the pathway for CTPs is efficient and effective. As the CTI develops there needs to be greater interaction with policy makers, as political will is essential for research to progress into meaningful efforts. For instance, 'top level' buy-in from policy makers has been a key driving force in the rapid adoption of CAR-T therapies in the UK, where commercial deals were established within ten days of approval[175]. This illustrates the value of support from policy makers in order to profoundly tackle and amend the areas that need changes and adjustments to better support CTP implementation. One area identified in the workshop, is the need to improve patient management and data management to aid in evidence generation and facilitate outcome-based payment mechanisms. This is a significant challenge in a system such as Canada and will require interprovincial efforts to appropriately and adequately share data. This is essential as CTPs such as CAR-T therapies necessitate a collaborative effort to avoid duplication and ensure both value and equity. In the UK, it is important to effectively harness the value of the one payer system, to efficiently capture patient data and effectively operate outcome-based reimbursement models.

Payment mechanisms remain a challenge that needs further clarification, in addition the sustainability of methods currently used to pay for expensive therapies will need to be evaluated, prior to the onset of more CTPs being approved. This will unavoidably entail creative payment models that are inspired by other industries such as the mining, finance and aeronautical industries, that finance high value projects and activities. This is vital as funding pots such as the Cancer Drug Fund are not a realistic solution for routine commissioning of all potential CTPs.

Ultimately, adoption is only necessary if the technologies in question can demonstrated value and address an unmet clinical need where other options have failed or are unavailable. This is because CTPs are very complex therapies that are concomitant with high risk and uncertainty; which can difficult to mitigate and would have burdensome high costs associated with disinvestment.

Chapter 8

Hypothesis: A cell therapy product treatment strategy can provide cost savings in comparison to current standard of care for PD

Assessment of the hypothetical DopaCell CTP demonstrated that under the assumptions made, a CTP would be a cost-effective treatment for PD. Compared to the other options such as deep brain stimulation (DBS) and mediation therapy, DopaCell could result in significant cost savings even with the use of immunosuppressants. The advantage of DopaCell compared to DBS, which is also cost saving, is the potential neurorestorative mode of action that would lead to quality of life improvements and slow down progression of the disease to its advanced and costlier stages.

The costs and finances of DopaCell are favourable for both the payer and manufacturer as both DopaCell and DopaCell adjusted are below the £30,000 cost-effectiveness threshold at prices up to £255,000 and £180,00, respectively. If the more realistic case of DopaCell adjusted is used, a £180,000 price tag per patient would allow for significant gross profits while affording health gains currently unattainable through the standard of care. Given the £15,000 to £180,000 price setting range, a commercial deal would be necessary to obtain a price agreement that equates to value for both the payer and the manufacturer. Arguably, the lack of disease modifying therapies for PD, allows DopaCell/DopaCell adjusted to provide value by addressing an unmet clinical need and non-health objectives such as lowering the economic burden of PD, which medication therapy currently does not do.

DopaCell/DopaCell adjusted is well placed for evaluation and adoption, however the success of such a therapy relies on an efficient manufacturing process and clinical trials that can generate robust evidence to appease both the regulators and the payers. Given the progress that has been made by BlueRock Therapeutics, it will be soon become evident how far a CTP for PD is for being a product and how cost-effective it can be.

Overall Summary

There is a clinical need for better treatments for PD that can reduce patient disease burden, improve quality of life and reduce the economic burden of PD on both patients and healthcare providers. One, promising mode of treatment is a CTP, this has the potential to be a both clinically and commercially viable option. There are currently several CTPs for PD at different development and clinical trial stages, illustrating the inclination towards this mode of treatment[84]. This has been intensified by the recent approval of products such CAR-T therapies, which have galvanised the CTI. Through the different aspects explored in the present work, key translational requirements for a manufactured dopaminergic neuroprogenitor CTP and CTPs in general have been identified.

Progress has been made in terms of understanding the key process parameters for the differentiation process, lessons such as the importance of density and small molecule concentrations have highlighted the sensitivity of the differentiation to process parameters. This was illustrated by the range of experiments that demonstrated poor growth and/or cell death due to cell densities and small molecule concentrations that were inadequate for the process. Process transfer activities should be robust and validated to ensure that process development activities are streamlined and do not have to establish the CPPs, which is a protocol development activity. This has been shown to be necessary in the present work due to differences in cell behaviour and outcomes while following shared and published protocol. This has been the case on two occasions, using the NIBSC protocol and also the Lund protocol, highlighting the importance of protocol standardisation. In particular, the present work has shown that different seeding densities and feeding strategies can affect cell quality in terms of cell growth, cell viability, cell phenotype and cell metabolic rates. Thus, addressing process transfer, protocol reproducibility, process control, CQA definition and standardisation are activities that have been identified as key requirements in the present work, that should be focussed on at the earliest convenience to enable successful CTP translation.

Speaking to regulators, HTA agencies and payers as early as possible and throughout the product and clinical development pathway is encouraged. In addition, iterative and informative process understanding should be carried out in order to facilitate robust and data driven product realisation. Early engagement with the adoption pathway is a crucial step for manufacturers to take as early as possible, both NICE and CADTH's scientific advice programmes provide a platform for manufacturers to better understand the HTA evidence requirements prior to submission. There are many considerations that manufacturers need to account for at an early stage to ensure that they are well placed for regulatory and reimbursement assessment. For instance, it is evident that different countries have different costs associated with the treatment of different diseases such PD. Thus, different HTA and reimbursement mechanisms need to be considered at an early stage as economic evaluations would differ in each

country. Therefore, using techniques such as the headroom method and early discussions with payers, developers can determine which markets to enter and in which order. An informed sequence of market authorisation and reimbursement in different countries could facilitate in evidence generation, prior to applying to other payers from different countries, which might require more rigorous evidence of clinical effectiveness and cost-effectiveness. Overall, western countries spend more money on the PD care and therefore have the potential to result in higher reimbursement as they are likely to benefit from greater savings, as a result of offsetting costs spent on continuous treatment of such a progressive chronic disease.

Price and affordability have been identified as factors hampering the adoption and reimbursement of CTPs. In some cases, it is unclear why the therapies are as expensive as they are, arguably the autologous nature of products such as CAR-T therapies inherently results in high cost of goods. In addition, due to their per patient manufacturing process, there are no economies of scale to offset production costs. However, it is probable that as the CTI develops and more therapies become available, costs will become lower. This is likely to the case for therapies such as DopaCell which would be allogenic with the potential of batch production that would reduce the per batch cost of goods. The challenge that will be faced at the juncture of a higher number of approved CTPs will be how to adequately implement and adopt such novel and destructive technologies into the healthcare systems. A broader framework to approaching implementation and adoption is a key requirement, one that demands coordinated efforts between policy makers, agencies, practitioners, patients, payers and industry promotors (i.e. the Cell and Gene Therapy Catapult and Canada's Centre for Commercialization of Regenerative Medicine) to better facilitate change in a concerted, efficient and effective manner. Thus, another key translational requirement for CTPs is the engagement of relevant stakeholders to ensure the challenges of CTP adoption are on their agendas, particularly at the policy level, in order for meaningful change and support to be given to the CTI

The present work has explored protocol understanding, optimisation and standardisation; economic evaluations and the state of reimbursement and adoption for cell therapies. The prominent message from these themes is that the scientific rigour and commercial viability of a product should be contemplated as early as possible. This is with the aim of facilitating the translation of basic scientific research into valuable and adoptable products, that can deliver on the promise of providing superior treatments and health benefits to patients in need.

Future Research

The translational requirements identified in the present work have progressed understanding of the vmDA differentiation process, the potential cost effectiveness of a therapeutic PD CTP and the state of reimbursement and adoption for CTPs. Simultaneously the present work has also opened up further areas of exploration.

For example, the H9 pluripotent cell culture parameters identified in Chapter 5 would need to be validated. Further comparisons between the control condition and route 2 would need to be carried out, ideally in a longitudinal experiment as a means of observing the effects that route 2 might have on the long-term behaviour and characteristics of the cells. Orthogonal analysis of the SGR, cell viability, metabolism and phenotype would need to be performed, additionally gene expression and karyotype analysis should be employed to ensure that no fundamental and/or detrimental effects arise as a consequence of the route 2 parameters.

Further research based on the present work will need to robustly identify the process and protocol parameters that can consistently deliver the desired vmDA CQA profile. Building on from the definitive screening studies in Chapter 6, the best conditions from the studies such as M1 and C1 would need to be further validated and optimised. Once optimised, rigorous characterisation studies would be employed to ensure that the CQA profile can adequately inform product purity, safety and potency assays. These assays could then be used to devise QC, QA and product release criteria within the preclinical stages of development. In addition, the robustness of the conceived protocol would need to be validated by assessing the cells in a preclinical setting, to ensure potency and functionality in an appropriate PD animal and/or disease model. This would ascertain whether or not the optimisation process would have yielded cells of equivalent or superior quality, functionality and potency.

Another area of further research is the identification of superior phenotypic markers that can better distinguish the desired vmDA cells from other contaminant neuronal populations and pluripotent cells. This is crucial as some of the current markers used in the panels are expressed in both pluripotent and differentiated cells (even those differentiated under suboptimal conditions). Potential markers for exploration would be GBX2, BARHL1 and LMX1A. In addition, studies could be conducted to further understand the implication of PAX6 presence/absence on the differentiation trajectory.

Chapter 7 highlights that the key steps to progressing the adoption and reimbursement of CTPs will require appropriate engagement with policy makers; identifying advocacy steps; identifying partners; improving the clinical evidence base; and improving the scientific/research agenda concerning CTP adoption and reimbursement. Targeted approaches in engaging politicians to pay attention and invest in the infrastructure required to facilitate the adoption and implementation of ATMPs with the

appropriate evidence base and HTA are necessary next steps. One such approach will be the final output of the white paper being written as a result of the work in Chapter 7. From a more technical perspective, further analysis is needed regarding creative payment models. This can be achieved by looking to other industries, for example, accounting, natural resource extraction, aerospace, risk fund hedging, high financing, other, and partnering with these groups as appropriate.

The work carried out in chapter 8 can be expanded upon by carrying out iterative headroom assessments as more information is obtained about the potency and efficacy of the product, during product development and eventually during the clinical trials. This will result in better informed data concerning the gross profit and price potentials of the PD CTP. Furthermore, as development progresses the cost of goods modelling would need to be iteratively carried out as additional development and manufacturing process data is obtained. Data such as batch/lot failure rates, facility costs and development amortisation would be gained and incorporated to ensure that the model is adequately representative of the development and manufacturing costs, which would ultimately be used for price setting negotiations

Chapter 10:

References

Chapter 10. References

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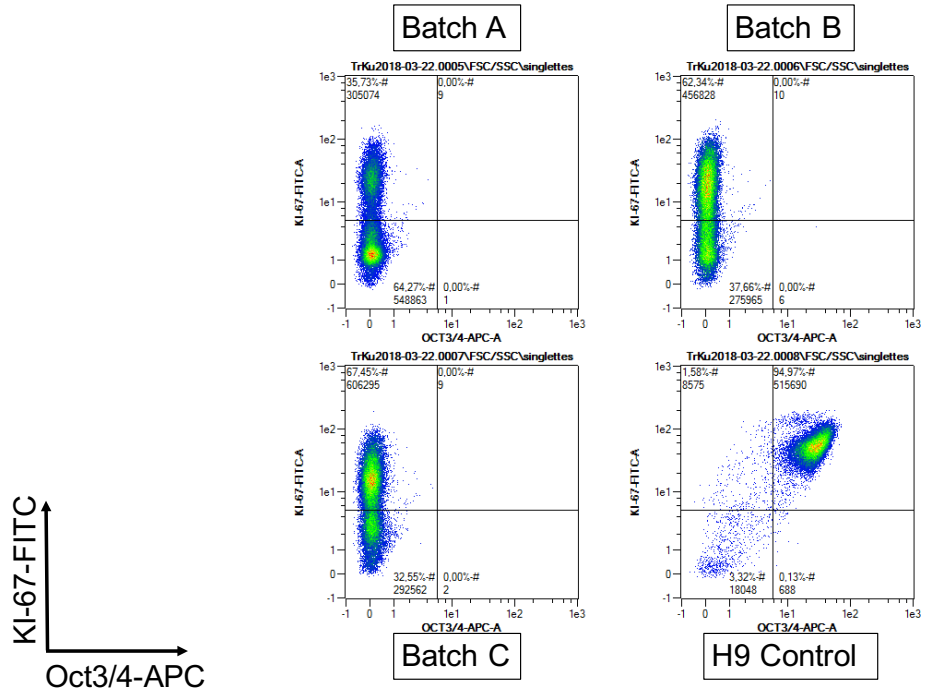
Chapter 11:

Appendices

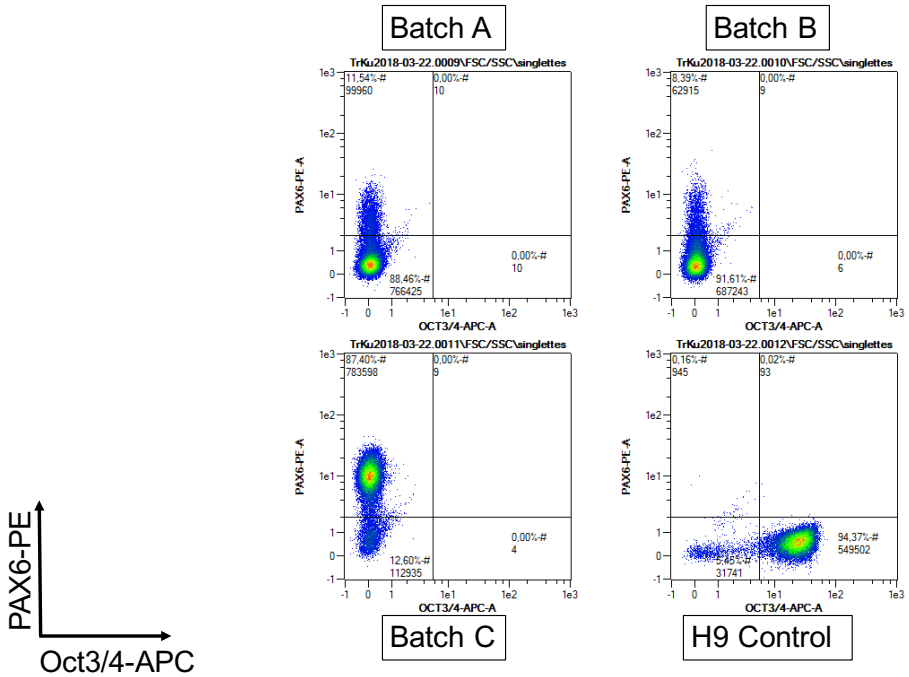
Chapter 11. Appendices

Appendix 1. Miltenyi Biotec Panel 1 flow cytometry analysis of H9 vmDA cells cultured at the Centre for Biological Engineering, Loughborough University.

