



CHARACTERISING THE ELECTROPHYSIOLOGICAL PROPERTIES OF CELLS IN HEALTH AND DISEASE

by

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Dedicated to my parents Sue and Dave, and my sister Hayley.

DECLARATION OF ORIGINALITY

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ABSTRACT

Biological cells possess intrinsic electrophysiological properties which are fundamental to cellular function. Changes in cell electrophysiology can act as a biomarker, for example to indicate transition from healthy to diseased cell states, changes in cell function, or cell differentiation. This thesis presents three studies which used dielectrophoresis (DEPtech 3DEP) and ζ -potential analysis (two fast, label-free, high-throughput, non-invasive, and low-cost tools) to examine the electrophysiological properties of two cell types, peripheral blood mononuclear cells (PBMCs) and chondrocytes, for novel medical applications.

The first study investigated the electrophysiological properties of PBMCs in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS); a debilitating disease of unknown pathophysiology with no reliable, validated, and quantitative diagnostic test. The dielectric and ζ -potential response of PBMCs to 1.5-hour hyperosmotic challenge differentiated ME/CFS donors from healthy controls with 81.80% sensitivity and 85.70% specificity. This shows potential as a quantitative diagnostic biomarker.

The second study examined whether the electrophysiological properties of PBMCs could act as a correlate of protection to SARS-CoV-2. Cytoplasmic conductivity in unchallenged PBMCs was significantly reduced in donors who had received three SARS-CoV-2 vaccine doses compared with unmatched COVID-19 naïve donors. Stimulation with the receptor binding domain of the SARS-CoV-2 spike protein resulted in significant differences in normalised values of membrane conductance in third-dose vaccinated donors, from COVID-19 naïve and second-dose donors.

The third study investigated chondrocytes, which are used extensively in cell-based cartilage-repair therapies. Chondrocytes rapidly dedifferentiate and become fibroblastic during monolayer cell culture – decreasing the success of reimplantation surgery. Significant changes in chondrocyte electrophysiological properties were observed over time in culture, laying the foundations for the identification of an electrophysiological biomarker that correlates with chondrocytic phenotype, to improve re-implantation outcome.

These studies demonstrate novel applications of dielectrophoresis and ζ -potential analysis – to quantitatively diagnose ME/CFS, identify changes in PBMCs following COVID-exposure, and changes in chondrocyte electrophysiology during dedifferentiation.

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NOMENCLATURE

$[X]_i$	Intracellular concentration of X
$[X]_o$	Extracellular concentration of X
E_X	Equilibrium potential
P_X	Combined relative permeabilities of channels permeable to X
E	Electric field
$+Q$	Positive charge
$-Q$	Negative charge
e	Electron charge ($1.602176634 \times 10^{-19}$ C)
F	Coulombic forces
F_{DEP}	Dielectrophoretic force
p	Dipole moment
$K(\omega)$	Clausius-Mossotti Factor
ϵ	Relative permittivity
ϵ_0	Permittivity of free space ($\approx 8.8541878 \times 10^{-12}$ F/m)
ϵ_{med}	Permittivity of the suspending medium
ϵ_m	Permittivity of the membrane
ϵ_c	Permittivity of the core
ϵ^*	Complex permittivity
ϵ_p^*	Complex permittivity of the particle
ϵ_m^*	Complex effective permittivity of membrane
ϵ_c^*	Complex effective permittivity of core
ϵ_{cell}^*	Complex effective permittivity of a cell
ϵ_{med}^*	Complex effective permittivity of suspension medium
ϵ_{1eff}	Effective permittivity
σ_m	Conductivity of membrane
σ_c	Conductivity of core
σ_m^*	Complex effective conductivity of membrane
σ_c^*	Complex effective conductivity of core
σ_{cell}^*	Complex effective conductivity of cell
σ_{1eff}	Effective conductivity

σ_{cyto}	Cytoplasmic conductivity
σ_{med}	Medium conductivity
t	Membrane thickness
f_{xo}	First cross-over frequency
f_{xo2}	Second cross-over frequency
ω	Radial frequency
F	Faraday gas constant (= 9.648×10^4 C/mol)
T	Temperature (Kelvin)
π	= 3.1415925...
j	= $\sqrt{-1}$
k	Boltzmann's constant (= 1.380649×10^{-23} J/K)
R	Ideal gas constant (8.314 J/K·mol)
$f(\kappa a)$	Henry's function
$1/\kappa$	Debye-Hückel screening length
C_m	Membrane capacitance
C_{eff}	Effective membrane capacitance
G_m	Membrane conductance
G_{eff}	Effective membrane conductance
c	Ion concentration (mol/m ³)
c_i	Concentration of ion i at the Stern layer
c_{oi}	Concentration of ion i in the bulk medium
z	Counterion valency
Λ	number of ions per m ³ for 1 S/m solution
η	Medium viscosity
r	Radius
U_E	Electrophoretic mobility
V_m	Membrane potential
ζ -potential	Zeta potential
ψ_{ext}	External surface potential
ψ_{st}	Stern potential
ψ_o	Surface potential

ABBREVIATIONS

AC	Alternating Current
ACE2	Angiotensin-Converting Enzyme 2
ACh	Acetylcholine
AChR	Acetylcholine receptor
ACI	Autologous Chondrocyte Implantation
APC	Antigen Presenting Cell
ARDS	Acute Respiratory Distress Syndrome
ATP	Adenosine Triphosphate
AUC	Area Under Curve
BCR	B-Cell Receptor
BK _{Ca}	Large-conductance Calcium and Voltage-Activated K ⁺ Channel
CaM	Calmodulin
CCC	Canadian Consensus Criteria
CCI	Characterised Chondrocyte Implantation
CD	Cluster of Differentiation
CI	Confidence Interval
CM	Clausius-Mossotti
CoP	Correlate of Protection
COVID-19	Coronavirus Disease 2019
CRAC	Calcium Release Activated Channel
cTFH	Circulating Follicular Helper T-cells
DAG	Diacylglycerol
DAMP	Damage Associated Molecular Pattern
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic Cell
DEP	Dielectrophoresis
DiSBAC	Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EDL	Electric Double Layer

EF	Extrafollicular
ELS	Electrophoretic Light Scattering
ELISA	Enzyme-Linked Immunosorbent Assay
ELISpot	Enzyme-Linked Immunosorbent Spot
ENaC	Epithelial Sodium Channels
E-protein	Envelope Protein
ER	Endoplasmic Reticulum
FBS	Foetal Bovine Serum
GC	Germinal Centre
GHK	Goldman-Hodgkin-Katz
GPCR	G-Protein Coupled Receptor
GWI	Gulf War Illness
ICC	International Consensus Criteria
ICS	Intracellular Cytokine Staining
IEG	Immediate-Early Genes
IFN- γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IP ₃	Inositol 1,4,5-trisphosphate
LDN	Low-Dose Naltrexone hydrochloride
LPS	Lipopolysaccharides
LSHTM	London School of Hygiene and Tropical Medicine
mAChR	Muscarinic Acetylcholine Receptor
MDV	Mean Difference Value
ME/CFS	Myalgic Encephalomyelitis/ Chronic Fatigue Syndrome
MHC	Major Histocompatibility Complex
M-protein	Membrane-protein
MS	Multiple Sclerosis
NAb	Neutralising Antibody
nAChR	Nicotinic acetylcholine receptor
NaCl	Sodium Chloride
NICE	National Institute for Health and Care Excellence
NK	Natural Killer
NKCC	Natural Killer Cell Cytotoxicity

N-protein	Nucleocapsid Protein
NTX	Naltrexone hydrochloride
OI	Orthostatic Intolerance
PAMP	Pattern Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PE	Phosphatidylethanolamine
PEM	Post-Exertional Malaise
PIP ₂	Phosphatidylinositol Bisphosphate
PKC	Protein Kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
Preg-S	Pregnenolone Sulphate
PRR	Pattern Recognition Receptor
PS	Phosphatidylserine
PSO	Post-Symptom Onset
RBC	Red Blood Cell
RBD	Receptor Binding Domain
RBM	Receptor-Binding Motif
RNA	Ribonucleic Acid
ROC	Receiver-Operator Characteristic
ROT	Electrorotation
RPMI	Roswell Park Memorial Institute
RyR	Ryanodine Receptors
SARS-CoV-2	Severe Acute Respiratory Coronavirus-2
SK _{Ca}	Small Conductance Calcium-Activated Potassium Channel
SNP	Single Nucleotide Polymorphism
SOCE	Store-Operated Calcium Entry
S-protein	Spike Protein
STIM	Stromal Interaction Molecule
TCR	T-Cell Receptor
TEA	Tetraethylammonium Chloride
TLR	Toll-Like Receptor
TNF- α	Tumour Necrosis Factor-alpha

TRP	Transient Receptor Potential
TRPM3	Transient Receptor Potential Melastatin 3
TWDEP	Travelling-Wave Dielectrophoresis
VOC	Variants of Concern
WHO	World Health Organisation

1. INTRODUCTION

This chapter introduces and summarises key concepts explored within the thesis. It is concluded with a breakdown of the thesis aims and objectives, research questions, and overall thesis structure.

1.1. The Electrical Properties of Cells

In the late 18th century, Luigi Galvani elicited muscle contraction in a preparation of frog legs, solely by holding the sciatic nerve cut from the opposite leg to the muscle using a non-conductive material. This evidence of an intrinsic electrical impulse without possible source of outside electricity, or as he then termed it ‘animal electricity’, laid the foundations of a new science, electrophysiology (Galvani, 1792, Piccolino, 1998, Funk et al., 2009, Harris, 2021, Hughes et al., 2021). Electrophysiology is the study of the electrical properties of biological cells and tissues (Park et al., 2013). From inspiring Mary Shelley’s character Frankenstein back in 1818, to becoming the basis of scientific journalist Sally Adee’s 2023 book “We Are Electric: The New Science of Our Body’s Electrome”, the idea that the human body is bioelectric, displaying and utilizing characteristics normally associated with electricity, is truly exciting. Bioelectricity is not confined to action potentials in excitable tissue and communication between the brain and nervous tissue (Adee, 2023). Bioelectricity plays an important role at each functional organizational level of the human body; in molecules and macromolecules, cell organelles, cells, tissues, organs, organ systems, and organisms (Funk and Scholkmann, 2023). How the bioelectric parameters of cells and the tissues they form come together is referred to as the electrome, and every single one of the 40 trillion cells in the human body possess their own “cellular electrome”.

The cellular electrome refers to the interaction of the electrical parameters of a cell (Hughes, 2023). The cell is encased in an insulative dielectric phospholipid plasma membrane which possesses resistance, conductance, and capacitance. Membrane conductance refers to the ability of a cell membrane to conduct charge. This consists of transmembrane conductance (movement of ions through the cell membrane via proteins which form aqueous pores spanning the impermeant lipid membrane – ion channels and transporters), and the conduction of ions across the membrane surface (Barker et al., 2017, Hoettges et al., 2019). Ion channel activity, charges on the membrane surface, and membrane morphology all affect membrane conductance (Hughes et al., 2022a). The cell membrane also forms a basic capacitor. The thin impermeable cell membrane separates electrolytes in the conductive cytoplasm and extracellular fluid, resulting in an accumulation of electric charge on either side of the dielectric membrane (Pethig, 2017). Membrane capacitance changes with the total cell surface area, and thus with changes in the cell radius and morphology, including membrane invaginations, ruffles, and microvilli. Membrane capacitance is also affected by cell surface modifications (Yale et al., 2018) and membrane composition (Golowasch and Nadim, 2013).

Encased by the phospholipid cellular membrane is a conductive cytoplasm (Hughes et al., 2021, Hughes et al., 2022a). Cytoplasmic conductivity (σ_{cyto}) refers to the concentration of ions within

the cell cytoplasm, most notably potassium (K^+), sodium (Na^+), calcium (Ca^{2+}), chloride (Cl^-), and magnesium (Mg^{2+}). The concentrations of ions are tightly controlled to facilitate intracellular signalling pathways necessary for cellular functions including gene expression, cell division, secretion, cell-volume regulation, and migration (Lewis et al., 1990). Additionally, ions are vital for the synthesis, folding, and stability of biomolecules and enzymes (Krzywoszyńska et al., 2020).

The transmembrane ion concentration gradients between the cytoplasm and extracellular environment give rise to a cell membrane potential (V_m). V_m is influenced by changes in intracellular and extracellular ion concentrations, the permeability of the cell membrane to ions, and the activity of ion pumps to maintain homeostatic ion concentrations. The electric potential at the hydrodynamic shear plane – the interface between ions which form a stable entity with the cell and ions beyond the plane within the continuous bulk solution, is called the zeta potential (ζ -potential) (Hughes et al., 2021, Hughes et al., 2022a). ζ -potential is a measure of the magnitude of the electrostatic attraction/repulsion between particles and is an indicator of the electrical stability of particles. A low absolute ζ -potential indicates low electrostatic repulsion and greater particle agglomeration (Fernandes et al., 2011, Leary, 2011, Malvern Panalytical, 2023).

Together these electrical properties are fundamental to cell function and physiological processes, including cell division (Strahl and Hamoen, 2010), cell proliferation (Sundelacruz et al., 2009), stem cell differentiation (Flanagan et al., 2008, Labeed et al., 2011, Adams et al., 2020), and cell volume regulation (Lewis et al., 2011).

1.2. Measuring the Electrical Properties of Cells

The electrical properties of cells can be measured using many different electrophysiological techniques. Patch-clamp is considered the gold-standard by many as it provides high-resolution measurements of ionic currents at a single-cell level. However, patch-clamp is highly invasive, destructive to the cell, time consuming, low throughput, and requires high expertise (Mansor and Ahmad, 2015). An alternative method by which the electrical properties of cells can be measured is through application of the electrokinetic phenomenon dielectrophoresis (DEP).

DEP is the movement of a suspended neutral polarizable particle in a non-homogenous electric field (Pohl, 1951, Pohl, 1958). The velocity and direction of movement of the particle within the non-uniform field depends on the shape, size, and polarizability of the particle relative to the polarizability of the suspension media, and the frequency of the alternating current (AC) electric field (Hoettges, 2010, Hughes, 2016, Cottet et al., 2019). Plotting the frequency-dependent relative

dielectrophoretic force experienced by cells within suspension across a range of frequencies (typically between 100 Hz to 50 MHz) produces a dielectrophoretic spectrum from which membrane conductance, membrane capacitance, and σ_{cyto} can be calculated (Kaler and Jones, 1990, Cottet et al., 2019). Specific membrane conductance and capacitance can be divided by membrane thickness to calculate effective membrane conductance (G_{eff}) and effective membrane capacitance (C_{eff}).

The 3DEP dielectrophoretic platform (DEPtech) is a commercial microfluidic platform which calculates the bioelectric properties of cells in real-time by simultaneously acquiring the relative DEP force experienced by at least 20 000 cells within suspension at 20 different frequencies in 30 seconds (Hoettges et al., 2019). Measuring the cellular bioelectric properties calculated from the high-resolution 20-point dielectrophoretic spectra has had a number of applications such as investigating the differentiation of neural stem cell progenitors (Labeed et al., 2011) and quantifying cellular apoptosis (Henslee et al., 2016). More recently, it has been used to characterize the properties of the cellular electrome in more detail (Hughes et al., 2021, Hughes et al., 2022a, Hughes et al., 2022b, Hughes, 2023).

ζ -potential cannot currently be derived from DEP spectra, and is instead measured using methods such as electrophoresis, electroosmotic flow, electroacoustic phenomena, sediment potential and streaming potential (Prakash et al., 2014). The Lab Blue Label zetasizer (Malvern Panalytical) is a commercially available machine which uses electrophoretic light scattering to measure the electrophoretic mobility of particles in solution, from which ζ -potential is calculated (Malvern Instruments 2015). Zetasizer instruments have been previously applied to measure changes in the ζ -potential of different types of human cells such as erythrocytes and HeLa cells both in health and during apoptosis (Bondar et al., 2012).

1.3. Measuring Changes in the Electrophysiological Properties of Cells for Medical Applications

As the electrophysiological properties of cells are indicative of cell function, quantifying changes in cellular electrophysiology has many applications including detecting transitions from healthy to diseased cell states, analysing changes in cell function, and tracking cell differentiation. Given that the DEPtech 3DEP and Malvern Zetasizer are low-cost, high throughput, non-invasive, and easily adoptable electrophysiological techniques, they are well-suited for characterising the electrical properties of cells for biomarker applications. For example, the 3DEP device has been used to characterise cellular dielectric changes in both oral cancer (Broche et al., 2007, Mulhall et al., 2011,

Liang et al., 2014, Graham et al., 2015, Hughes et al., 2023) and bladder cancer (Hoque et al., 2020) diagnostic applications.

Novel medical applications explored in this thesis include investigating whether changes in the electrical properties of healthy cells could be used as a biomarker for myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), as a correlate of protection to SARS-CoV-2, and to detect changes in chondrocyte dedifferentiation for cell-based cartilage-repair therapies.

1.3.1. Investigating an Electrical Biomarker for Myalgic Encephalomyelitis/ Chronic Fatigue Syndrome

ME/CFS is a chronic, complicated, and highly debilitating disease affecting up to 24 million people globally, characterized by unexplained disabling fatigue not relieved by rest and exacerbated by mental and physical exercise (Lacerda et al., 2018, Moneghetti et al., 2018, Lande et al., 2020). Pathological changes present across multiple organ systems including abnormalities in cellular metabolism, immune dysfunction, neuroendocrine disturbances, atypical blood pressure, and heart rate regulation; manifesting as many different symptoms including post-exercise malaise (PEM), cognitive dysfunction, muscle pain, and sleep abnormalities (Locher, 2015, CDC, 2021). ME/CFS significantly reduces the quality of life of sufferers, impacting their ability to go to work or school and engage in social activities, with one in four bed- or house-bound for long periods of time (CDC, 2018).

Despite global endeavours to identify a reliable biomarker, there is no validated quantitative laboratory test available for disease diagnosis (Hornig et al., 2015, Esfandyarpour et al., 2019). One challenge in finding a biomarker is the heterogeneity of the disease; the severities, frequencies and combinations of multi-systemic symptoms varies between patients, with a patients' own symptoms often changing too (Locher, 2015, Collatz et al., 2016, Nacul et al., 2020). Changes in natural killer (NK) cell function, altered cytokine profiles, complement activation, patterns in DNA methylation, and immune dysregulation are known to occur, but they have been unreproducible across studies as biomarkers, possibly due to the heterogeneity of ME/CFS sufferers and varied laboratory methodologies (Lande et al., 2020).

Without a standardized quantitative test, diagnosing ME/CFS is difficult. Doctors currently follow different sets of ME/CFS diagnostic criteria, use clinical guidelines, physical examinations, and medical histories to exclude differential diagnoses (Lande et al., 2020, NHS, 2021). The National Institute for Health and Care Excellence (NICE) advise diagnosis of ME/CFS to be explored when a

patient experiences extreme tiredness that has lasted at least six months, decreased their ability to do tasks and activities they used to do, and can not be explained by other causes (Locher, 2015, NHS, 2021). However, ME/CFS diagnostic criteria lack sensitivity, specificity, and are subjective, meaning many patients struggle with symptoms of ME/CFS for years before diagnosis and follow unsuccessful treatments which can be detrimental. It is estimated a further 84 to 91 percent of ME/CFS cases are currently undiagnosed, causing significant burden to public health (Locher, 2015, Moneghetti et al., 2018, Esfandypour et al., 2019). Furthermore, lack of consistency between ME/CFS diagnostic criteria used in different institutions is detrimental to research into this disease (Nacul et al., 2020).

Recent evidence shows that differences in the electrical properties of peripheral blood mononuclear cells (PBMCs) from individuals who have ME/CFS compared with healthy controls may pave the way towards a diagnostic tool. Esfandypour *et al.* (2019) recently developed a nano-electronics assay, with which they measured the electrical impedance of PBMCs from donors with moderate and severe ME/CFS and healthy controls when incubated in their own plasma supplemented with NaCl to impart hyperosmotic stress. The impedance response of PBMCs to the hyperosmotic stressor in ME/CFS donors was significantly different to that of healthy controls over a two-hour period (Esfandypour et al., 2019). However, the device used measured overall impedance – a single combination measurement consisting of cell membrane capacitance, σ_{cyto} , cell-cell interactions and cell-sensor surface adhesion (Esfandypour et al., 2019). Detailed electrical properties of unchallenged and hyperosmotically challenged PBMCs in ME/CFS, and how the properties compare to healthy and disease controls, remains uncharacterised.

1.3.2. Correlate of Protection to SARS-CoV-2

First reported in December 2019, Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) is a novel beta coronavirus strain which causes severe pneumonia-associated respiratory syndrome (Yang et al., 2020b, Zhou and Ye, 2021). The clinical presentation of SARS-CoV-2 infection varies amongst individuals, but can lead to acute respiratory distress syndrome in severely affected individuals, resulting in respiratory failure, sepsis and shock, multiple organ failure, and mortality (World Health Organisation, 2020, Olajide et al., 2021, Paludan and Mogensen, 2022). To protect the population from the medical, social, and economic impact of the Coronavirus Disease 2019 (COVID-19) pandemic, international collaboration of researchers led to the accelerated development and implementation of prophylactic SARS-CoV-2 vaccines including Pfizer/BioNTech BNT162b2 and Oxford/AstraZeneca ChAdOx1-S. Although vaccination has proven highly effective in preventing

symptomatic disease and reinfection (Polack et al., 2020, Amit et al., 2021, Petter et al., 2021, Voysey et al., 2021), both the humoral and cellular immune response have been demonstrated to decrease over time (Ibarrondo et al., 2020, Patel et al., 2020, Self et al., 2020, Seow et al., 2020, Gaebler et al., 2021, Morales-Núñez et al., 2021, Tseng et al., 2022, O'Shea et al., 2023).

To evaluate the efficacy of vaccination, ascertain population level immunity, inform immunisation programmes and develop recommendations on public health and social measures (Gilbert et al., 2022a, Misra and Theel, 2022, Mcvernon and Liberman, 2023), significant research has focused on identifying an easily adoptable and measurable correlate of protection (CoP) to SARS-CoV-2. Most studies focus on humoral immune responses. However, the cellular immune response to SARS-CoV-2 is also important in SARS-CoV-2 protection and clearance (Seow et al., 2020, GeurtsvanKessel et al., 2022). Limitations of current cellular T-cell response assays include a lack of standardisation across assays, and methods such as enzyme-linked immunosorbent spot (ELISpot) assays are time-intensive. Given that the electrical properties of PBMCs change following antigen stimulation, it is hypothesised that differential electrical responses to SARS-CoV-2 stimulation could pave the way towards a new cellular CoP.

1.3.3. Dedifferentiation of Chondrocytes During Cell Culture

Globally more than 250 million people suffer from cartilage injury and degradation (Chen et al., 2022b). However, cartilage has very limited regenerative capabilities because it is avascular and the migration ability of chondrocytes is limited (Costa et al., 2018, Mao et al., 2019). Therefore, cell-based tissue engineering therapies are used to repair damaged cartilage, such as autologous chondrocyte implantation (ACI). Significant *in vitro* cell culture expansion of isolated chondrocytes is required to obtain sufficient numbers for ACI. However, during monolayer cell culture chondrocytes rapidly dedifferentiate and become fibroblastic (Mao et al., 2019), with phenotypic changes reported to occur as early as passage one (Darling and Athanasiou, 2005). As chondrocytes become more fibroblastic, the success of re-implantation decreases, and consequently up to 70% of patients develop fibrocartilaginous fill (Bartlett et al., 2005, Huang et al., 2016, Mao et al., 2019); altering cartilage stretch and mechanical loading (Das et al., 2008). To optimise patient outcomes, characterised chondrocyte implantation is licensed in Europe (Saris et al., 2008, Vanlauwe et al., 2011). Methods used to monitor changes in chondrocyte phenotype include changes in gene expression (Ma et al., 2013), single-cell RNA sequencing, live-cell metabolic assays (Chen et al., 2022b) and flow cytometry immunohistochemical staining (Wang et al., 2001). Additional methods of assessing chondrocyte phenotype would prove highly beneficial. Characterisation of the electrical properties of cells have

been demonstrated to evaluate neural stem cell phenotype during differentiation (Labeed et al., 2011, Zhao et al., 2016b, Adams et al., 2018), but has not yet been applied to chondrocyte dedifferentiation during cell culture.

1.4. Thesis Aims and Objectives

The primary aim of this thesis was to explore changes in the electrophysiological properties of healthy cells as biomarkers for biomedical applications. To meet this aim, this thesis presents three studies which used dielectrophoresis and ζ -potential analysis to examine the electrical properties of two cell types; PBMCs and chondrocytes, across three applications.

The first study (**Chapter 3**) aimed to develop an electrical biomarker for ME/CFS, answering the research question: does the electrical response of PBMCs to hyperosmotic stress differentiate individuals who have ME/CFS from no ME/CFS donors? To achieve this, the first objective was to use the 3DEP and Zetasizer to measure baseline values of G_{eff} , C_{eff} , σ_{cyto} , and ζ -potential in unchallenged PBMCs donated from individuals diagnosed with severe ME/CFS, mild/moderate ME/CFS, healthy controls, and multiple sclerosis (disease control). The second objective was to measure the electrical response of PBMCs to NaCl and mannitol hyperosmotic challenge over 2-hours to identify which electrical parameters differentiate donor cohorts and could act as a potential diagnostic biomarker.

The second study (**Chapter 4**) aimed to investigate whether the electrical properties of PBMCs could function as a CoP to SARS-CoV-2, answering the research question: is the electrical response of PBMCs to SARS-CoV-2 stimulation different in COVID-19 naïve individuals compared with recovered or vaccinated donors, which could act as a CoP to SARS-CoV-2? The first objective was to identify the baseline properties of G_{eff} , C_{eff} , and σ_{cyto} in COVID-19 naïve, recovered COVID-19, and donors who had received their first dose, second dose, and third dose of a SARS-CoV-2 vaccine. Changes in the electrical properties of PBMCs following stimulation with the receptor binding domain of the SARS-CoV-2 spike protein were compared between donor cohorts to identify whether differential electrical responses occurred between donor cohorts, which could function as a CoP.

The third study (**Chapter 5**) aimed to investigate how the electrical properties of chondrocytes change over time during cell culture. This question was proposed to investigate how values of G_{eff} , C_{eff} , σ_{cyto} , and ζ -potential of chondrocytes change during cell culture for potential future applications as a marker of chondrocyte dedifferentiation, to improve the success of cell-based cartilage repair therapies. The second chapter aim was to identify how the electrical properties of chondrocytes

change when suspended in media of different ionic conductivities, to gain more insight into how the dielectric parameters of an adherent cell type are interconnected.

1.5. Thesis Structure

The structure of this thesis is as follows:

Chapter 2: The Electrophysiological Properties of Mammalian Cells

This chapter is an overarching literature review on the electrophysiological properties of cells, focussing on PBMCs and chondrocytes. The review begins with an introduction to the generic structure of a eukaryotic cell, before focussing on the structure and function of PBMCs and chondrocytes. The review subsequently focuses on the properties of the highly resistive dielectric cell plasma membrane and how it establishes a cellular electromotive force, minimalistically modelled as a bioelectric circuit possessing resistance, capacitance, transmembrane conductance, a cell membrane potential (V_m) and ζ -potential. How the electrical properties of PBMCs and chondrocytes facilitate their respective functions is subsequently described. Electrophysiological techniques used to measure bioelectric parameters are then explored, including the theory and biomedical application of the phenomenon dielectrophoresis and dielectric characterization of cells using the 3DEP device.

Chapter 3: Electrophysiological Changes in White Blood Cells Under Hyperosmotic Stress in ME/CFS

This first research chapter explores the differences in the cellular electromotive force of PBMCs isolated from individuals with ME/CFS compared with healthy controls and multiple sclerosis (disease control). It begins with a literature review evaluating the current diagnostic criteria used clinically to diagnose ME/CFS, the necessity for a quantitative diagnostic biomarker, and the current developments towards achieving this. The methods used to measure the electrical properties of PBMCs are then outlined, including the standard protocol for 3DEP, Zetasizer, and cell radius data collection. Differences in the electrical properties of PBMCs between donor cohorts when unchallenged and during hyperosmotic stress are then investigated. The performance of different electrical parameters as diagnostic biomarkers for ME/CFS are then assessed.

Chapter 4: The Electrophysiological Properties of White Blood Cells From COVID-19 Naïve vs. COVID-19 Exposed Donors

The second research chapter starts with a literature review which evaluates the necessity of a CoP to SARS-CoV-2, followed by an overview of humoral and cellular CoP and their limitations. Differences in the electrical properties of PBMCs when unchallenged and after stimulation with the receptor binding domain of the SARS-CoV-2 spike protein are then explored. The performance of the parameter which best differentiates individuals from different cohorts is then assessed and evaluated.

Chapter 5: Investigating The Dielectric Properties of Primary Chondrocytes During Cell Culture and in Different Medium Conductivities

This last experimental research chapter starts with a literature review on the clinical relevance of chondrocyte dedifferentiation during cell culture, then delves into how the extracellular environment affects the cellular electrome of healthy cells. This is followed by an outline of the data collection methods, including an outline of primary bovine chondrocyte cell culture and the collection of G_{eff} , C_{eff} , σ_{cyto} , and ζ -potential for cells acutely suspended in media of three different conductivities from passages 1 – 6, with up to five biological repeats. Differences in the electrical properties of chondrocytes over cell culture duration is explored.

Chapter 6: Key Findings, Future Work, and Conclusion

This final chapter discusses the key findings of the experimental Chapters 3-5, recommends direction of future work, and ends with a final conclusion.

Appendices: Appendix A, Appendix B, Appendix C, Appendix D

Appendix A – supplementary data to the research chapters 3 – 5.

Appendix B – DEP Modelling MATLAB Code developed by Professor Mike Hughes and Komal Kaur used to model the dielectrophoretic spectra obtained by the 3DEP machine to calculate G_{eff} , C_{eff} , and σ_{cyto} .

Appendix C – an outline of engagement to research throughout the duration of my PhD.

Appendix D – COVID impact statement to describe how the COVID-19 pandemic impacted the thesis.

2. THE ELECTROPHYSIOLOGICAL PROPERTIES OF MAMMALIAN CELLS

This literature review chapter delves into the biophysical characterization of cells as an electric circuit, introducing the 'Cellular Electrome'. The function of PBMCs and chondrocytes are explored, including how their electrical properties facilitate their respective functions. The techniques and tools used to measure the electrophysiological properties of cells are discussed, including the Malvern Zetasizer which uses electrophoretic light scattering to measure extracellular ζ -potential. There is particular emphasis on the phenomenon *dielectrophoresis* and the 3DEP machine, which applies DEP to measure G_{eff} , C_{eff} , and σ_{cyto} .

2.1. Introduction to Eukaryotic Cells

Cells are the fundamental units of life, and the functional and structural units of living organisms. With the advancement of microscopes, Robert Hooke is largely attributed for beginning the study of cells (cell biology) and famously first coined the word 'cell' (Hooke, 1665, Edwards, 2013). Later in the 1830s, two colleagues Schleiden and Schwann were the first to propose a unified definition of the cell, now known as cell theory (Bisceglia, 2014). Original cell theory stated all living organisms are composed of cells, and the cell is the basic functional and structural unit of life (Bisceglia, 2014). Rudolf Virchow in 1855 further contributed that new cells arise from pre-existing cells (Edwards, 2013). Some organisms possess only one cell, like simple bacteria and amoebae, whereas other organisms are multicellular, like humans who have approximately 10^4 cells (Bisceglia, 2014, Pethig, 2017).

2.1.1. The Fundamental Structure of a Mammalian Eukaryotic Cell

The fundamental structure of a mammalian eukaryotic cell (shown in **Figure 1**) consists of a lipid-based cell membrane which encases an internal gel-like fluid called the cytoplasm. Within the cytoplasm is an intracellular network of membranes which define specific intracellular compartments called membrane-bound organelles; subcellular structures surrounded by a membrane which perform a specific function (Perouansky, 2006).

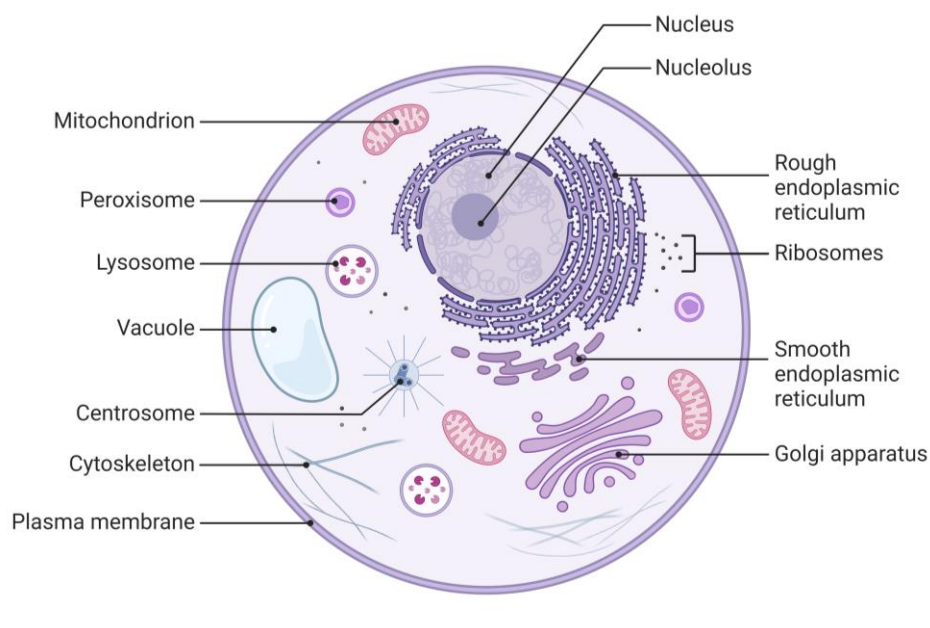


Figure 1 | A diagram of a generic mammalian eukaryotic cell and its organelles. Reprinted from "Structural Overview of an Animal Cell", by Biorender.com (2023). Retrieved from <https://app.biorender.com/biorender-templates>.

The Plasma Membrane

The main function of the plasma membrane is to serve as a semi-porous barrier that holds the cell together, spatially separates, and tightly controls the transport and diffusion of ions, proteins, lipids, nutrients, and pathogens intra- and extracellularly to provide a regulated intracellular environment. Other key functions include facilitating cellular interactions and cell signalling pathways (Alberts et al., 2015). To fulfil these functions, the fluid mosaic model proposed by SJ Singer and GL Nicolson in 1972 describes the fundamental structure of a plasma membrane to consist of a dynamic lipid bilayer, with a mosaic pattern of both integral transmembrane proteins and peripheral membrane proteins which only adhere to the surface (Singer and Nicolson, 1972, Goni, 2014).

The amphipathic nature of lipid molecules, consisting of a polar hydrophilic head group and a nonpolar hydrophobic tail, drives the spontaneous formation of lipid bilayers in aqueous environments to be most energetically favourable. Their hydrophilic head groups face the intracellular and extracellular compartments, and the hydrophobic acyl chains align laterally to form a hydrophobic core (Ma et al., 2017). Lipid molecules constitute ~ 50% of the mass of a plasma membrane, of which glycerophospholipids are predominant in eukaryotic cell membranes. Glycerophospholipids have a polar head group and two hydrophobic hydrocarbon fatty acid (acyl) chains (Alberts et al., 2015). Cholesterol intercalates between phospholipids, orientated so their hydroxyl groups are close to the polar head of the phospholipid and their steroid rings interact and immobilize the hydrocarbon groups closest to the polar heads. Cholesterol thus decreases the deformability and permeability of the membrane to small molecules and prevents the crystallization of hydrocarbon chains. The fluidity of the membrane is also affected by the length and saturation of the fatty acid tails, as they influence the ability for phospholipids to pack together (Cooper and Hausman, 2013, Alberts et al., 2015).

Glycoproteins are also located within the lipid bilayer, and are proteins which carry N- and O-glycosidically-linked carbohydrate chains (Brockhausen and Kuhns, 1997). All vertebrate cells possess a dense glycan layer on their cell surface, which is often terminated with sialic acids. Expression of glycoproteins affects the physiology of a cell, and varies between different cell types, functioning in cell-to-cell communication, adhesion, migration, and recognition by other cells and antibodies (Dagur et al., 2019). For example, the terminal sialic acid residues on lymphocytes assist in maintaining homing patterns to tissues (Brockhausen and Kuhns, 1997). Furthermore, the expression of glycoproteins changes in disease. For example, cells expressing P-glycoprotein are increased in patients with proliferative lupus nephritis, and there are glycoproteomic shifts in cancers such as leukaemia and lymphoma (Haverland et al., 2017, Tsujimura et al., 2017, Marx, 2021).

Intracellular Organelles

Some examples of membrane-bound organelles include the nucleus, mitochondria, endoplasmic reticulum (ER), Golgi apparatus, and lysosomes (Clark et al., 2019). The nucleus contains the cell's genetic information and is the principal site of DNA replication, transcription, and RNA synthesis, and is surrounded by a double membrane punctuated with large proteinaceous pore complexes, referred to as the nuclear envelope (Tripathi and Prasanth, 2011, Guo and Fang, 2014, Zidovska, 2020). The ER is the cell's largest organelle and is a continuous membrane system physically connected to the nuclear envelope, consisting of rough ER and smooth ER domains (Öztürk et al., 2020). The rough ER is an organized network of membrane sheets enveloping a common lumen, studded with millions of ribosomes bound to the cytosolic facing surface, serving as the site of protein synthesis. The smooth ER is a network of interconnected cylindrical tubules which synthesize and metabolize lipids, phospholipids, and steroids. The smooth ER is so called because it is smooth and not studded with ribosomes. Otherwise known as the 'powerhouse' of the cell, the mitochondria possess two membranes and are the primary site of metabolic respiration. Although the primary role of mitochondria is oxidative phosphorylation to produce cellular ATP, other key functions include ion homeostasis, apoptosis, and the production and consumption of reactive oxygen species (Brand et al., 2013).

Other organelles such as ribosomes are not membrane-bound. The function and structure of organelles found in the cell are described in more detail in **Appendix A.1 Table 20**. For a cell to function as a unit, there is extensive co-ordination and interplay between organelles, where closely situated organelles tether to bridge inter-organelle membrane contacts which facilitate the exchange of lipids, proteins, and metabolites (Schrader et al., 2020).

Throughout the human body different cells possess unique and specific properties to perform their specialized and intricate functions (Bisceglia, 2014), such as different glycoproteins expressed on their cell membranes. Cell types explored in this thesis include peripheral blood mononuclear cells and chondrocytes.

2.1.2. Peripheral Blood Mononuclear Cells

Whole blood consists of plasma, a watery liquid containing salts, proteins, enzymes, ions, hormones, and clotting factors (Miltenyi Biotec, 2023), within which blood cells including erythrocytes (red blood cells), platelets, and leukocytes (white blood cells) are suspended. Less than 1% of all blood cells are leukocytes – erythrocytes are approximately 1000 times more abundant (Shevkoplyas et al.,

2005). However, leukocytes are very important and form part of the immune system, whereby they circulate the blood to protect the body against pathogens and injury.

Peripheral blood mononuclear cells (PBMCs) are a heterogeneous subpopulation of leukocytes which all possess a single round nucleus, including monocytes, macrophages, natural killer (NK) cells, T-lymphocytes (T-cells), B-lymphocytes (B-cells), and dendritic cells (DCs). All PBMCs originate in the bone marrow from haematopoietic stem cells which differentiate during haematopoiesis into blood cells of myeloid and lymphoid lineages, a tightly regulated process which functions to continuously replenish all classes of blood cells. Developed PBMCs are subsequently secreted into the peripheral circulation whereby they are integral to the immune system (Delves and Roitt, 2011).

The immune system refers to physical barriers, chemical barriers and cellular processes which function to protect the body from pathogenic micro-organisms, cancer, toxins and viruses (Marshall et al., 2018). Aside from the physical protection provided by closed skin and mucosal membranes, and chemical barriers such as acid, mucus and enzymes which prevent pathogenic growth, there are two fundamental lines of defence: the innate and the adaptive immune system (IQWiG, 2006, Marshall et al., 2018). The innate immune system is the body's first line of defence; it responds to non-specific intruding pathogens, initiating a rapid immediate response within minutes or hours of encountering an antigen. The innate immune system has no immunological memory, thus does not recognize a pathogen even if it has encountered it before (Marshall et al., 2018). The adaptive immune response is the host's second line of defence. As opposed to the innate immune response, it is antigen-dependent and antigen-specific, with a slower lag-time of around 96-hours between antigen exposure and maximum immune response. However, the adaptive immune response does have the capacity for immunological memory, whereby subsequent re-exposure to the same antigen results in a quicker and more efficient adaptive response. The innate and adaptive responses are not exclusive mechanisms, but instead work synergistically and complement one another for maximal host protection (Delves and Roitt, 2011, Marshall et al., 2018). A summary of the key structure and functions of different PBMCs within the innate and adaptive immune response is included in **Table 1**.

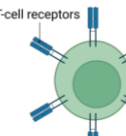
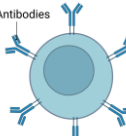
	Monocytes	Macrophages	Dendritic Cells	Lymphocytes		
				Natural Killer Cells	T-Cells	B-Cells
Immune Response	Innate	Innate	Innate/Adaptive	Innate	Adaptive	Adaptive
Structure	 (Created with Biorender.com) Kidney-shaped nucleus	 (Created with Biorender.com) Vary depending on subtype	 (Created with Biorender.com) Dendritic tree-like shape	 (Created with Biorender.com) Single-lobed nucleus	 (Created with Biorender.com) Round nucleus	 (Created with Biorender.com) Round nucleus
Percentage of PBMCs	5 – 10%	Varies	1 – 2%	10 – 30%	25 – 60% CD4 ⁺ 5 – 30% CD8 ⁺	5 – 10%
Lifespan	Hours/days	Months/years	Days/week	7 – 10 days	Weeks/years	Days (plasma) Years/lifetime (memory)
Function	Differentiate into macrophages and dendritic cells. Phagocytosis.	Phagocytosis. Chemokine and cytokine production. Tissue surveillance. Antigen presentation to T-cells.	Antigen-presenting cell. Phagocytosis.	Release perforin and granzymes to induce apoptosis and kill infected cells.	T-helper cells (CD4⁺): Activate macrophages, B-cells and dendritic cells. Cytotoxic T-cells (CD8⁺): Kill target cells via 1) Cytokine secretion. 2) Releasing cytotoxic granules. 3) Activating caspase cascade to trigger apoptosis. Regulatory T-cells: Suppress the immune response to maintain self-tolerance.	Plasma cells: Secrete antibodies. Memory B-cells: Long-lived quiescent cells which respond quickly when an antigen is re-encountered.

Table 1 | Summary of the structure and functions of PBMCs (Peckham et al., 2003, Cheng et al., 2010, Marshall et al., 2018, Khodadadi et al., 2019, Palm and Henry, 2019, Miltenyi Biotec, 2023).

Function of PBMCs within the Innate Immune System

Monocytes, macrophages, DCs and NK cells are part of the innate immune response. As part of the innate immune system, they express Pattern Recognition Receptors (PRRs) and proteins on their cell membranes, intracellular organelle membranes and within the cytoplasm (Li and Wu, 2021). PRRs recognise and bind to molecular patterns associated with pathogens which are not found in the host (Pattern Associated Molecular Patterns: PAMPs) and molecules released from damaged/necrotic cells (Damage Associated Molecular Patterns: DAMPs). There are many different types of cell-associated and soluble PRRs, including Toll-like receptors (TLRs), C-type lectin receptors, retinoic acid-inducible gene-I-like receptors, nucleotide oligomerization domain-like receptors, and absent in melanoma-2-like receptors (Mogensen, 2009, Delves and Roitt, 2011). Recognition and binding of PRRs to PAMPs and/or DAMPs induces intracellular signalling cascades which orchestrate pro-inflammatory and microbicidal responses to contain and eliminate pathogens (Amarante-Mendes et al., 2018).

Monocytes are the largest leukocyte with a diameter of between 12 – 20 μm , possess a convoluted bilobed nucleus (kidney-shaped), and have a half-life of between 1 – 3 days (Yang et al., 2014, Espinoza VE, 2023). Monocytes express TLRs and scavenger receptors, and on detecting and binding to PAMPs or DAMPs they leave the bone marrow, migrate, and infiltrate the infected/damaged tissue within 12 – 24 hours (Espinoza VE, 2023). In the tissue, monocytes can differentiate into tissue macrophages or DCs (Shi and Pamer, 2011). Macrophages have different shapes upon maturation, depending on their polarizability. In response to stimuli including cytokines, metabolic factors, and microbial products, monocyte derived macrophages become polarized into either classical M1 subtypes (which are driven by bacterial lipopolysaccharides (LPS) and interferon- γ (IFN- γ) and have pro-inflammatory and tissue-destructive phenotypes). Alternatively, macrophages are activated M2 subtypes (driven by interleukin-4 (IL-4)) which are tissue-reparative (Cassol et al., 2009, Hoare et al., 2016). Dysregulated or exaggerated responses of monocytes and macrophages results in host tissue damage, chronic inflammatory disease, autoimmunity, fibrosis, or tumorigenesis (Hoare et al., 2016, Selezneva et al., 2022).

Monocytes, macrophages, and DCs are all professional phagocytes responsible for the removal of pathogens. Phagocytosis refers to the ingestion and elimination of particles over 0.5 μm in diameter, including pathogens and apoptotic cells. Phagocytes express opsonin receptors (immunoglobulin Fc receptors and complement receptors), which bind to pathogens coated in either antibodies or complement proteins to promote efficient phagocytosis (Uribe-Querol and Rosales, 2020). Phagocytosis is also triggered when PAMPs and DAMPs bind to certain PRRs (Li and Wu, 2021).

Once recognized by phagocytic receptors, activation of intracellular signalling pathways results in internalization of the particle. Phagocytosis involves a change in membrane composition and reorganization of the actin cytoskeleton so the part of the membrane where the phagocytic receptor bound to the pathogenic ligand depresses and forms a phagocytic cup. Pseudopods form around the pathogen and the protrusions fuse at the distal end such that the pathogen is completely internalized within a newly formed vesicle known as the phagosome. The new phagosome matures by combining with early endosomes and lysosomes to become a very acidic phagolysosome with a pH of 4.5. Hypochlorous acid (a potent microbicidal) and hydrolytic enzymes including lysozymes, cathepsins, proteases and lipases within the phagolysosome degrade ingested pathogens to form many antigen fragments. The efficiency of phagocytosis is increased by cytokines, inflammatory mediators, and bacterial products. Phagocytosis is also regulated by cell differentiation, with macrophages having a higher phagocytic efficiency than monocytes (Uribe-Querol and Rosales, 2020).

Antigen fragments obtained during phagocytosis are loaded onto major histocompatibility complexes (MHC) II molecules and transported to the surface of the cell to be presented to lymphocytes. Only once an antigen is processed and presented on an MHC II molecule can CD4⁺ T-cells recognise the antigen and become activated. Monocytes, macrophages, and DCs constitutively express MHC II and are professional antigen-presenting cells (APCs), forming a conduit between the innate and adaptive immune response (Delves and Roitt, 2011, Kambayashi and Laufer, 2014, Hoare et al., 2016, Chakraborty et al., 2020). DCs are the main APC, and possess numerous cytoplasmic processes to increase their surface area-to-volume ratio, facilitating contact with large numbers of surrounding cells (Roghani, 2022).

Representing 5 – 15% of circulating PBMCs, natural killer (NK) cells are large granular effector lymphocytes, which under physiological conditions circulate in a resting state (Paul and Lal, 2017, Sharrock, 2019). NK cells share a common progenitor with T-cells, but do not express a T-cell receptor and are considered within the innate immune response due to a lack of antigen-specific cell surface receptors (Vivier et al., 2011). NK cells target pathogen-infected and cancerous host cells via an integrated balance of activating co-stimulatory and inhibitory signals which determine NK cell activation and function (Wu et al., 2020b). Healthy nucleated host cells express major histocompatibility complex class I (MHC I) molecules which bind to immunoglobulin-like receptors expressed on NK cells to inhibit NK function and provide self-tolerance to healthy host cells (Sharrock, 2019). However, in virus-infected or cancerous cells, MHC expression can become reduced or absent when interferon responsive gene products interfere with protein translation. In response to stresses such as DNA damage, the MHC complex can also present proteins which are not normally expressed on host cells. Resultant loss of inhibitory binding between the MHC class I and NK cell receptors results

in activation of NK-mediated killing by either the death receptor pathway or the granule-dependent pathway, leading to apoptosis within 30 – 60 minutes (Delves and Roitt, 2011, Paul and Lal, 2017). Once activated, NK cells secrete cytokines, which modulate the function of other immune cells. These cytokines include IFN- γ , granulocyte macrophage colony-stimulating factor, tumour necrosis factor- α (TNF- α), and chemokines such as CCL2, CCL4 and CXCL8 (Paul and Lal, 2017).

Function of PBMCs within the Adaptive Immune System

The adaptive immune response is primarily mediated by T- and B-cells which both display highly specific receptors on their plasma membranes.

80% of blood lymphocytes are T-cells. All T-cells express a specific T-cell receptor (TCR)-CD3 complex on their cell membrane. The TCR-CD3 complex is composed of an $\alpha\beta$ TCR heterodimer (sometimes $\gamma\delta$) non-covalently associated with CD3 $\epsilon\gamma$, CD3 $\epsilon\delta$ and CD3 $\zeta\zeta$ dimers (Mariuzza et al., 2020). The TCR α and β immunoglobulin-domain polypeptide chains consist of a constant region and a highly diverse variable domain (generated by somatic V(D)J recombination) which bind to peptide-MHC complexes for antigen recognition with incredibly high specificity and sensitivity (Hwang et al., 2020). MHC class I proteins expressed on all nucleated cells present endogenous intracellular peptides to T-cells. In comparison, MHC class II proteins are only expressed by APCs present exogenous extracellular peptides generated through proteolytic degradation of both self- and foreign proteins within cells, as previously described above (Marshall et al., 2018, Mariuzza et al., 2020).

A naïve T-cell which has not been previously engaged in an immune response undergoes clonal expansion to build great numbers of cells which target a specific antigen, and differentiate to an effector T-cell capable of mounting an immune response after receiving two key signals (Delves and Roitt, 2011). The first signal is TCR peptide-MHC binding, which is propagated via the CD3 co-receptor complex immune tyrosine activation motifs (ITAMs). Phosphorylation of ITAMs by the Src family protein tyrosine kinase Lck initiates down-stream T-cell signalling cascades (Delves and Roitt, 2011, Hwang et al., 2020, Mariuzza et al., 2020). The second signal is co-stimulation including CD80/CD86 on DCs engaging with CD28 on T-cells, which is mediated by increased IL-2 secretion (Delves and Roitt, 2011).

T-cells differentiate to either CD8⁺ (cytotoxic) T-cells or CD4⁺ (T-helper) T-cells following stimulation (Marshall et al., 2018). Cytotoxic T-cells bind to peptides presented on MHC class I proteins, and are effector cells which kill virally infected cells, cells containing intracellular bacteria, and cancerous cells (Sauls, 2022). Cytotoxic T-cells kill cells via two distinct pathways, the Fas/Fas

ligand pathway and perforin/granzyme pathway which both culminate in the activation of caspases (cytotoxic proteases). Cytotoxic granules are transported via the microtubules system to the immunological synapse, where perforin facilitates entry of an array of cathepsin-like proteases (granzymes) into the target cell to induce cell death (Delves and Roitt, 2011). CD8⁺ T-cells also release cytokines IFN- γ , TNF- α and TNF- β to inhibit viral replication, increase MHC class I expression and activate macrophages (Janeway CA Jr, 2002).

CD4⁺ T-helper cells (Th) consist of multiple different subtypes based on their cytokine secretion, including Th1, Th2, Th17, TH cells, and regulatory T-cells. The strength of TCR stimulation determines the differentiation path; for example, strong TCR stimulation in CD4⁺ T-cells favours Th1 cell differentiation as opposed to Th2 cell differentiation (Delves and Roitt, 2011, Hwang et al., 2020). Th1 lymphocytes enhance responses to intracellular bacteria and viruses through IFN- γ secretion, activating macrophages and assisting in CD8⁺ lymphocyte expansion. Furthermore, Th1 lymphocytes enhance the production of opsonizing antibodies by facilitating B-cell differentiation, enhancing phagocytosis efficiency (Delves and Roitt, 2011, Marshall et al., 2018). The Th2 response involves release of cytokines IL-4, IL-5, and IL-13, helping the differentiation, proliferation, and class switching of B-cells (Constant and Bottomly, 1997). The Th17 response promotes inflammatory responses and neutrophil recruitment through secretion of IL-17 (Delves and Roitt, 2011). Following infection, most effector cells are phagocytosed, but some are retained as memory cells. Memory cells initiate a faster and stronger immune response on re-exposure to an antigen (Delves and Roitt, 2011, Marshall et al., 2018).

B-cells constitute 5 – 10% of total PBMCs and facilitate the humoral antibody response – they are involved in antibody production, cytokine secretion, antigen-presentation, and modulating T-cell differentiation (Feher et al., 2022). Mature B-cells co-express the B-cell receptor (BCR) isotypes transmembrane IgD and IgM molecules on their cell surface which bind to specific antigens (Treanor, 2012, Tsai et al., 2019). The expression of cell surface proteins varies between cell subtypes. Specific proteins can be used for lymphoid cell typing and classification, for example between progenitor cells, immature B-cells, and memory B-cells (Haverland et al., 2017).

Similar to T-cells, B-cell activation and differentiation requires two signals. The first signal is derived from antigen coupling to BCRs. The second signal can be either T-cell independent or T-cell dependent. T-cell independent antigens include lipopolysaccharides and glycolipids, which cause differentiation to short-lived plasma cells with low-affinity antibodies. The five types of antibodies include IgA, IgD, IgE, IgG, and IgM. The secretion of cytokines from local Th cells direct which type of antibody is produced. Antibodies bind to antigens on the pathogen surface to promote pathogen

elimination via effector cells, phagocytosis (acting as an opsonin), and complement activation (Marshall et al., 2018). In contrast, T-cell dependent responses are induced by follicular helper T-cells, whereby the CD40 ligand of T-cells binds to CD40 expressed on B-cells. Binding to CD40 gives rise to either short lived plasma cells or facilitates B-cell entry into the germinal centre to differentiate into memory B lymphocytes or plasma cells with high antigen affinity (Delves and Roitt, 2011, Tsai et al., 2019). Memory B lymphocytes have a long lifespan and continue to express antigen-binding receptors to produce antibodies and eliminate antigens quickly on re-encounter (Marshall et al., 2018).

2.1.3. Chondrocytes

Cartilage is a strong, flexible, load-bearing connective tissue that serves as a shock absorber, protects joints and bones, and provides low friction properties for smooth movement of arthrodial joints (Kwon et al., 2016, Van Gelder et al., 2023, Zhou et al., 2023). There are three types of cartilage in the body including hyaline cartilage, elastic cartilage, and fibrocartilage (Chang, 2024), each specialised to perform different functions. Hyaline cartilage is the most common, and is located on the articular surfaces of joints, in addition to the nasal septum, larynx, and tracheal rings. Fibrocartilage is found in intervertebral discs, the pubic symphysis, and in ligaments, whereas elastic cartilage is found in the external ear, the epiglottis, and in parts of laryngeal cartilages (Young et al., 2014).

Cartilage is avascular and has a very low cell content – it is composed of a dense extracellular matrix (ECM) and a sparse distribution of chondrocytes. Chondrocytes are the only resident cells found within cartilage (Sophia Fox et al., 2009), and occupy ~ 1 – 5% of total cartilage volume (Akkiraju and Nohe, 2015, Rim et al., 2020). Chondrocytes are specialised cells which function to synthesise, maintain, and remodel the ECM, including the production of collagen II (Barrett-Jolley et al., 2010, Mobasheri et al., 2012, Lewis et al., 2013, Akkiraju and Nohe, 2015). Chondrocytes are typically quiescent, and function to maintain the ECM in low-turnover equilibrium (Otero et al., 2011). The ECM is the non-cellular component present in tissues, consisting primarily of water, collagen, and proteoglycans, in addition to non-collagenous proteins, and glycoproteins (Sophia Fox et al., 2009, National Institutes of Health, 2023). The ECM functions to physically maintain cells and influence cellular progresses, such as migration, differentiation, and proliferation (Kular et al., 2014).

Chondrocytes are approximately 10 – 13 μm in diameter (Phull et al., 2016). *In situ*, chondrocytes exist in lacunae; fibre-free and proteoglycan-rich cavities (Karamanos et al., 2021) and appear singly with a round/elliptical morphology (Karim et al., 2018). As chondrocytes partake in

protein synthesis, chondrocytes have a prominent ER, developed Golgi apparatus, and glycogen granules scattered in the cytoplasm. Interactions with the ECM are mediated via cytoplasmic processes (Young et al., 2014). The chemical and mechanical environment of chondrocytes, such as pro-inflammatory cytokines and growth factors regulate the metabolic activities of chondrocytes (Akkiraju and Nohe, 2015, Karamanos et al., 2021).

Each type of cartilage varies by the amount and type of fibres embedded in abundant proteoglycan ground substance; a porous hydrated gelatinous material which resists compressive forces and bares compression (Young et al., 2014, Maleckar et al., 2018). Mature hyaline cartilage consists of small aggregates of chondrocytes within a matrix of ground substance reinforced by few collagen fibres. Fibrocartilage consists of hyaline cartilage with thick layers of dense abundant collagen fibres, whereas elastic cartilage contains numerous bundles of branching elastin fibres, particularly dense in the vicinity of the chondrocyte (Young et al., 2014). Elastic and collagen fibres (mainly type II collagen) provide tensile strength. Trapped proteoglycans (including aggrecan) account for the solid, flexible consistency of cartilage, and help resist compressive forces and bare compression (Maleckar et al., 2018, Young et al., 2014).