180322 – Flow QC Analysis mesDA batches Loughborough
Panel 1: Pax6-PE – Oct3/4-APC – KI-67-FITC

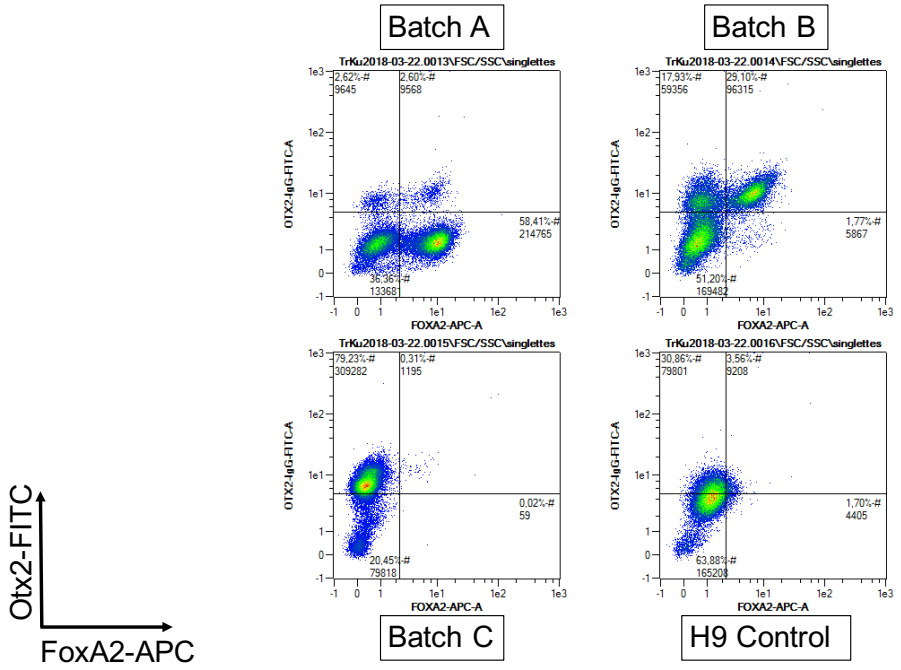


180322 – Flow QC Analysis mesDA batches Loughborough
Panel 1: Pax6-PE – Oct3/4-APC – KI-67-FITC

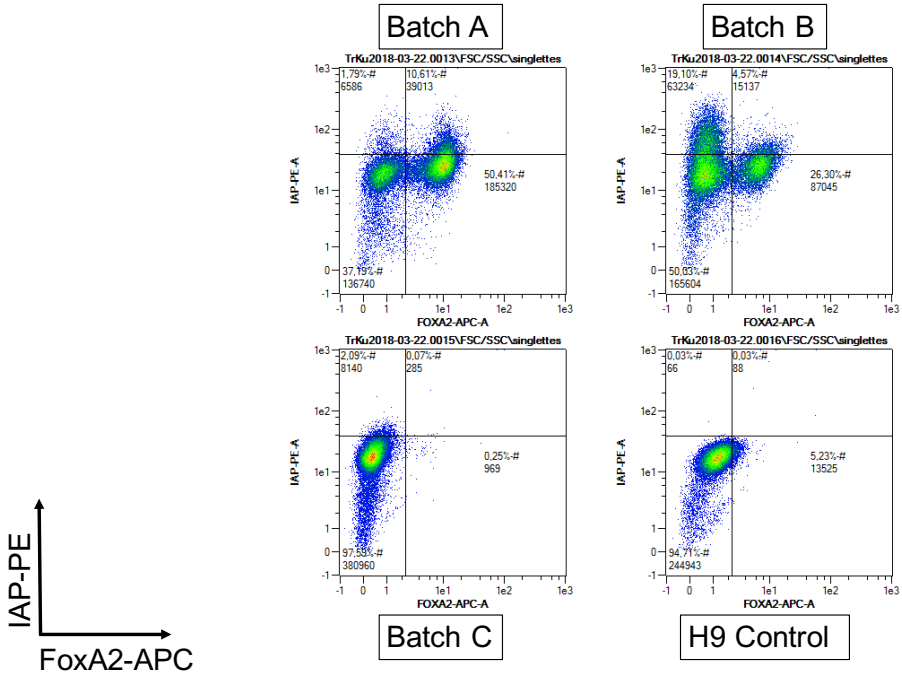


Appendix 2. Miltenyi Biotec Panel 2 flow cytometry analysis of H9 vmDA cells cultured at the Centre for Biological Engineering, Loughborough University.

180322 – Flow QC Analysis mesDA batches Loughborough
Panel 2: IAP-PE 1:30 – FoxA2-APC 1:11 – Otx2 -FITC

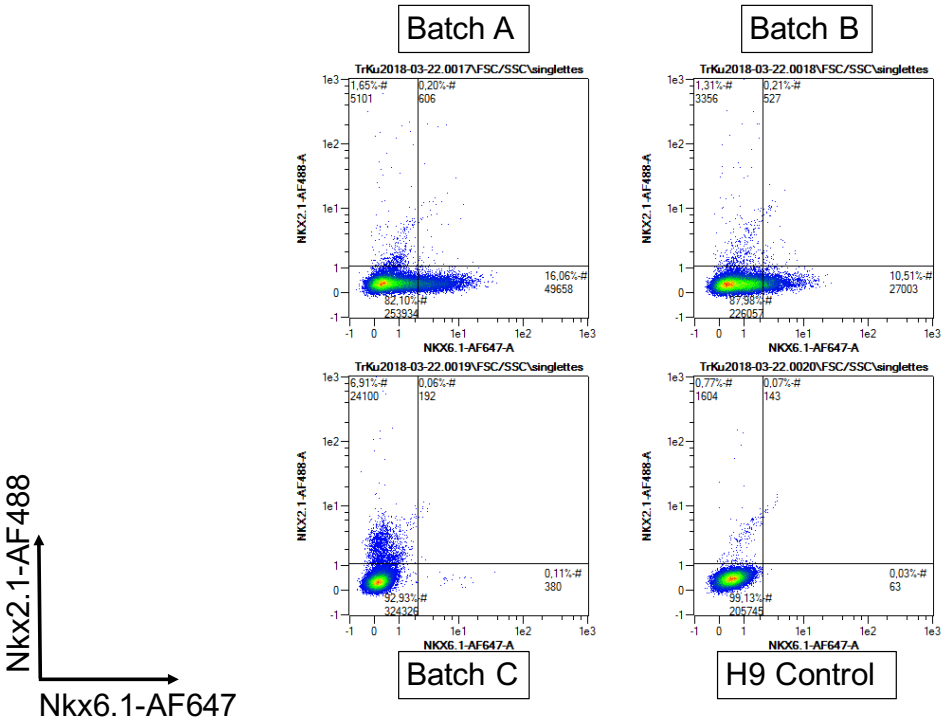


180322 – Flow QC Analysis mesDA batches Loughborough
Panel 2: IAP-PE 1:30 – FoxA2-APC 1:11 – Otx2 -FITC

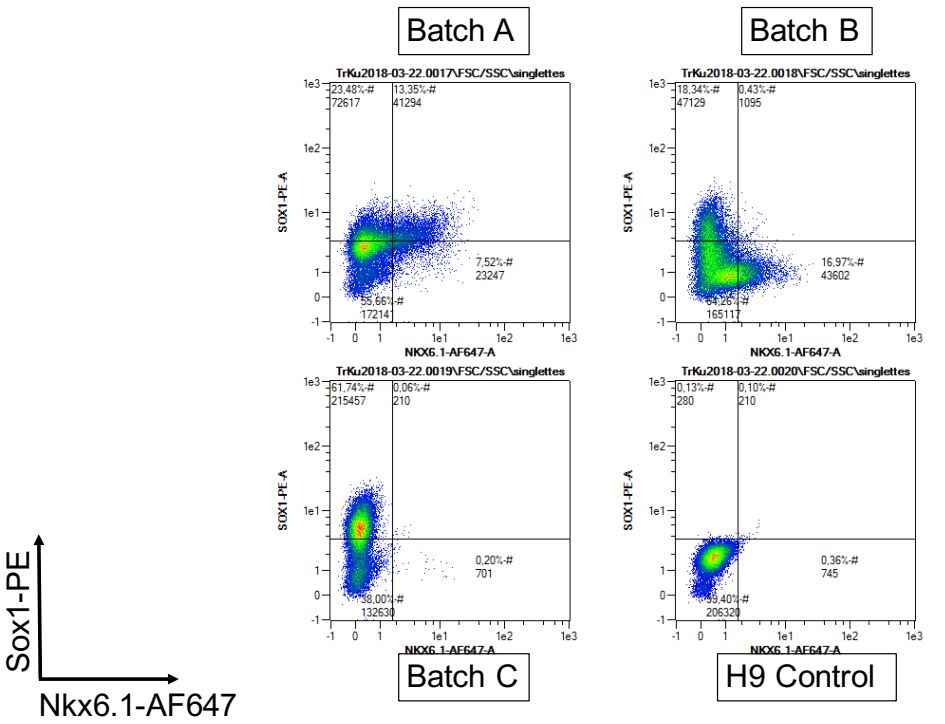


Appendix 3. Miltenyi Biotec Panel 3 flow cytometry analysis of H9 vmDA cells cultured at the Centre for Biological Engineering, Loughborough University.

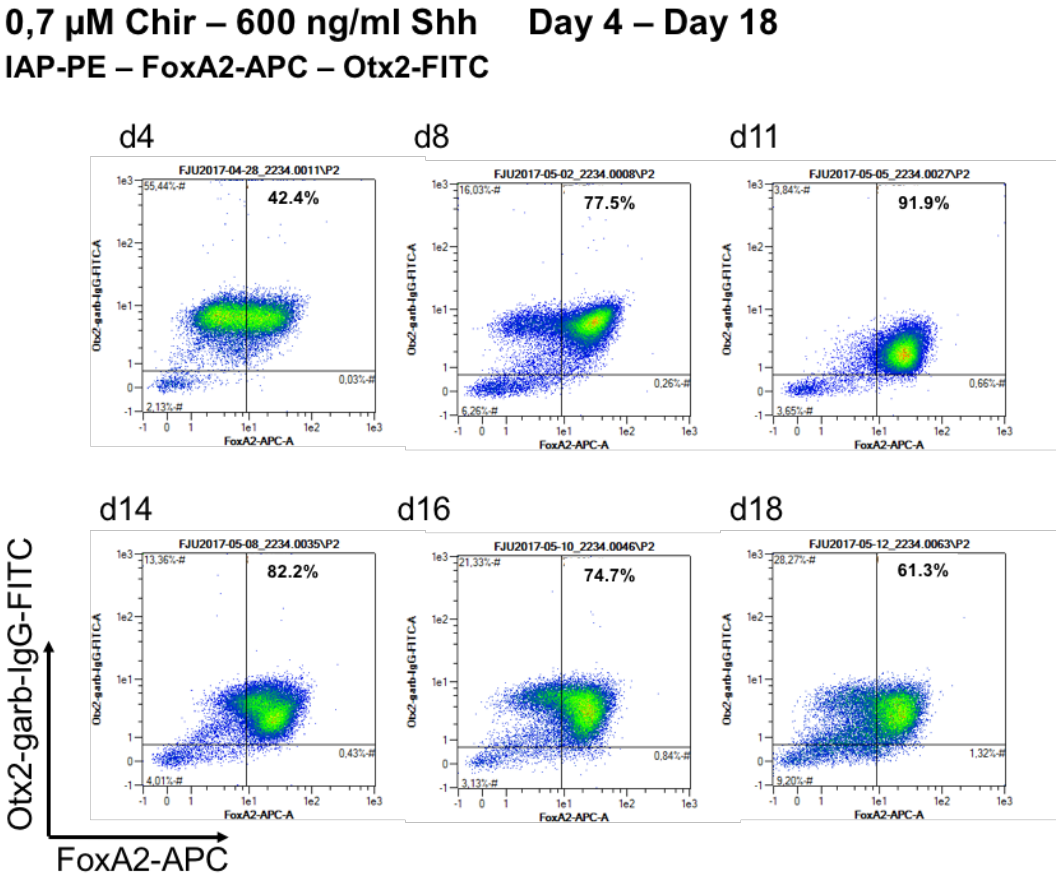
180322 – Flow QC Analysis mesDA batches Loughborough
Panel 3: Nkx2.1-AF488 - Nkx6.1-AF647– Sox1-PE



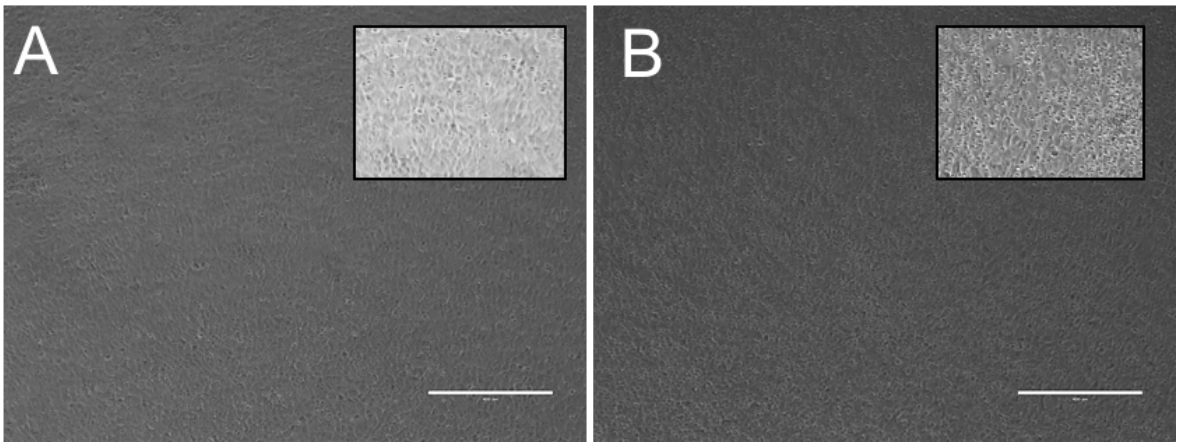
180322 – Flow QC Analysis mesDA batches Loughborough
Panel 3: Nkx2.1-AF488 - Nkx6.1-AF647– Sox1-PE



Appendix 4. Miltenyi Biotec Panel 2 flow cytometry analysis of H9 vmDA cells cultured by Miltenyi Biotec personnel. Plots show cells at six different time points with varying levels of FOXA2 and OTX2 expression.



Appendix 5. Images of cells on day 12 of the differentiation process. (**A**) Cells seeded at less than the recommended 800,000 cells/cm^2. (**B**) Cells seeded at the recommended 800,000 cells/cm^2. Cells in **A** have less debris and flowing cells in comparison to cells at the higher density **B.** Scale bar = 400 µm.



Appendix 6. Payment by Results case studies. Extracted from Gateway Ref: 18135 – A simple guide to Payment by Results.