Imbalance in the function of chondrocytes can lead to osteoarthritis. Osteoarthritis is a degenerative disease characterised by the degradation of cartilage, the formation of osteophytes, and stiffening of joints. A breakdown of proteoglycans results in a reduction to bare compression and an acceleration in collagen loss (Akkiraju and Nohe, 2015). Cell culture of isolated chondrocytes is useful for studying cellular responses to alterations in the environment, such as in osteoarthritis (Otero et al., 2011). An image of primary chondrocytes in cell culture is shown in **Figure 2**. It is also important for increasing the number of chondrocytes for cell-based cartilage-repair therapies. Significant *in vitro* expansion of isolated chondrocytes is required to obtain a sufficient cell number before re-implantation (Mao et al., 2019).

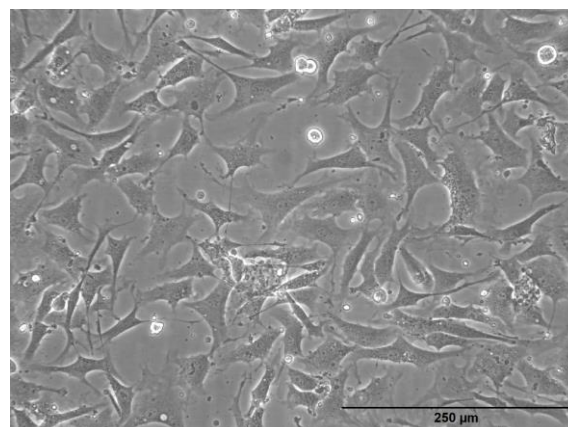


Figure 2 | Passage 0 primary bovine chondrocytes in cell culture.

During standard monolayer cell culture protocols, chondrocytes rapidly dedifferentiate and change phenotype to become more fibroblastic (Layman et al., 1972, Lin et al., 2008, Schulze-Tanzil, 2009, Mao et al., 2019, Ghosh et al., 2021), and senescent (Piñeiro-Ramil et al., 2023). Changes in the relative gene expressions of collagen type I and II, and superficial zone protein have been shown to occur as early as passage one using quantitative real-time PCR in monolayer-passaged articular chondrocytes (Darling and Athanasiou, 2005). Past passage four, chondrocytes no longer produce cartilage-specific matrix proteins including collagen type II and cartilage-specific proteoglycans (Schulze-Tanzil et al., 2002), and increase their expression of collagen type I, indicating chondrocyte dedifferentiation (Kang et al., 2007). Dedifferentiated chondrocytes possess a flattened cell morphology and increased formation of stress fibres (Ghosh et al., 2021). As passage number increases, chondrocyte growth rate and viability decrease, and apoptosis increases (Kang et al., 2007). Dedifferentiation is thought to be attributable to epigenetic factors such as DNA methylation (Duan et al., 2015, Duan et al., 2017), senescence (Ashraf et al., 2016), the microenvironment, and oxidative stress (Chen et al., 2022b).

2.2. The Cell as an Electric Circuit

It was in 1902 that Julius Bernstein, without any knowledge of the structure of a cell or the presence of a plasma membrane, hypothesized cells have a thin membrane which is largely impermeable to ions, and surrounds a conducting electrolyte (Bernstein and Tschermak, 1906, Pethig, 2017). Furthermore, Bernstein hypothesized an electrical potential exists across the thin membrane when in a resting state, and in excitable cells electrical stimulation results in a transient increase in membrane permeability to K^+ ions, thus a reduction in transmembrane potential difference (Pethig, 2017). Not long later, between 1910 and 1913, Rudolf Höber provided the first electrical evidence validating Bernstein's hypothesis of the cell possessing an electrolytic cytoplasm containing free ions. Through a series of experiments, Höber determined the conductivities of frog muscle tissue and compacted red blood cells were approximately ten times greater at high electric frequencies of ~5 MHz than at low frequencies of ~150 Hz. At low frequencies, the current could not penetrate the dielectric insulating membrane and flowed around the cell, whereas at high frequencies the current reached the conducting core. When the cell membrane was perforated, the conductivity recorded at low frequencies increased to that of MHz frequencies (Höber, 1910, Höber, 1912, Höber, 1913, Pethig and Schmueser, 2012).

Through the work of electrophysiologists including Lund, Nernst, Goldman, Hodgkin, Huxley, and Katz to name a few, the bioelectric model of the cell has become more understood, though is still an evolving working model. In its minimalist form, a cell consists of a charged core surrounded by a dielectric membrane possessing surface charge on its inner and outer surface (Hughes et al., 2021). As shown in **Figure 3**, the dielectric plasma membrane has capacitance (membrane capacitance), conductance (both transmembrane conductance and surface conductance), and surrounds a conductive cytoplasm containing different concentrations of different ions. Transmembrane ion concentration gradients give rise to a cell membrane potential (V_m), and the charge at the cell membrane surface produces an extracellular zeta potential (ζ -potential) (Hughes et al., 2021, Hughes et al., 2022a).

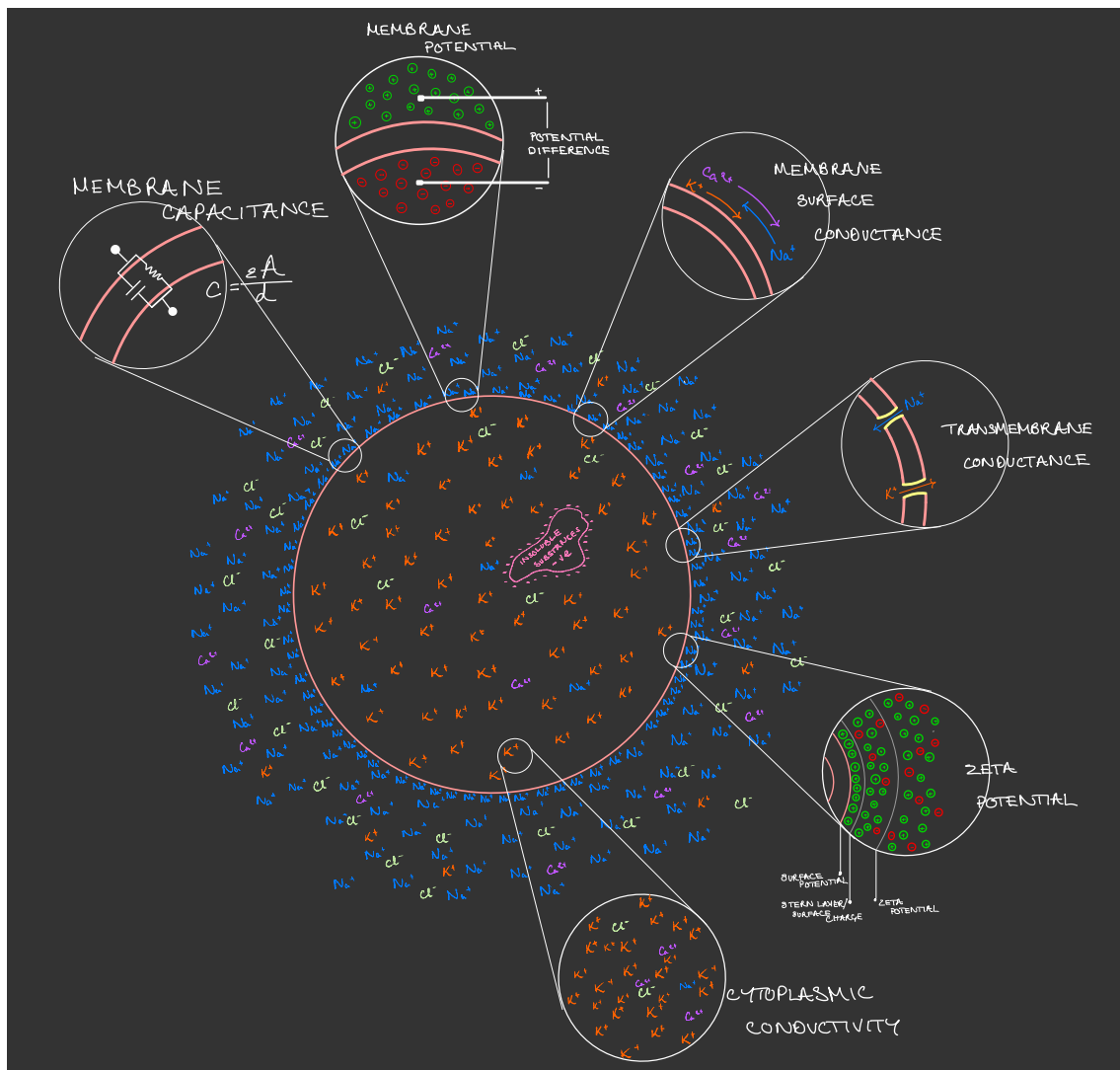


Figure 3 | The Cellular Electrome. Illustration by Oreoluwa Griffiths.

2.2.1. Membrane Conductance

Membrane conductance refers to the ability of a cell membrane to conduct charge, and is a composite of the movement of ions across the membrane (transmembrane conductance) and the conduction of ions across the membrane surface (surface conductance) (Hoettges et al., 2019). Small uncharged polar molecules including water, oxygen, and carbon dioxide diffuse freely across the dielectric plasma membrane, but the transport of hydrophilic and charged species e.g. ions and larger molecules such as sugars and amino acids are greatly regulated (Pethig, 2017). This regulation is due to the fact phospholipids themselves are incredibly resistive; in saline solutions pure phospholipids have been recorded to have a resistivity of $10^{13} \Omega\text{m}$ and phospholipid bilayers are estimated to have a specific resistivity (R_m ; resistance per unit square area of membrane) of $40 \text{ k}\Omega\text{m}^2$. Biological membranes have a much lower resistivity at around $0.01 - 1 \Omega\text{m}^2$ due to the presence of other structures embedded in the bilayer such as ion channels, transporters, and protein-bounded aquaporins which facilitate regulated movement of impermeable molecules across cell and organelle membranes (Pethig, 2017, Mahtani and Treanor, 2019).

Ion channels are integral membrane proteins which assemble into multi-molecular aggregates to form an aqueous channel pore to facilitate the passage of ions across a plasma membrane. Ions move either by facilitated diffusion (passive movement along concentration and electrochemical gradients between either side of the plasma membrane e.g. cytoplasm and extracellular fluid), or by transporters which use active transport (ATP driven pumps against the concentration gradient) (Feske et al., 2015). There are a huge number of different ion channels and transporters encoded within the human genome – the HUGO Gene Nomenclature Committee database lists 330 ion channel genes encoding a diverse range of channels which differ in which ions they specifically transport and the physiological processes they regulate (Moreau et al., 2008, Mahtani and Treanor, 2019). Ion channels which are always open, even in unstimulated or ‘resting’ cells are referred to as leak channels. Gated ion channels activate in response to specific mechanical or chemical stimuli, such as ligand binding (ligand-gated ion channels), changes in voltage across the membrane (voltage-gated ion channels), mechanical stress (mechano-sensitive ion channels), temperature, osmotic pressure, or lipid binding the ion channels. In response to their activating stimulus, gated ion channels undergo conformational changes to transition between an open and closed state (gating) to allow specific ions through in single-file fashion in response to stimuli. The physical width of a channel and the amino acids which line the interior of the channel specify which ions travel through (Zaydman et al., 2012, Nature Education, 2014, Mahtani and Treanor, 2019).

The passive resistance of a cell membrane refers to its permeability to ions (Pethig, 2017). The permeability of the membrane for a given ion is controlled by the number and activity of ion channels for that ion, and represents the electrical transmembrane conductance of a membrane. Changes in membrane conductance is indicative of ion channel presence and function (Ramahi and Ruff, 2014). The density of different ion channels typically ranges from 50 – 500 per μm^2 , and their conductance is around 1 – 100 pS (Pethig, 2017). Factors which affect ion channel function include pharmacological inhibition, pH, temperature, phosphorylation of ion channel residues, and intracellular messengers (Zuberi, 2001). Other factors which affect membrane conductance include charges on the surface of the membrane, and membrane morphology (Hoettges et al., 2019, Hughes et al., 2022a).

Changes in membrane conductance facilitate cellular functions. Henslee et al. (2017) found human red blood cells display circadian rhythms in membrane conductivity dependent on dynamic regulation of K^+ transport (Henslee et al. 2017). Furthermore, changes in membrane conductance due to a gain or loss of ion channel function and alters cell function may lead to disease states. For example, cystic fibrosis is caused by defective transmembrane chloride ion transport in epithelial cells due to mutations in the CFTR membrane protein (Dalemans et al., 1991).

2.2.2. Cytoplasmic Conductivity

The σ_{cyto} of a cell reflects the concentration of free ions within the cytoplasm. The intracellular concentration of ions varies between different cell types. The main ions considered include K^+ , Na^+ , Cl^- , Ca^{2+} , and Mg^{2+} . The intracellular concentrations of these ions within the cytoplasm and within specific organelles is tightly controlled and regulated to facilitate cellular functions including cell development, activation, maintenance, secretion, and gene transcription (Lewis et al., 1990, Mahtani and Treanor, 2019, Thiel et al., 2021).

As shown in **Figure 3**, K^+ is the most abundant intracellular cation. 98% of the body's total K^+ is located intracellularly, resulting in cytoplasmic concentrations between 140 – 150 mM compared with an extracellular concentration of between 3.5 – 5 mM (Zacchia et al., 2016, Tchounwou and Udensi, 2017). In contrast, Na^+ is the most abundant extracellular cation (Yu et al., 2010, Kowacz and Pollack, 2020) with an extracellular ion concentration of approximately 140 mM and an intracellular concentration of 5 – 15 mM (Melkikh and Sutormina, 2008). Together, both K^+ and Na^+ regulate cell volume by regulating water balance and the acid-base balance (Kowey, 2002).

After K^+ , Mg^{2+} is the second most abundant intracellular cation (Romani, 2011). The total intracellular Mg^{2+} concentration is around 10 mM, but approximately 90% is bound or sequestered

resulting in free ion cytosolic concentrations of between 0.5 – 1 mM (Murphy, 2000). Mg^{2+} is essential for many important cellular processes, metabolic cycles, and signalling pathways, acting as a cofactor to over 600 enzymes involved in biomolecular synthesis, folding and stability (Romani, 2011, Krzywoszyńska et al., 2020). Mg^{2+} also regulates ion channel function and the transport of K^+ , Ca^{2+} , Na^+ and PO_4^{3-} , facilitates ribosome function, and interacts with cell lipids to ensure the cell membrane is intact (Melkikh and Sutormina, 2008). The concentration of Mg^{2+} in extracellular fluid and plasma is ~ 1.2 – 1.4 mM (Romani, 2011).

Similar to Mg^{2+} , the extracellular concentration of Ca^{2+} is 1 – 2 mM (Putney and Tomita, 2012, Schwarz and Blower, 2016). Ca^{2+} is an ambivalent secondary signalling molecule involved in many signal transduction pathways. Ca^{2+} in the cytoplasm is ~ 100 nM, though there are significant intracellular Ca^{2+} stores within organelles including the ER and Golgi apparatus (Schwarz and Blower, 2016, De Loof and Schoofs, 2020, Krzywoszyńska et al., 2020). Depending on the cell type, the ER luminal concentrations can reach 1 mM (Putney and Tomita, 2012, Raffaello et al., 2016). Movement of Ca^{2+} into and out of intracellular stores and/or influx/efflux across the cell membrane via Ca^{2+} pumps, channels, transporters and buffering proteins ensures cytosolic Ca^{2+} concentrations are tightly regulated both in space and time to specifically regulate cellular processes including exocytosis, gene transcription, proliferation, metabolism, apoptosis, and differentiation (Mekahli et al., 2011, Raffaello et al., 2016, Krzywoszyńska et al., 2020). Disturbances in these mechanisms leading to abnormally high cytoplasmic Ca^{2+} is toxic to cells and induces multiple pathologies (Mekahli et al., 2011, Lock et al., 2019). For example, mutations in subunits of voltage-gated Ca^{2+} channels in the plasma membrane can lead to paralysis and migraines (Mekahli et al., 2011).

The transmembrane ion concentration gradients between the cytoplasm and extracellular environment give rise to a cell membrane potential (V_m) (Mahtani and Treanor, 2019).

2.2.3. Membrane Potential

Electrical potential is defined as the energy required to move a Coulomb of charge in an electric field, measured in Volts (Lyklema and Leeuwen, 1991, Benarroch and Asally, 2020). The difference in intracellular and extracellular ion concentrations across the cell membrane (unbalanced charge distribution) described in **Section 2.2.2.** results in an electrical potential known as V_m or transmembrane potential (Yang and Brackenbury, 2013, Hughes et al., 2021). V_m is essentially the voltage that if applied to the membrane reduces transmembrane conductance to equal zero (Hughes et al., 2022a). Resting V_m refers to the voltage at which there is a net ionic current of zero across the

membrane. Resting V_m is typically between -10 mV and -100 mV for different cells types (Kadir et al., 2018). Chondrocytes have a resting V_m of approximately -45 mV (Lewis et al., 2011) compared with inactivated lymphocytes which are approximately -59 mV (Feske et al., 2015), red blood cells which are nearer to -10 mV (Hughes et al., 2021), and neurons between -60 mV to -70 mV (Rivas-Ramírez et al., 2020, Khadria, 2022). The value of V_m is measured relative to the outside of the cell, and if the cell potential becomes more positive or negative, the cell is described as depolarized or hyperpolarized respectively (Yang and Brackenbury, 2013).

Resting V_m is governed by the uneven distribution of ions across the cell membrane, and the permeability of the cell membrane to different ions (Bonzanni et al., 2019). The direction and extent ions move across the cell membrane through ion channels depends on the electrochemical gradient of an ion, which combines both the movement down ion concentration gradients between the intra- and extracellular space, and the drive of electrical gradients as the negative resting V_m either attracts or repels ions into the cell depending on their charge (Feske et al., 2015, Khadria, 2022). According to the adopted “constant field model”, resting V_m is maintained by ion channels, transporters and pumps which control the passive and active transport of ions to achieve a non-equilibrium steady-state between concentration and electrical gradients (Sundelacruz et al., 2009, Bonzanni et al., 2019). In both excitable and non-excitable cells, there is a large expenditure required to maintain V_m , estimated at 10 – 50% of the cellular energy budget (Lazzari-Dean et al., 2021).

The potential at which the electrical and chemical gradients for an ion are balanced is known as the equilibrium potential, and is calculated using the Nernst equation (**Equation 1**):

$$E_x = \frac{RT}{zF} \ln \frac{[X]_o}{[X]_i} \quad \text{Equation 1}$$

Whereby E_x is the equilibrium potential (mV); R is the ideal gas constant; F is Faraday’s constant; T is absolute temperature; $[X]_o$ and $[X]_i$ are the extracellular and intracellular concentration (mmol/L) respectively. The equilibrium potential is positive or negative depending on whether the ion is diffusing into or out of the cell.

If a cell was permeable to only one type of ion, V_m would equal the equilibrium potential of that ion. However, cells are permeable to multiple different ions, with a permeability constant (e.g. P_{Na}) for each ion depending on the number of ion channels within the membrane and whether the ion channels are open. The greater the conductance of an ion, the more the ion influences the cellular V_m (Ramahi and Ruff, 2014). As the cell membrane is more permeable to K^+ than Na^+ , the resting V_m falls closer to the equilibrium potential of K^+ than Na^+ (Chrysafides SM, 2023). The primary ions involved in

generating resting V_m include Na^+ and K^+ , although other ions including Cl^- , Ca^{2+} , H^+ and organic anions also contribute (Lazzari-Dean et al., 2021). The Goldman-Hodgkin-Katz (GHK) equation (**Equation 2**):

$$V_m = \frac{RT}{F} \ln \left(\frac{P_{Na^+}[Na^+]_{out} + P_{K^+}[K^+]_{out} + P_{Cl^-}[Cl^-]_{in}}{P_{Na^+}[Na^+]_{in} + P_{K^+}[K^+]_{in} + P_{Cl^-}[Cl^-]_{out}} \right) \quad \text{Equation 2}$$

Whereby R is the ideal gas constant; T is temperature; F is the Faraday constant; $[X]_{out}$ and $[X]_{in}$ equal the extracellular and intracellular concentrations of the ions; and P_x is the permeability coefficient of the ion. The GHK equation incorporates the relative permeability of the four most notable ions to determine the resting V_m of a cell. The GHK equation achieves this by calculating the reversal potential; the point at which the net ionic current reverses. The GHK equation is the de facto descriptor of V_m (Hughes et al., 2022a).

V_m is traditionally thought of in respect to action potentials in excitable cells, when Hodgkin and Huxley (1952) first characterized how rapid changes in the permeability of the membrane to ions led to the generation of action potentials in nerves (Hodgkin and Huxley, 1952, Kadir et al., 2018, Khadria, 2022). However, V_m is a key biophysical signal in both excitable and non-excitable cells, as well as across organelle membranes, in which V_m can vary within seconds or days (Yang and Brackenbury, 2013, Lazzari-Dean et al., 2021). V_m regulates a multitude of cellular processes and behaviours in both eukaryotic and prokaryotic cells including cell division (Strahl and Hamoen, 2010), stem cell differentiation (Labeed et al., 2011), cell proliferation (Sundelacruz et al., 2009), cell cycle progression (Cone, 1970, Cone and Cone, 1976), cell volume regulation (Lewis et al., 2011), electrotaxis (Zhang et al., 2016), and cell migration (Yang et al., 2020a). Furthermore, differences in V_m is heavily implicated in cancer progression, for example motile cancer cells possess a more depolarized V_m than terminally-differentiated healthy cells (Yang and Brackenbury, 2013).

In addition to the Goldman-Hodgkin-Katz model of charge separation, the Donnan potential also contributes to the negative electrical membrane potential, arising from fixed charges such as DNA, RNA, ribosomes, and proteins (Benarroch and Asally, 2020, Aydogan Gokturk et al., 2022). The asymmetrical distribution of negatively charged phospholipids (described in **Section 2.2.5**) further contributes to the negative membrane potential. The sum determines the total transmembrane potential difference (Benarroch and Asally, 2020).

2.2.4. Membrane Capacitance

The impermeability of the lipid bilayer cell membrane to ions means it functions as a very thin dielectric (~7 nm), separating electrolytes within the conductive cytoplasm from the extracellular fluid. The cell membrane thus forms a basic capacitor, with electric charge accumulating on either side of the membrane and electrostatically interacting across the microscopic distance they are separated (Pethig, 2017). The capacitive model of the cellular membrane is described in further detail in **Section 2.2.5.**

One factor which affects membrane capacitance is cell surface area. Biophysical theory indicates membrane capacitance is directly proportional to the surface area of the membrane for a membrane of constant thickness, with capacitance increasing with surface area (Golowasch and Nadim, 2013). Invaginations, ruffles, and microvilli increase the total surface area of the cell membrane and thus whole cell capacitance (Wang et al., 2002) but not the capacitance per area (as the lipid permeability and membrane thickness does not change). Increasingly invasive cancer cells are shown to have increased membrane ruffling, resulting in increased whole cell membrane capacitance (Jiang, 1995, Salmanzadeh et al., 2013).

Membrane capacitance is also affected by distance between the bilayer (membrane thickness), with greater charge separation decreasing capacitance (Golowasch and Nadim, 2013). The absolute thickness of the double layer depends on the plasma membrane lipid composition, but is constrained by the size of the phospholipid head groups and fatty acid tails (Yale et al., 2018). Lipid rafts within the cell membrane cause local changes in membrane thickness (~ 10% thicker than non-raft membranes), resulting in changes in membrane capacitance (Golowasch and Nadim, 2013).

Cell surface modifications contribute to membrane capacitance (Desai et al., 2009, Muratore et al., 2012, Yale et al., 2018). Yale et al. (2018) showed N-glycosylation (the formation of highly branched N-glycans) of neural stem cell membranes increase membrane capacitance. Changes in cell surface area were not visible by phase contrast microscopy, but cell surface glycosylation is known to affect microvilli and lipid raft formation, associated with membrane invagination and increased cell surface area (Yale et al., 2018). Membrane capacitance is therefore indicative of cell morphology and can be used to identify different cell types, such as neural stem and progenitor cells which have different differentiation fates (Labeed et al., 2011, Adams, 2019).

2.2.5. The Electric Double Layer

The surface of a cell is typically negatively charged, due to sialic acid residues on the cell surface (Eylar et al., 1962), and the presence of negatively charged glycolipids (lipids containing one or more sugar residues/ oligosaccharides) exclusively localized to the non-cytosolic monolayer (extracellular leaflet) of the plasma membrane (Ma et al., 2017). The negatively charged surface attracts counterions and repels co-ions and other negatively charged bodies, forming what is known in surface and colloidal science as an electrical double layer (EDL) (Hughes et al., 2021). An EDL forms whenever two conducting phases meet at an interface; surface charge accumulates at the interface and the resultant change in the electrostatic surface potential causes the redistribution of mobile charges in the vicinity, lowering the total free energy of the system (Gillespie et al., 2020). The propagation of ion redistribution and surface chemistry effects into neighbouring phases electrostatically neutralizes and stabilizes colloids (Park and Seo, 2011, Gillespie et al., 2020).

The simplest model of the EDL was first put forward by Helmholtz in the 1850s, where he described the ion distribution at the charged surface-electrolyte interface to be a parallel-plate capacitor (Helmholtz, 1853). In mammalian cells, the typically negative electrostatic surface charge (due to lipids, amino acids, and sugars on the cell surface) represents one plate of a capacitor, and a fixed layer of electrostatically attracted counterions (known as the Helmholtz plane) would represent the other plate (Lyklema, 2005, Pethig, 2017, Nishino et al., 2020). Gouy and Chapman advanced the Helmholtz model, combining the Poisson equation and Boltzmann distribution to describe a diffuse layer whereby the thermal motion of ions in the electrolyte and screening by counterions are considered. The Gouy-Chapman model thus relates electrical potential $\psi(r)$ with charge density distribution (Gouy, 1910, Chapman, 1913, Pethig, 2017, Sposito, 2018, Gillespie et al., 2020). Otto Stern (1924) combined both models, describing two distinct layers – a surface layer (Stern layer) and a diffuse layer (Stern, 1924). The Stern layer consists of strongly bound immobile counterions specifically adsorbed to the negatively charged surface, unaffected by the ionic concentration of the dispersion medium (Lyklema, 2005). $\psi(r)$ linearly decreases between the surface potential (ψ_0) and the voltage at the end of the Stern layer (ψ_{st}). The Gouy-Chapman diffuse layer surrounds the internal Stern layer, containing free ions which are less firmly associated. The Gouy-Chapman layer consists of a higher concentration of counterions; although negative ions are present due to screening by the Stern layer (Park and Seo, 2011). Within the diffuse layer, $\psi(r)$ decreases exponentially from the edge of the Stern layer to zero within the bulk medium (Hughes et al., 2021). The Gouy-Chapman-Stern model of the spatial distribution of ions within the EDL of a negatively charged colloid is shown in **Figure 4**.

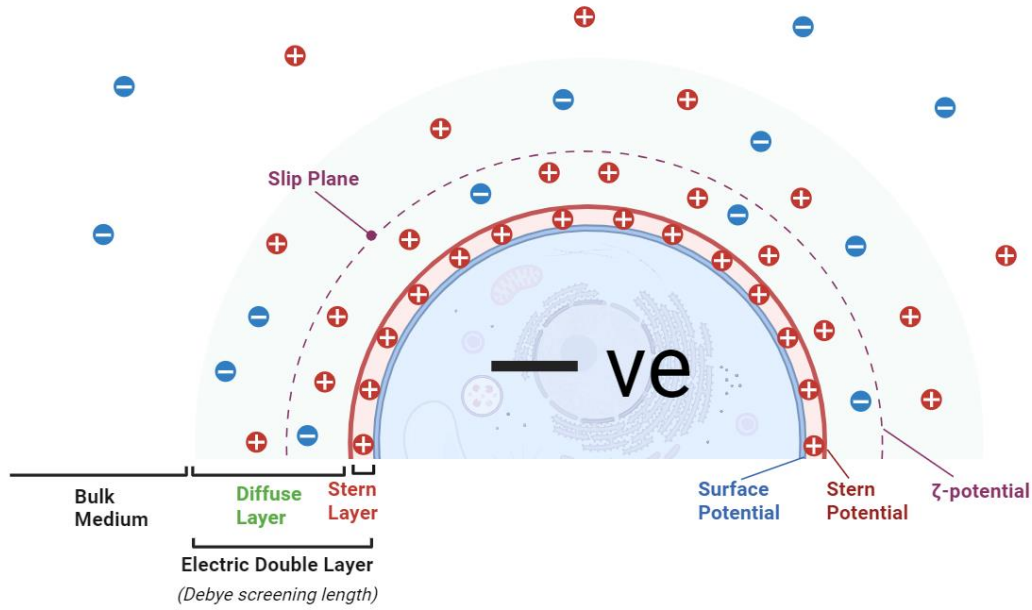


Figure 4 | The Gouy-Chapman-Stern model of the EDL. The distribution of ions surrounding a charged particle is determined by electrostatic interactions and thermal agitation, acting to electrically neutralize the particle. Charged ions of the dispersion medium adsorb onto the surface of a colloidal particle. Counterions are attracted to the negative surface charge and strongly bind, forming the Stern layer. Surrounding the Stern layer is a diffuse layer, consisting of the hydrodynamic shear plane. The electrical potential at the slip plane is referred to as the zeta potential (ζ) (Park and Seo, 2011). Figure: Created with BioRender.com.

When the colloidal particle moves within the dispersion medium, a proportion of counterions remain attached and move with the particle as a stable entity, forming the hydrodynamic plane of shear (known as the slip plane; ~ 1 nm outside the membrane) (Lyklema, 2005, Pethig, 2017, Hughes et al., 2021). The magnitude of the electrical potential between the slip plane and the mobile ions of the bulk fluid is the ζ -potential (Hughes et al., 2021).

The thickness of the EDL is measured by the Debye screening length, which is proportional to the reciprocal of the square root of electrolyte ion concentration, calculated using **Equation 3** shown below (Debye, 1923, Hughes et al., 2021).

$$\frac{1}{\kappa} = \sqrt{\left(\frac{\epsilon RT}{2czF^2}\right)} \quad \text{Equation 3}$$

Whereby ϵ is the relative permittivity; R is ideal gas constant; T is the temperature in Kelvin, c is the electrolyte concentration mol/m³, and z is the counterion valency.

The cytosolic leaflet of the phospholipid bilayer has a negative surface charge which attracts a layer of positive charge, and as such a second electric double layer forms within the cell interior. The cytosolic leaflet has a negative charge due to the composition of the phospholipid bilayer. The composition of the bilayer and distribution of lipids both between the two leaflets of the membrane,

and laterally within each monolayer, is heterogenous (Ma et al., 2017). The four major phospholipids in the mammalian plasma membrane are phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine and sphingomyelin, which are asymmetrically distributed between the outer and inner monolayer and maintained by ATP-dependent flippase and floppase enzymes (Hankins et al., 2015, Ma et al., 2017). At physiological pH, PS and other anionic phospholipids including PE and phosphatidylinositol (PI) species (phosphatidylinositol 4,5-bisphosphate (PIP₂) and phosphatidylinositol 3,4,5-triphosphate (PIP₃)) carry a net negative charge due to the low acid dissociation constant (pKa) of the phosphate groups in their hydrophilic head. These phospholipids are primarily located on the intracellular leaflet of the membrane and generate a static negative surface potential of -25mV. This negative electrostatic potential attracts positively charged molecules (such as peripheral membrane proteins) and repels negatively charged molecules from the cytoplasm by Coulomb's law, thus forming a second electric double layer forms, within the cell interior (McLaughlin, 1989, Graber et al., 2012, Ma et al., 2017).

In contrast, sphingomyelin and zwitterionic phosphatidylcholine are electrically neutral and primarily located in the extracellular leaflet (Lyklema, 2005, Alberts et al., 2015, Ma et al., 2017) which generates two leaflets with different electrostatic surface potentials (Hankins et al., 2015). Greater mechanical stability of the membrane is ensured by the asymmetric distribution of PS, as it interacts with subjacent cytoskeletal proteins (Ma et al., 2017).

2.2.6. Zeta Potential

As previously described, the electrical potential at the notional boundary between ions which form a stable entity with a particle (if the particle were to move, would move with it), and ions beyond the boundary in the continuous bulk solution (which would stay with the bulk dispersant – the hydrodynamic plane of shear), is the ζ -potential (Kaszuba et al., 2010). ζ -potential is a measure of the magnitude of the electrostatic attraction/repulsion between particles, and is an indicator of the electrical stability of particles i.e. whether they are monodisperse or agglomerate (Fernandes et al., 2011, Leary, 2011, Malvern Panalytical, 2023). A high ζ -potential magnitude of more than ± 30 mV is indicative of large repulsive electrostatic forces leading to monodispersity. A low absolute ζ -potential of $< \pm 5$ mV indicates low-electrostatic repulsion, thus the attraction between particles due to Van der Waals forces may be larger, resulting in particle agglomeration (Leary, 2011).

ζ -potential can be measured using electrophoresis, electroosmotic flow, electroacoustic phenomena, sediment potential, and streaming potential (Prakash et al., 2014). Micro-electrophoresis

is commonly used to measure the ζ -potential, in which two electrodes apply a uniform electric field across a cell containing dispersed particles resulting in the movement of charged particles to the electrode of opposite charge. The electrophoretic mobility of a particle refers to the velocity of a particle in a unit electric field, and can be determined using Laser Doppler electrophoresis by measuring small frequency shifts in the light scatter when the particle moves in the applied electric field (Kaszuba et al., 2010). The electrophoretic mobility of a particle is related to the ζ -potential by the Henry equation (**Equation 4**), where U_E is electrophoretic mobility; ϵ is dielectric constant of the dispersant; ζ is zeta potential; $f(\kappa a)$ is Henry's function; and η is medium viscosity (Henry and Lapworth, 1931).

$$U_E = \frac{2\epsilon\zeta f(\kappa a)}{3\eta} \quad \text{Equation 4}$$

The main factor which affects ζ -potential is the pH of the media (Lu and Gao, 2010). In a plot of ζ -potential against pH, at low pH ζ -potential tends to be positive whereas at high pH ζ -potential is less positive or negative. The point whereby ζ -potential equals zero is called the isoelectric point (Hiremath et al., 2021). ζ -potential is also affected by the ionic strength of the media or conductivity of the solution and temperature (Lu and Gao, 2010).

The Lab Blue Label Zetasizer (Malvern Panalytical) is a commercially available machine which uses electrophoretic light scattering (ELS) to measure the electrophoretic mobility of particles in solution, from which ζ -potential is calculated (Malvern Instruments 2015). The Blue Label Zetasizer (Malvern Panalytical, UK) device used solely measures the extracellular ζ -potential, and the intracellular ζ -potential is outside the scope of this thesis.

Biological Relevance of Zeta Potential

Measuring ζ -potential has a number of applications in pharmaceuticals, medicine, ceramics, electronics, and mineral processing (Malvern Panalytical, 2023). ζ -potential analysis of cells, protein, lipids, nucleic acid, and polysaccharides is used to understand their characteristic charge behaviour (Prakash et al., 2014) and varies between different cell types, such as red blood cells (RBCs) -15.7 mV (Tokumasu et al., 2012) and HeLa cells -19.4 ± 0.8 (Bondar et al., 2012). As serum proteins are negatively charged, the negative surface potential of a cell prevents aggregation and non-specific binding (Hughes et al., 2021). The ζ -potential can also be indicative of cell function. The ζ -potential of chondrocytes is regulated by cell metabolism and modulates interactions with the extracellular matrix (Kuo and Lin, 2006, Maleckar et al., 2018). ζ -potential could be used as an early marker of apoptosis,

as the increase in the content of PS on the cell surface has been shown to lead to a decrease in ζ -potential (Bondar et al., 2012).

Analysing ζ -potential is useful for predicting long-term stability of suspensions and emulsions by determining the presence or absence of charged groups/moieties on the cell surface to optimize drug formulations (Kaszuba et al., 2010, Lu and Gao, 2010, Prakash et al., 2014). Understanding the dispersion and aggregation properties of drugs is important in the development of pharmaceuticals for the preparation of stable parenteral and oral drugs to determine shelf-life and dosages (Prakash et al., 2014). Ullah *et al.* (2022) measured ζ -potential to establish surfactant concentration during formulation development of pH responsive nano-pharmaceuticals used for the targeted delivery of methotrexate (an anti-cancer drug) (Ullah et al., 2022).

Other applications of ζ -potential analysis in cells include the study of cell membrane composition and the cell membrane permeation to peptides and ionic surfactants. For example, neuraminidase treatment (used to remove the sialic acid from the cell surface) significantly decreases ζ -potential in RBCs from -15.7 mV to -6.06 mV (Tokumasu et al., 2012). Fan et al. (2014) used ζ -potential analysis to investigate the effect of the fungicidal lipopeptide surfactin on the asymmetry of partitions in the outer and inner membrane leaflets of the cell membrane. At low concentrations, Fan et al. found surfactin binds solely to the outer leaflet (Fan et al., 2014). When RBCs are infected with *Plasmodium falciparum*, the placement of electron-dense 'knobs' result in a decrease in ζ -potential (Tokumasu et al., 2012).

2.2.7. The Cellular Electrome of Peripheral Blood Mononuclear Cells

PBMCs, like all other excitable and non-excitable cells, express a multitude of receptors, transporters, and ion channels. The opening of ion channels results in the influx and efflux of ions across the plasma membrane, or the release of ions from intracellular organelles including mitochondria, lysosomes, or the ER. Ion influx and efflux regulate their V_m , cell function, gene expression, cell development, proliferation, migration, and apoptosis (Feske et al., 2015). Ion channels expressed by PBMCs include K^+ channels (voltage-gated, Ca^{2+} activated and background), Ca^{2+} channels (voltage-gated and store-operated), voltage-gated proton channel, Transient Receptor Potential (TRP) channels and voltage-gated Na^+ channels (NaV) channels (Feske et al., 2015, Feher et al., 2022).

TRP ion channels are homo-tetrameric or hetero-tetrameric cation-permeable pores preferentially selective to Ca^{2+} , assembled from six-transmembrane polypeptide subunits (Clapham et

al., 2001, Parenti et al., 2016, Yue and Xu, 2021). The permeation profile depends on the splice variant of the gene (Marshall-Gradisnik et al., 2015b). Based on the TRP channel amino acid sequences and structural similarities they can be divided into six subsets including TRPC, TRPV, TRPA, TRPML, TRPP, and TRPM channels (Marshall-Gradisnik et al., 2016b, Khalil et al., 2018). TRP channels are widely expressed on almost all cell types throughout the body (Khalil et al., 2018) including lymphocytes, DCs, macrophages, neutrophils, and mast cells (Parenti et al., 2016). TRP channels are localised to plasma membrane and intracellular organelle membranes, and are activated by extracellular and intra-cellular stimuli (Yue and Xu, 2021). TRP channels function as transduction molecules, responding to a range of physical and chemical stimuli including changes in shear stress, temperature, osmolarity, pH, and reactive molecules (Parenti et al., 2016).

2.2.7.1 Lymphocytes

The resting V_m of inactivated lymphocytes is approximately -59 mV (Verheugen et al., 1995), which is primarily regulated by K^+ channels (voltage-gated $K_v1.3$ and Ca^{2+} activated $K_{Ca3.1}$), ORAI1, TRPM4, and Cl^- channels expressed in the plasma membrane (Feske et al., 2015). The assortment of ion channels expressed by T-cells and B-cells are shown in **Figure 5** and **Figure 6** respectively.

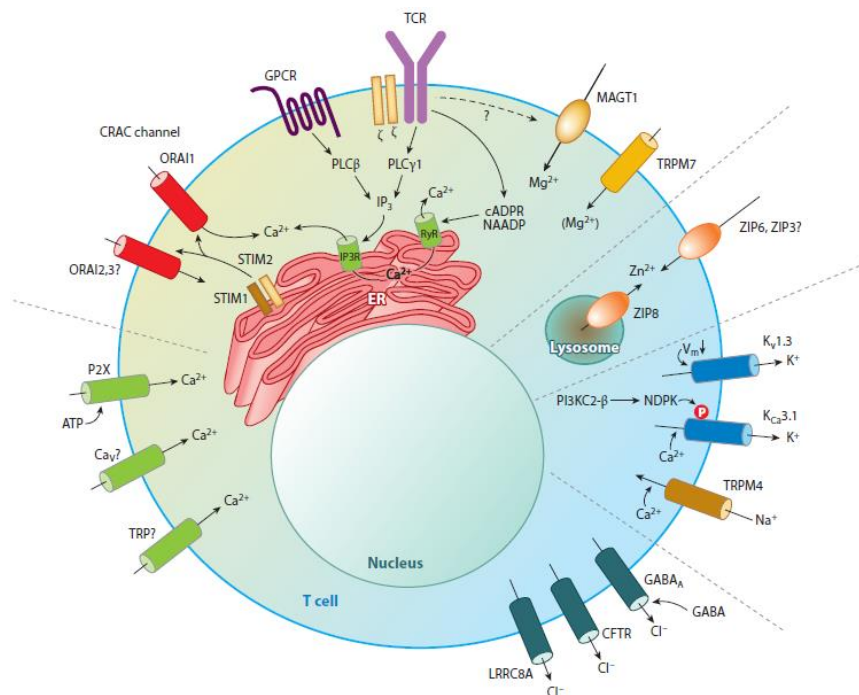


Figure 5 | Ion channels expressed in T-cells include K^+ channels (voltage-gated, Ca^{2+} activated and background), Ca^{2+} channels (voltage-gated and store-operated), voltage-gated proton channel, Transient Receptor Potential (TRP) channels and voltage-gated Na^+ channels (NaV) channels. Figure reprinted from: (Feske et al., 2015).

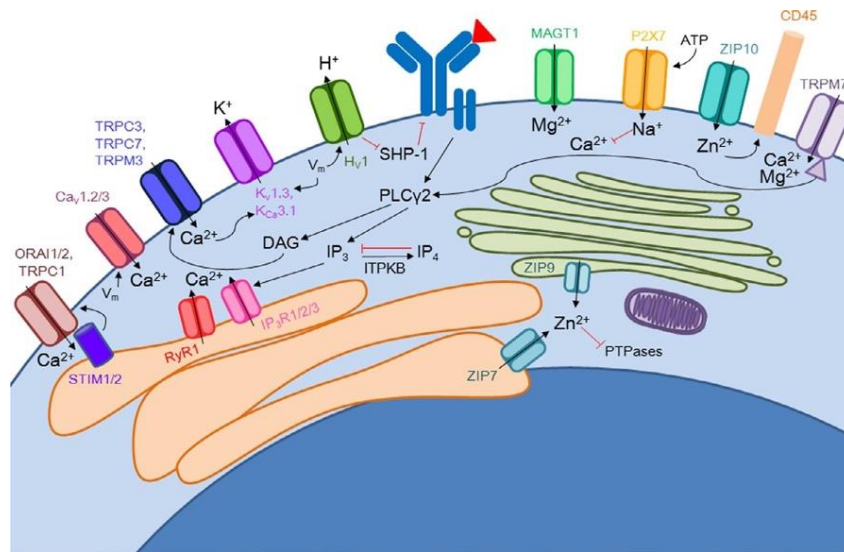


Figure 6 | Functional ion channels expressed in B-cells. Following TCR and BCR activation, the cytosolic enzyme phospholipase C (PLC) is activated. PLC hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP₂) to yield two ubiquitous intracellular signalling molecules inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ binds to the receptors IP₃R and ryanodine receptors (RyR) located on the ER, after which ER stores of Ca²⁺ are released into the cytoplasm. Transmembrane proteins stromal interaction molecules (STIM)-1 and STIM-2 bind and activate the inwardly rectifying plasma membrane calcium release activated calcium (CRAC) channels, composed of Orai1 and Orai2 subunits through which extracellular Ca²⁺ enters. During Ca²⁺ influx, V_m increases, and the cell membrane depolarizes. Kv1.3 and KCa3.1 channels are activated by membrane depolarisation and increases intracellular Ca²⁺ respectively, facilitating K⁺ efflux which maintains a hyperpolarised V_m to drive further Ca²⁺ influx. Figure reprinted from: (Mahtani and Treanor, 2019).

K⁺, Ca²⁺, P2X receptors, TRP channels, Mg²⁺, and Zn²⁺ transporters control cytoplasmic concentrations of Ca²⁺, Mg²⁺, and Zn²⁺ which function as important secondary messengers to regulate lymphocyte effector functions (Mahtani and Treanor, 2019, Carden et al., 2021) including cell activation, cell signalling, development, and proliferation (Feher et al., 2022).

Ca²⁺ Signalling During Lymphocyte Activation

The most characterised and studied pathway in lymphocyte electrophysiology is Ca²⁺ influx following TCR and BCR activation (Feske et al., 2015). In resting T and B-cells, the cytosolic concentration of Ca²⁺ is low (Vig and Kinet, 2009) at ~ 50–100 nM – approximately 10⁴ times less than that of plasma Ca²⁺ concentrations. However, following antigen recognition in T and B lymphocytes, the triggered TCR or BCR stimulation facilitates increases in intracellular Ca²⁺ levels to ~1 μM (Feske et al., 2012). Ca²⁺ signalling is vital for lymphocyte activation and the modulation and function of enzymes and transcription factors involved in cytokine production, differentiation, proliferation, survival, and apoptosis (Oh-Hora and Rao, 2008, Fenninger and Jefferies, 2019).

The concentration of cytoplasmic Ca^{2+} is regulated by mechanisms including store-operated calcium entry (SOCE). Phospholipase C (PLC) enzymes are a family of cytoplasmic proteins activated by either tyrosine kinase receptors, TCRs, BCRs, F_c receptors, integrin adhesion molecules or G-protein coupled receptors (GPCRs) (depending on the isoform) by cognate ligands (Bill and Vines, 2020). Once activated, PLC hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield two ubiquitous intracellular signalling molecules inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (shown in **Figure 6**). When IP_3 binds to the receptors IP_3R and ryanodine receptors (RyR) located on the ER, ER stores of Ca^{2+} are released into the cytoplasm (Ma et al., 2017, Mahtani and Treanor, 2019). The transmembrane proteins stromal interaction molecules (STIM)-1 and STIM-2 are located on the ER membrane with their N-terminus domain in the lumen of the ER bound to Ca^{2+} . On depletion of ER Ca^{2+} stores, Ca^{2+} dissociates from the N-terminus, resulting in a conformational change in the STIM N-terminus domain followed by the cytoplasmic C-terminus domain, acting as an intraluminal Ca^{2+} sensor. The conformational changes resultant of the transient Ca^{2+} drop causes the oligomerization of STIM1 and STIM2 and their translocation to plasma membrane-ER junctions. Now in close proximity to the plasma membrane, here STIM1 and STIM2 bind and activate the inwardly rectifying plasma membrane calcium release activated calcium (CRAC) channels, composed of ORAI1 and ORAI2 subunits. ORAI1 is a tetra-spanning plasma membrane protein ubiquitously expressed in T-cells which forms the pore of the channel. The first transmembrane domain (M1) lines the pore of ORAI1 and possesses a glutamate amino acid residue which binds Ca^{2+} and causes the CRAC channel to be highly selective for Ca^{2+} (Feske et al., 2015). This results in the influx of Ca^{2+} from the ECM to replenish stores. As CRAC channels are thus activated by depletion in ER Ca^{2+} stores, they are referred to as SOCE channel (Zhang et al., 2005, Feske et al., 2015, Martin-Romero et al., 2018, Fenninger and Jefferies, 2019, Mahtani and Treanor, 2019). The effective concentration of PIP_2 at the cytoplasmic leaflet of the plasma membrane is reversely modulated by the electrostatic protein-lipid interactions of polybasic-charged proteins and the negatively charge surface charge of the cytoplasmic leaflet (Ma et al., 2017). DAG and cytosolic Ca^{2+} act in coordination to activate protein kinase C (PKC); a family of enzymes which phosphorylate substrates at serine/threonine residues to influence cellular processes such as gene expression and cell proliferation (Lim et al., 2015).

During Ca^{2+} influx, V_m increases, and the cell membrane depolarizes. To ensure Ca^{2+} influx via CRAC channels is maintained to replenish ER Ca^{2+} stores, the K^+ channels $\text{K}_v1.3$ and $\text{K}_{Ca}3.1$ co-localize to the immunological synapse following lymphocyte activation. Both channels facilitate K^+ efflux to maintain a hyperpolarized V_m following Ca^{2+} influx, to drive further Ca^{2+} influx through CRAC channels (Feske et al., 2015). The depolarisation caused by Ca^{2+} influx activates the voltage-gated K^+ channel $\text{K}_v1.3$, resulting in K^+ efflux. $\text{K}_v1.3$ is a homotetramer composed of four α -subunits. Each subunit

possesses six transmembrane segments (S1-S6) and a P-loop. Four arginine residues in the S4 transmembrane segment detect depolarization of the plasma membrane, resulting in a conformational change to open the ion channel and K⁺ efflux (Feske et al., 2015). In contrast, K_{Ca}3.1 is activated by the increase in intracellular Ca²⁺. K_{Ca}3.1 is a voltage-independent ion channel and is activated when intracellular Ca²⁺ binds to the intracellular messenger protein calmodulin (CaM). CaM is constitutively associated to the intracellular domain of the C-terminus of K_{Ca}3.1, thus binding of Ca²⁺ to CaM causes a conformational change and opening of the ion channel for intermediate-conductance K⁺ efflux (Feske et al., 2015, Sforza et al., 2018). Efflux of K⁺ via both K_v1.3 and K_{Ca}3.1 acts to maintain a negative V_m to sustain Ca²⁺ influx (Feske et al., 2015). However, the expression of these two channels differs between different subsets of B-cells e.g. IgD⁺CD27⁺ class-switched memory B cells highly express K_v1.3, whereas in IgD⁺CD27⁺ memory B cells K_{Ca}3.1 is the main K⁺ channel (Feske et al., 2015).

Other channels activated by PLC following TCR and BCR activation includes TRP channels such as TRPM4, although whether this is store-operated or capacitative Ca²⁺ entry remains controversial (Putney and Tomita, 2012, Hogan and Rao, 2015, Bacsá et al., 2020). As opposed to other TRP channels, TRPM4 is more permeable to Na⁺ than Ca²⁺ with channel activation resulting in Na⁺ influx and depolarization of the plasma membrane. Na⁺ influx regulates calcium oscillations following lymphocyte activation and leads to oscillations in Ca²⁺ transients (Vig and Kinet, 2009, Feske et al., 2015).

Ca²⁺ signalling is key for NK cell function, including cytotoxic activity, formation of the immune synapse, the granule-dependent pathway of apoptosis, microtubule reorganisation and cytokine gene transcription (Schwarz et al., 2013, Marshall-Gradisnik et al., 2016a). Defective Ca²⁺ signalling leads to defective cytotoxic granule exocytosis and weak cell lysis (Li et al., 2022c). SOCE is also the dominant pathway of Ca²⁺ influx in NK cells (Li et al., 2022c), with ORAI1 critical for exocytosis of lytic granules to target cells (Maul-Pavicic et al., 2011). However, the Ca²⁺-permeable TRPM2 channel, located at NK cell plasma and lysosomal membranes gated by ADP-ribose, has also been shown to be important for cytolytic granule polarisation and degranulation (Rah et al., 2015). The function of TRPM3 channels in immune cells is unknown. However, TRPM3 channels were recently found to be expressed on NK cells (Nguyen et al., 2016). Moreover, TRPM3 expression has been shown to increase when intracellular Ca²⁺ stores are depleted, and impaired channel function impairs Ca²⁺ mobilisation (Nguyen et al., 2017). Interestingly, impaired TRPM3 function in NK cells has recently been implicated in the pathology of Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) (Cabanac et al., 2019a, Eaton-Fitch et al., 2022) and post COVID-19 condition patients (Sasso et al., 2022).

2.2.7.2. Macrophages

Due to the dynamic nature of macrophage plasma membranes, especially when activated, electrophysiological measurements using patch-clamp is difficult (Selezneva et al., 2022). Measurements of macrophage membrane potentials have shown a high degree of variation, with values reported of $-14.5 \text{ mV} \pm 5 \text{ mV}$ (Gallin and Gallin, 1977) and $-42 \pm 14 \text{ mV}$ (McCann et al., 1983). This variation is thought to be attributable to different macrophage populations, and reflect the functional characteristics of those measured (Selezneva et al., 2022). Mitochondrial membrane potential, generated by proton pumps of the electron transport chain, has been shown to regulate macrophage function (Wang et al., 2021a).

As shown in **Figure 7**, macrophages express many receptors similar to lymphocytes, including $K_v1.3$, $K_{Ca}3.1$, TRPM4, and ORAI1 (Feske et al., 2015).

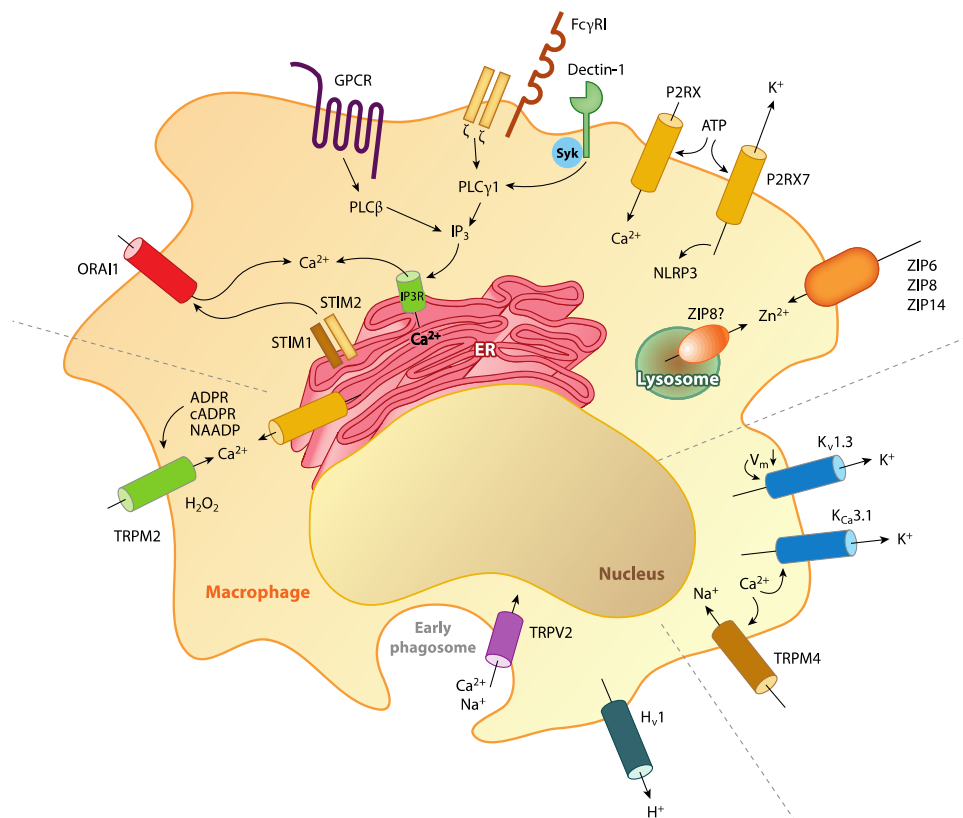


Figure 7 | Ion channels expressed by macrophages include $K_v1.3$, $K_{Ca}3.1$, TRPM4, and ORAI1. Figure reprinted from: (Feske et al. 2015).

A multitude of receptors expressed by macrophages including Fc receptors, TLRs and G-protein coupled chemokine receptors activate ion channels. Both cross-linking of Fc receptors and engagement of chemokines to chemokine receptors activates PLC, resulting in increased cytosolic Ca^{2+} concentrations via SOCE. $K_v1.3$, $K_{Ca}3.1$, and TRPM4 are again used to regulate V_m . Intracellular Ca^{2+} is

important in macrophage function and regulates functions such as TNF- α production, nitric oxide production, phagocytosis, and phagolysosome fusion (Feske et al., 2015).

The ζ -potential of freshly isolated monocytes has been measured at -20.9 ± 0.7 mV, and the ζ -potential of unstimulated macrophages was measured at -21.8 ± 0.48 mV. The ζ -potential of macrophages is thought to become significantly more negative during incubation with bacteria, due to the rearrangement of surface proteins during phagocytic activity (Chakraborty et al., 2020). Chakraborty et al. (2020) demonstrated that both peripheral blood monocytes and macrophages differentiated from monocytes using monocyte colony stimulating factor, have significantly less negative ζ -potential values in comparison to monocytes activated with lipopolysaccharide or IL-1 β (unstimulated macrophages: -21.8 ± 0.48 mV; LPS stimulated: -17.8 ± 0.87 mV; IL-1 β stimulated: -17.13 ± 0.48 mV). Furthermore, Chakraborty et al. (2020) identified that naïve phenotype macrophages have a significantly more negative ζ -potential (-22.72 ± 1.32 mV) than M1 pro-inflammatory (-18.25 ± 1.29 mV) and moderately more negative ζ -potential than M2 anti-inflammatory macrophages (-20.01 ± 1.34 mV). Using flow cytometry, MCH-II was found to be upregulated in M1 macrophages, and CD11b was increased in both M1 and M2 macrophages (Chakraborty et al., 2020).

2.2.8. The Cellular Electrome of Chondrocytes

While chondrocytes are non-excitable cells, their plasma membranes express a large and diverse array of ion channels, transporters, and pumps collectively known as the chondrocyte channelome, which facilitate their functions (Lewis et al., 2013, Mobasheri et al., 2019, Abdallat et al., 2021). The channelome includes K⁺ channels (K_{ATP}, BK, K_v (voltage-gated K⁺ channels K_v1.1, K_v1.3 and K_v1.4) and SK), Na⁺ channels (epithelial channels (ENaC, V-gated channels)), TRP Ca²⁺ permeable cation channels (including TRPV4, TRPV5 and TRPV6), non-selective cation channels, and Cl⁻ channels, shown in **Figure 8** (Barrett-Jolley et al., 2010, Lewis et al., 2013, Mobasheri et al., 2019, Takács et al., 2023).