	Patient A	Patient B
Treatment	Elective caesarean during a 7 day spell in April	Emergency admission for fragility hip fracture in April
Code	ICD-10 codes are **O300 (twin pregnancy** and **Z37.2 (twin both live born)** OPCS-4 code is **R17.2 (elective lower uterine segment caesarean delivery)** Submitted to SUS in May	ICD-10 codes are **S7200 (fractured neck of femur)** and **W19.0 (unspecified fall at home)** OPCS-4 codes are **W37.1 (primary total prosthetic replacement of hip joint using cement)** and **Z94.3 (left sided operation)** Submitted to SUS in May
Group	HRG payment currency is **NZ13A (planned lower uterine caesarean section with complications)**	HRG payment currency is **HA12C (major hip procedures category 1 for trauma without complications and comorbidities)**
Tariff	Elective and non-elective spell tariff is **£2,704**	Base tariff is **£5,323**
Tariff adjustments	The expected length of stay for NZ13A is 5 days. A **long stay payment** of £394 is payable for each additional day's stay, in this case 2 days.	There is a best practice tariff for fragility hip fracture which applies to HA12C and some other HRGs. An **additional best practice payment** of £1,335 is payable where care complies with clinical characteristics of best practice. In this case, surgery within 36 hours of arrival in A&E, under expert care of a consultant geriatrician.
MFF	**Guy's and St Thomas'** has an MFF payment index of **1.2770**	**Leeds Teaching's** MFF is **1.0461**
Reimbursement	Total payment is: (£2,704 + (2 x £394)) x 1.2770 = **£4,459** SUS extract in July informs monthly reconciliation between NHS Lambeth and Guy's and St Thomas'	Total payment is: (£5,323 + £1,335) x 1.0461 = **£6,965** SUS extract in July informs monthly reconciliation between NHS Leeds and Leeds Teaching

Appendix 7. CHART workshop agenda

Challenges in the Adoption of Regenerative Medicine Therapies

June 24-25, 2019
Toronto, Canada

Overview

This workshop will bring together key stakeholders and policy-makers in academia, medicine, government and industry to discuss key challenges that will need to be overcome for advanced therapy medicinal products (ATMPs) to successfully traverse the reimbursement and adoption pathway. The workshop will focus primarily on issues faced by Canada, the United Kingdom and other government-funded health-care systems, including:

- appropriate data generation to support decision-making for ATMPs;
- overcoming barriers to adoption, such as hospital infrastructure, drug costs and financing; and
- payment structures and cost offsetting considerations for ATMPs.

The workshop is hosted by Medicine by Design, a regenerative medicine initiative at the University of Toronto, in collaboration with CCRM, the Toronto Health Economics and Technology Assessment Collaborative (THETA) and Loughborough University (U.K.).

Outcomes

The primary goal of the workshop is to create a network of stakeholders in Canada and the U.K. that will drive discussion and research and influence policy-making around ATMP health technology assessment (HTA), reimbursement and adoption. It will do this by strengthening relationships between key stakeholders, who will collaborate over time to understand challenges to the adoption of ATMPs and facilitate focused research, development and appropriate payment system modelling.

Our short-term goals are to produce a white paper and doctoral thesis research exploring these issues from a Canada-U.K. perspective, and catalyze further workshops and symposia. We will also explore opportunities to access funding for ongoing, collaborative work.

Organizing Committee

Medicine by Design	• Allison Brown, Director, Strategy & Translation • Ann Perry, Manager, Communications & Operations • Jackie Denholm, Operations Assistant
CCRM	• Siofradh McMahon, Senior Manager, Clinical Translation & Regulatory Affairs
Loughborough University	• James Kusena, PhD candidate, Centre for Biological Engineering and Wolfson School of Mechanical and Manufacturing Engineering
THETA	• Dr. Murray Krahn, Program Director • Karen Bremner, Research Associate

Agenda

	Monday, June 24, 2019 MaRS Collaboration Centre (101 College St.) South Tower, Main Level Collaboration Room 2 (CR2)	
8:30 am-9 am	**REGISTRATION**	
9 am-9:10 am	**Welcome**	**Speaker:** **Dr. Vivek Goel** Vice-President, Research & Innovation University of Toronto (Canada)
9:10 am-10:15 am	**Session 1: Introduction and context** • Current/forthcoming challenges regarding pricing and reimbursement for ATMPs • Definition of ATMPs, product development, regulations and reimbursement context • HTA decision frameworks • Brief introduction of speakers, discussants and guests	**Speakers:** **Dr. Murray Krahn** Program Director THETA (Canada) **James Kusena** PhD candidate Loughborough University (U.K.) **Siofradh McMahon** Senior Manager, Clinical Translation & Regulatory Affairs CCRM (Canada)
10:15 am-10:30 am	**COFFEE**	
10:30 am-12 pm	**Session 2: Evidence of clinical effectiveness** • What is the evidence supporting the benefits and safety of ATMPs? • What is the structure of the data/evidence? • How do we ascertain the clinical benefits for ATMPs? Does their promise of cure/long-term effect make them different? • What is the strength of the evidence? • How can these technologies/therapies be evaluated when the sample size is very small? • How to satisfy both payers and regulators by producing adequate data? *Speakers: 15 minutes each* *Discussants: 5 minutes each* *Open discussion: 50 minutes*	**Speakers:** **Pilar Pinilla-Dominguez** Senior Scientific Advisor National Institute for Health and Care Excellence (NICE) (U.K.) **Dr. John Kuruvilla** Hematologist Princess Margaret Cancer Centre (Canada) **Discussants:** **Tania Bubela** Dean, Faculty of Health Sciences Simon Fraser University (Canada) **Alex Klarer** Biomedical Engineer Hitachi Chemical Advanced Therapeutics Solutions, LLC (USA)
12 pm-1:30 pm	**LUNCH**	
1:30 pm-3 pm	**Session 3: Health economics** • How to evaluate the cost effectiveness of ATMPs? • What are the methodological challenges? *Speaker: 15 minutes* *Discussants: 5 minutes each* *Open discussion: 60 minutes*	**Speaker:** **Ana Duarte** Research Fellow, Centre for Health Economics University of York (U.K.) **Discussants:** **Grace Hampson** Senior Principal Economist The Office of Health Economics (U.K.)

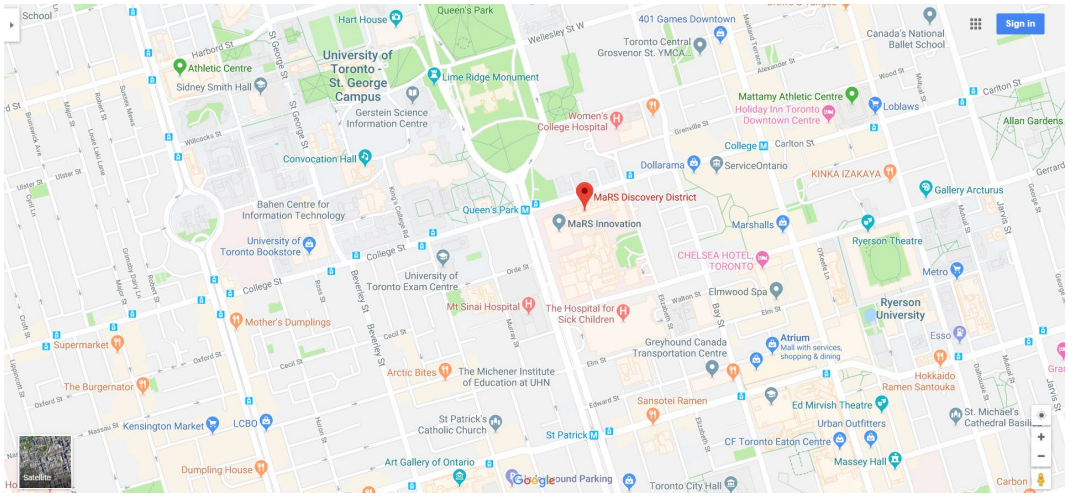
		Pilar Pinilla-Dominguez Senior Scientific Advisor NICE (U.K.) **William Wong** Assistant Professor, School of Pharmacy University of Waterloo (Canada)
3 pm-3:15 pm	**COFFEE**	
3:15 pm-4:45 pm	**Session 4: Social values toward regenerative medicine** • What is the public/patient perception of ATMPs? • Patient preferences/experiences/perspectives • Bioethical perspectives *Speaker:15 minutes* *Discussants: 5 minutes each* *Open discussion: 65 minutes*	**Speakers:** **Jennifer Gibson** Director, Joint Centre for Bioethics University of Toronto (Canada) **Discussants:** **Fiona Miller** Professor, Institute of Health Policy, Management and Evaluation University of Toronto (Canada) **Tania Bubela** Dean, Faculty of Health Sciences Simon Fraser University (Canada)
4:45 pm-5 pm	**Wrap-up discussion**	**Speakers:** **Dr. Murray Krahn** Program Director THETA (Canada) **James Kusena** PhD candidate Loughborough University (U.K.) **Siofradh McMahon** Senior Manager, Clinical Translation & Regulatory Affairs CCRM (Canada)
5 pm-7 pm	**BREAK**	
7 pm	**Dinner for speakers and discussants**	**Mercatto** MaRS (Elizabeth Street side of building) http://college.mercatto.ca/

	Tuesday, June 25, 2019 MaRS Collaboration Centre (101 College St.) South Tower, Main Level Collaboration CAFÉ	
8:30 am-9:45 am	**Session 5: Health technology assessment overview** • HTA and clinical processes - do they differ? • What is the data and how is it packaged into the HTA process? • The specifics of an evaluation, what is the HTA journey like? • What is the decision-making process/formula? *Speakers: 15 minutes each* *Discussants: 5 minutes each* *Open discussion: 35 minutes*	**Speakers:** **Heather Logan** Senior Advisor, Pharmaceutical Reviews Canadian Agency for Drugs and Technologies in Health (CADTH) (Canada) **Pilar Pinilla-Dominguez** Senior Scientific Advisor NICE (U.K.) **Discussants:** **Suzanne McGurn** Assistant Deputy Minister, Drugs and Devices and Executive Officer, Ontario Public Drug Programs Government of Ontario (Canada) **Rebecca Yu** Vice-President, Market Access & External Affairs Takeda Canada (Canada)
9:45 am-10 am	**COFFEE**	
10 am-11:30 am	**Session 6: Payment system mechanisms** • Are there any novel payment mechanisms? • Do we need novel systems or adjustment of codes? • What are the funding mechanisms/risk sharing agreements? *Discussants: 5 minutes each* *Open discussion: 65 minutes*	**Discussants:** **Ana Duarte** Research Fellow, Centre for Health Economics University of York (U.K.) **Rebecca Yu** Vice-President, Market Access & External Affairs Takeda Canada (Canada)
11:30 am-12:30 pm	**LUNCH**	
12:30 pm-2:30 pm	**Session 7: Adoption and implementation** • How do we facilitate the effective adoption and implementation of ATMPs, and ensure we gather the relevant evidence? • How do sessions 1-6 help us address the adoption challenges (as tangible action points)? • Do we need changes in infrastructure? (ATTC – discussion point?) • What are the barriers to adoption and implementation? *Speakers: 15 minutes each* *Discussants: 5 minutes each* *Open discussion: 75 minutes*	**Speakers:** **James Rose** Head of Clinical Innovation Adoption Oxford Academic Health Science Network (U.K.) **Discussants:** **Dr. John Kuruvilla** Hematologist Princess Margaret Cancer Centre (Canada) **Linnea Doyle** Senior Director, Market Access & Government Relations Gilead Sciences, Inc. (Canada) **Shahira Bhimani** Director, Health Solutions MaRS Partnerships (Canada)
2:30 pm-3 pm	**Wrap-up** • Next steps • Summary • White paper	**Speakers:** **Dr. Murray Krahn** Program Director THETA (Canada)

	• Next meeting?	**James Kusena** PhD candidate Loughborough University (U.K.) **Siofradh McMahon** Senior Manager, Clinical Translation & Regulatory Affairs CCRM (Canada)
3 pm	**WORKSHOP CONCLUDES**	
3 pm-4 pm	• Organizing committee debrief	

Location

All sessions will take place at the MaRS Discovery District (101 College St.), in the Collaboration Centre (main floor, South Tower). The closest subway station is Queen's Park. For out-of-town participants, MaRS is a short walk (less than 10 minutes) from the Chelsea Hotel.



Appendix 8. CHART workshop participants directory

Challenges in the Adoption of Regenerative Medicine Therapies

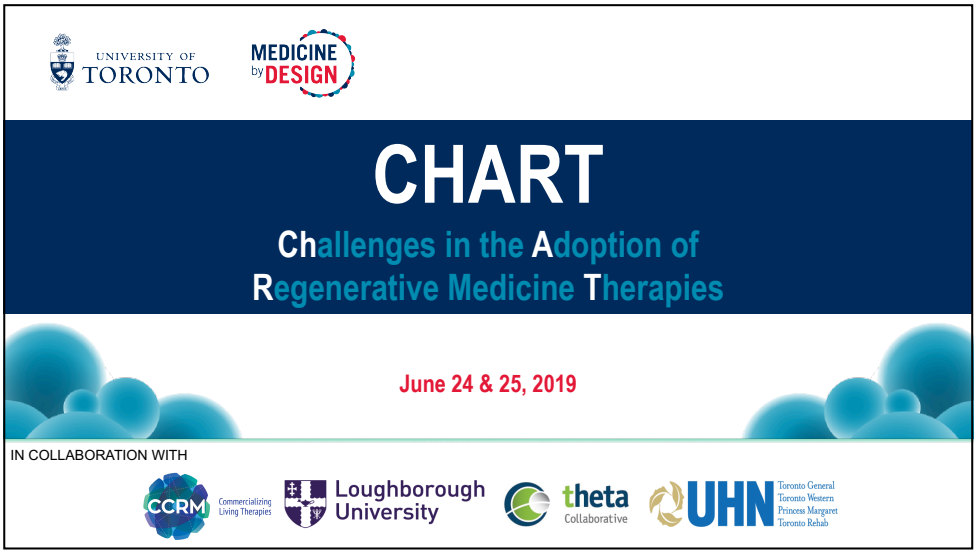
June 24-25, 2019
Toronto, Canada

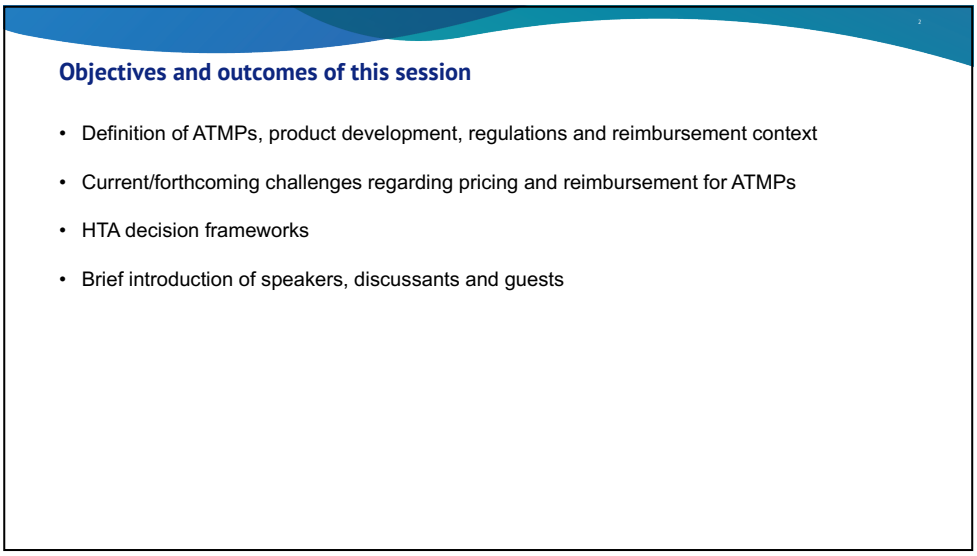
Participants

	Name	Title	Organization	Country	Workshop Role
1	Céline Bauwens	Scientific Manager, Medicine by Design	University of Toronto	Canada	Invited attendee
2	Dr. Donna Berry	Program Manager, Garron Family Cancer Centre & ENACT Committee	The Hospital for Sick Children	Canada	Invited attendee
3	Shahira Bhimani	Director, Health Solutions	MaRS Partnerships	Canada	Discussant – Session 7
4	Diane Bigras	Associate Director, Market Access and Government Affairs for Ontario	Gilead Sciences, Inc.	Canada	Invited attendee
5	Karen Bremner	Research Associate	Toronto Health Economics and Technology Assessment Collaborative	Canada	Organizing committee
6	Allison Brown	Director, Strategy & Translation, Medicine by Design	University of Toronto	Canada	Organizing committee
7	Tania Bubela	Dean, Faculty of Health Sciences	Simon Fraser University	Canada	Discussant – Session 2 Discussant – Session 4
8	Jackie Denholm	Operations Assistant, Medicine by Design	University of Toronto	Canada	Organizing committee
9	Kathryn Deuchars	Director, Ontario Personalized Medicine Network and Senior Manager, Sector Innovation and Programs	Ontario Genomics	Canada	Invited attendee
10	Linnea Doyle	Senior Director, Market Access & Government Relations	Gilead Sciences, Inc.	Canada	Discussant – Session 7
11	Ana Duarte	Research Fellow, Centre for Health Economics	University of York	U.K.	Speaker – Session 3 Discussant – Session 6
12	Kristina Ellis	MSc candidate (Supervisor: William Wong)	University of Waterloo	Canada	Invited attendee
13	Michael Farkouh	Chair, Peter Munk Centre of Excellence in Multi-national Clinical Trials	University Health Network	Canada	Invited attendee
14	Aren Fischer	Market Access Manager	Takeda Canada	Canada	Invited attendee
15	Jennifer Gibson	Director, Joint Centre for Bioethics	University of Toronto	Canada	Speaker – Session 4
16	Dr. Vivek Goel	Vice-President, Research & Innovation	University of Toronto	Canada	Speaker – Welcome
17	Peter Goodhand	Chief Executive Officer	Global Alliance for Genomics and Health	Canada	Invited attendee
18	Grace Hampson	Senior Principal Economist	The Office of Health Economics	U.K.	Discussant – Session 3