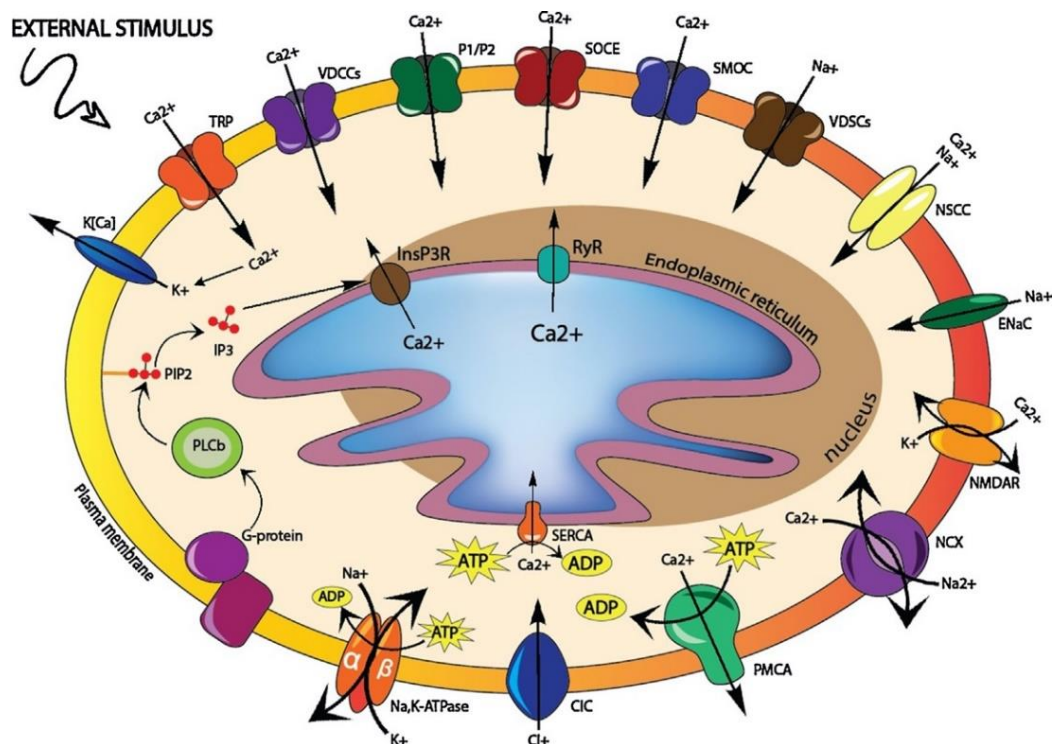


Figure 8 | The chondrocyte channelome. Ion channels expressed include K^+ channels (K_{ATP} , BK, K_v (voltage-gated K^+ channels $K_v1.1$, $K_v1.3$ and $K_v1.4$) and SK), Na^+ channels (epithelial channels (ENaC, V-gated channels)), TRP Ca^{2+} permeable cation channels (including TRPV4, TRPV5 and TRPV6), non-selective cation channels and Cl^- channels. (Figure reproduced from: (Mobasheri et al., 2019). Copyright © 2019 Elsevier Masson SAS. All rights reserved.)

As with PBMCs, Ca^{2+} is an important intracellular secondary signalling molecule in chondrocytes. Changes in intracellular Ca^{2+} concentrations are essential for modulating chondrogenesis, proliferation, cell death, and baseline catabolic and anabolic activities (Takashi et al., 2015, Suzuki et al., 2016). An influx of extracellular Ca^{2+} via voltage-gated channels activates the Ca^{2+} /calmodulin-dependent phosphatase signalling pathway, causing dephosphorylation of the cytoplasmic transcription factor NF-AT and its cooperative binding to transcription factors leading to gene expression (Xu et al., 2009). Kurita et al. (2016) showed Cl^- currents contribute to regulating resting V_m and thus regulate intracellular Ca^{2+} . Impairment of the CIC-7 channel significantly increases intracellular Ca^{2+} and has been shown to lead to cell death (Kurita et al., 2015).

K_{Ca} channels are Ca^{2+} -gated K^+ channels which are classified into three groups based on their conductance. This includes big conductance (BK), intermediate conductance (IK) and small conductance (SK) channels. BK channels have a tetrameric structure of pore-forming six transmembrane domain α subunits which can act alone or with regulatory β and/or γ subunits (Suzuki et al., 2016). BK channels are activated in response to stretch (Lewis et al., 2013) by either increases

in intracellular Ca^{2+}_i or Ca^{2+} independent mechanisms such as coupling to mechanoreceptors such as integrins (Barrett-Jolley et al., 2010, Mobasheri et al., 2010). Expression of the ion channel BK (KCNMA1) in chondrocytes has been validated by electrophysiological and immunohistochemistry methods. One function of BK channels includes maintaining resting V_m and regulating cell volume (Lewis et al., 2013).

2.3. Dielectrophoresis

2.3.1. Measuring the Electrical Properties of Cells

Patch-clamp is considered the traditional gold-standard technique used to measure the electrical properties of cells as it provides high-resolution direct measurement of V_m or transmembrane current at a single-cell level (Neher and Sakmann, 1976, Mansor and Ahmad, 2015, Hill and Stephens, 2021). Furthermore, it is very versatile, with the capability to measure the kinetics of individual ion channels in cells and action potentials, depending on the set-up configuration (Hill and Stephens, 2021). However, the technique is invasive as it uses a sharpened glass electrode to form a tight giga-ohm (G Ω) seal with the cell membrane, often rupturing the cell membrane to access the cell cytoplasm. Patch-clamp is also technically challenging, laborious, time-consuming, and a low throughput technique as it measures only a single cell at a time (Sundelacruz et al., 2009, Bonzanni et al., 2019, Kawai et al., 2020).

Recently developed voltage-sensitive fluorescent dyes measure relative changes in V_m (instead of absolute) via changes in spectra properties in response to voltage changes in cells. Slow membrane potential dyes such as Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol (DiSBAC) can be used for optical quantification of non-excitable cell types (Adams and Levin, 2012a, Bonzanni et al., 2019). Advantages include ease of use, and the simultaneous monitoring of many cells including those that are moving (Sundelacruz et al., 2009). However, limitations include bleaching of fluorescent dyes and self-quenching. Also many dyes do not discriminate which membranes they cross such as the mitochondria, so sufficient subcellular resolution must be used to identify the voltage of which specific membrane is being measured (Adams and Levin, 2012b).

As such, the phenomenon dielectrophoresis is gaining increased popularity to characterise the electrical properties of cells including cell membrane capacitance, conductance, and σ_{cyto} as it is a high-throughput, label-free, fast, accurate, low-cost technique that does not rely on genotype-dependent biomarkers, and is non-contact (Salmanzadeh et al., 2013, Abd Rahman et al., 2017).

2.3.2. Dielectrophoresis

First defined by Pohl (Pohl, 1951), dielectrophoresis (DEP) is an electrokinetic phenomenon described as the movement of a suspended neutral polarisable particle in a non-homogenous electric field (Pohl, 1951, Pohl, 1958). DEP is a phenomenon similar to electrophoresis, whereby charged particles move in a uniform electric field towards an electrode of opposite polarity. However, DEP uses a non-uniform electric field and affects any polarisable particle with different dielectric properties to the medium the particle is suspended in (Pethig, 2017). The velocity and direction of particle motion depends on many factors: the polarizability of the dielectric particle, the shape and size of the particle, the frequency of the alternating electric field applied, and the electric properties of the suspension media. As such, particles with different dielectric characteristics can be manipulated and the electrophysiological properties of the particle extrapolated from the magnitude and direction of the resultant translational dielectrophoretic force $\mathbf{F}_{DEP}$ they experience; thus their movement during DEP (Hoettges, 2010, Hughes, 2016).

When a neutral dielectric particle is exposed to an electric field, it becomes electrically polarized as equally positive and negative charges (+Q, -Q) are induced. The charges finitely separate and distribute on opposite sides of the particle, as shown in **Figure 9**, and a dipole moment $\mathbf{p}$ forms. If the applied electric field $\mathbf{E}$ was uniform, coulombic forces $\mathbf{F}$ acting on the two positively and negatively charged sides of the particle's dipole would be equal. A torque on the particle would cause the induced electric poles to align with the direction of the field to minimize electrical potential energy, but there would be no lateral movement (Pethig, 2017). Whereas in DEP, the induced dipole interacts with the field gradient and the resultant forces on either side of the particle's dipole are different as the force is greater where the electric field strength is stronger. At the same time, the particle's dipole interacts with the dipole of the suspension medium. The vector difference between the two forces is a net dielectrophoretic force $\mathbf{F}_{DEP}$ acting on the dipole, which induces motion of the particle, as it pulls the dipole in the direction of increasing field gradient (Pohl, 1951, Pohl, 1958, Pohl and Hawk, 1966, Hoettges, 2010, Hughes, 2016, Pethig, 2017). The magnitude and direction of the resultant translational dielectrophoretic force $\mathbf{F}_{DEP}$ on a small, polarized particle can be calculated by:

$$\mathbf{F}_{DEP} = (\mathbf{p} \cdot \nabla) \mathbf{E} \quad \text{Equation 5}$$

for a dipole moment $\mathbf{p}$ and an electric field gradient $\nabla \mathbf{E}$. If the electric field was uniform ($\nabla \mathbf{E} = 0$), there would be no net force on the dipole. A negative field gradient shows that the magnitude of the field decreases as distance from the electrode increases (Pethig, 2017). The direction and strength of $\mathbf{F}_{DEP}$ depends on the polarizability of the dielectric particle relative to the suspending medium for a given frequency (Pethig and Markx, 1997). The magnitude and polarity of a particle's effective

electrical polarizability can significantly change as a function of the applied electric field frequency (Pethig, 2017). When the particle is more polarizable than the surrounding medium, the particle is attracted up the electric field gradient, known as positive DEP (**Figure 9A**). Whereas if the particle is less polarisable than the surrounding medium, the particle is repelled from the regions of high electric field gradient, known as negative DEP (**Figure 9B**) (Hoettges, 2010, Broche et al., 2011, Hughes, 2016). However, if the particle had the same dielectric properties as the medium, the electric field would not be perturbed by the suspended particle; DEP only occurs in particles with different dielectric properties to the medium they are suspended in (Hoettges, 2010).

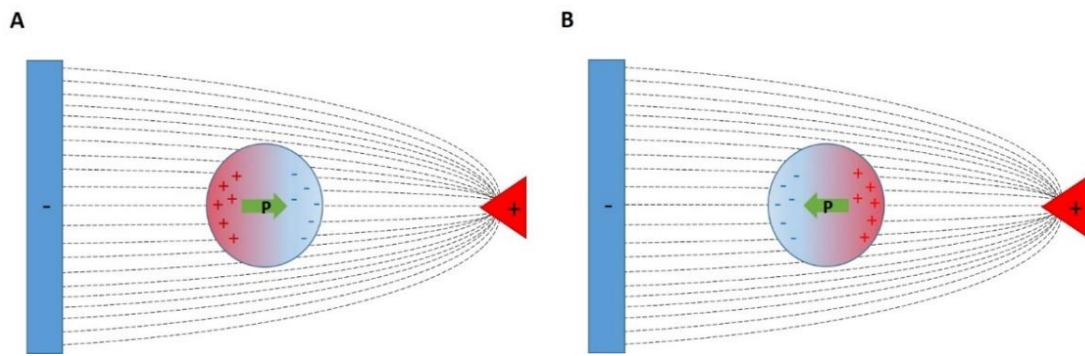


Figure 9 | Dielectrophoresis | When a suspended dielectric particle is in a non-uniform electric field, it becomes polarised. The strength of the forces acting on the induced dipole moment on opposite sides of the particle are different due to the electric field gradient. The net dielectrophoretic force results in particle movement up or down the field gradient depending on the relative polarizability of the particle to the medium. | **A** | **Positive Dielectrophoresis**. If the particle is more polarizable than the suspending medium, the particle moves up the field gradient. | **B** | **Negative Dielectrophoresis**. If the particle is less polarizable than the suspending medium, the particle moves down the field gradient (Hoettges, 2010, Hughes, 2016).

To derive F_{DEP} for particles/cells, each cell is modelled as a homogenous dielectric particle (lossy dielectric particle), assumed to be perfectly spherical, form a simple dipole moment, and have no net charge or conductive losses (Pethig, 2010). The induced dipole moment $\mathbf{p}$ is given by:

$$\mathbf{p} = 4\pi r^3 \epsilon_0 \epsilon_{med} \text{Re}[K(\omega)] \mathbf{E} \quad \text{Equation 6}$$

for a particle of radius r , permittivity of free space ϵ_0 , the permittivity of the suspending medium ϵ_{med} , and electric field $\mathbf{E}$. By inserting **Equation 6** into **Equation 5**, F_{DEP} can be written as:

$$\mathbf{F}_{DEP} = 2\pi r^3 \epsilon_0 \epsilon_{med} \text{Re}[K(\omega)] \nabla E^2 \quad \text{Equation 7}$$

$K(\omega)$ is the Clausius-Mossotti (CM) factor, which represents the variation in the relative polarizability of the particle and the medium. The difference in the electrical properties of materials when an electrical field is applied is known as the Maxwell-Wagner interfacial polarization (Pethig, 2017, Hughes et al., 2023). For a homogenous sphere, the real part of the CM factor $\text{Re}[K(\omega)]$ is given by:

$$K(\omega) = \frac{\epsilon_p^* - \epsilon_{med}^*}{\epsilon_p^* + 2\epsilon_{med}^*} \quad \text{Equation 8}$$

With complex permittivity defined as: $\varepsilon^* = \varepsilon - \frac{j\sigma}{\omega}$

Equation 9

For real permittivity ε , real conductivity σ , $j = \sqrt{-1}$, and a radial frequency $\omega = 2\pi f$. ε_p^* is the complex permittivity of the particle, and ε_{med}^* is the complex permittivity of the suspending medium. Therefore, by inserting **Equation 8** into **Equation 7**, F_{DEP} can be calculated by:

$$F_{DEP} = 2\pi r^3 \varepsilon_0 \varepsilon_{med} \text{Re} \left[\frac{\varepsilon_p^* - \varepsilon_{med}^*}{\varepsilon_p^* + 2\varepsilon_{med}^*} \right] \nabla E^2$$

Equation 10

For a perfect sphere the Clausius-Mossotti factor $K(\omega)$ is always between -0.5 and 1.0. The $K(\omega)$ factor is negative at frequencies where the particle is less polarizable than the suspension media ($\varepsilon_p^* < \varepsilon_{med}^*$), and a negative dipole moment is induced – known as negative DEP. In contrast, $K(\omega)$ is positive when the particle is more polarizable than the surrounding medium ($\varepsilon_p^* > \varepsilon_m^*$) and a positive dipole moment is induced – known as positive DEP (Huang et al., 1992). The frequency at which $K(\omega)$ is zero, as ε_p^* is equal to ε_{med}^* , is known as the cross-over frequency, whereby dielectrophoresis of the cell changes between positive and negative (Pethig, 2017). This frequency is unique between different cell types, and can thus be used to characterise, manipulate, and separate cells (Jones, 2003, Hoettges, 2010, Pethig, 2017).

As can be indicated from electric field appearing as ∇E^2 , reversing the polarity of the applied voltage does not affect the direction of the DEP force. If the electric field re-orientates, the induced dipole re-orientates – the direction of the field does not make a difference. The forces acting on the induced dipole only depend on the strength of the electric field gradient and are independent of the polarity of the field, thus AC electric fields can be used during DEP (Pethig and Markx, 1997). Advantages of using AC fields include eliminating simultaneous electrophoresis due to charged particles and preventing electrolysis and other electrochemical reactions at the electrodes (Pohl, 1958, Hoettges, 2010).

2.3.3. Characterizing the Electrical Properties of Cells by Dielectrophoresis

Since the velocity and direction of particle motion during DEP depends on the electrophysiological properties of the particle, the medium, and the frequency of the applied electric field, these factors make DEP an attractive technique for manipulating bioparticles and characterizing cellular electrical properties (Hoettges, 2010, Hughes, 2016). By determining the strength and direction of F_{DEP} over a wide range of different electric field frequencies (typically kHz to MHz), a mathematical model can be generated to characterize the electrophysiological properties of a cell.

Plotting the $Re[K(\omega)]$ against frequency produces a DEP spectrum with frequency dependent behaviour specific to the cell from which the electrical properties of the cells are derived including membrane conductance, membrane capacitance, and σ_{cyto} (Hughes, 2023). When modelling mammalian cells, the CM factor is often assumed to take the form of a simple ‘single-shell’ model, derived from Maxwell-Wagner interfacial polarization between the surrounding media and the cell modelled as a homogenous particle consisting of a cytoplasmic core surrounded by a cell membrane (the shell), shown in **Figure 10**.

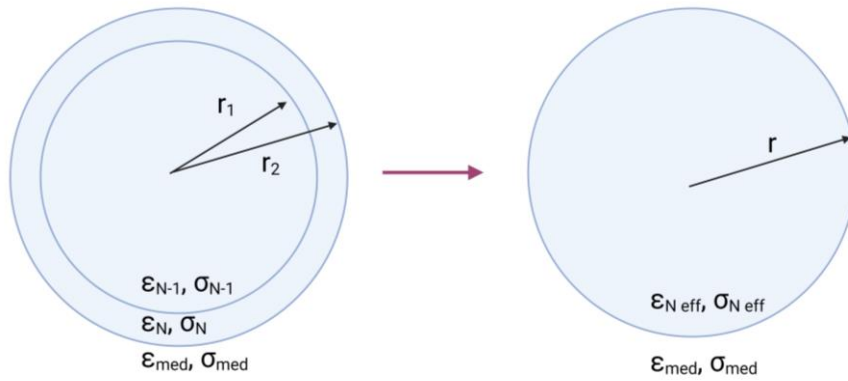


Figure 10 | Single-shell model. Figure: Created with BioRender.com (Adapted from (Huang et al., 1992, Kruckek, 2022)).

The complex effective permittivity ε_{cell}^* (**Equation 11**) and complex effective conductivity σ_{cell}^* (**Equation 12**) of a cell, comprising of a thin membrane m of thickness t surrounding a core c of radius r (Hughes, 2023) are as follows:

$$\varepsilon_{cell}^* = \varepsilon_m^* \frac{\left(\frac{r}{r-t}\right)^3 + 2 \frac{\varepsilon_c^* - \varepsilon_m^*}{\varepsilon_c^* + 2\varepsilon_m^*}}{\left(\frac{r}{r-t}\right)^3 - \frac{\varepsilon_c^* - \varepsilon_m^*}{\varepsilon_c^* + 2\varepsilon_m^*}} \quad \text{Equation 11}$$

$$\sigma_{cell}^* = \sigma_m^* \frac{\left(\frac{r}{r-t}\right)^3 + 2 \frac{\sigma_c^* - \sigma_m^*}{\sigma_c^* + 2\sigma_m^*}}{\left(\frac{r}{r-t}\right)^3 - \frac{\sigma_c^* - \sigma_m^*}{\sigma_c^* + 2\sigma_m^*}} \quad \text{Equation 12}$$

The CM for a cell is therefore calculated as:

$$K(\omega) = \frac{\varepsilon_{cell}^* - \varepsilon_{med}^*}{\varepsilon_{cell}^* + 2\varepsilon_{med}^*} \quad \text{Equation 13}$$

Assumptions of the single-shell CM model include that the cytoplasm up to the inner leaflet of the plasma membrane is electrically homogenous, perfectly spherical, and has no conductive losses or net charge. Furthermore, the model assumes that cytoplasmic conductivity is electrically independent of cell environment, neglects the effects of the EDL, and assumes the electrical parameters derived from the DEP spectrum do not affect each other (Hughes et al., 2022a, Hughes, 2023).

However, biological cells are not homogenous – a single phospholipid bilayer surrounds most organelles, and the nucleus and mitochondria are surrounded by two lipid bilayers. A smeared-out sphere approach can be used to form a multi-shell model of a cell (Irimajiri et al., 1979), where concentric dielectric spheres of complex ε_N permittivity, complex σ_N conductivity and radius r_N are combined to calculate the combined effective $\varepsilon_{N\text{ eff}}^*$ and $\sigma_{N\text{ eff}}^*$ of a cell (Huang et al., 1992, Kruckek, 2022). For example, a cell wall (in yeast and bacteria), nuclear membrane and nucleolus can be represented as concentric spherical shells with distinct radii. Successively combining the shells results in an effective complex permittivity for the whole cell (Chung et al., 2011). The multi-shell model has been used to interpret the dielectric properties of nuclear membrane in lymphocytes (Asami et al., 1989), and a four-shell model has been applied to yeast cells (Hölzel, 1997). However, very high AC frequencies and accurate DEP spectra have to be obtained for multi-shell modelling, thus it is difficult to obtain unique results from these models (Hughes, 2023).

The spectrum has two dispersions – transition from negative to positive at low frequencies and one transition from positive to negative at high frequencies. The cross-over frequencies within the DEP spectra are derived when the real part of **Equation 13** ($\text{Re}[K(\omega)]$) is equal to zero. At low AC frequencies, the low-conductivity plasma membrane shields the cell from the applied electric field, so the value of $K(\omega)$ is indicative of plasma membrane properties, morphology, and cell shape. The equation for the first cross-over frequency (f_{xo}) is shown as **Equation 14**:

$$f_{xo} = \frac{1}{2\pi} \sqrt{\frac{(\sigma_m - \sigma_c)(\sigma_c + 2\sigma_m)}{(\varepsilon_c - \varepsilon_m)(\varepsilon_c + 2\varepsilon_m)}} \quad \text{Equation 14}$$

For which $\sigma_m = rG_m$ and $\varepsilon_m = rC_m$, with r equal to cell radius, G_m equal to membrane conductance, and C_m equal to membrane capacitance (Chung et al., 2011).

However, when the AC frequency is higher, the electric field starts penetrating the cell interior, thus indicating cytoplasmic features (Russo et al., 2021). The second cross-over frequency (f_{xo2}) is determined by the dielectric properties of the intracellular properties including the cytoplasmic conductivity σ_{cyto} (Chung et al., 2011).

2.3.4. Dielectrophoretic Techniques and Their Applications

Dielectrophoretic methods used to measure the dielectric properties of cells include conventional DEP, travelling-wave DEP (TWDEP), electrorotation (ROT) and electro-orientation (Li et al., 2022b). Whereas stationary electric fields are used in conventional DEP, closely related techniques ROT and TWDEP utilize moving electric fields (Goater and Pethig, 1999).

ROT is a widely-used technique whereby polarized particles subjected to a rotating electric field by multiple out-of-phase force-generating electrodes experience a lagging induced dipole moment, resulting in a rotational torque which aims to align the dipole moment with the field, thus causing cellular spin (Arnold and Zimmermann, 1982, Arnold and Zimmermann, 1988, Huang et al., 1992, Goater and Pethig, 1999, Li et al., 2022b). The electric polarizability of the cell and the suspension medium determines the unique cellular rotational rate for a given voltage and frequency of field rotation. From a frequency-dependent electrorotation spectrum, the relative dielectric and conductive properties of a given particle (Huang et al., 1992, Goater and Pethig, 1999, Li et al., 2022b). As opposed to using the real part of the CM factor as in conventional DEP, the ROT torque is proportional to the imaginary part (Huang et al., 1992). Advantages of ROT include each cell is analysed separately at single-cell resolution; therefore, it is easier to assess inter-cell variations (Schwan, 1991, Georgieva et al., 1998). However, ROT is a slower technique which measures small cell numbers at a time and it is difficult to take into account variations in field strength (De Gasperis et al., 1999).

The first application of DEP in 1966 separated living and dead yeast cells (Pohl and Hawk, 1966). Now 57 years later, DEP has and continues to be, applied across a variety of mechanical, biomedical, and biotechnological fields. DEP has been used to characterize the specific dielectric properties of many different biological cells, such as human leukocyte subpopulations using ROT (Yang et al., 1999), red blood cells (Beving et al., 1994), and human mesenchymal stem cells (Adams et al., 2014). Optically induced DEP has been used to determine membrane capacitance and membrane conductance of Raji cells, MCF-7 cells (breast cancer), HEK293 cells (Human Embryonic Kidney), and K562 cells (lymphoblasts) (Liang et al., 2017). Additionally, DEP and electrorotation have been used for measuring specific membrane capacitance of ovarian cancer cells (Salmanzadeh et al., 2013). DEP and ROT have also been used to assess the viability of yeast cells (Hölzel and Lamprecht, 1992, Huang et al., 1992) and the dielectric properties of microalgae cells (Lin et al., 2021).

One common biomedical application of DEP includes the selective spatial manipulation, separation, and electrophysiological characterization of bacteria (Markx et al., 1996, Peitz and van Leeuwen, 2010), circulating tumour cells from clinical blood specimens (Gascoyne et al., 2009), breast

cancer cells from isolated PBMCs (Adekanmbi et al., 2020), neural stem cells for astrocyte-biased enrichment (Jiang et al., 2019), and viable from unviable cells (Pethig and Markx, 1997) based on differences in their dielectric properties. One way this can be achieved is by dielectrophoretic field-flow fractionation, where DEP forces are produced by microelectrodes and used to levitate cells in a thin chamber such that the vertical DEP force is equal to the sedimentation force causing the cells to reach an equilibrium height. Particles of different dielectric and density properties levitate to different heights. Cells are separated by the hydrodynamic velocities of a fluid moving through the chamber, which transports cells levitating at different equilibrium heights at different velocities (Wang et al., 2000). Use of dielectrophoretic field-flow fractionation to separate cells possessing different dielectric properties have been used in applications such as the separation of human breast cancer from T-cells (Wang et al., 2000). As opposed to flow cytometry and magnetic bead separation, separation by DEP is a label-free method of cellular manipulation which does not rely on genotype-dependent biomarkers (Salmanzadeh et al., 2013).

Some research groups have integrated both DEP and ROT into a single system. Li et al. (2022) describe the application of a device which uses negative DEP to trap a single cell within an elevated electrode structure, which itself applies ROT forces to measure the dielectric properties of *Chlamydomonas reinhardtii* (a lipid-accumulating microalga) with single-cell resolution (Li et al., 2022b). Similarly, Trainito et al. (2019) also combined the use of DEP and ROT to determine the dielectric properties of sequentially-staged cancer cells with single-cell resolution (Trainito et al., 2019).

With the advancement of microfabrication technologies, the integration of electrokinetic phenomena such as DEP into lab-on-chip platforms have proven very useful in cellular manipulation and characterization (Trainito et al., 2019). Modern microelectrode arrays of planar and three-dimensional electrode structures are capable of generating strong DEP forces on application of small voltages (Mohamed et al., 2019). An example of a DEP platform is the 3DEP device, which uses conventional DEP to characterize the dielectric properties of cells.

In contrast with the biomedical applications discussed, some examples of mechanical applications of DEP include the deposition of carbon nanotubes onto micro-fabricated electrodes (Duchamp et al., 2010), the manipulation of self-assembled amyloid peptide nanotubes (Castillo et al., 2008), and using abrasive powders to polish local areas of three-dimensional surfaces (Kim et al., 2004).

2.3.5. The 3DEP

Developed at the University of Surrey, the 3DEP (DEPtech) dielectrophoretic platform is a commercial cytometry platform capable of analysing the bioelectric properties of typically 20 000 cells simultaneously in ten seconds with high resolution. The assessment of thousands of cells at one time reduces the chance of type 1 errors (Hoettges et al., 2019). The system consists of a disposable 3DEP chip injected with a cell suspension of between 10^4 to 10^8 cells/mL, which is inserted into a reader instrument to take measurements (DEPtech, 2019). The 3DEP chip, shown in **Figure 11A**, is made of alternating layers of copper and glass fibre reinforced epoxy laminates. Each chip contains twenty wells (each 1 mm in diameter), with exposed surfaces gold-plated for biocompatibility and stability. The wells are hydraulically connected by a gasket on the underside of the chip; injecting approximately 80 μ L solution into one well fills all the wells simultaneously (Hoettges et al., 2008). On insertion of the 3DEP chip into the zero-insertion force mount of the 3DEP machine (**Figure 11B**), the motorized clamp locks the 3DEP chip and secures it in position. Each well is served by a different electrode connected to separate DDS-based signal generators so the user can energize each well with independent AC frequencies of 20V_{pp} up to 45 MHz (typically covering five points per decade between 1 kHz to 45 MHz) for parallel acquisition of a 20-point spectrum in seconds. A 20-point spectrum is assumed to have sufficient resolution to observe all main features of a DEP spectrum, including the low, middle, and high frequency plateaus, and two dispersions (Hoettges et al., 2019).

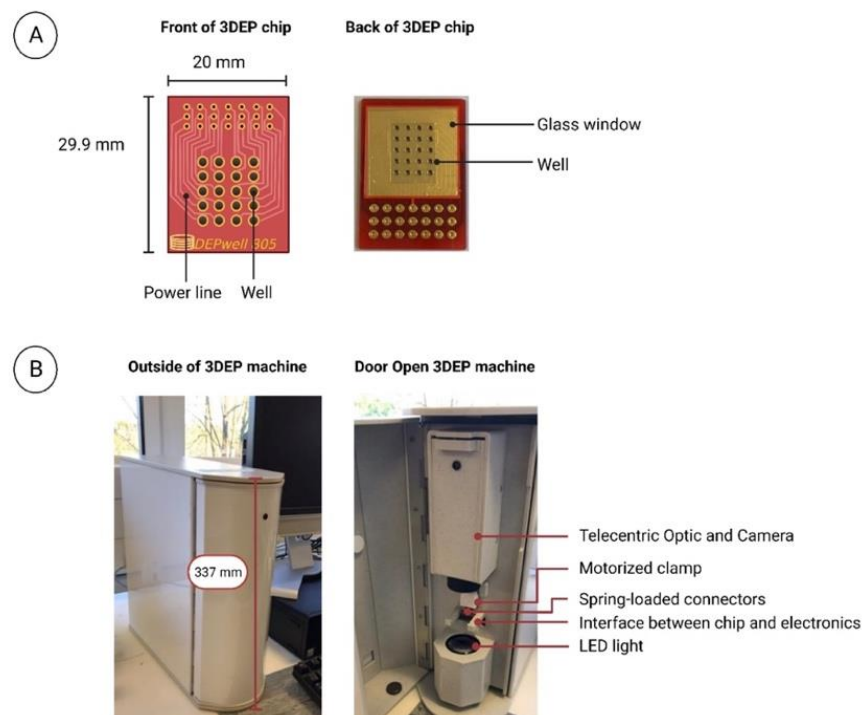


Figure 11 | Diagrams of **|A|** the 3DEP chip **|B|** the 3DEP machine. Figure adapted from (Kruchek, 2022).

First the 3DEP door is shut to minimise external light interference. All wells of the chip are then illuminated simultaneously with a collimated LED light source and monitored using a complementary metal-oxide semiconductor camera containing a bi-telecentric optic (shown in **Figure 11**). The camera takes pictures every second for a user-defined period, commonly between 10 and 30 seconds. An image analysis algorithm on the host PC tracks the movement of cells within each well; the AC frequency applied to each well causing the cells to move by either positive or negative DEP. During positive DEP the cells are attracted to the electrodes and move from the centre of the well to the edge of the well. During negative DEP, the cells are repelled by the electrodes and move to the centre of the well. As the system is one-dimensional, all cells move in the same direction no matter where they are situated within the well (DEPtech, 2019, Hoettges et al., 2019). The work-flow of a 3DEP experiment is demonstrated in **Figure 12**, which includes a typical DEP spectrum displayed in the 3DEP software (DEPtech 2013, version 1.5.1.68) in step 5.

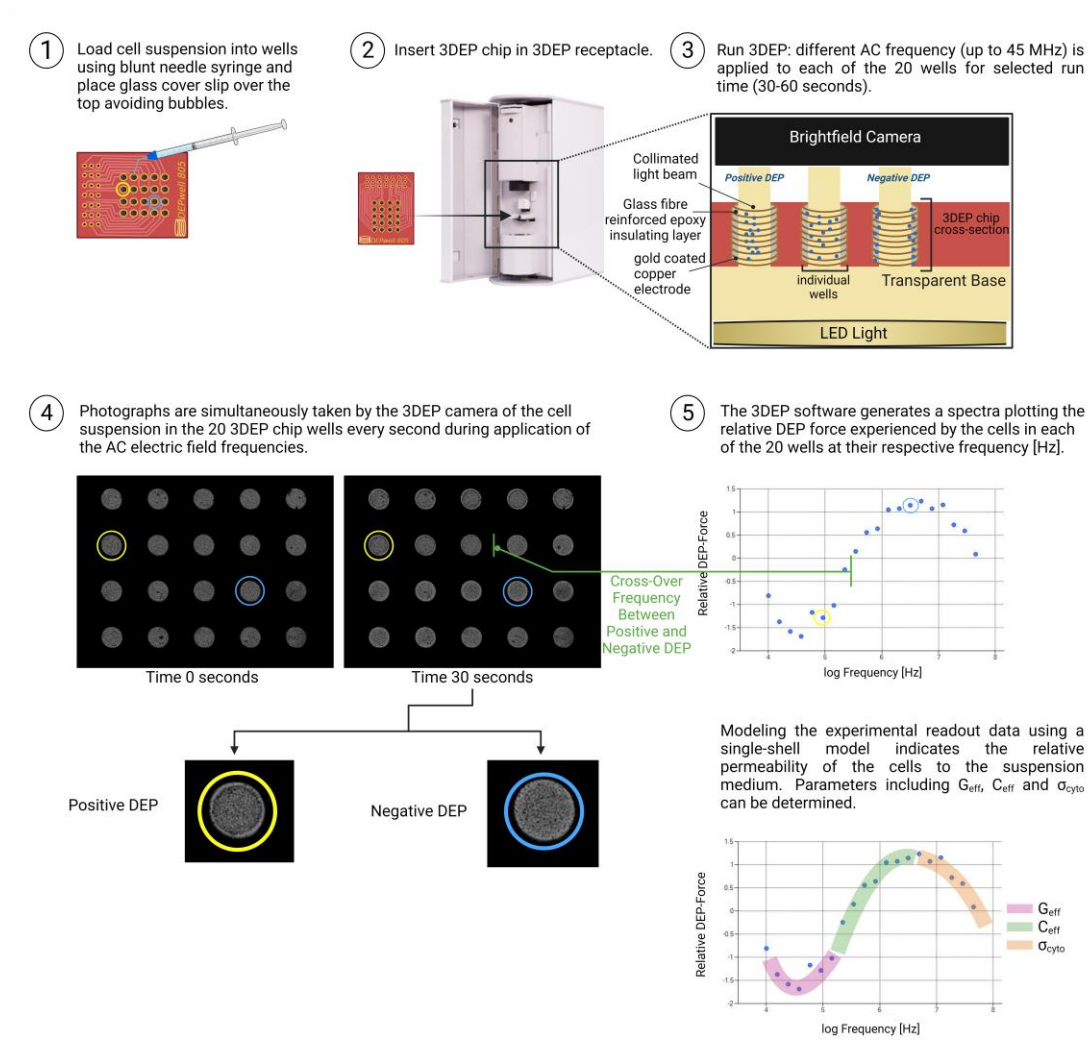


Figure 12 | Standard operation of the 3DEP – an overview of how the 3DEP works. Figure adapted from (Kruckek, 2022).

During data processing, the 3DEP software divides each well into 10 concentric bands, and the light absorbance is measured to determine the movement of cells within each band. Exclusion of the outermost band due to edge effects is common for noise reduction in DEP spectra. The central band is excluded when cells of small radii are analysed due to minimal movement in the user-defined period. The change in absorbance is proportional to CM factor, and the CM model is fitted using a Levenberg-Marquardt best-fit algorithm (DEPtech, 2019, Hoettges et al., 2019). Values of membrane G_{eff} , C_{eff} , and σ_{cyto} can be calculated from the CM single-shell model using the MATLAB script attached in **Appendix B**. G_{eff} and C_{eff} are calculated by dividing membrane conductance and membrane capacitance respectively by membrane thickness, removing the influence of membrane thickness on the CM model.

Values of G_{eff} are typically higher than measured by patch-clamp, as G_{eff} includes both transmembrane conductance indicative of ion channel activity, and the tangential surface conductance related to movement of surface charge within the EDL related to medium conductivity (σ_{med}) (Hughes, 2023). Values of whole cell capacitance can be calculated by multiplying C_{eff} by the surface area of the cell (assumed to be spherical of cell radius r). Values of whole cell capacitance derived from the 3DEP compare to values acquired by patch clamp (Hoettges et al., 2019). During 3DEP analysis, cells are suspended in medium and typically of relatively low σ_{med} compared to physiological conditions to minimize noise of the 3DEP spectra. Following measurement and exposure to the low-intensity electric field approximately 70% of cells inserted into the 3DEP chip can be recovered (Hoettges et al., 2019).

The 3DEP device has been used to characterise the electrical properties of cells in many applications, including the characterization of neural stem cells (Labeed et al., 2011), detection of cellular apoptosis (Labeed et al., 2006), measuring membrane capacitance of neural stem cells during differentiation (Yale et al., 2018), and in the characterization of circadian rhythms in blood cells (Henslee et al., 2017b, Beale et al., 2019). Moreover, the 3DEP has been shown to efficiently and accurately detect electrical changes which occur in cells between disease and health states. Hoque et al. (2020) demonstrated a sensitivity of 75% and specificity of 88% at differentiating healthy controls from bladder cancer patients when comparing the DEP spectra of voided cells using the 3DEP. It has also shown similar sensitivities, specificities, and diagnostic accuracy at detecting oral cancer from non-invasive brush-samples (Broche et al., 2007, Graham et al., 2015, Hughes et al., 2023). Additionally, the 3DEP has been used to characterise changes in the electrical properties of chondrocytes during osteoarthritis (Abdallat et al., 2021).

2.4. Conclusion

All biological cells, including PBMCs and chondrocytes, possess intrinsic electrical properties fundamental to their function. Changes in the electrical properties of cells can indicate altered cell morphology, phenotype, function, cell-cell interactions, and protein-cell interactions. Electrophysiological characterisation of cells can be applied to a range of biomedical applications, including detecting diseased cell states, changes in cell function, and tracking cell differentiation.

A snapshot of the dielectric properties of thousands of cells can be measured rapidly and accurately using the DEPtech 3DEP device and the Malvern Zetasizer. The 3DEP machine uses dielectrophoresis to measure values of G_{eff} , C_{eff} , and σ_{cyto} . The Malvern Zetasizer uses ELS to measure extracellular ζ -potential. Both low-cost, rapid, non-contact, high-throughput devices can be used to build an overview of cell electrophysiology and are suited for biomedical applications.

3. ELECTROPHYSIOLOGICAL CHANGES IN WHITE BLOOD CELLS UNDER HYPEROSMOTIC STRESS IN ME/CFS

This first experimental chapter explores the electrophysiological properties of PBMCs in ME/CFS.

3.1. Chapter Overview, Aims, and Objectives

ME/CFS is a complex, debilitating condition with an unknown aetiology (Cliff et al., 2019), characterised by persistent fatigue, post-exercise malaise, and pain (Germain et al., 2021). No effective quantitative laboratory test or biomarker is currently validated for ME/CFS diagnosis. Doctors instead rely on subjective, unspecific, and unreliable consensus diagnostic criteria, meaning it can take years before a diagnosis is reached, with frequent misdiagnosis, stigma, and trivialisation of the disease. A break-through paper in 2019 by Esfandarypour et al. reported evidence of an electrophysiological biomarker for ME/CFS; the overall impedance of PBMCs, incubated in the same donor's plasma supplemented with NaCl to impart hyperosmotic stress, from ME/CFS patients was significantly different to healthy controls when continuously measured over 1.5 hours (Esfandarypour et al., 2019).

This chapter describes a pilot study which aimed to further investigate changes in the electrical properties of PBMCs in response to hyperosmotic stress in ME/CFS compared with healthy controls. Values of G_{eff} , C_{eff} , σ_{cyto} , and ζ -potential of PBMCs donated from individuals diagnosed with severe ME/CFS, mild/moderate ME/CFS, healthy controls, and multiple sclerosis (MS) were analysed every 30 minutes over 2-hours during incubation in hyperosmotic NaCl media. This was to examine the electrical changes in PBMCs to hyperosmotic stress in more depth than the nanoneedle device used by Esfandarypour et al. (2019), which collected a single combined measurement of overall impedance consisting of cell membrane capacitance, σ_{cyto} , cell-cell interactions and cell-sensor surface adhesion (Esfandarypour et al., 2019). MS donors were recruited as disease controls to evaluate the specificity of ME/CFS electrical changes during hyperosmotic challenge, which was not included in the study by Esfandarypour et al., but is important when considering potential use as an electrical diagnostic biomarker. Moreover, the DEPtech 3DEP and Malvern Zetasizer are rapid and low-cost techniques which could potentially be used clinically for diagnostic applications, compared with the nanoneedle device which was not engineered for routine use. All experiments were repeated using hyperosmotic mannitol media as the stressor, to investigate whether different osmotic stressors produce similar ME/CFS electrical fingerprints.

3.2. Myalgic Encephalomyelitis/ Chronic Fatigue Syndrome (ME/CFS)

3.2.1. About ME/CFS

ME/CFS is a complex multisystemic disease with evidence of pathological changes in cellular metabolism, immunity, cardiovascular, autonomic dysfunction, and neurological dysfunction (Locher, 2015, Collatz et al., 2016, Du Preez et al., 2021a). ME/CFS is characterised by profound unexplained disabling fatigue which is not relieved by rest and is exacerbated by mental or physical activities (Lacerda et al., 2018, Moneghetti et al., 2018, Lande et al., 2020, Castro-Marrero et al., 2021, Germain et al., 2021), cognitive impairment, sleep abnormalities, pain, and orthostatic intolerance (Germain et al., 2021).

For many ME/CFS patients, their symptoms represent a significant disability (Valdez et al., 2019). The quality of life of individuals with severe ME/CFS is usually poor; quality of life functional and health-related scores have been lower in ME/CFS than in MS, cancer, osteoarthritis, and heart disease (Nacul et al., 2011, Chu et al., 2019). The unadjusted EuroQol 5-dimensional 3-level (a quality-of-life measure) mean scores of patients with ME/CFS is 0.47 compared with that of lung cancer at 0.69 (Falk Hvidberg et al., 2015). At least one in four people with ME/CFS remain bed or house-bound for long periods of time (Clayton, 2015, Pendergrast et al., 2016, CDC, 2018, Williams and Isaacson-Barash, 2021). In some very severe ME/CFS cases, the patient is physically incapable of sitting up or swallowing, relies on tube feeding, in-home assistance, and is sensitive to light and sound so requires a dark and quiet environment (Rowe et al., 2017, CDC, 2019, Williams and Isaacson-Barash, 2021). Suicide risk is high due to such poor quality of life, with one example of a patient completely bed-bound for years who felt isolated, struggled with severe exhaustion and pain, was often unable to speak or have the cognitive energy to focus, who consequently decided to end her life (Williams and Isaacson-Barash, 2021).

The prevalence of ME/CFS globally is increasing, estimated to affect between 0.4% and 2.5% of the population (Rowe et al., 2017, Słomko et al., 2019, Lim et al., 2020), including approximately 3 million individuals in the European continent (Nacul et al., 2021) and approximately 2.28 million in the United States (Valdez et al., 2019). ME/CFS affects all ages, genders, ethnicities, and socioeconomic groups (Cortes Rivera et al., 2019), though affects two to four times more women than men (Bakken et al., 2014, Castro-Marrero et al., 2017, Cortes Rivera et al., 2019, Słomko et al., 2019, Lim et al., 2020, Nacul et al., 2021). ME/CFS is most common in young to middle-aged adults with the peak age of onset between 20-45 years old (Bakken et al., 2014, Chu et al., 2019, Cortes Rivera et al., 2019, Słomko et al., 2019).

Despite research efforts, the aetiology of ME/CFS is not established (Bakken et al., 2014, Rowe et al., 2017, Castro-Marrero et al., 2019, Valdez et al., 2019). Mechanisms proposed to trigger ME/CFS include infection (such as Epstein-Barr Virus), stress, trauma, and autoimmunity (Bakken et al., 2014). The prognosis of ME/CFS is variable between patients. The symptoms presented, the severity, and duration varies between individuals, and disease manifestation within an individual may change over time (Bakken et al., 2014, Nacul et al., 2020). The median rate of full recovery is 5% (Cairns and Hotopf, 2005), with improvements in symptoms more commonly reported (Cortes Rivera et al., 2019). Death in ME/CFS is mostly due to another co-existing illness such as cancer or cardiovascular abnormalities (Cortes Rivera et al., 2019).

Currently, there is no cure for ME/CFS and no FDA-approved drugs for effective treatment (Castro-Marrero et al., 2019). However, some symptoms and co-morbidities can be treated or managed by methods such as increasing salt and fluid uptake for orthostatic intolerance, sleep hygiene techniques and medication for insomnia, and pain relief medication (Rowe et al., 2017, Nacul et al., 2020, Williams and Isaacson-Barash, 2021). Alternative therapies and major lifestyle changes can increase quality of life (Williams and Isaacson-Barash, 2021). Energy management plans which structure cognitive, physical, emotional, and social activities based on the patient's judgement to ensure total energy expenditure fall within their limits reduces the risk of post-exertional malaise. Total energy expenditure should be maintained and adjusted based on symptoms, and graded exercise therapy should not be undertaken as it worsens patients' symptoms (NICE, 2021).

3.2.2. Diagnosis of ME/CFS

Diagnosing ME/CFS is a difficult and often very lengthy process. Despite global endeavours to identify a reliable biomarker, there is still no effective and validated quantitative clinical test to diagnose the condition (Hornig et al., 2015, Esfandyarpour et al., 2019, Valdez et al., 2019).

Current Method of ME/CFS Diagnosis

Doctors first use clinical guidelines, physical examinations, medical histories, and blood tests to eliminate other conditions which share similar symptom presentation, such as anaemia, underactive thyroid, kidney, and liver problems (Lande et al., 2020, NHS, 2021). The British National Institute for Health and Care Excellence (NICE) advise exploration of a ME/CFS diagnosis when an individual has experienced unexplained tiredness for more than three months, a decreased ability to undertake occupational, educational, social, or personal activities from pre-illness levels, and only

when differential diagnoses have been excluded (Institute of Medicine, 2015, NHS, 2021). Doctors then follow different sets of diagnostic criteria developed through expert consensus which specify mandatory symptoms to confirm a diagnosis, including the Canadian Consensus Criteria (CCC), the Fukuda CFS criteria (1994), NICE Clinical Guidelines for CFS/ME (2007), Revised Canadian ME/CFS criteria (2010), and ME-International Consensus Criteria (2011) (Carruthers et al., 2003, Carruthers et al., 2011, Institute of Medicine, 2015). A comparison of the specific symptoms required for different diagnostic criteria is outlined in **Figure 13** (Open Medicine Foundation Canada, 2023).

	Holmes CDC, 1988	Fukuda CDC, 1994	Canadian Consensus Criteria, 2003	International Consensus Criteria, 2011	Institute of Medicine, 2015
Persistent fatigue	Required	Required	Required		Required
Cognition problems			Two symptoms from these categories	One symptom from these categories	This or orthostatic intolerance
Motor-sensory problems					
Short-term memory problems					
Pain	Eight symptoms required from these categories	Four symptoms required from these categories	Required		Required
Disturbed sleep			Required	Required	Required
PEM			Required	One symptom from these categories	
Flu-like symptoms					One symptom from these categories
Susceptibility to infection					
Food intolerance					
Gastro-intestinal problems			One symptom from these categories		
Genitourinary problems					
Orthostatic intolerance					
Respiratory problems			One symptom from these categories	One symptom from these categories	This or cognition problems
Cardiovascular problems					
Temperature intolerance					
Thermostatic instability					

Figure 13 | Comparison of the symptoms required to obtain a diagnosis of ME/CFS across different diagnostic criteria. Figure adapted by Dr Fatima Labeed, from (Open Medicine Foundation Canada, 2023).

The CCC (outlined in **Figure 14**; Created with BioRender.com) is a commonly used set of criteria preferentially used in many investigative diagnostic biomarker studies, including the study by Esfandyarpour et al. (2019), to prevent recruitment and inclusion of individuals who may not have ME/CFS and therefore skew the data. CCC is also one of the criteria used to recruit donors who can donate samples for research studies at the UK ME/CFS Biobank (CureME, 2023).

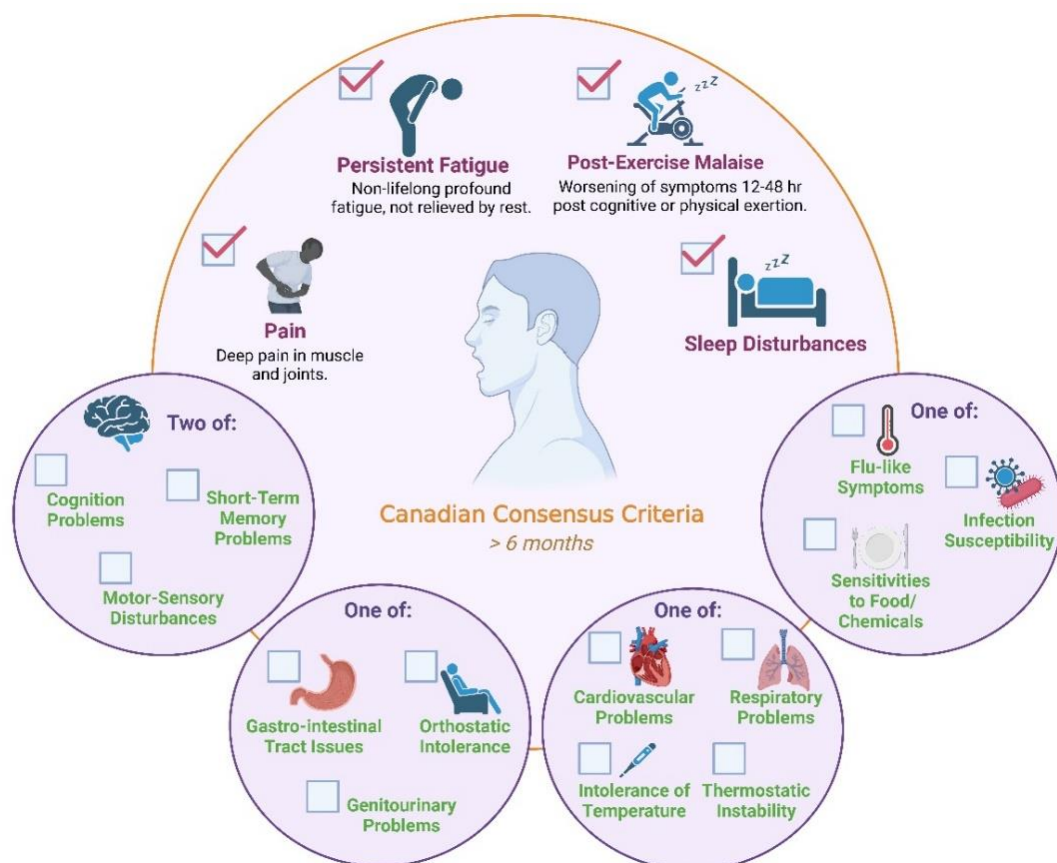


Figure 14 | A diagram of the requirements for ME/CFS diagnosis by the Canadian Consensus Criteria (Created with BioRender.com).

The symptom of “persistent fatigue”, required by all diagnostic criteria except the International Consensus Criteria (see **Figure 13**), refers to a non-lifelong profound fatigue that is not relieved by rest and results in a greatly lowered ability or impairment to do educational, occupational, or social activities than patients were able to before illness. Another required symptom across multiple criteria is post-exertional malaise (PEM). PEM describes the worsening of symptoms 12 to 48 hours after cognitive or physical exertion that was tolerated before disease onset. PEM may include difficulty breathing, sleeping, headaches, and severe tiredness, with a slow return to baseline not caused by sedentary lifestyle or deconditioning. As the onset of PEM is delayed, to sometimes 24-hours post-

exertion or even longer, PEM helps to differentiate ME/CFS from other diseases such as MS, lupus, and major depressive disorder where there is no delay before fatigue and malaise (Nijs et al., 2010, Jason et al., 2015b). The ability to exercise is limited in most patients as they experience PEM (Moneghetti et al., 2018). Other common symptoms include non-restorative sleep (patients wake up unrefreshed) (Institute of Medicine, 2015, CDC, 2021), cognitive impairment (includes trouble thinking quickly, remembering, and paying attention to detail), and orthostatic intolerance (OI); a worsening of symptoms on moving to/maintaining upright posture, with improvement on lying down. OI can be measured using a NASA lean test or tilt table (CDC, 2021). Other common symptoms include deep pain in muscles and joints, headaches, digestive issues, immune dysfunction, and infection (Institute of Medicine, 2015).

Limitations of Diagnostic Criteria

Although consensus-based definitions are necessary when no diagnostic tests are available, their subjectivity, questionable specificity, uncertain external validity, and criterion variance causes diagnostic unreliability (Jason et al., 2015b, Lande et al., 2020). The clear heterogeneity in symptoms between different criteria outlined in **Figure 13** means homogenous sets of patients are not identified (Carruthers et al., 2011). For example, the International Consensus Criteria (ICC) and CCC both identify a smaller subset of patients with more severe symptoms than Fukuda, as both ICC and CCC require more symptoms to make a diagnosis. In patients who exhibit more symptoms, the rate of psychiatric co-morbidities may increase inadvertently; symptoms of the Fukuda criteria overlap with depression (Carruthers et al., 2011, Jason et al., 2015b). Therefore, which diagnostic criteria is chosen for donor recruitment in research studies may affect the results; for example, ME/CFS prevalence estimation studies (Cortes Rivera et al., 2019). Symptom heterogeneity also increases the difficulties in identifying the underlying pathologies of ME/CFS. To reduce diagnostic unreliability, there is a need for a more empirical-based definition or improved biomarkers to reduce the heterogeneity of criteria used for diagnosis (Jason et al., 2015a, Jason et al., 2015b).

Necessity of a Clear Diagnostic Test

Although recognised as a serious condition with potentially severe disability, there has been significant disbelief, trivialisation, marginalisation, and misunderstanding of ME/CFS by society including medical personnel, colleagues, and family due to the absence of a quantitative validated biomarker. As routine blood tests of individuals with ME/CFS often return nothing outside of normal limits, many doctors dismiss, trivialise, or misdiagnose their symptoms (Rowe et al., 2017, Williams and Isaacson-Barash,

2021). Misinformation or lack of knowledge in healthcare providers, particularly those in primary care, means diagnosis is a lengthy and costly process often taking years (Esfandyarpour et al., 2019, Nacul et al., 2021). It is estimated 84 – 91% of patients affected by ME/CFS are undiagnosed (Espinosa and Urra, 2019, Valdez et al., 2019).

Consequences of prolonged diagnosis include difficulties in receiving the appropriate level of care from the health services, dismissal of their disease by employers and educators, neglect by the welfare system, and social isolation (Nacul et al., 2021). This is important as the reduced ability to perform daily tasks (for example, showering or preparing food) results in difficulties maintaining a job or attending school and participating in social activities (CDC, 2018). As such, between 35 – 69% of people with ME/CFS are unemployed (Castro-Marrero et al., 2019, Cortes Rivera et al., 2019) with only 19% working full-time (Lim et al., 2020) at the detriment of enduring symptoms and partaking in no social activities or interests, to rest when they are not working (Nacul et al., 2021). Individual income losses amount to approximately \$20 000 for each household per year (Cortes Rivera et al., 2019), and the economic burden due to loss of productivity and medical bills is estimated to cost €40 billion annually in Europe (Pheby et al., 2020).

Dismissal or misdiagnosis of ME/CFS also means individuals do not receive the correct care for prolonged periods of time (Nacul et al., 2021), and this is fundamental as one key risk factor which determines patient prognosis is the standard of early management of the condition (Cortes Rivera et al., 2019). For example, a patient was continually dismissed or misdiagnosed with conditions such as depression or menopause for over ten years. Doctors disbelieved her and told her she was the “epitome of good health” due to normal blood test results. This led to mismanagement of her condition as she became a yoga instructor in an attempt to improve her energy levels, though this only exacerbated symptoms of undiagnosed ME/CFS. Earlier diagnosis and correct management of her condition could have prevented her deterioration; today she is severely disabled, spending 21 – 23 hours a day in bed, with no career or independence, fully reliant on her parents to be caregivers (Williams and Isaacson-Barash, 2021).

ME/CFS is often misdiagnosed as different psychiatric conditions. Young patients with very severe ME/CFS who are unable to swallow or eat are sometimes wrongly diagnosed with the psychiatric illness Pervasive Refusal Syndrome (Otasowie et al., 2021). Misdiagnosis can lead to the adoption of incredibly detrimental regimes, including forced exercise and separation from their family (Rowe et al., 2017). Cognitive ME/CFS symptoms including loss of short-term memory and poor concentration can result in misdiagnosis of attention deficit disorder without hyperactivity (Rowe et

al., 2017). Nausea and gastrointestinal symptoms resulting in the inability to eat properly can lead to an eating disorder diagnosis (Rowe et al., 2017).

As well as accelerating ME/CFS diagnosis and preventing misdiagnosis, and achieving a better understanding of aetiology, the development of a clear diagnostic biomarker would provide relief and validation to patients that their disease is recognised, and their symptoms result from abnormal biological mechanisms. The development of a clear diagnostic marker will relieve fears of imminent mortality and the presence of a perhaps malignant disease causing their symptoms, accelerate correct management, and treatment of symptoms (Rowe et al., 2017). Additionally, it will decrease the number of patients who are over-investigated, reducing the risks and cost of unnecessary exploratory tests (Nacul et al., 2021).