	Name	Title	Organization	Country	Workshop Role
19	Rosemary Hannam	Senior Research Associate, Centre for Health Sector Strategy, Rotman School of Management	University of Toronto	Canada	Invited attendee
20	Timothy Key	Manager, Technology & Venture Development	MaRS Innovation	Canada	Invited attendee
21	Alex Klarer	Biomedical Engineer	Hitachi Chemical Advanced Therapeutics Solutions, LLC	USA	Discussant – Session 2
22	Dr. Murray Krahn	Program Director	Toronto Health Economics and Technology Assessment Collaborative	Canada	Moderator Organizing committee
23	Dr. John Kuruvilla	Hematologist	Princess Margaret Cancer Centre	Canada	Speaker – Session 2 Discussant – Session 7
24	James Kusena	PhD candidate	Loughborough University	U.K.	Moderator Organizing committee
25	Heather Logan	Senior Advisor, Pharmaceutical Reviews	Canadian Agency for Drugs and Technologies in Health	Canada	Speaker – Session 5
26	John-Paul Marino	Director, Market Access	Gilead Sciences Inc.	Canada	Invited attendee
27	Lisa Masucci	PhD candidate (Supervisor: Dr. Murray Krahn)	University of Toronto	Canada	Invited attendee
28	Suzanne McGurn	Assistant Deputy Minister, Drugs and Devices, and Executive Officer, Ontario Drug Programs	Government of Ontario	Canada	Discussant – Session 5
29	Siofradh McMahon	Senior Manager, Clinical Translation & Regulatory Affairs	CCRM	Canada	Moderator Organizing committee
30	Fiona Miller	Professor, Institute of Health Policy, Management and Evaluation	University of Toronto	Canada	Discussant – Session 4
31	Allan Miranda	Head	JLABS @ Toronto	Canada	Invited attendee
32	Ann Perry	Manager, Communications & Operations, Medicine by Design	University of Toronto	Canada	Organizing committee
33	Pilar Pinilla-Dominguez	Senior Scientific Advisor	National Institute for Health and Care Excellence (NICE)	U.K.	Speaker – Session 2 Discussant – Session 3 Speaker – Session 5
34	Nezar Rghei	Vice-President, Strategic Partnerships & Resource Development	Ontario Genomics	Canada	Invited attendee
35	James Rose	Head of Clinical Innovation Adoption	Oxford Academic Health Science Network	U.K.	Speaker – Session 7
36	Michael Sefton	Executive Director, Medicine by Design	University of Toronto	Canada	Invited attendee
37	Soror Sharifpoor	Research Program Manager, Translational Biology & Engineering Program	Ted Rogers Centre for Heart Research	Canada	Invited attendee
38	Nick Timmins	Vice-President, Process Sciences	BlueRock Therapeutics	Canada	Invited attendee
39	Christine Williams	Deputy Director	Ontario Institute for Cancer Research	Canada	Invited attendee
40	William Wong	Assistant Professor, School of Pharmacy	University of Waterloo	Canada	Discussant – Session 3
41	Rebecca Yu	Vice-President, Market Access & External Affairs	Takeda Canada	Canada	Discussant – Session 5 Discussant – Session 6

Appendix 9. CHART workshop, Session 1 presentation slides: Introduction





Organising committee


Dr. Murray Krahn - Program Director THETA (Canada)


James Kusena – PhD candidate, Loughborough University


Siofradh McMahon – Senior Manager, Clinical translation and Regulatory Affairs, CCRM


Karen Bremner – Research associate THETA (Canada)


Ann Perry – Manager, Communications and Operations, Medicine by Design


Allison Brown – Director, Strategy and Translation, Medicine by Design


Jackie Denholm – Operations assistant, Medicine by Design

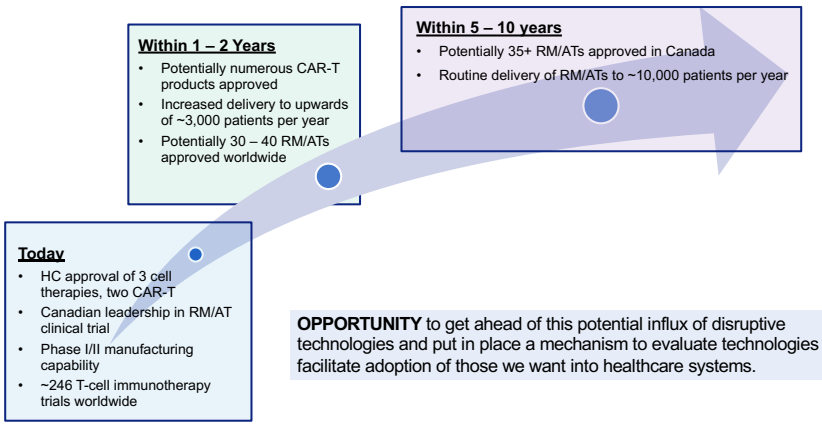
ATMPs: definition and regulatory processes

- EU ATMP definition is being used throughout to describe these products

"An advanced therapy medicinal product (ATMP) is a medicinal product which is either: a gene therapy medicinal product. a somatic cell therapy medicinal product. a tissue engineered product."

- Regulatory processes/approval to enter the market
  - UK: EMA and national regulators
  - Canada: Regulated by Health Canada as "drugs"
    - *(with a few exceptions for transplant material and minimally manipulated autologous homologous use cells)*
- Health Technology Assessment
  - UK: NICE
  - Canada: CADTH and INESSS (Quebec)
    - CAR-T therapies reviewed through the technology pathway (i.e. not drug)

The Opportunity



OPPORTUNITY to get ahead of this potential influx of disruptive technologies and put in place a mechanism to evaluate technologies and facilitate adoption of those we want into healthcare systems.

Key Steps in the Adoption of ATMPs

Research and Development	Regulatory Review and Approval	Reimbursement	Adoption
New technology development	New Drug Submission	Health Technology Assessment (Canada: CADTH / INESSS; UK: NICE)	Infrastructure
Clinical trials	Health Canada/EMA review	Patented Medicines Pricing Review Board (PMPRB)	Training
Manufacturing process development	Agreed commercial manufacturing process	(Pan-Canadian Pharmaceutical Alliance)	Monitoring
Scale up and logistics			Patient management
			Care guidelines

Challenges in pricing and reimbursement

Bluebird prices gene therapy Zynteglo at €1.575M in Europe, to be paid over 5 years

No end in sight to rising drug prices, study finds

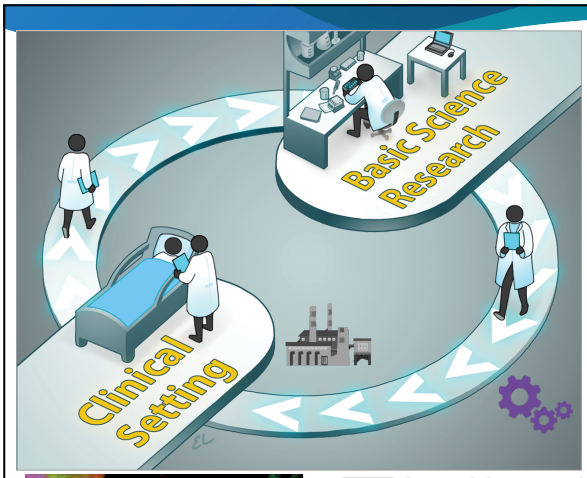
CAR T-cell therapy total cost can exceed $1.5 million per treatment

May 29, 2019

Richard T. Maziarz

PHILADELPHIA — When factoring in all the costs associated with chimeric antigen receptor T-cell therapy, hospitals may charge as much as $1.5 million or more to avoid losing money, according to **Richard T. Maziarz, MD,** professor of medicine at Oregon Health & Science University's Knight Cancer Institute.

$2.1m Novartis gene therapy to become world's most expensive drug



EPSRC & MRC Centre for Doctoral Training Regenerative Medicine

Loughborough University

Centre for Biological Engineering

Healthcare Engineering Group focusing on:

- Protocol optimisation
- Protocol standardisation
- Process automation
- Regulatory frameworks
- **Cost and financial headroom**
  - **Economic evaluations**
  - **Headroom**
  - **Reimbursement pathway**
  - **Adoption pathway**

The challenge

VALUE

Regenerative Medicine Value Systems: Navigating the Uncertainties

Some key questions, as these therapies are being developed:

- Can they actually be afforded?
- How do we pay for them?
- How are they going to be implement?

People we work with:

CATAPULT Cell and Gene Therapy

Innovate UK Technology Strategy Board

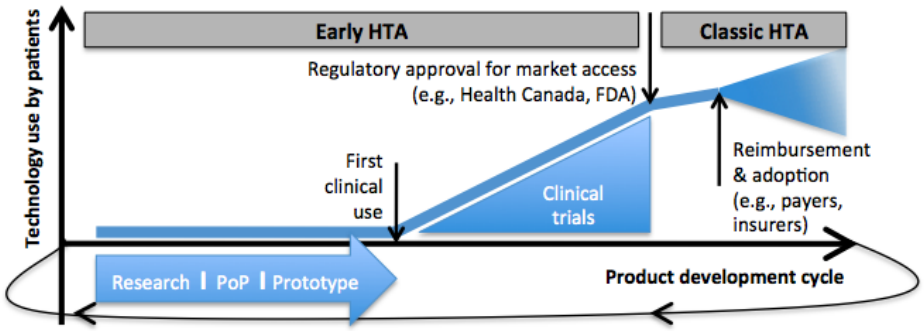
Innovate UK Knowledge Transfer Network

UK Regenerative Medicine Platform

MEDICINE by DESIGN

HTA decision frameworks and the structure of the next 2 days

EARLY & CLASSIC HTA IN TECH DEVELOPMENT



International Journal of Technology Assessment in Health Care, 34:3 (2018), 290–299.

doi:10.1017/S0266462318000235

Method

DEVELOPMENT OF THE ONTARIO DECISION FRAMEWORK: A VALUES BASED FRAMEWORK FOR HEALTH TECHNOLOGY ASSESSMENT

1. BENEFITS AND HARMS

				Rank
Benefits and Harms	Benefit	Magnitude	*Insert measures of effectiveness. For example, gain in quality-adjusted life year (QALY), relative risk reduction, or odds ratio*	
		Certainty	*Insert measures of certainty that the benefit is true. For example, confidence intervals (for random/systematic error) or GRADE assessment (for risk of bias).*	
	Harm	Magnitude	*Insert measures of harm. For example, relative risk or odds ratio for adverse event.*	
		Certainty	*Insert measures of certainty that the harm is true. For example, confidence intervals (for random/systematic error) or GRADE assessment (for risk of bias).*	
	Patient perspective	Strongly for/against or not a determinant	*Patient inputs on how patients perceive the net benefits and harms.*	
	SUMMARY	Takes into account both the magnitude and certainty of benefits and harms, and the ways in which patients perceive these benefits and harms, to produce the likelihood that this technology/ intervention will produce net benefit or harm.	*Highly likely to produce net benefit* / *Moderately likely to produce net benefit* / *Uncertain benefit/ harm* / *Moderately likely to produce net harm*	

2. VALUE FOR MONEY

✓ Check mark (" ✓ ") indicates formal analysis completed. X mark (" ✗ ") indicates no formal analysis completed.

	Type of analysis							
Economics	Value for money	Cost effectiveness	CE Threshold	*Highly likely to be CE (80%-100%)*	*Moderately likely to be CE (60%-79%)*	*Uncertain CE (40%-59%)*	*Moderately likely to not be CE (20%-39%)*	*Highly likely to not be CE (0%-19%)*
			$50K/QALY					
			$100K/QALY					
	Adequacy	**Downgrade Consideration**	*Adequate*			*Not adequate*		
		Appropriateness of cost and outcome measures						
		Comprehensiveness of cost and outcome valuation/ aggregation						
	SUMMARY	Taking account of both the probability of cost effectiveness, and the adequacy of the measures used, select the overall likelihood that this technolog(ies)/ intervention is cost effective.	CE Threshold	*Highly likely to be CE (80%-100%)*	*Moderately likely to be CE (60%-79%)*	*Uncertain CE (40%-59%)*	*Moderately likely to not be CE (20%-39%)*	*Highly likely to not be CE (0%-19%)*
			$50K/QALY					
			$100K/QALY					

3. Patient Centred Care

✓	Check mark ("✓") indicates formal analysis completed. X mark ("✗") indicates no formal analysis completed.						
Patient-Centred Care	Patients: Values & Preferences	Aligned with patient values & preferences	*Do patients have specific values, preferences or needs related to the condition, treatment or life impact that are relevant to this assessment? (NB. Values and preferences of family, informal caregivers or the public to be considered, as appropriate)*				
		Consistent with commitments to autonomy, privacy, confidentiality	*Are there concerns regarding accepted ethical or legal standards related to patient autonomy, privacy or confidentiality that are relevant to this assessment?*				
	Populations: Equity & Coordinated Care	Enhances equity in access or outcomes	*Are there disadvantaged populations or populations in need whose access to care or health outcomes might be improved (or not worsened) that are relevant to this assessment?*				
		Coordinates care	*Are there challenges in the coordination of care for patients that might be improved (or not worsened) that are relevant to this assessment?*				
	SUMMARY	Taking account of these considerations, select the degree to which the evidence supports the use of the technology(ies)/ intervention.	*Strongly supports*	*Somewhat supports*	*Neutral/ Unknown*	*Does not support*	*Strongly discourages*

4. Health System Feasibility

Cost considerations	*What are the relevant cost considerations associated with implementing this technology/intervention (e.g., budget impact)?*
Organizational implications	*What are the relevant non-cost implications (e.g., logistical, human resources) associated with implementing this technology/intervention?*

Appendix 10. CHART workshop, Session 2 presentation slides: Evidence of clinical effectiveness

NICE National Institute for Health and Care Excellence

Session 2: Evidence of clinical effectiveness: NICE's experience on the evaluation of cell and gene therapies

Pilar Pinilla-Dominguez, Senior Scientific Adviser, NICE Scientific Advice

Challenges in the Adoption of Regenerative Medicine Therapies Workshop

24 June, 2019

Key questions

- What is the evidence supporting the benefits and safety of ATMPs?
- What is the structure of the data/evidence?
- How do we ascertain the clinical benefits for ATMPs? Does their promise of cure/long-term effect make them different?
- What is the strength of the evidence?
- How can these technologies/therapies be evaluated when the sample size is very small?
- How to satisfy both payers and regulators by producing adequate data?