3.2.3. Developments Towards a Consistent Diagnostic Biomarker

A diagnostic biomarker for ME/CFS would effectively and objectively differentiate those with ME/CFS from healthy controls. This necessitates a parameter which can be measured repeatably at low-cost, with high precision, sensitivity, specificity, and reliability using a technique/equipment that is easily accessible. The optimal biomarker would be a parameter(s) which can be measured quickly, have little variability, and a high signal-to-noise ratio (Califf, 2018, Maksoud et al., 2023). As previously stated, there is no definitive diagnostic test for ME/CFS. However, there are a multitude of different studies which have identified pathological changes occurring in ME/CFS, thus suggesting potential diagnostic biomarker candidates. Pathological changes include blood and plasma abnormalities, immune dysfunction (including NK, complement and cytokine dysfunction), neurology, genetics, cardiac function, metabolic dysfunction, and changes in cellular function. Maksoud et al. (2019) performed a systematic review collating and appraising studies proposing potential ME/CFS biomarkers. The quality, efficiency, and translatability differed between all identified ME/CFS biomarkers, with limited reproducibility of findings between studies. Although biological abnormalities are recognised in individuals with ME/CFS, none currently identified are specific enough for diagnostic application (Querec et al., 2023). Development of a diagnostic method is further complicated by the heterogeneity of the disease, with the severities, frequencies, and combinations of multi-systemic symptoms varying between patients, with a patient's own symptoms often changing too (Collatz et al., 2016).

3.2.3.1. Metabolic and Mitochondrial Dysfunction

Metabolic dysfunction is a recognised factor involved in the pathogenesis of ME/CFS. PEM occurs in 95% of people with ME/CFS and does not occur in other malaise or fatigue disorders (Rutherford et al., 2016), thought to be due to mitochondrial dysfunction and disruption in both ATP synthesis or utilisation of ATP. However, direct investigations into specific parameters have had contradictory results (Missailidis et al., 2020b).

Many studies have identified mitochondrial dysfunction in ME/CFS (Myhill et al., 2009, Tomas et al., 2017, Missailidis et al., 2020b, Missailidis et al., 2020a, Sweetman et al., 2020, Tomas et al., 2020b). Lower measures of oxidative phosphorylation have been reported in thawed PBMC samples from individuals with ME/CFS compared with controls. Tomas et al. (2017) found reduced maximal respiration best differentiated mitochondrial function between both cohorts. A reduced maximal respiration would suggest that the mitochondria of PBMCs in ME/CFS are unable to fulfil the cells basal cellular energy demands and elevate their respiration rate to compensate for high metabolic demands during increased physiological stress (Tomas et al., 2017). However, Nguyen et al. (2019) found there was no statistical difference in mitochondrial respiration in NK cells of ME/CFS patients compared with age and sex-matched non-fatigued healthy controls (Nguyen et al., 2019). The sample size was limited however, with only six in each cohort and the use of different methodologies to Tomas et al. (2017).

Nguyen et al. (2019) also identified a significant reduction in the ability of NK cell mitochondria in ME/CFS to increase glycolytic flux, similar to the study by Mandarano et al. (2020) which found basal glycolysis to be reduced in both CD4⁺ and CD8⁺ T cells in ME/CFS (Mandarano et al., 2020). In contrast, normal functioning of the glycolysis pathway has been identified in PBMCs (Tomas et al., 2017) and skeletal muscle cells (Tomas et al., 2020a). In addition, Tomas et al. (2020b) found that both moderate and severe ME/CFS have reduced mitochondrial function. Furthermore, severe ME/CFS were shown to have glycolytic impairments with higher rates of respiratory acidification (Tomas et al., 2020b).

It has been proposed that ME/CFS individuals have a problem with ATP utilisation as opposed to ATP production (Lawson et al., 2016). However, many studies support a model of deficient ATP production in ME/CFS (Castro-Marrero et al., 2013, Tomas et al., 2017, Mandarano et al., 2020). The rate of ATP synthesis by Complex V (involved in oxidative phosphorylation) is significantly reduced in ME/CFS lymphoblasts (Missailidis et al., 2020a). This is supported by the increased expression of a large number of mitochondrial proteins in ME/CFS, proposed to be an attempt to compensate for deficiencies in ATP production and mitochondrial function. Sequential window acquisition of all

theoretical mass spectra has identified the differential expression of proteins involved in oxidative phosphorylation (Complex V), the electron transport chain (Complex 1), and the oxidative stress response in ME/CFS (Sweetman et al., 2020).

Furthermore, protein expression of the ATP synthase subunit beta (ATPB) is significantly increased in ME/CFS, suggested to be in attempt to increase ATP production (Ciregia et al., 2016). Combining the differential expression of both ATPB and aconitate hydratase (ACON; the enzyme which converts citrate to isocitrate in the Krebs cycle), Ciregia et al. (2016) identified these biomarkers to have a sensitivity of 85%. Whether changes in mitochondrial protein expression could function as a diagnostic marker for ME/CFS requires validation with larger ME/CFS cohorts and incorporation of disease-controls to assess specificity. As mitochondrial dysfunction is associated with a plethora of diseases, ranging from neurodegenerative diseases such as Parkinson's disease (Chen et al., 2019), to cardiovascular diseases (Poznyak et al., 2020), autoimmune diseases such as MS (Barcelos et al., 2019), and psychiatric disorders including schizophrenia (Ni and Chung, 2020), it is important to thoroughly test the specificity of any potential diagnostic marker (Myhill et al., 2009, Nicolson, 2014).

Missailidis et al. (2020b) investigated the diagnostic potential of abnormal mitochondrial respiratory function, activity of the cellular stress-sensing kinase Target Of Rapamycin Complex 1 (TORC1) and increased lymphocyte-death rate in culture of ME/CFS lymphoblasts compared with healthy controls. The sensitivity of these three parameters as diagnostic biomarkers was very high at over 90%. The specificity of each parameter was low with approximately 40% of control samples incorrectly identified as ME/CFS. Nonetheless, combining all three parameters together using multiple logistic-regression to one cell-based biomarker provided a sensitivity and specificity of almost 100% (Missailidis et al., 2020a). However, limitations of the protocol used include the time, expense, and expertise required to test all three parameters for diagnostic applications. Furthermore, it is unknown how specific these parameters are to other illnesses which cause chronic fatigue (Missailidis et al., 2020b). For example, paediatric Dengue fever also decreases frozen PBMC viability (Perdomo-Celis et al., 2016).

3.2.3.2. Immune-Dysfunction

Immune dysfunction has been identified in the pathology of ME/CFS, with lymphocytes as a model to investigate illness pathology (Maksoud et al., 2023). A leading hypothesis of ME/CFS onset is an abnormal immune response to common antigens, resulting in altered functioning of the central nervous system (Espinosa and Urra, 2019). A significant number of studies have demonstrated that

there is wide-spread immunological disruption in ME/CFS, with a systematic review by Maksoud et al. (2019) finding immune dysfunction to be the most prevalent biomarker-type investigated.

Natural Killer (NK) Cell Dysfunction

NK cell dysfunction was once considered a hallmark of ME/CFS and a biomarker which correlates with disease severity (Institute of Medicine, 2015). However, differences in natural killer cell cytotoxic (NKCC) activity between individuals with ME/CFS and healthy controls is inconsistent. A significant reduction in CD69 receptor (a classical early marker of lymphocyte activation) expression post-stimulation compared with healthy controls has been reported in NK cells of ME/CFS donors (Mihaylova et al., 2007). A number of studies have found NKCC activity and function to be significantly reduced in ME/CFS (Caligiuri et al., 1987, Ojo-Amaize et al., 1994, Brenu et al., 2012, Hardcastle et al., 2015, Marshall-Gradisnik et al., 2016a, Eaton-Fitch et al., 2019). In contrast, multiple other studies found no diminished NKCC activity (Curriu et al., 2013, Theorell et al., 2017, Cliff et al., 2019, Querec et al., 2023). These inconsistencies in findings could be due to small sample sizes, different recruitment criteria yielding population differences, and varied methodologies. For example, both Theorell et al. (2017) and Cliff et al. (2019) used freeze-thawed cells as opposed to fresh samples, involving the use of dimethyl sulfoxide (DMSO); a solvent shown to be toxic to cells at concentrations < 10% (Maksoud et al., 2023). Furthermore, Theorell et al. (2017) used PBMCs and not isolated lymphocytes. Levels of intracellular perforin have also been reported to be both diminished (Maher et al., 2005) and increased (Brenu et al., 2011) in ME/CFS. The disparity across studies indicates that although NK cell dysfunction has potential to be an effective model, NKCC requires greater repeatability and standardisation of protocols before use as a clinical diagnostic tool.

Cytokine Dysfunction

Cytokines facilitate the activation and proliferation of leukocytes, direct migration and influence leukocyte function (Khaiboullina et al., 2015). The profile of cytokines and chemokines change during ME/CFS, including an increase in pro-inflammatory cytokines in patients (Fletcher et al., 2009, Yang et al., 2019, Domingo et al., 2021). This is in accordance with the fact that administering pro-inflammatory cytokines causes characteristic severe fatigue, fever, impaired cognitive processing, musculoskeletal achiness, and disturbed sleep – all symptoms of ME/CFS (Nakamura et al., 2013). However, the use of cytokines as a diagnostic biomarker is controversial.

The repertoire of cytokines measured and the results observed vary greatly between studies. Fletcher et al. (2009) identified IL-4, IL-5, LT α , and IL-12 to be elevated in ME/CFS donors compared

with healthy controls and possess large areas under curves using Receiver-Operator Characteristic (ROC) curve analyses, indicating good potential as biomarkers. Like Fletcher et al. (2009), Khaiboullina et al. (2015) also identified IL-4 to increase in ME/CFS donors. In contrast, Landi et al. (2016) found levels of plasma IL-4 to decrease (Landi et al., 2016) and Groven et al. (2020) found IL-4 to significantly decrease in ME/CFS donors than controls. Groven et al. (2020) reported IL-1 β , IL-10, IL-17, and TNF α to significantly decrease in ME/CFS patients, which contrasted with other studies which found these cytokines to increase in patients with ME/CFS (Maes et al., 2012, Kadhum, 2018).

Finding a consistent, stable, and replicable circulating cytokine profile to act as a specific ME/CFS diagnostic is very unlikely due to how sensitive and easily effected cytokines are to biological mechanisms as well as widespread differences in cytokine laboratory methodology (Fletcher et al., 2009, Blundell et al., 2015, VanElzakker et al., 2018, Yang et al., 2019). For example, Bioassay, ELISA, and multiplex assay results are not comparable, even when using kits of the same assay (VanElzakker et al., 2018). When comparing the ability of four multiplex kits and multiple lots of the same kit to detect 13 cytokines from the same sample at six different laboratories, Breen et al. (2011) found at least one significant laboratory or lot effect for each cytokine. Variance was observed in the same laboratory and across laboratories (Breen et al., 2011). Directly measuring cytokine levels is influenced by measuring levels in plasma vs. serum, time between blood draw and separation of plasma or serum, repeated freezing and thawing, and storage temperature. Measuring cytokine levels of PBMCs stimulated *in vitro* varies with stimulants used and culture conditions (Fletcher et al., 2009).

Furthermore, the heterogenous nature of ME/CFS associated with ME/CFS subtypes, severity, and duration, in addition to inconsistencies in cytokine methodologies and repeatability suggests cytokine profiling as a quantitative diagnostic biomarker harbours a lot of limitations and may not be feasible. A multi-centre study by Hornig et al. (2015) identified markedly different plasma immune signatures in early ME/CFS patients compared with healthy controls. However, these distinct alterations were not present in longer duration ME/CFS patients. Early ME/CFS had prominent activation of pro-inflammatory cytokines such as TNF α and IL-1 α , and anti-inflammatory cytokines such as IL-1, compared with controls. However, the same cytokines were lower in long-duration ME/CFS cases than healthy controls. Stronger correlations in cytokine alterations were found with the duration of illness than severity of illness, indicative of ME/CFS possessing a non-static immunopathology (Hornig et al., 2015). Furthermore, evidence suggests ME/CFS patients with varying clinical presentations and comorbidities may have distinct differences in cytokine and chemokine levels (Hornig et al., 2017).

Changes in cytokine levels is not necessarily specific for ME/CFS, and more likely indicative of immune activation with chronic inflammation, supported by observed elevation in the pro-inflammatory cytokines LT α , IL-1 α , IL-1 β , and IL-6 in ME/CFS compared with controls (Fletcher et al., 2009). The chronic condition fibromyalgia shares similar symptomology to ME/CFS and Groven et al. (2020) found no difference in the cytokines and chemokines immune markers investigated between ME/CFS and fibromyalgia patients. The neuroimmune demyelinating disorder MS is another chronic debilitating disorder which shares a similar symptomology with ME/CFS, including severe disabling fatigue (Jason, 2017). MS is characterised by altered pro-inflammatory and anti-inflammatory cytokines resulting in great inflammation within the central nervous system (Reale et al., 2012). Reale et al. (2012) identified serum IL-1 β and IL-17 pro-inflammatory cytokines to be elevated in both ME/CFS and MS compared with controls. However, Hornig et al. (2016) identified a unique cytokine profile which was able to differentiate donors with MS from ME/CFS (Hornig et al., 2016). Gulf War Illness (GWI) has similar symptom presentation to that of ME/CFS, and similar to ME/CFS plasma IL-5 (Whistler et al., 2009) and IL-6 (Petrescu et al., 2018) has been reported to be elevated in GWI compared with controls. However, Khaiboullina et al. (2015) has found evidence towards different immune profiles in ME/CFS compared with GWI, even though symptoms overlap.

3.2.3.3. Electrical Properties of Immune Cells in ME/CFS

Since it was first suggested that the symptoms of ME/CFS are secondary to acquired abnormalities in voltage-gated or ligand-gated ion channels (Chaudhuri and Behan, 1999, Chaudhuri et al., 2000), growing evidence supports intracellular secondary messenger signalling and ion channel dysfunction in ME/CFS pathophysiology. A number of studies have recently implicated acetylcholine receptors (both nicotinic and muscarinic) and TRP channel action in ME/CFS donors, particularly TRPM3 (Marshall-Gradisnik et al., 2016b, Cabanas et al., 2019b, Eaton-Fitch et al., 2021, Eaton-Fitch et al., 2022).

Dysfunction of Acetylcholine Receptors and Transient Receptor Potential Channels in Immune Cells in ME/CFS

The classical neurotransmitter acetylcholine (ACh) is a ubiquitous signalling molecule well characterised within the central and peripheral cholinergic nervous system. Evidence suggests there is dysregulation of the autonomic sympathetic and parasympathetic nervous system in ME/CFS, including sensitivity to pain and sensory stimuli (Loebel et al., 2016, Fernandez-Guerra et al., 2021), and abnormal peripheral cholinergic function during ACh challenge (Khan et al., 2004, Spence et al.,

2004). The parasympathetic nervous system partly regulates the interaction between the nervous and immune systems (Fujii et al., 1998), with ACh involved and important in inflammation and immune responses (Skok et al., 2005, Cox et al., 2020, Mashimo et al., 2021).

ACh present in whole blood (Watanabe et al., 1986) has been demonstrated to mainly originate from PBMCs (not polymorphonuclear lymphocytes) (Kawashima et al., 1993). ACh and choline acetyltransferase, the enzyme which catalyses the synthesis of ACh from choline, has been shown to be present in immune cells including T lymphocytes (Fujii et al., 1996, Fujii et al., 1999), B lymphocytes, and NK cells (Rinner et al., 1998, Reardon et al., 2013, Cox et al., 2020). Immunocytochemical analysis, specific ligand binding, and mRNA expression studies confirmed muscarinic and nicotinic acetylcholine receptors (mAChRs and nAChRs respectively) are expressed in immune cells (Sato et al., 1999, Kawashima and Fujii, 2000, Kawashima, 2004, Skok et al., 2005, Fujii et al., 2017), with expression varying with cell type and activation status (Kawashima et al., 2007, Qian et al., 2011).

AChRs are important for Ca^{2+} signalling and consequently immune cell function. mAChRs are G-protein coupled receptors (metabotropic), of which there are five subtypes (M1, M2, M3, M4, and M5) (Kawashima et al., 2012, Cox et al., 2020). M1, M3, and M5 couple with G_q , and when stimulated mediate the activation of PLC, resulting in an increase in $[\text{Ca}^{2+}]_i$ and PKC activation (see **Chapter 2**). Stimulation of M3 or M5 mAChRs in T-cells facilitate the release of Ca^{2+} from intracellular stores via IP_3 , resulting in CRAC channel Ca^{2+} influx and sustained $[\text{Ca}^{2+}]_i$ oscillations which enhances the expression of c-fos and IL-2 (Mashimo et al., 2016). In contrast, M2 and M4 couple to $G_{i/o}$, which when activated mediate the inhibition of adenylyl cyclase and a decrease in cAMP synthesis (Kawashima et al., 2012, Fujii et al., 2017). nAChRs are fast ligand-gated (ionotropic) cation channels and homo- or heteropentameric structures, which when activated increase membrane permeability to Na^+ , K^+ , and Ca^{2+} leading to membrane depolarisation and excitation (Kawashima and Fujii, 2000, Fujii et al., 2017). Activation of mAChRs and nAChRs are important for immune cell function, including increased cytotoxicity, cell proliferation, and the activation of B and T lymphocytes (Fujii et al., 2017). In B-cells, activation of AChRs has been shown to mediate cell development, activation, and antibody immune responses (Skok et al., 2005, Skok et al., 2006, Skok et al., 2007, Koval et al., 2009, Koval et al., 2011). B-cell produced ACh inhibits the local recruitment of neutrophils (Reardon et al., 2013) and limits steady-state haematopoiesis (Schloss et al., 2022). In macrophages, activation of AChRs modulates the expression of cytokines (Papatriantafyllou, 2013). T-cells up-regulate the synthesis of ACh in the blood when activated (Fujii et al., 1998).

Given the importance of ACh and Ca^{2+} signalling in immune cell function, the impaired immune cell function and sensitivities reported by patients with ME/CFS to environmental irritants and toxins suggest dysfunction of AChRs channels in ME/CFS, for which there is clear and growing evidence. Single nucleotide polymorphism (SNP) and genotype analysis of DNA extracted from whole blood samples identified seventeen SNPs in AChRs which are significantly associated with ME/CFS, nine of which were associated with mAChRM3 (Marshall-Gradisnik et al., 2015a). In the same cohort, thirteen SNPs significantly associated with ME/CFS were identified in TRP ion channel genes, nine of which were located within the gene sequences of TRPM3 (the others in TRPC4, TRPA1) (Marshall-Gradisnik et al., 2015b). NK cells isolated from ME/CFS donors have been shown to possess numerous SNPs and genotypes in nAChRs, TRPM3, and TRPM8 (Marshall-Gradisnik et al., 2016a). SNPs have been identified in isolated B-cells of ME/CFS donors; of 78 SNPs in nAChRs and mAChRs genes of isolated B-cells of ME/CFS, 35 were within mAChM3. SNPs were also found in TRPM3 (Marshall-Gradisnik et al., 2016b). Given the interdependence of TRP and mAChRs, it is possible that specific mAChRM3R and TRPM3 SNP genotypes contribute to the pathology and heterogenic phenotypes of ME/CFS (Marshall-Gradisnik et al., 2016c).

The Australian National Centre for Neuroimmunology and Emerging Diseases confirmed ME/CFS is a TRP channelopathy class of metabolic disorders (Marshall-Gradisnik, 2022). TRP ion channels are homo- or hetero-tetrameric cation-permeable pores preferentially selective to Ca^{2+} , assembled from six-transmembrane polypeptide subunits (Clapham et al., 2001, Parenti et al., 2016, Yue and Xu, 2021). However, the permeation profile depends on the splice variant of the gene (Marshall-Gradisnik et al., 2015b). Based on the TRP channel amino acid sequences and structural similarities they can be divided into six subsets: TRPC, TRPV, TRPA, TRPML, TRPP and TRPM channels (Marshall-Gradisnik et al., 2016b, Khalil et al., 2018). TRP channels are widely expressed on almost all cell types throughout the body (Khalil et al., 2018) including immune cells such as lymphocytes, DCs, macrophages, neutrophils, and mast cells (Parenti et al., 2016). TRP channels are localised to plasma membrane and intracellular organelle membranes, and are activated by extracellular and intra-cellular stimuli (Yue and Xu, 2021). They function as transduction molecules, responding to a range of physical and chemical stimuli including changes in shear stress, temperature, osmolarity, pH, and reactive molecules (Parenti et al., 2016). The sensitivities reported by patients with ME/CFS to environmental irritants and toxins, are consistent with TRP channel dysfunction.

As previously mentioned, SNPs in TRPM3 have been identified in ME/CFS (Marshall-Gradisnik et al., 2015b, Marshall-Gradisnik et al., 2016b). The location of SNPs (whether they occur in coding, non-coding, or intergenic regions of genes) and the influence of splicing mechanisms determine whether SNPs lead to human disease (Marshall-Gradisnik et al., 2015a). There is substantial

evidence demonstrating TRPM3 dysfunction in ME/CFS patients. Immunophenotyping assays have demonstrated expression of TRPM3 ion channels to be significantly reduced and significantly increased in different NK cells and CD19⁺ B-cells subsets when compared with healthy controls (Nguyen et al., 2016, Nguyen et al., 2017). Furthermore, the activity and function of TRPM3 in NK cells is impaired in ME/CFS. When opened, TRP channels induce depolarisation of the cell and Ca²⁺ influx, and the activation of intracellular signalling pathways (Marshall-Gradisnik et al., 2016b). Whole-cell patch clamp demonstrated the amplitude of TRPM3 currents after stimulation with pregnenolone sulphate (a fast reversible TRPM3 activator) was significantly reduced in isolated NK cells from ME/CFS patients compared with healthy controls (Cabanas et al., 2018, Cabanas et al., 2019a). Additionally, TRPM3-associated impairments in the mobilisation, influx, and storage of intracellular Ca²⁺ have been reported in B-cells and NK cell subtypes (Nguyen et al., 2016, Nguyen et al., 2017). Upregulation of TRPM3 in B-cells and NK cell subsets has been suggested to be a compensation mechanism to impaired Ca²⁺ influx, mobilisation, and storage (Nguyen et al., 2016). As Ca²⁺ is required for many NK cell functions, including cytotoxic activity, formation of the immune synapse, the granule-dependent pathway of apoptosis, microtubule reorganisation, and cytokine gene transcription (Schwarz et al., 2013, Marshall-Gradisnik et al., 2016a), impaired TRPM3-dependent Ca²⁺ signalling may contribute to the NK cell dysfunction seen in ME/CFS. Furthermore, TRPM3 is involved in the transmission of heat and pain, nociception, thermoregulation, and has been implicated in inflammatory pain syndromes and proinflammatory cytokine secretion (Marshall-Gradisnik et al., 2015b), seen in ME/CFS.

μ-opioid receptors (μOR) are a family of GPCRs whose subunits can directly bind and inhibit TRPM3. Naltrexone hydrochloride (NTX) is a long-lasting antagonist of μOR, and acts to negate TRPM3 inhibition (Eaton-Fitch et al., 2022). Low-dose NTX (LDN) is taken in ME/CFS patients to treat pain attributable to insufficient opioid peptide secretion and excess release of pro-inflammatory cytokines, with 73.9% of patients reporting improved symptoms (Polo et al., 2019). Interestingly, using whole-cell patch clamp, TRPM3 channel activity when modulated with the TRPM3-agonist pregnenolone sulphate, is restored in IL-2 stimulated NK cells of ME/CFS donors following 24-hour incubation with NTX (Cabanas et al., 2019b). Moreover, the function of TRPM3 channels in ME/CFS patients taking LDN is similar to that of healthy controls, with TRPM3-like ionic currents in NK cells (Cabanas et al., 2021). Moreover, restoration of TRPM3 function following *in vitro* overnight treatment of NK cells with NTX has been shown to translate to re-established TRPM3-dependent Ca²⁺ influx (Eaton-Fitch et al., 2022).

Dysfunction of other TRP channels has been identified, including the increased expression of the sensory ion channel TRPV1 (Light et al., 2012) and TRPM2 (Balinas et al., 2019) in ME/CFS. TRPM7

may be implicated (Du Preez et al., 2021a), with crosstalk between IL-2 and TRPM7 (Du Preez et al., 2021b), and alterations of TRPM7-dependent Ca^{2+} influx in ME/CFS (Du Preez et al., 2023).

Evidence of the fundamental role TRPM3 ion channel dysfunction in NK cells in ME/CFS pathophysiology validates this disabling condition to be biological as opposed to psychological (Marshall-Gradisnik, 2020). However, there are limitations to consider this finding as a diagnostic tool. Previous studies have recruited a limited number of participants and did not have diseased-controls to determine specificity. Epigenetic and genetic diagnostic biomarkers require validation and repeatability of specific SNPs, as well as consistency as to which cell type to quantify. Furthermore, quantification of TRPM3 function to diagnose ME/CFS using patch-clamp is a low-throughput method requiring high expertise, time, and expense. Greater optimisation of a diagnostic protocol is required.

3.2.3.4. Ionic Differences

Levels of Mg^{2+} in red blood cells have been reported to be reduced in ME/CFS (Cox et al., 1991). Dysregulated levels of Na^+ and K^+ have also been reported, including increased muscle Na^+ content in ME/CFS patients compared with controls (Petter et al., 2022) and decreased exercise-induced K^+ outflow from muscles (Jammes et al., 2020). Furthermore, there is evidence of dysregulation of the Na^+/K^+ and Ca^{2+} -ATPase pump in ME/CFS donors, proposed to be attributable to increased production of reactive oxygen species causing an increased fluidity of the sarcoplasmic reticulum membrane (Fulle et al., 2003).

3.2.3.5. Electrical Changes Detected in PBMC and Plasma Samples Under Hyperosmotic Stress

A great break-through towards the identification of a diagnostic biomarker for potential clinical application was published by Esfandyarpour et al. (2019), in which they found real-time monitoring of the electrical response of hyperosmotically challenged PBMCs (PBMCs were incubated in the donor's plasma supplemented with NaCl) differentiated patients with ME/CFS from healthy controls. PBMCs donated by ME/CFS patients (mix of moderate and severe ME/CFS donors) and healthy controls were incubated in the donors own plasma, which was increased to a concentration of 200 mM NaCl to impart hyperosmotic stress on the cells. As shown in **Figure 15**, Esfandyarpour et al. (2019) found introduction of a hyperosmotic stressor to healthy control samples resulted in a transient decrease in impedance before returning to baseline, where the impedance did not change for approximately 2.5 hours. Similarly, after the hyperosmotic stressor was added to the ME/CFS samples, a transient decrease in impedance was measured until a return to baseline at around 40 minutes. However, in contrast to the healthy controls, the impedance continued to markedly rise

above the initial baseline values with an increase in impedance magnitude of $|Z|$ 74.92% $\pm$ 0.69, in-phase impedance (Z_{re}) of 301.67% $\pm$ 3.55, and out-of-phase impedance (Z_{im}) of 64.73% $\pm$ 0.62. This significantly different response to hyperosmotic stress between healthy control and ME/CFS donors represents a unique biomarker and indicator of ME/CFS (Esfandypour et al., 2019).

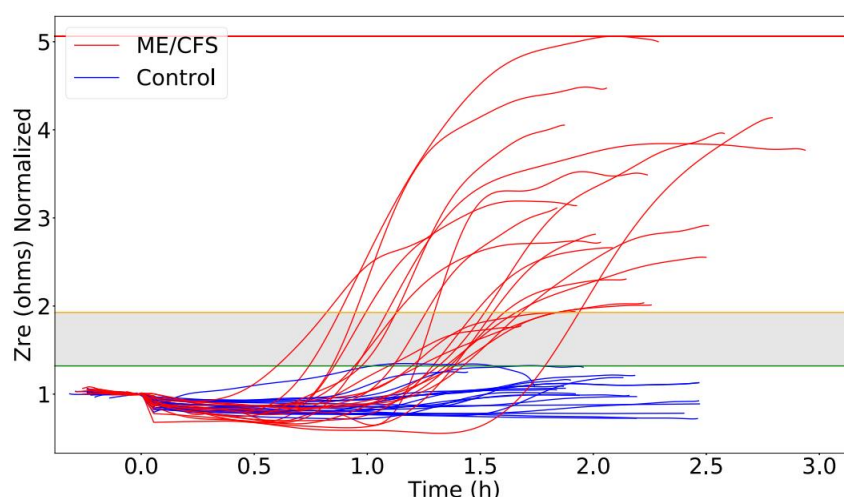


Figure 15 | Electrical impedance of hyperosmotically challenged PBMCs from the paper by Esfandypour et al. (2019). Electrical impedance (Z_{re}) against time curves of 20 ME/CFS donors (red lines) and 20 healthy controls (blue lines) of PBMCs incubated in their own plasma supplemented to 200 mM NaCl. Graph reprinted from (Esfandypour et al., 2019).

The nanoelectronic assay Esfandypour et al. (2019) used was developed for high-throughput real-time detection of proteins, nucleic acids, and gene quantification (Esfandypour et al., 2012, Esfandypour et al., 2013a, Esfandypour et al., 2013b, Esfandypour et al., 2013c, Esfandypour et al., 2016). The device consists of thousands of nanoneedle biosensors arranged in parallel within a microfluidic channel (trough). An individual nanoneedle biosensor **Figure 16A** has a width of approximately 3 – 5 μm and consists of three film layers: a 30-nm thin insulator layer separating two 100-nm-thin gold conductive layers. Additional protective oxide layers were deposited above (20 nm thin) and below (250 nm thin) the sensor, to prevent the top conducting electrode getting into contact with the solution and to electrically insulate the bottom conducting electrode from the substrate. The active sensor region was the interface of the middle oxide layer with the electrolyte (Esfandypour et al., 2013b, Esfandypour et al., 2019). The assay measures the electrical impedance between the two conductive electrode layers to detect any modulation in impedance attributable to the presence and/or interactions of biomolecules at the active sensing regions (Esfandypour et al., 2014, Esfandypour et al., 2019).