NICE

2

Key questions for NICE

Benefit

How well does the technology work compared with established practice in the health service?

How much does this course of action cost compared with established practice in the health service?

Cost

NICE

3

Exciting times

Pipelines of novel (bio)pharmaceuticals and advanced therapy medicinal products (ATMPs) with potential for major benefits – cures?

Immunotherapies / Chimeric antigen receptor T-cell	Gene therapies	Therapies targeting biomarkers	Combination therapies

Challenges

High costs	Uncertain long-term benefits and adverse effects	Timely patient access while the evidence is still emerging

NICE

4

ATMPs: what is different/difficult?

Potential for a cure	• High upfront cost and uncertain long-term effects
ATMPs frequently developed by SMEs/academics	• Lack of funding • Multi-stakeholder expertise • Expensive manufacturing • Complicated logistics • Limited experience of HTA requirements
Limited evidence	• RCTs – impossible? • Limited generalisability and external validity • Small sample sizes • Surrogate/intermediate rather than final/clinical outcomes • Short trials – maintenance of effect? Uncertain long-term effects • Unknown future adverse effects

NICE

5

ATMPs at NICE – not new

NICE Technology Appraisal 16: Autologous cartilage transplantation for full thickness cartilage defects in knee joints

Published date: 16 December 2000

NICE

6

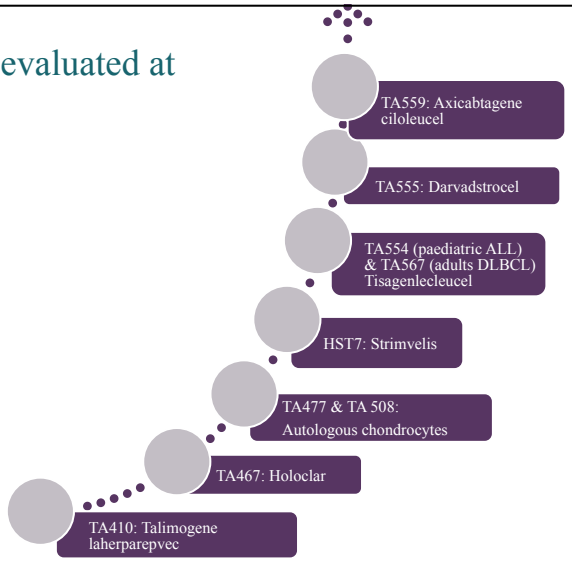
Many ATMPs withdrawn from market

Alipogene tiparvovec (Glybera)	• Not evaluated by NICE
Sipuleucel-T (Provenge)	• Originally not recommended by NICE in TA332
Characterised viable autologous cartilage cells (Chondrocelect)	• MA withdrawn during TA447
Matrix-applied characterised autologous cultured chondrocytes (MACI)	• MA suspended during TA447

NICE

7

ATMPs evaluated at NICE



NICE

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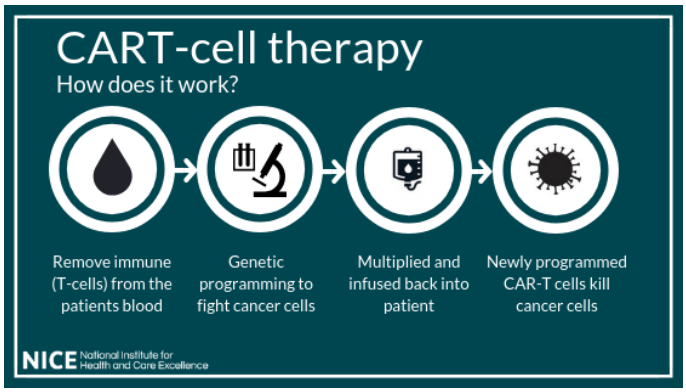
NICE Technology Appraisals for CAR-T: case studies



NICE

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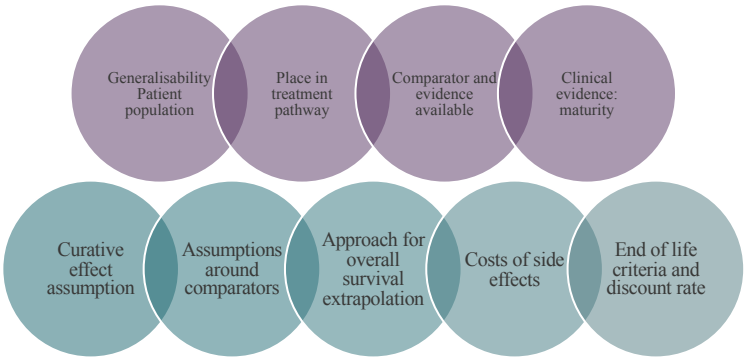
Key issues in clinical and cost effectiveness in CAR-T cell appraisals



NICE

10

Clinical effectiveness

Single-arm trials

No appropriate comparative source of data for comparison: differences in patient characteristics and prognostic factors

Short-term evidence:

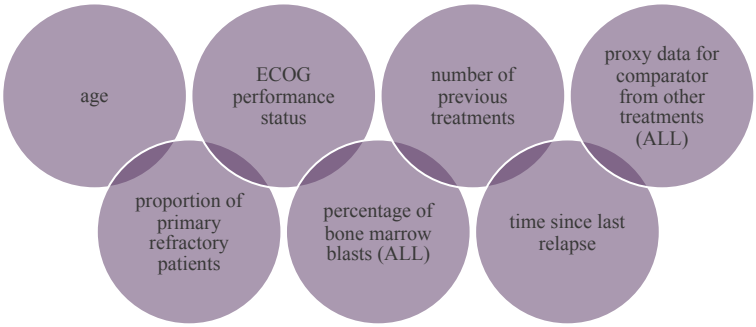
- Median overall survival just or not reached
- Curative assumptions proportion of patients
- Proportion of patients who subsequently have a transplant
- Uncertain adverse effects

NICE

11

Clinical effectiveness

✓ No appropriate comparative source – differences in patient characteristics and prognostic factors between patients in the trials and comparative evidence include:



NICE

ALL = acute lymphoblastic leukaemia

12

Clinical effectiveness issues with high impact on cost effectiveness

- ✓ Short term evidence: overall survival
  - Tisagenlecleucel-T – ALL (up to 25 years): **median follow up less than 3 years**; median overall survival only reached in the single centre trial, high degree of censoring
  - Axicabtagene ciloleucel – DLBCL: **Median overall survival not reached,** with overall survival rates of 52% at 18 months (95% CI 41 to 62).
  - Tisagenlecleucel-T – DLBCL: Median overall survival between 11 and 22 months; **high degree of censoring**

Clinical experts highlighted the promising results; acknowledging the short-follow up

NICE

ALL = acute lymphoblastic leukaemia
DLBCL: diffuse large B-cell lymphoma

13

Clinical effectiveness issues with high impact on cost effectiveness

Curative assumptions: cure point and proportion of patients	• **No robust evidence of curative effect** → assumed cure point between 2 and 5 years
Proportion of patients who subsequently have a transplant (ALL)	• number of patients who would have an SCT after having CAR-T is **highly uncertain** • interim analyses of the ELIANA study showed that of patients having tisagenlecleucel, 11% had a subsequent SCT
Uncertain adverse effects (DLBCL)	• Number of patients who will need intravenous immunoglobulin treatment for B-cell aplasia and for how long

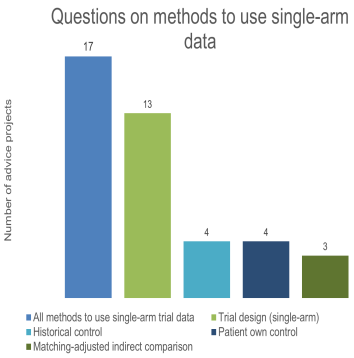
NICE

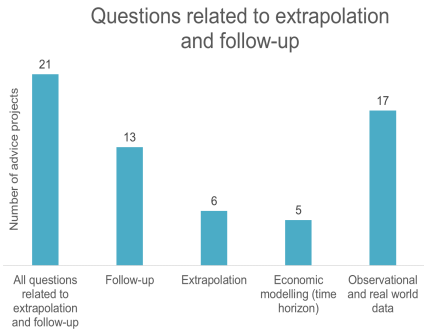
ALL = acute lymphoblastic leukaemia
SCT: stem cell transplant
DLBCL: diffuse large B-cell lymphoma

14

Experience in NICE scientific advice

- 25 ATMP NICE SA with 15 gene and 10 cell therapies





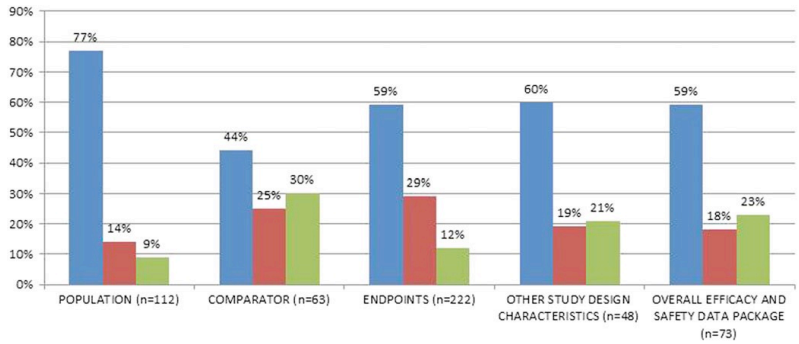
Updated by March 2019

NICE

15

Alignment between regulators and HTA bodies

- Retrospective qualitative analysis of minutes of discussion meetings held at the EMA between 2010 and May 2015 (n=31)



Tafuri et al. (2016) How aligned are the perspective of EU regulators and HTA bodies? A comparative analysis of regulatory-HTA parallel scientific advice. *Br J Clin Pharmacol*

NICE

16

www.nice.org.uk/scientificadvice

Follow us on Twitter
@NICESciAdvice

Pilar Pinilla-Dominguez
Senior Scientific Adviser
Pilar.Pinilla-dominguez@nice.org.uk

NICE

17

Appendix 11. CHART workshop, Session 3 presentation slides: Health Economics

NICE National Institute for Health and Care Excellence

Session 3: Health economics; Discussant points

Pilar Pinilla-Dominguez, Senior Scientific Adviser, NICE Scientific Advice

Challenges in the Adoption of Regenerative Medicine Therapies Workshop

24 June, 2019

Some challenges for HTA and decision-making

- Non-comparative trials
- Heterogeneity of populations
- Surrogate endpoints with unclear relationship with OS and HRQL
- Small sample sizes
- Early regulatory approval with short-term follow-up and ethical consequences

NICE

2

Challenges: how to deal with them?

- **Small sample size:** Limited generalisability & external validity
- **Single arm-trials:** Methods for comparison require strong assumptions:
  - Matched adjusted indirect comparisons (MAIC)
  - Simulated treatment comparison (STC)
  - Before and after comparisons, historical controls…
- **Surrogate outcomes:** validity and relationship with clinical and patient relevant outcomes
- **Short-term trial:** need for extrapolation and assumptions:
  - Parametric distributions, proportional hazard assumptions, splines, cure models (mixture cure models) and assumptions about cure fraction
- **Discount rate:** costs incurred in short term and benefits observed lifetime

NICE 3

Key questions

NICE 4

Appendix 12. CHART workshop, Session 5 presentation slides: Health Technology Assessment (CADTH)

Health Technology Assessment of CAR T-cell Therapies

CHALLENGES IN THE ADOPTION OF REGENERATIVE MEDICINE THERAPIES
JUNE 24-25, 2019

Heather Logan
Senior Advisor, Pharmaceutical Reviews

CADTH

Disclosure

- No conflicts of interest to declare.
- CADTH receives funding from Canada's federal, provincial, and territorial governments, with the exception of Quebec.
- CADTH is an independent, not-for-profit organization responsible for providing Canada's health care decision-makers with objective evidence to help make informed decisions about the optimal use of drugs, medical devices, diagnostics, and procedures in our health care system.

CADTH

OUTLINE

HTA
- What, Who ,When, How

HTA of CAR T-cell
- Is it different?
  - Typical drug HTA?
  - Typical clinical intervention HTA?

CADTH

What is HTA?

WHO definition
- the systematic evaluation of properties, effects, and/or impacts of health technology
- health technology is the *application of organized knowledge and skills in the form of medicines, medical devices, vaccines, procedures and systems developed* to solve a health problem and improve quality of life.
- A multidisciplinary process to evaluate the *social, economic, organizational and ethical issues* of a health intervention or health technology.
- **The main purpose is to inform policy decision making**

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Who Does HTA?

HTA bodies

- NICE
- ICER
- CADTH
- INESSS

Hospital based units

- Hospital value analysis committees
  - Central difference is that HTA bodies conduct evaluation of a class of devices, while hospital based value analysis is done at the product level

4

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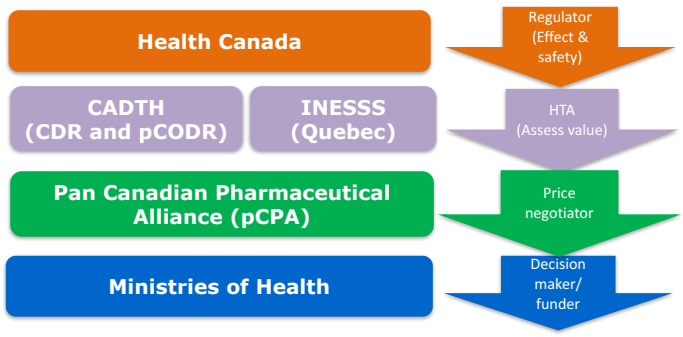
Overview of Drug Review in Canada



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CADTH

CADTH

is an independent, not-for-profit organization responsible for providing Canada's health care decision-makers with objective evidence about the optimal use of drugs and medical devices.

Our Programs and Services

DRUG REIMBURSEMENT RECOMMENDATIONS
- CADTH Common Drug Review (CDR)
- CADTH pan-Canadian Oncology Drug Review (pCODR)

HEALTH TECHNOLOGY MANAGEMENT PROGRAM
- Rapid Response Service
- Health Technology Assessment Service
- Optimal Use Service
- Environmental Scanning
- Horizon Scanning

OTHER PROGRAMS AND SERVICES
- Scientific Advice

KNOWLEDGE MOBILIZATION AND LIAISON OFFICERS
- Located in jurisdictions across Canada
- Understand the needs and priorities of local decision-makers
- Provide advice and tools to help turn evidence into policy and practice

CADTH

When: Current framework

- Canada is a federal system that is highly decentralized.
- Regulatory approval to enter the marketplace is conducted federally by Health Canada
- Majority of drug reviews conducted concurrent with regulatory review. Not true for devices/clinical interventions.
  - Drugs: HTA and pan-Canadian price negotiation are required to enter marketplace
  - Interventions: Neither HTA nor pan-Canadian price negotiation are required. Typically, once in the market place, funding is determined and provided at the provincial/territorial level
- Administration of health care delivery is done regionally within provinces

8 CADTH

Choice of HTA

Is the value proposition different for a pharmaceutical versus a clinical intervention/device?

- Decision problem for both is whether it should be adopted

If the mechanism for adoption is predominantly that it should be funded and placed in the formulary

- HTA for a pharmaceutical

If the mechanism for adoption is funding and a complex implementation system,

- HTA for a clinical intervention

9 CADTH

How are they different?

HTA DRUGS

Determination of ***value***: Should this be adopted

Deliberative Framework:

- Relevance and Unmet Need
- Benefits
- Harms
- Patient perspective
- Economic impact

HTA CLINICAL INTERVENTION

Determination of ***value:*** Should this be adopted

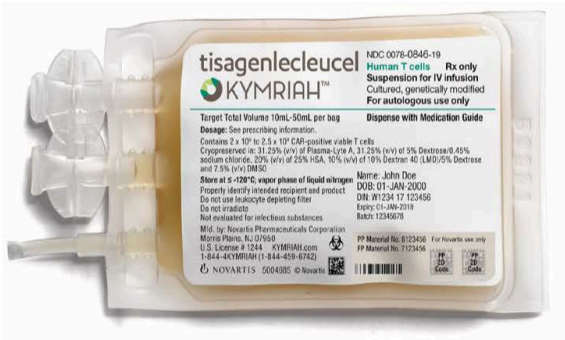
Deliberative Framework:

- Relevance and Unmet Need
- Benefits
- Harms
- Patient perspective
- Economic impact
- Implementation
- Legal
- Ethical
- Environmental impact

10

CADTH

Specific HTA for CAR T-cell



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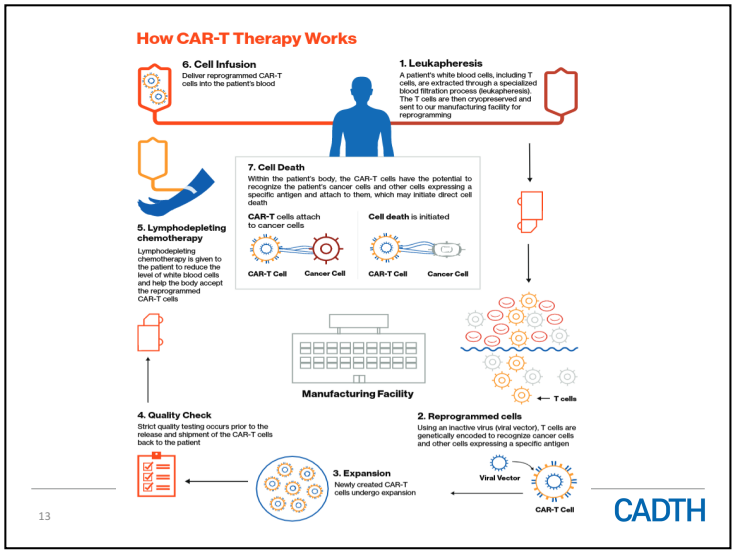
Is this a complex intervention?

12

CADTH



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CADTH

Decision Problem

How should the provision of tisagenlecleucel for children and young adults with relapsed or refractory B-cell acute lymphoblastic leukemia (r/r-ALL) and adults with relapsed or refractory diffuse large B-cell lymphoma (r/r-DLBCL) be structured?

Evidentiary Needs

1. Clinical effectiveness
2. Cost effectiveness
3. Ethical issues
4. Implementation considerations
5. Patient & care giver perspective

What is Assessed?

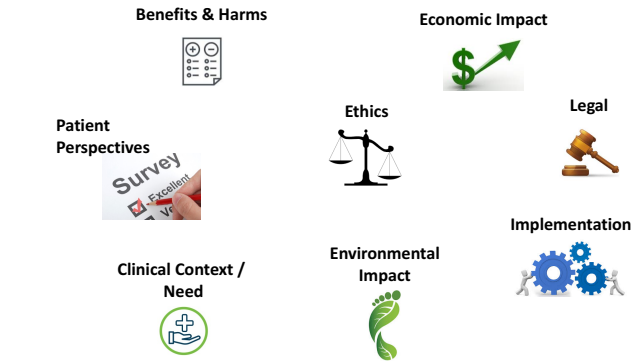
EVIDENCE PACKAGE that includes comparative evidence on

- Relevance and Unmet Need
- Benefits
- Harms
- Patient perspective
- Economic impact
- Implementation
- Legal
- Ethical
- Environmental impact

Health Technology Expert Review Panel (HTERP)

- 7 core members
  - Chair
  - Public member
  - Ethicist
  - Health economist
  - Three health care practitioners
- Up to 5 expert members

HTERP Deliberative Framework



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HTERP Overall Recommendation

On the condition that there is a reduction in price, the expert committee recommends the provision of tisagenlecleucel….

19

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HTERP Implementation Recommendations

1. The creation of interprovincial agreements to ensure equitable access to eligible patients in all jurisdictions, including consideration of financial and logistic support for required travel and short-term relocation.

2. the development of clear and transparent eligibility criteria that are acceptable to patients' and clinicians' needs, based on the approved indications

3. The collection of standardized outcomes data in a pan-Canadian registry of patients, which uses a defined set of outcomes and definitions to generate real-world evidence, for consideration in future reassessments to assess longer-term effectiveness, safety, and cost-effectiveness

Conclusion

- Cell and gene therapies are complex interventions
- HTA requires consideration of the many implementation and ethical issues in the deliberation.
- Disruptive technologies, at the point of first implementation, require additional discussion and planning

Appendix 13. CHART workshop, Session 5 presentation slides: Health Technology Assessment (NICE)

22/07/2019

NICE National Institute for Health and Care Excellence

Session 5: Health technology assessment at NICE

Pilar Pinilla-Dominguez, Senior Scientific Adviser, NICE Scientific Advice

Challenges in the Adoption of Regenerative Medicine Therapies Workshop

25 June, 2019

Key questions

- HTA and clinical processes - do they differ?
- What is the data and how is it packaged into the HTA process?
- The specifics of an evaluation, what is the HTA journey like?
- What is the strength of the evidence?
- What is the decision-making process/formula?

NICE

2

1

Key questions for NICE

Benefit

How well does the technology work compared with established practice in the health service?

How much does this course of action cost compared with established practice in the health service?

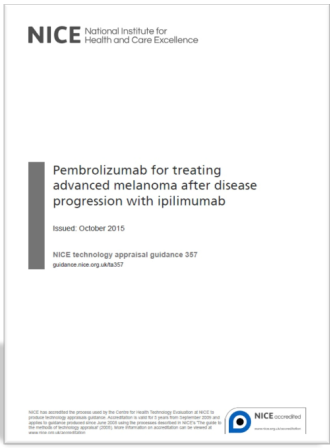
Cost

NICE

3

NICE Technology Appraisals

- Formal referral by the Secretary of State for Health (Department of Health and Social Care) to NICE required
- A review of clinical and economic evidence leading to recommendations on the appropriate use of new and existing technologies for the NHS in England



NICE

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NHS Constitution 2012

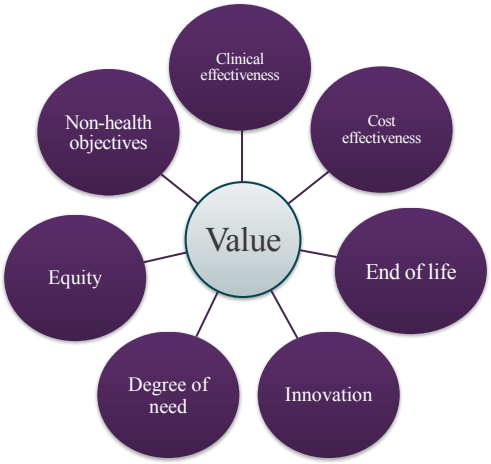
You have the right to drugs and treatments that have been recommended by NICE for use in the NHS, if your doctor says they are clinically appropriate for you.

THE NHS CONSTITUTION
the NHS belongs to us all

NICE

5

What does NICE value?



NICE

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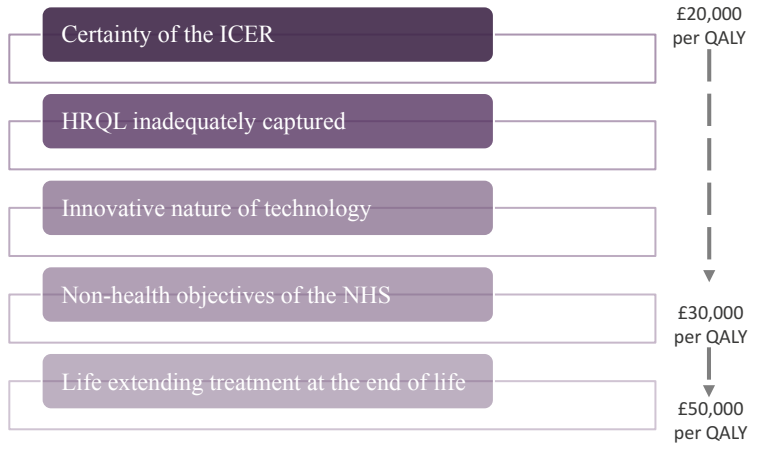
Flexible decision making



NICE

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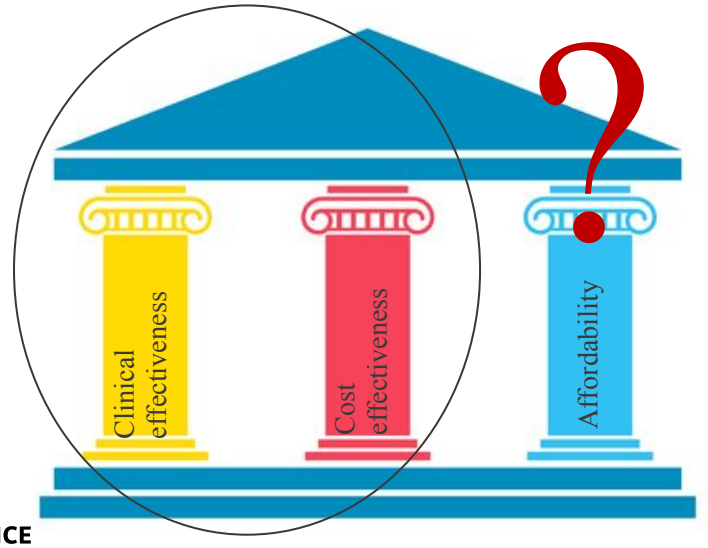

NICE

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What's new at NICE

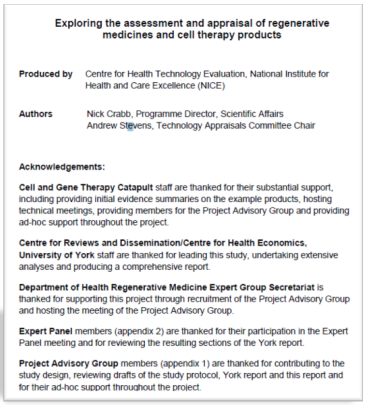
By 2020, NICE will expand its scope by reviewing all new drugs approved by drug licensing agencies in addition to most new uses of already approved drugs

Cost-recovery for NICE technology appraisals and highly specialised technology (HST) appraisals started in April 2019

NICE

11

NICE Regenerative Medicines Study (2016)



Exploring the assessment and appraisal of regenerative medicines and cell therapy products

Produced by Centre for Health Technology Evaluation, National Institute for Health and Care Excellence (NICE)

Authors Nick Crabb, Programme Director, Scientific Affairs
Andrew Stevens, Technology Appraisals Committee Chair

Acknowledgements:

Cell and Gene Therapy Catapult staff are thanked for their substantial support, including providing initial evidence summaries on the example products, hosting technical meetings, providing members for the Project Advisory Group and providing ad-hoc support throughout the project.

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Department of Health Regenerative Medicine Expert Group Secretariat is thanked for supporting this project through recruitment of the Project Advisory Group and hosting the meeting of the Project Advisory Group.

Expert Panel members (appendix 2) are thanked for their participation in the Expert Panel meeting and for reviewing the resulting sections of the York report.

Project Advisory Group members (appendix 1) are thanked for contributing to the study design, reviewing drafts of the study protocol, York report and this report and for their ad-hoc support throughout the project.

- Prompted by a recommendation from the Department of Health Regenerative Medicine Expert Group
- Included a broad exploration of the applicability of NICE TA methods to regenerative medicines
- Hypothetical example product based on early clinical data for related real products supplemented with hypothetical evidence

NICE Source: https://www.nice.org.uk/Media/Default/About/what-we-do/Science%20policy%20and%20research/Regenerative-medicine-study-march-2016.pdf

12

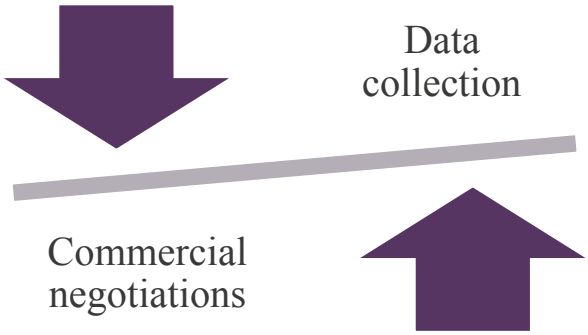
NICE Regenerative Medicines Study (2016)

- ✓ NICE appraisal methods and decision frameworks applicable
- ✓ Key to quantify and present clinical outcome and decision uncertainty

NICE

13

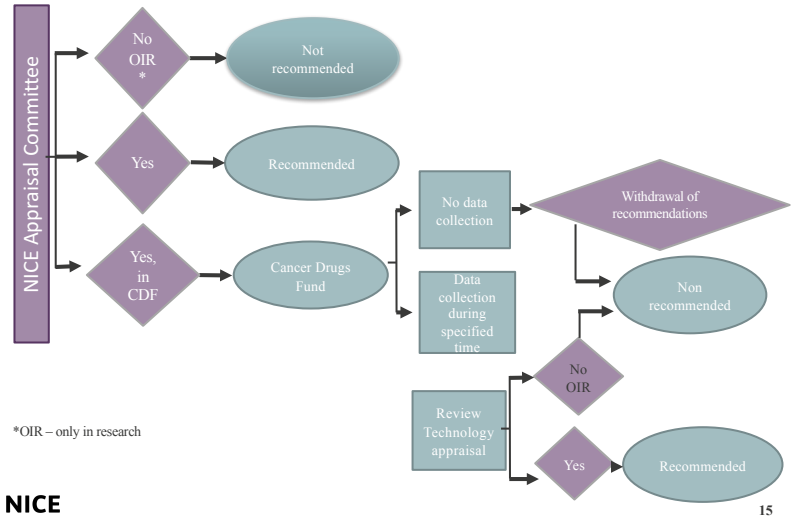
Managing uncertainty and risk



NICE

14

Cancer Drugs Fund overview



*OIR – only in research

NICE

15

Cost effectiveness results: most plausible incremental cost-effectiveness ratio (ICER) for the CAR-T appraisals

Tisagenlecleucel-T – ALL (up to 25 years)	Tisagenlecleucel-T – DLBCL	Axicabtagene ciloleucel – DLBCL
>£30,000 per QALY gained	>£50,000 per QALY gained	>£50,000 per QALY gained

NICE

ALL = acute lymphoblastic leukaemia
DLBCL: diffuse large B-cell lymphoma

16

Discount rate: the NICE Guide to the methods of technology appraisals says…

Cost-effectiveness results should reflect the present value of the stream of costs and benefits accruing over the time horizon of the analysis.

The same annual discount rate should be used for both costs and benefits (currently 3.5%)

A discount rate of 1.5% for costs and benefits may be considered when treatment effects are both substantial in restoring health to full or near full health and sustained over a very long period (normally at least 30 years), and if the committee is satisfied that the introduction of the technology does not commit the NHS to substantial irrecoverable costs

NICE

17

End of life (EOL) criteria

- ✓ The treatment is indicated for patients with a short life expectancy (<24 months)
- ✓ There is sufficient evidence to indicate that the treatment has the prospect of offering an extension to life, (>3 months) compared with current NHS treatment.
- ✓ The estimates of the extension to life are sufficiently robust
- ✓ The assumptions used in the reference case economic modelling are plausible, objective and robust.

NICE

18

Other factors

3.5% for both costs and benefits

- No robust/mature evidence to support curative effect

EOL criteria

- Tisagenlecleucel-T – ALL (up to 25 years): not met → the life expectancy evidence is uncertain
- Tisagenlecleucel-T – DLBCL: met
- Axicabtagene ciloleucel – DLBCL: met

NICE

EOL: end of life
ALL = acute lymphoblastic leukaemia
DLBCL: diffuse large B-cell lymphoma

19

Cancer Drugs Fund

	Committee's rationale in CAR-T appraisals
Starting point: drug not recommended for routine use	✓ Not recommended
1. Why is drug not recommended? Is it due to clinical uncertainty?	✓ Yes
2. Does drug have plausible potential to be cost-effective at the current price, taking into account end of life criteria?	✓ Yes
3. Could data collection reduce uncertainty	✓ Yes
4. Will ongoing studies provide useful data? and 5. Is CDF data collection feasible?	✓ Yes
Recommend enter CDF	

Indicate research question, required analyses and number of patients in NHS in England needed to collect data

NICE

20

CDF data collection

Collection of data on	Sources	Period of data collection
• Overall survival • Convergence of progression-free survival and overall survival curves • Rate of subsequent SCT • Immunoglobulin usage	• Ongoing clinical trials • CDF through SACT data	• 5 years

NICE

21

Pricing and managed access agreements

Tisagenlecleucel-T – DLBCL	Tisagenlecleucel-T – ALL	Axicabtagene ciloleucel – DLBCL
• **List price:** £282,000 per infusion (company submission) • **Commercial arrangement:** (managed access agreement with a simple discount [size of the discount is commercial in confidence] and rebate scheme)	• **List price:** £282,000 per infusion (company submission) • **Commercial arrangement** (the size of the discount is commercial in confidence)	• **List price:** submitted as commercial in confidence • **Commercial arrangement** (details of the arrangement are commercial in confidence)

NICE

ALL = acute lymphoblastic leukaemia
DLBCL: diffuse large B-cell lymphoma

22

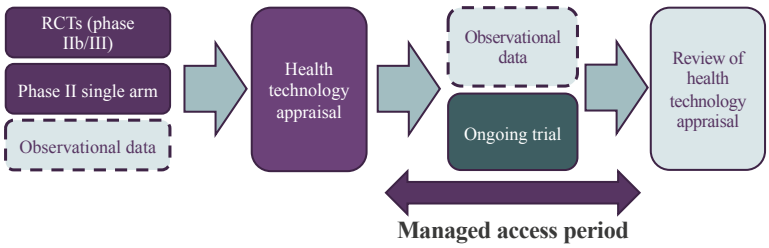
The outcomes

Axicabtagene ciloleucel (Yescarta, Kite-Gilead) for treating diffuse large B-cell lymphoma and primary mediastinal B-cell lymphoma after 2 or more systemic therapies	• TA559: recommended for use within the Cancer Drugs Fund
Tisagenlecleucel- T (Kymriah, Novartis) for treating relapsed or refractory diffuse large B-cell lymphoma after 2 or more systemic therapies	• TA567 –recommended for use within the Cancer Drugs Fund
Tisagenlecleucel-T (Kymriah, Novartis) for treating relapsed or refractory B-cell acute lymphoblastic leukaemia in people aged 3 to 25 years	• TA554: recommended for use within the Cancer Drugs Fund

NICE

23

What outcome data is collected whilst a drug is available on the CDF?



NICE

24

Planning regulatory and HTA pathway together

NICE

25

Support initiatives for innovative products such as ATMPs

PRIME
- Designation granted by the European Medicines Agency

EAMS
- Designation granted by the UK Medicines and Healthcare Regulatory Agency

Accelerated Access Collaborative
- Designation supporting rapid national uptake

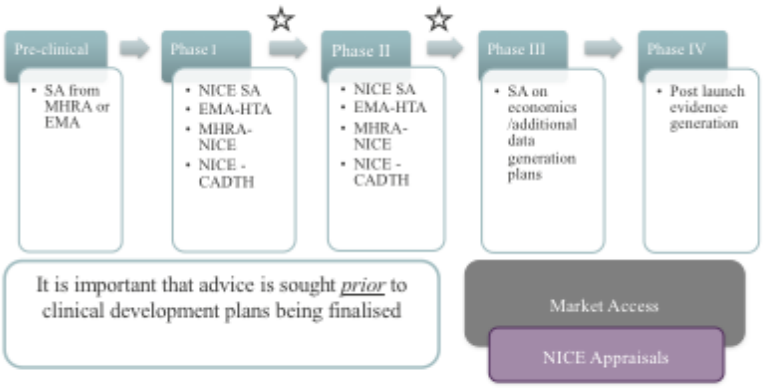
Scientific advice

NICE Office for Market Access

NICE

26

When to seek Scientific Advice?



NICE

27

We've been asked in scientific advice projects on cell and gene therapies

Given the highly innovative nature of this therapy, what clinical endpoints will be acceptable to NICE in its evaluation? How would the following phase II clinical trial endpoints be evaluated and are they acceptable?

Will this observational study be considered sufficiently robust to be used as a comparator to the single-arm phase II study?

This therapy is delivered in a single, one-off intervention, as compared with many other oncology treatments which have a number of cycles and/or long-term therapy – does this fact warrant any special consideration in the modelling?

In the absence of any other long-term data on adverse events, what kind of sensitivity analyses would be expected to fully explore the uncertainty associated with the decision problem? What data would be required on toxicity management?

NICE

28

Key issues to consider early

- Clinical trial design and feasibility to conduct comparisons with established practice
- Relevant outcomes, their relationship with health-related quality of life and mortality, and frequency of data collection
- Proposed modelling approaches (based on clinical plausibility and data available to date) to deal with uncertainty at the time of submission
- Long-term evidence generation plans → increasing relevance of post-marketing authorisation studies including clinical effectiveness and adverse effects data
- Data collection in the relevant setting

NICE 29

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Senior Scientific Adviser
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Appendix 14. CHART workshop, Session 7 presentation slides: Adoption and Implementation

Oxford Academic Health Science Network

Exploring Adoption and Implementation of ATMPs:

A UK Perspective

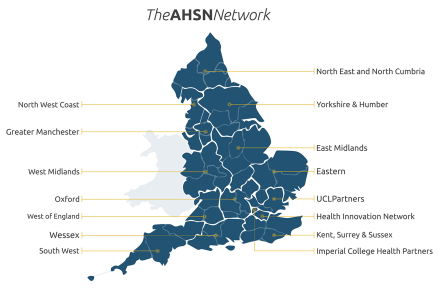
James Rose, Head of Innovation Adoption, Oxford AHSN

CHART Workshop June 2019

Oxford Academic Health Science Network

Oxford Academic Health Science Network

- Brings together Academia, Industry and Healthcare
  - 700 life science companies
  - 6 large acute hospital trusts
  - 5 universities
  - 3 million citizens
- **Focus on spread innovation** at "pace and scale"
- improving health and generating economic growth



James Rose | June 25 2019 | Adoption and Implementation of ATMPs 2

Is the UK currently an attractive market for ATMPs?

Oxford Academic Health Science Network

- UK a leader in basic/clinical research – NHS for many years struggled with translation and adoption
- Investment in developing people, networks, infrastructure, capacity and capabilities
- Almost £250m (C$421m) provided by the UK government to drive translation, commercialisation and adoption of ATMPs
  - CGT Catapult
  - CGT Manufacturing Centre
  - Advanced Therapies Treatment Centres designed to help translation to NHS
- Uncertainty around Brexit remains
- **NHS is a "Single-payer" market - advantages of potential centralised commissioning, coordination, standardisation, risk management and data access to support adoption**

Source UK Government 2018 :https://bit.ly/2QemRu3

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10 licensed ATMPs / 7 with a positive NICE TA

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Name - Manufacturer	Indication	Authorisation	NICE TA
Strimvelis® - GSK	ADA-SCID	2015	APPROVED
Imlygic® - Amgen	Melanoma	2015	APPROVED
Holoclar® - Chiesi	Severe limbal stem cell deficiency	2015	APPROVED
Zalmoxis® - Molmed	Stem cell transplantation (high-risk blood cancer)	2016	APPROVED
Spherox® - co.don	Cartilage defects in the knee joint	2017	APPROVED
Alofisel® - Tigenix	Perianal fistulas in Crohn´s disease	2018	REJECTED
Yescarta® - Kite/Gilead	B-cell lymphoma	2018	APPROVED CDF
Kymriah® - Novartis	ALL, DLBCL	2018	APPROVED CDF
Zynteglo® – Bluebird	Transfusion-dependent thalassaemia (TDT)	2019	PENDING
Luxturna® - Spark	Inherited Retinal Disease	2019	PENDING

Data accurate as of June 2019

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EMA expecting a steep rise in submissions and authorisations in coming years

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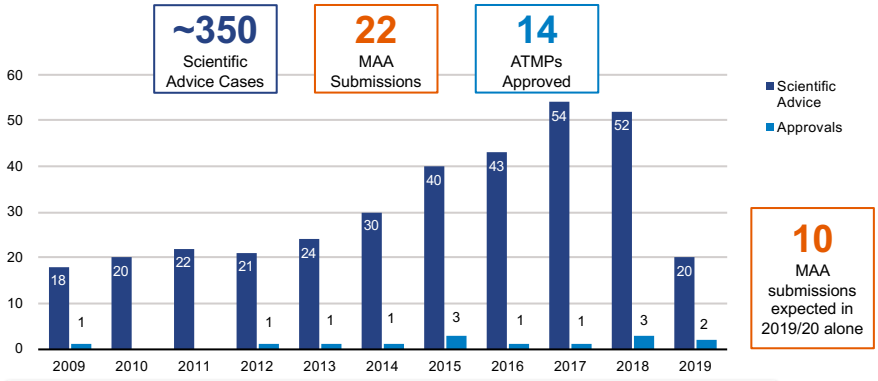


Collectively recognise the need to be ready!

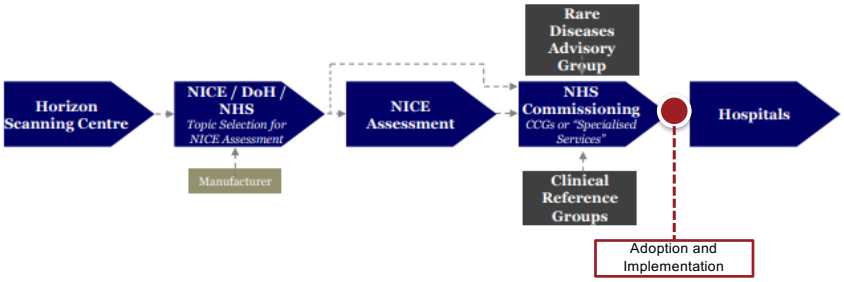
Source

The pathway to market access is established…

Oxford Academic Health Science Network

Pathway to adoption and implementation less so

Top-level roadmap to market access for licensed ATMPs (England*)

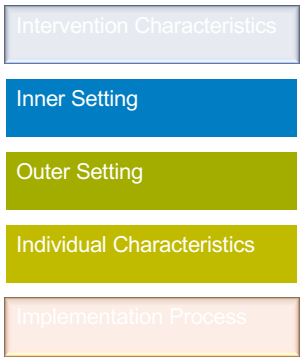


Source: Kefalas CGT 2014 https://ct.catapult.org.uk/sites/default/files/publication/Achieving-market-access-for-cell-therapies-a-UK-perspective-May-2014.pdf

The Consolidated Framework for Implementation Research

Source: Damschroder et al, Implementation Science 2009 https://implementationscience.biomedcentral.com/articles/10.1186/1748-5908-4-50

The Consolidated Framework for Implementation Research



Source: Damschroder et al, Implementation Science 2009 https://implementationscience.biomedcentral.com/articles/10.1186/1748-5908-4-50

Inner Setting - Institutional barriers to adoption

Oxford Academic Health Science Network

Looking at example of recent CAR-T implementation – 11 centres in UK

Inner Setting - Institutional barriers to adoption

Oxford Academic Health Science Network

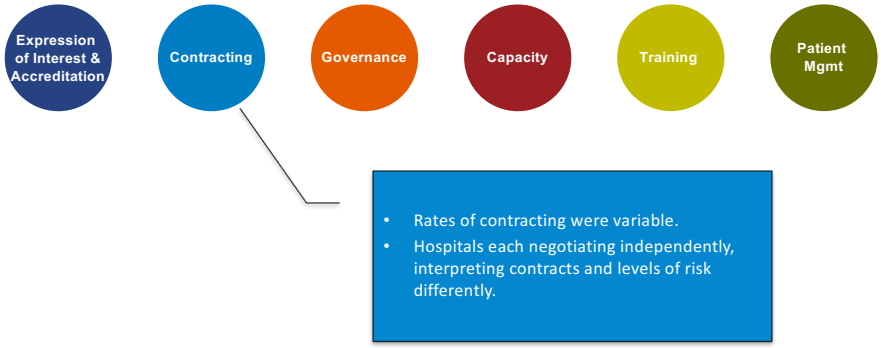
Looking at example of recent CAR-T implementation – 11 centres in UK

- Thousands man hours invested in accreditation
- Huge resource commitment to SOPs, policies, pathways and guidelines

Inner Setting - Institutional barriers to adoption

Oxford Academic Health Science Network

Looking at example of recent CAR-T implementation – 11 centres in UK



- Rates of contracting were variable.
- Hospitals each negotiating independently, interpreting contracts and levels of risk differently.

Inner Setting - Institutional barriers to adoption

Oxford Academic Health Science Network

Looking at example of recent CAR-T implementation – 11 centres in UK

- Critical to build-in appropriate governance and oversight around ATMPs
- Role of Pharmacy has been important – natural fit with current QA responsibilities.

Inner Setting - Institutional barriers to adoption

Oxford Academic Health Science Network

Looking at example of recent CAR-T implementation – 11 centres in UK

Governance | Capacity | Training | Patient Mgmt

- Critical to build-in appropriate governance and oversight around ATMPs
- Role of Pharmacy has been important – natural fit with current QA responsibilities.

Inner Setting - Institutional barriers to adoption

Oxford Academic Health Science Network

Looking at example of recent CAR-T implementation – 11 centres in UK

Expression of Interest & Accreditation | Contracting | Governance | Capacity | Training | Patient Mgmt

- Capacity was one of biggest issues for CAR-T sites
  - Apheresis unit capacity
  - Stem cell freezing
  - Staff
  - Ward Capacity
  - ITU capacity

Inner Setting - Institutional barriers to adoption

Oxford Academic Health Science Network

Looking at example of recent CAR-T implementation – 11 centres in UK

- All CAR-T sites in UK reported challenge in releasing staff for the required training time.
- Not considered sustainable for providers to redesign processes and train dozens of staff for each ATMP

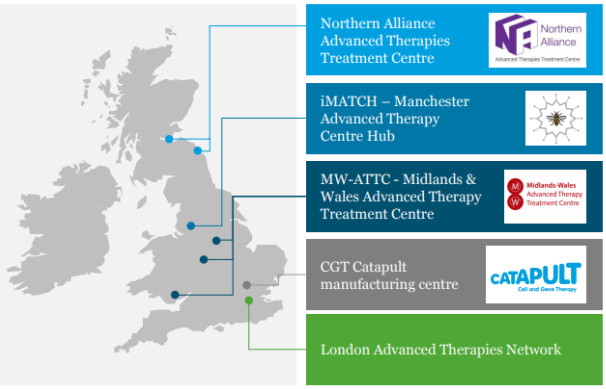
Inner Setting - Institutional barriers to adoption

Oxford Academic Health Science Network

Looking at example of recent CAR-T implementation – 11 centres in UK

- Developing appropriate referral and patient review pathways
- Regular national panel reviews and approves requests
- Weekly MDT meetings to discuss CAR-T patient management at site level

Outer Setting – Creating a network of "Innovation niches"

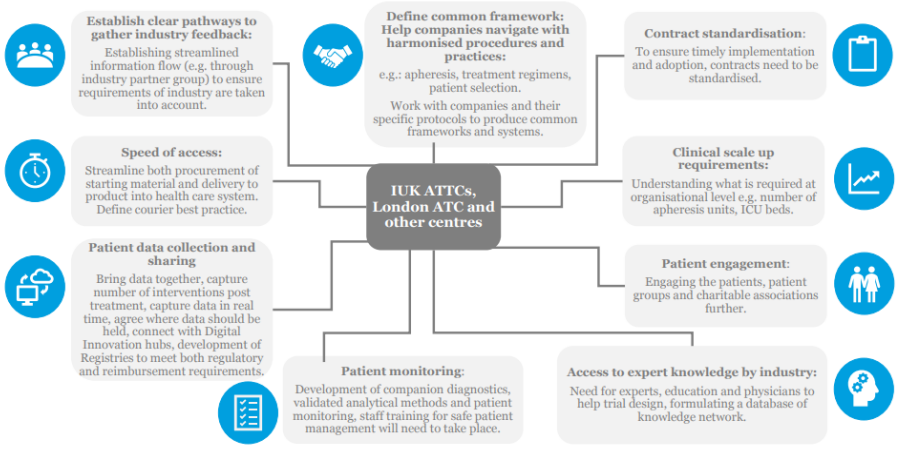


The network of Advanced Therapy Treatment Centres will develop and deliver systems for the delivery of cutting edge cell and gene therapies.

- The network will increase the ability of the NHS to deliver disruptive medicines
- The centres will develop systems and processes within the trusts and hospitals capable of delivering advanced therapies at scale to patients across the NHS
- The learnings and systems from the initial centres will be rolled out to other centres in the UK

OUTER SETTING

Challenges the ATTCs are exploring



Individuals – Understanding different perspectives

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Patients	Clinicians	Managers	Policy Makers
			
• Generating and maintaining healthy public perception • Joint decision making • Patient education	• Clinical champions • Clarity around roles and responsibilities • Embedding appreciate governance	• Fit with Trusts strategic priorities • Compelling business case • Levels of risk vs benefit	• Political Will to support sector • Alignment of policy with practice and patient need? • Addressing needs of global workforce

Individuals – Understanding different perspectives

Oxford Academic Health Science Network

Patients	Clinicians	Managers	Policy Makers
			
• Patient education	• governance	• Fit with strategic priorities • Compelling business case • Levels of risk vs benefit	• Political will to support sector • Alignment of policy with practice and patient need? • Addressing needs of global workforce

- Significant Hospital resource required for implementation around CAR-T.
- Estimated ~£42000 investment before first patient treated at one site (550 staff hours)

Individuals – Understanding different perspectives

Oxford Academic Health Science Network

Patients	Clinicians	Managers	Policy Makers
• Generating and maintaining healthy public perception • Joint decision making • Patient education	• governance	• Levels of risk vs benefit	• Political will to support sector • Alignment of policy with practice and patient need? • Addressing needs of global workforce

NHSE and CGT Catapult currently looking closely at the future workforce.
- Pharmacy's role
- Medical training
- Apprenticeships

Summary Points

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- ATMP Implementation is not straightforward - needs coordination and collaboration to ensure sustainability
- ATTCs in the UK will offer "innovation niches" for more efficient adoption and implementation approaches to be tested
- NHSE played key role in CAR-T adoption/ delivery (e.g. service spec development, patient panel, capacity management etc) – contributions to other parts of process may be necessary
- For patients to benefit from future ATMPs, changes required in infrastructure, data collection and workforce training will be required
- Opportunities to learn from outside our own innovation system very important

Appendix 15. Deep Brain Stimulation cost data used to inform the final values used in the analysis

	Device Cost		Surgery Costs		Hospital Costs		Pre Op Costs		Misc Costs		
Source	**Value**	**Info**	**Value**	**Info**	**Value**	**Info**	**Value**	**info**	**Value**	**info**	**Total**
Moon *et al* (2017)	£12,202.00	-	£7,772.00	-	£1,903.00	Averaged over 2 years	£641.00	-	£616.00	battery change	£23,134.00
DBS Policy (2013)	£12,202.00	-	£7,000.00	-	£2,000.00		£700.00	-	£4,000.00	battery change/ follow up	£26,058.00
Yianni *et al* (2013)	£11,104.00	-	£6,115.00	-	£1,593.00		£856.00	-	£1,059.00	follow up	£20,727.00
Eggington *et al* (2014)	£12,202.00	-	£7,131.00	-	£1,593.00		£641.00	-	£616.00	battery change	£22,183.00
Pietzch *et al* (2014)	Broken down cost data not provided										£24,680.00
Average	**£11,927.50**		**£7,004.50**		**£1,772.25**		**£709.50**		**£1,572.75**		**£22,986.50**

Appendix 16. Deep Brain Stimulation health utility data used to inform the final values used in the analysis

Source	Value	Gain from baseline of 0.42	Info	Baseline	QALY over 10 years	Increment over 10 years
Yianni *et al* (2013)	0.76	0.47	base line was 0.29	0.29	7.6	**4.7**
Moon *et al* (2017)	0.644	0.224	Improvement by 0.16 so intial health state would be 0.484	0.42	6.44	**2.24**
DBS Policy (2013)	0.59	0.17	70% reduction in motor complications . Quality of life improvement of 30% over medication	0.42	5.9	**1.7**
Eggington *et al* (2014)	0.72	0.3	from H&Y 3 50 -75 % off to H&Y 2 off 0- 25%	0.42	7.2	**3**
Pietzch *et al* (2014)	0.589	0.169		0.42	5.89	**1.69**
Average	**0.6606**	**0.2666**		**0.394**	**6.606**	**2.666**

Appendix 17. Deep Brain Stimulation sensitivity analysis of the costs associated with the therapy for the payer.

Sensitivity Analysis Table					
	Device Cost	**Surgery Costs**	**Hospital Costs**	**Pre Op Costs**	**Miscellaneous Costs**
level 1	£20,005	£21,235	£22,543	£22,809	£22,593
level 2	£22,987	£22,987	£22,987	£22,987	£22,987
level 3	£25,968	£24,738	£23,430	£23,164	£23,380
	Device Cost	Surgery Costs	Hospital Costs	Pre Op Costs	Miscellaneous Costs
Adjusted Sensitivity Table	£(2,982)	£(1,751)	£(443)	£(177)	£(393)
	£-	£-	£-	£-	£-
	£2,982	£1,751	£443	£177	£393
Range	5963.75	3502.25	886.125	354.75	786.375
Rank	**1**	**2**	**3**	**5**	**4**
Ordered Sensitivity Table	Device Cost	Surgery Costs	Hospital Costs	Miscellaneous Costs	Pre Op Costs
	£(2,981.88)	£(1,751.13)	£(443.06)	£(393.19)	£(177.38)
	£-	£-	£-	£-	£-
	£2,981.88	£1,751.13	£443.06	£393.19	£177.38

Appendix 18. Medication therapy cost data used to inform the final values used in the analysis

	Medication Costs		**Care Cost**		**Hospital Costs**		**Loss of Productivity Costs**		**Miscellaneous Costs**		
Source	**Value**	**Info**	**Value**	**Info**	**Value**	**Info**	**Value**	**info**	**Value**	**info**	**Total**
Parkinson's UK (2017)	£2,297.00	per year	£3,704.00	-		-	£11,093.00	-	£1,000.00	-	**£18,094.00**
Weir *et al* (2018)	£2,300.00	Can go up to £4000 in the tenth year of the disease	£3,700.00	-	£2,722.00	-	£11,000.00	-		-	**£19,722.00**
DBS Policy (2013)	£2,297.00	-	£9,776.00	-		-	£11,093.00	-		-	**£23,166.00**
Eggington *et al* (2014)	£3,725.00	-	£3,704.00	-	£1,593.00	-	£11,093.00	-		-	**£20,115.00**
Pietzch *et al* (2014)	£1,948.00	-	£3,704.00	-	£1,593.00	-	£11,093.00	-		-	**£18,338.00**
Average	**£2,654.75**		**£5,221.00**		**£2,157.50**		**£11,069.75**		**£1,000.00**		£22,103.00

Appendix 19. Medication therapy health utility data used to inform the final values used in the analysis

QALY	Value	Gain from base of 0.42	Info	Baseline	QALY over 10 years	Increment over 10 years		Meds QALY
Moon *et al* (2017)	0.644	0.08	Improvement by 0.16 so intial health state would be 0.484	0.564	5	0.8		**0.5**
DBS Policy (2013)	0.59	0.06	70% reduction in motor complications . Quality of life improvement of 30% over medication	0.53	4.8	0.6		**0.48**
Eggington *et al* (2014)	0.66	0.242	this is assumed that the drugs carry on working in the best way for period analysed	0.418	6.62	2.42		**0.662**
Pietzch *et al* (2014)	0.57	0.15		0.42	5.7	1.5		**0.57**
Average	**0.616**	**0.133**		**0.483**	**5.53**	**1.33**		**0.553**

Appendix 20. Medication therapy sensitivity analysis data of the associated costs for the payer.

Sensitivity Analysis Table					
	Medication Costs	**Care Cost**	**Hospital Costs**	**Loss of Productivity Costs**	**Miscellaneous Costs**
level 1	£21,439	£20,798	£21,564	£11,033	£21,103
level 2	£22,103	£22,103	£22,103	£22,103	£22,103
level 3	£22,767	£23,408	£22,642	£24,870	£22,603
	Medication Costs	Care Cost	Hospital Costs	Loss of Productivity Costs	Miscellaneous Costs
Adjusted Sensitivity Table	£(664)	£(1,305)	£(539)	£(11,070)	£(1,000)
	£-	£-	£-	£-	£-
	£664	£1,305	£539	£2,767	£500
Range	1327.375	2610.5	1078.75	13837.1875	1500
Rank	**4**	**2**	**5**	**1**	**3**
Ordered Sensitivity Table	Loss of Productivity Costs	Care Cost	Miscellaneous Costs	Medication Costs	Hospital Costs
	£(11,069.75)	£(1,305.25)	£(1,000.00)	£(663.69)	£(539.38)
	£-	£-	£-	£-	£-
	£2,767.44	£1,305.25	£500.00	£663.69	£539.38

Appendix 21. DopaCell/DopaCell adjusted cost data used to inform the values used in the analysis

	Therapy Costs		**Surgery Costs**		**Hospital Costs**		**Pre Op Costs**		**Misc Costs**		
Development cost	**Sell Price**	**Info**	**Value**	**Info**	**Value**	**Info**	**Value**	**info**	**Value**	**info**	**Total**
£10,000.00	£15,000.00	Sale price to payer	£7,000.00		£1,700.00		£712.00		£8,000.00	Immunosuppressants	**£32,412.00**

Appendix 22. DopaCell adjusted health utility data used to inform the values used in the analysis

Value	Gain from base of 0.42	Info	Baseline	QALY over 10 years	Increment over 10 years
0.72	0.3	Based on transitioning from H&Y III to H&Y II	0.42	7.2	3

Appendix 23. DopaCell/DopaCell adjusted sensitivity analysis data of the costs associated with the therapy for the payer

Sensitivity Table					
	Therapy Costs	**Surgery Costs**	**Hospital Costs**	**Pre Op Costs**	**Misc Costs**
level 1	£11,250	£38,662	£39,987	£40,234	£24,412
level 2	£15,000	£40,412	£40,412	£40,412	£40,412
level 3	£18,750	£42,162	£40,837	£40,590	£48,412
	Therapy Costs	Surgery Costs	Hospital Costs	Pre Op Costs	Misc Costs
Adjusted Sensitivity Table	£(3,750)	£23,662	£24,987	£25,234	£9,412
	£-	£25,412	£25,412	£25,412	£25,412
	£3,750	£27,162	£25,837	£25,590	£33,412
Range	7500	3500	850	356	24000
Rank	2	3	4	5	1
Ordered Sensitivity Table	Misc Costs	Therapy Costs	Surgery Costs	Hospital Costs	Pre Op Costs
	£9,412.00	£(3,750.00)	£23,662.00	£24,987.00	£25,234.00
	£25,412.00	£-	£25,412.00	£25,412.00	£25,412.00
	£33,412.00	£3,750.00	£27,162.00	£25,837.00	£25,590.00

Appendix 24. Input data for headroom method, cost-effectiveness and financial analysis

	$HRQoL_{ND}$	**$HRQoL_{CT}$**	**Duration**	**WTP (£)**	**C (£)**	**N (patients)**	**V (number of years with N patients)**	**C_{DD} (£) per patient**
DopaCell	1	0.553	10	30000	32412	100	1	**10000**
Dopacell (adjusted)	0.72	0.553	10	30000	32412	100	1	**10000**
Medication	0.553	0.553	10	30000	22103	100	1	**2654.75**
Deep Brain Stimulation	0.6606	0.553	10	30000	22986.5	100	1	**11927.5**