The low-cost device measured ultrasensitive and precise values of impedance in real-time, possessing a sampling frequency of 5 Hz and collecting ~40 000 data points over 3-hours. However,

one main limitation of the device is that the values of impedance calculated are the lumped combination of all the separate and resistive components of the PBMCs and plasma (**Figure 16B**), including the overall cell impedance (consisting of membrane capacitance and σ_{cyto}), as well as the impedances caused by media-sensor surface interactions, cell-cell interactions, cell-sensor surface adhesion, solution resistance, and other plasma components such as proteins, lipids, and exosomes (Esfandyarpour et al., 2019). Furthermore, the device was not engineered for routine use.

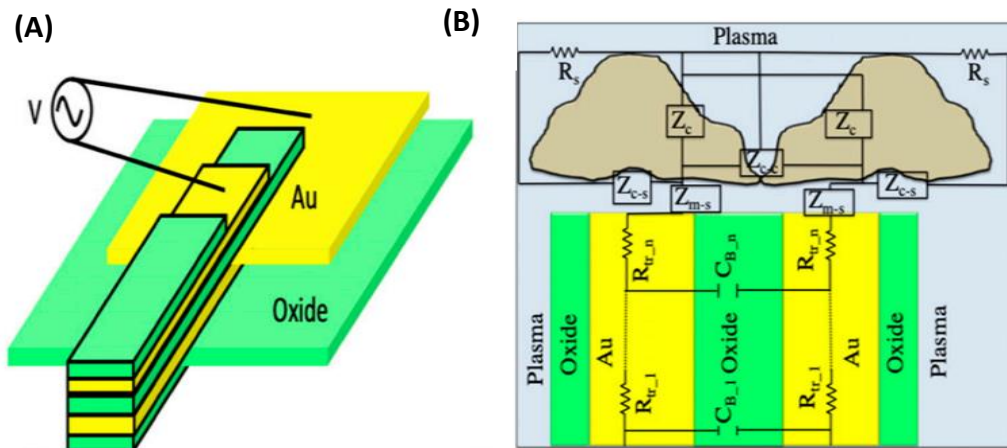


Figure 16 | Illustrations of the nanoneedle biosensor used by Esfandyarpour et al. (2019). **|A|** Schematics of a single nanoneedle biosensor not drawn to scale. **|B|** An illustration of the electrical circuit model at the sensor-solution active sensing region interface. Z_{m-s} is media-sensor impedance; Z_{c-s} is cell-sensor surface adhesion impedance; Z_c is cell impedance; Z_{c-c} is cell-cell interaction impedance; R_s is solution resistance; $R_{u,n}$ is conducting electrode resistance; $C_{B,n}$ is insulating electrode capacitance. Illustrations reprinted from (Esfandyarpour et al., 2019).

3.3. Methods

3.3.1. Sample Acquisition

A favourable ethical opinion for this project was obtained from the UCL Biobank Ethical Review Committee – Royal Free London NHS Foundation Trust (RFL B-ERC) (Reference number: NC2020.23. NRES EC number: 16/WA/0289).

This project was conducted in collaboration with researchers from the London School of Hygiene and Tropical Medicine (LSHTM). Frozen PBMC vials donated from individuals with a severe ME/CFS diagnosis ($n = 8$), mild/moderate ME/CFS diagnosis ($n = 9$), healthy controls ($n = 7$), and MS diagnosis ($n = 5$) were obtained from the UK ME/CFS Biobank and transported to the University of Surrey via medical courier on acquisition of a Material Transfer Agreement. All frozen samples were classified as NHS REC samples and stored at the cryostore at the Faculty of Health and Medical Science

liquid nitrogen facility. Samples were transported individually from the cryostore on an ad hoc basis to the CryoCube F101h, 230V/50 Hz, UK (Fisher Scientific, UK, #16309636) -80 °C freezer and stored for a maximum of four days before experimentation to facilitate data collection without compromising sample integrity (Ticha et al., 2021). All experimental investigation and data analysis was conducted at the University of Surrey.

3.3.2. Subjects

The general characteristics of each donor cohort is described in **Table 2**, detailing the number of donors, the most common age group (age at time of blood donation), their sex assigned at birth, and ethnicity. Individual donor characteristics are detailed in **Figure 17** to cluster donors within each cohort more specifically. As per the UK ME/CFS Biobank donation inclusion criteria, individuals diagnosed with ME/CFS possess a clinical diagnosis following the CCC (Carruthers et al., 2003) and/or Centers for Disease Control (CDC)-1994 and/or ICC (Carruthers et al., 2011). With no quantitative biomarker, disease severity was based on symptom severity. As defined by Carruthers et al. (2011), pre-illness activity levels must have been reduced by approximately 50% to be diagnosed with mild ME/CFS. Individuals with moderate ME/CFS were mostly housebound, severe ME/CFS individuals were mostly bedridden, and very severe ME/CFS patients were completely bedridden (Carruthers et al., 2011). MS donors required a clinical diagnosis from an NHS consultant neurologist following NICE guidelines. Donor exclusion criteria include other severe illness such as cancer, history of acute or chronic infectious diseases such as hepatitis B or HIV, psychiatric diagnoses, pregnancy, taking any medication known to alter immune function, anti-viral medication, or vaccination within the previous 3 months. Furthermore, healthy controls were excluded if they possessed a current or past fatiguing illness/major morbidity. MS donors were excluded if they possessed any other conditions which could cause chronic fatigue (CureME, 2023). The main symptom of all severe ME/CFS, mild/moderate ME/CFS, and MS donors is fatigue, with the exception of one MS donor whose main symptom was immune-type symptoms.

Table 2 | Baseline characteristics of total n = 29 donors whose PBMCs were obtained from the UK ME/CFS Biobank; consisting of individuals with severe ME/CFS (n = 8), mild/moderate ME/CFS (n = 9), healthy controls (n = 7) and MS (n = 5). Continuous variables are expressed as mean ($\pm$ SD) and categorical variables as frequency (fractions of the donor cohort).

	Severe ME/CFS Diagnosis	Mild/Moderate ME/CFS Diagnosis	Healthy Controls	MS Diagnosis
Number of donors (n)	8	9	7	5
Sex	Female 6/8	Female 6/9	Female 5/7	Female 4/5
Mode Age Group(s)	45 – 50 yrs (n = 4)	55 – 60 yrs (n = 4)	50 – 55 yrs (n = 3)	35 – 40 yrs (n = 2) 50 – 55 yrs (n = 2)
Ethnicity	Caucasian 8/8	Caucasian 8/8	Caucasian 6/7 Chinese 1/7	Caucasian 5/5

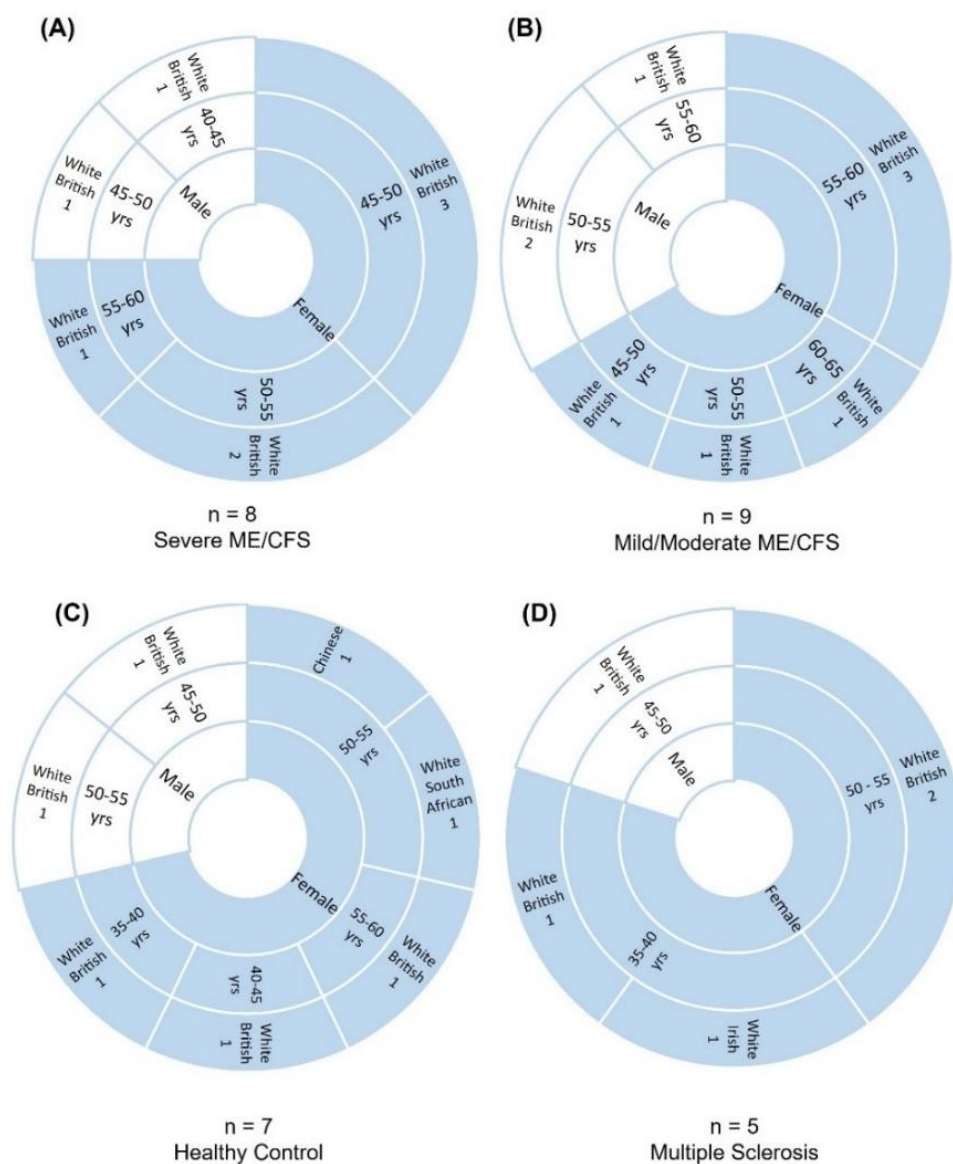


Figure 17 | Sunburst diagrams of **|A|** Severe ME/CFS diagnosis (n = 8) **|B|** Mild/Moderate ME/CFS diagnosis (n = 9) **|C|** Healthy Controls (n = 7) **|D|** Multiple Sclerosis diagnosis (n = 5) donor characteristics to identify individuals of each cohort by sex, age, and ethnicity.

3.3.3. Medium Preparations

Control Media: RPMI-1640 Medium GlutaMAX supplement (ThermoFisher, UK, #61870010) supplemented with 10% heat inactivated Gibco™ Foetal Bovine Serum (Fisher Scientific, UK, #11523387), 1% penicillin/streptomycin (Sigma-Aldrich, UK, #P4333), and 10 mM HEPES (ThermoFisher, UK, #15630080).

Hyperosmotic NaCl Treatment: Control media described above was supplemented with NaCl (Sigma-Aldrich, UK, #S9625) to a total concentration of 200 mM.

Hyperosmotic Mannitol Treatment: Control media described above was supplemented with 193 mM D-mannitol (Sigma-Aldrich, UK #M9647).

DEP Medium: Dielectrophoretic cell characterisation using the 3DEP (DEPtech, UK) was performed in sterile DEP medium. Deionised water was diluted with 8.5% (w/v) sucrose (Sigma-Aldrich, UK, #S9378), 0.5% (w/v) dextrose (Sigma-Aldrich, UK, #G8270), 250 μ M MgCl₂ (ThermoFisher, UK, #447155000), and 100 μ M CaCl₂ (ThermoFisher, UK, #L13191.0I). σ_{med} was measured using the Hanna Instruments 9811-51 pH/EC/TDS/°C meter (Hanna Instruments, #HI-9811-51) at room temperature (18 – 22 °C), and adjusted to 193 mS/m by adding phosphate-buffered saline (PBS) (Sigma-Aldrich, UK #D8537) through titration. The medium was then filter sterilised and stored at 2 – 5 °C for a maximum of two weeks, and pre-warmed to 37 °C before experiments.

All media were filter sterilised using a 0.22 μ m filter (Millex Syringe-driven filter 0.22 μ m, Sigma-Aldrich, UK, #SLGP033RS) or Bottle Top Filtration Unit 0.22 μ m (StarLab, UK, #CC8221-5226) depending on media volume). Values of media osmolality, pH, and conductivity were measured before commencing each experiment with mean measurements ($\pm$ SD) detailed in **Table 3**. Media osmolality was measured using the Gonotec® Osmomat Freezing Point Osmometer Model 3000 Basic (Wolflabs, UK, #32.B), media conductivity was measured using the Hanna Instruments HI-99301 EC/TDS/Temperature Meter – High Range (Hanna Instruments, # HI-99301) and media pH measured using the Hanna Instruments 9811-51 pH/EC/TDS/°C meter (Hanna Instruments, #HI-9811-51). Measurements were taken at the temperature the media was used at: control media, hyperosmotic NaCl media, and hyperosmotic mannitol media measured at 37 °C and DEP media measured at 18 – 22 °C. All equipment was calibrated prior to measurement and three technical repeats of media characteristics measured before each experiment.

Table 3 | Mean ($\pm$ SD) values of media osmolality (mOsm/kg), pH, and conductivity (mS/m).

Medium	Osmolality (mOsm/kg)	pH	Conductivity (mS/m)
Control Medium	285 ($\pm$ 6.8)	7.76 ($\pm$ 0.27)	1088 ($\pm$ 23.00)
Hyperosmotic NaCl	460 ($\pm$ 7.4)	7.79 ($\pm$ 0.18)	1684 ($\pm$ 8.38)
Hyperosmotic Mannitol	479 ($\pm$ 22.2)	7.74 ($\pm$ 0.14)	1020 ($\pm$ 66.54)
DEP Medium	282 ($\pm$ 3.5)	7.53 ($\pm$ 0.15)	193 ($\pm$ 0.15)

3.3.4. PBMC Preparation

Following transportation from liquid nitrogen to the -80°C freezer, the frozen PBMC vial (containing 1 mL frozen PBMC at approximately 5×10^6 cells/mL) was thawed following the LSHTM PBMC Cryopreservation and Thawing Standard Operating Protocol. The vial was placed in a water bath of 37°C until only a small amount of ice remained. Using aseptic techniques, the thawed liquid contents of the vial were transferred to a 15 mL falcon tube containing 10 mL of control medium (pre-warmed to 37°C) within a laminar flow hood. 0.5 mL of control medium was added back to the remaining frozen contents of the vial and mixed until the remainder had thawed, and was then transferred to the same 15 mL falcon tube. The cell suspension was centrifuged (PrO-Research Centrifuge, Centurion Scientific Ltd, UK) at 400 g for 5 minutes at room temperature and the supernatant discarded. The cell pellet was resuspended in 2 mL of control medium to calculate cell concentration using a C-Chip Disposable Haemocytometer, DHC-N01 (Labtech, Uckfield, UK) and cell viability using Trypan Blue Solution, 0.4% (ThermoFisher, UK, #15250061) following the methods outlined in **Section 3.3.7**. Control media was then added to the cell suspension to a volume of 12 mL and incubated at 37°C 5% CO_2 for two hours to facilitate cell recovery post-thawing. Post-incubation the cell suspension was resuspended thoroughly and evenly aliquoted into autoclave sterilised Eppendorf Safe-Lock micro-centrifuge tubes (Sigma-Aldrich, UK, #EP0030123620-500EA) for treatment (depending on cell concentration into 14-18 aliquots).

3.3.5. Treatment of PBMCs to Induce Hyperosmotic Stress

PBMC cell suspension aliquots underwent centrifugation at 400 g for five minutes at room temperature. The supernatant was removed, and the cell pellets resuspended in 1 mL control media, hyperosmotic NaCl media, or hyperosmotic mannitol media, and incubated at 37°C 5% CO_2 . The electrical properties of PBMCs were measured at time 0 and after 1.5-hours incubation in control and

challenged cells using the 3DEP (DEPtech, UK), and every 30 minutes from time 0 up to 2 hours for each treatment condition using the Zetasizer Lab Blue Label (Malvern Panalytical, UK) following the methods in **Section 3.3.6**. To increase the resolution of changes, in a small number of donors (n = 2 severe ME/CFS, n = 2 mild/moderate ME/CFS, n = 2 healthy controls, and n = 3 MS) the 3DEP was used to measure the electrical properties every 30 minutes up to 1.5 hours during hyperosmotic NaCl incubation.

3.3.6. Data Collection

At each data collection timepoint, the required PBMC aliquot was removed from incubation and centrifuged at 400 g for five minutes at room temperature. The supernatant was discarded, and the cell pellet resuspended in 1 mL DEP medium. To wash off residual high conductivity treatment medium the suspension was centrifuged again at 400 g for five minutes, the supernatant discarded, and the pellet resuspended in DEP medium at a volume to ensure a concentration of 1×10^6 cells/mL.

ζ-Potential Data Collection

The volume required to extract 120 000 cells from the cell suspension was transferred to a micro-centrifuge tube and made up with DEP medium to 800 μ L. This secondary diluted cell suspension was carefully filled into a capillary cell (Malvern Panalytical, UK, #DTS1070) ensuring the absence of air bubbles. Two plugs were positioned carefully onto the top of the capillary cell and inserted into the Blue Label Lab Zetasizer (Malvern Panalytical, UK). Once the temperature of the capillary cell reached equilibrium, up to five technical repeats of ζ -potential were measured.

3DEP Data Collection

The remaining primary stock cell suspension was loaded into a 3DEP chip and analysed using the 3DEP (see **Figure 12**) as follows. Approximately 80 μ L of cell suspension was injected into a 3DEP chip using a blunt bent needle positioned onto a syringe. All 20 electrode wells of the chip were loaded evenly and free of bubbles. The wells were covered with a 18x18 mm glass cover slide, and the chip inserted into the 3DEP reader. The signal generator in the 3DEP reader supplied a 20V_{pp} sinusoidal signal to the chip, and the wells were energised by 20 frequencies (a different frequency to each well) spaced on a Log10 scale between 10 kHz and 45000 kHz. Runtime duration during which the electric field was applied was 30 seconds, with images taken of all wells every second using a 4x magnification microscope. Changes in well light intensity over time were thus visible. Between three and five

technical repeats were obtained for each sample. The wells of the chip were flushed with DEP medium in between repeats.

C-Chip Data Collection

The cell suspension was gently but thoroughly resuspended to ensure an even cell density. 10 μ L of cell suspension and 5 μ L cell suspension mixed 1:1 with Trypan Blue Solution, 0.4% (ThermoFisher, UK, #15250061) were both inserted into different wells of the C-Chip Disposable Haemocytometer DHC-N01 (Labtech, Uckfield, UK). The haemocytometer was analysed under the Nikon TS100 inverted phase contrast microscope. Photographs were taken at x10 and x20 magnification using Vimba Viewer (V2.4.0, Allied Vision) for cell radius, cell concentration, and cell viability data analysis.

3.3.7. Data Analysis

ζ - Potential Data

ζ -potential data measured by the Malvern Panalytical Zetasizer were recorded using the Zetasizer Ultra-Pro ZS Xplorer (Malvern Panalytical, v3.00) software. ζ -potential values were exported into a Microsoft Excel document, in which the mean ($\pm$ SEM) ζ -potential of all technical repeats was calculated.

3DEP Data

Raw data intensity values of bands 7 to 9 (calculated by the 3DEP software) for each frequency were exported from the 3DEP software (DEPtech 2013, version 1.5.1.68) in .csv file format and collated in Microsoft Excel. The mean intensity value for each frequency was calculated. Collective spectra of technical repeats were modelled in MATLAB (MathWorks, R2022b) to obtain values of G_{eff} , C_{eff} , and σ_{cyto} . Regression analysis was used to assess the goodness of fit of the model to the data, quantified using a coefficient of determination (R^2 value). The MATLAB code written by Professor Michael Hughes and Komal Kaur is shown in **Appendix B**. For the scope and applications of this thesis, single-shell models were used. Any values of light intensity determined by the 3DEP software as outliers due to the presence of bubbles or low cell concentration were automatically excluded. Modelling of DEP spectra in MATLAB was used over 3DEP software modelling to allow for increased sensitivity and specificity. Only DEP spectra modelled with an R squared value (Pearson correlation coefficient) of above 0.7 were included.

To determine whether there were quantifiable differences in the average DEP spectra detectable without spectrum modelling (optimizing time and eliminating the requirement of cell radius measurements), the average raw data was analysed using an ABCD method: first five data points of the DEP spectra (frequency range 10.0 kHz to 58.8 kHz) were averaged (A), the second five data points (frequency range 91.5 kHz to 538 kHz) were averaged (B), the third five data points (frequency range 837 kHz to 4.92 MHz) were averaged (C) and the last five data points (frequency range 7.66 MHz to 45.0 MHz) were averaged (D). All technical repeats were included, and no outliers were removed to eliminate the possibility of operator bias. ABCD data averages were normalised, and two-way ANOVAs were performed to determine whether there were differentiating trends between cohorts and treatments.

Cell Radius and Concentration

Cell radius, cell concentration and cell viability of the cells within the C-Chip Haemocytometer images were analysed using the software Image J (National Institutes of Health, V1.54d). The scale of the image was set by equating a set number of pixels to the known distance of a haemocytometer square (depicted in **Figure 18**). Cell diameters were measured by drawing a line through the centre of a cell consistently at 45° to account for any non-spherical PBMCs. The average diameter of 50 to 100 cells was measured and exported into Microsoft Excel, in which the mean ($\pm$ SEM) radius was calculated. The mean cell radius calculated using ImageJ was inserted into the MATLAB script (**Appendix B**) for DEP spectra modelling. Cell concentrations were measured following the North-West rule to prevent over-estimations.

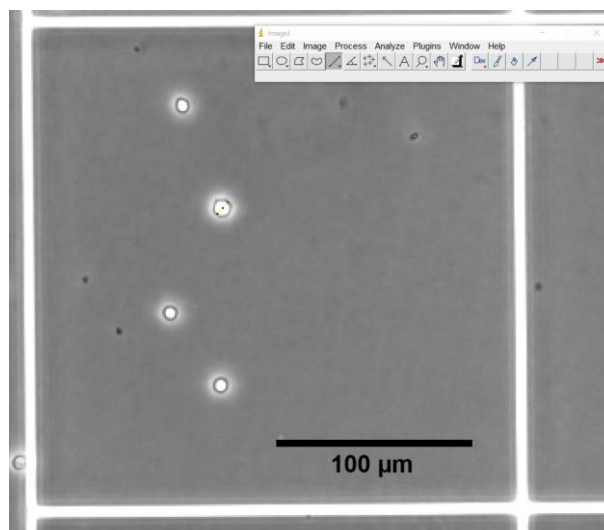


Figure 18 | Cell radius analysis of PBMCs on a c-chip haemocytometer measured using ImageJ software.

Data Analysis

The raw values of G_{eff} , C_{eff} , σ_{cyto} , and ζ -potential were normalised by donor by subtracting the values of unchallenged PBMCs from hyperosmotically challenged PBMCs. The percentage change in ζ -potential between 1.5-hour hyperosmotic NaCl incubation and 1.5-hour physiological incubation was also calculated. Logistic regression was performed using GraphPad Prism 9 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com) to combine potential biomarkers.

Statistical Analysis

Measurements of G_{eff} , C_{eff} , σ_{cyto} , ζ -potential, cell radius, and ABCD data values were each plotted against time of incubation using GraphPad Prism 9 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). The Shapiro-Wilk test for normality was used to determine whether values of G_{eff} , C_{eff} , σ_{cyto} , ζ -potential, and cell radius were normally distributed ($p > 0.05$).

To determine whether the values of G_{eff} , C_{eff} , σ_{cyto} , ζ -potential, and cell radius in unchallenged PBMCs were statistically different between donor cohorts, ordinary one-way ANOVAs followed by Tukey's multiple comparisons test were used for normally distributed data ($*p < 0.05$), and the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons tests was used for non-normally distributed data ($*p < 0.05$).

To determine whether values of ζ -potential changed over time during hyperosmotic challenge, three-way ANOVAs followed by Tukey's multiple comparisons ($*p < 0.05$) were used to determine whether ζ -potential was affected by the duration of hyperosmotic challenge, unchallenged vs challenge, and whether diagnosed with ME/CFS or not.

To determine whether any of the electrical parameters significantly changed from unchallenged baseline values following 1.5-hour hyperosmotic NaCl or hyperosmotic mannitol challenge within or between cohorts, mixed effects models were performed with Geisser-Greenhouse correction followed by Tukey's multiple comparisons ($*p < 0.05$). Paired mixed-effects analysis matched models followed by Tukey's multiple comparisons were used to compare normalised values for hyperosmotic NaCl vs. hyperosmotic mannitol treatment for each donor cohort ($*p < 0.05$).

To determine whether differences in the electrical properties of PBMCs between donor cohorts performed in line or better than diagnostic biomarkers proposed in published studies, Receiver-Operator Characteristic (ROC) curves were plotted in GraphPad Prism software. The 'best' threshold of optimal sensitivity and specificity at differentiating individuals with ME/CFS diagnosis to those with no ME/CFS diagnosis was the threshold closest to (0.0, 100.0) on the ROC curve. Combining

biomarkers into one diagnostic biomarker was performed using binary logistic regression in GraphPad Prism 9. The area under the curve (AUC) for the ROC curve, the p-value of the AUC, the confidence interval (CI), and the sensitivity, and specificity of the biomarker at the 'best threshold' were recorded.

3.4. Results and Discussion

3.4.1. Comparing Electrical Properties of Unchallenged PBMCs Between Donor Cohorts

The first objective of this pilot study was to determine whether the baseline electrophysiological properties of PBMCs from individuals with a severe ME/CFS diagnosis, mild/moderate ME/CFS diagnosis, MS diagnosis, and healthy controls were different when unchallenged in control media. Baseline parameters in G_{eff} , C_{eff} , σ_{cyto} , and ζ -potential in individuals with ME/CFS and MS have not previously been explored, thus were investigated to determine whether there were any significant differences in any of the parameters which may contribute to ME/CFS pathology.

To investigate this, frozen PBMC cell aliquots were thawed and incubated for two hours in RPMI cell culture medium to recover, and the initial baseline electrical values were obtained using the 3DEP and Zetasizer. Values of G_{eff} , C_{eff} , and σ_{cyto} , obtained from MATLAB-fitted models (**Appendix B**) of DEP spectra which possessed an R^2 value of over 0.7 of unchallenged PBMCs from severe ME/CFS diagnosis ($n = 6$), mild/moderate ME/CFS diagnosis ($n = 5$), healthy control ($n = 7$), and MS diagnosis donors ($n = 4$) are recorded in **Table 4** and plotted in **Figure 19A-C**. An R^2 value of 0.7 or greater is considered to have a strong effect size and a high correlation (Ratner, 2009), and was chosen as a cut-off threshold to ensure values of G_{eff} , C_{eff} , and σ_{cyto} are derived from well-fitted models. A higher R^2 cut-off was not chosen because the noise of 3DEP spectra would have resulted in the elimination of data from multiple participants. Values of ζ -potential of unchallenged PBMCs from donors with a severe ME/CFS diagnosis ($n = 8$), mild/moderate ME/CFS diagnosis ($n = 9$), healthy controls ($n = 7$) and MS diagnosis ($n = 5$) are recorded in **Table 4** and plotted in **Figure 19D**.

Table 4 | Values of G_{eff} , C_{eff} , and σ_{cyto} of unchallenged PBMCs from severe ME/CFS (n = 6), mild/moderate ME/CFS (n = 5), healthy controls (n = 7), and MS donors (n = 4). Values of ζ -potential were collected from severe ME/CFS (n = 8), mild/moderate ME/CFS (n = 9), healthy controls (n = 7), and MS donors (n = 5). Mean values $\pm$ SEM.

	G_{eff} (kS/m ²)	C_{eff} (mF/m ²)	σ_{cyto} (S/m)	ζ -Potential (mV)
Severe ME/CFS	21.4 $\pm$ 5.3	9.8 $\pm$ 2.3	0.52 $\pm$ 0.03	-14.4 $\pm$ 1.0
Mild/Moderate ME/CFS	19.4 $\pm$ 6.1	9.0 $\pm$ 2.0	0.43 $\pm$ 0.04	-14.9 $\pm$ 0.8
Healthy Controls	30.8 $\pm$ 9.1	12.0 $\pm$ 0.9	0.47 $\pm$ 0.05	-14.3 $\pm$ 2.5
MS	19.3 $\pm$ 4.5	8.0 $\pm$ 2.6	0.40 $\pm$ 0.03	-10.7 $\pm$ 3.7

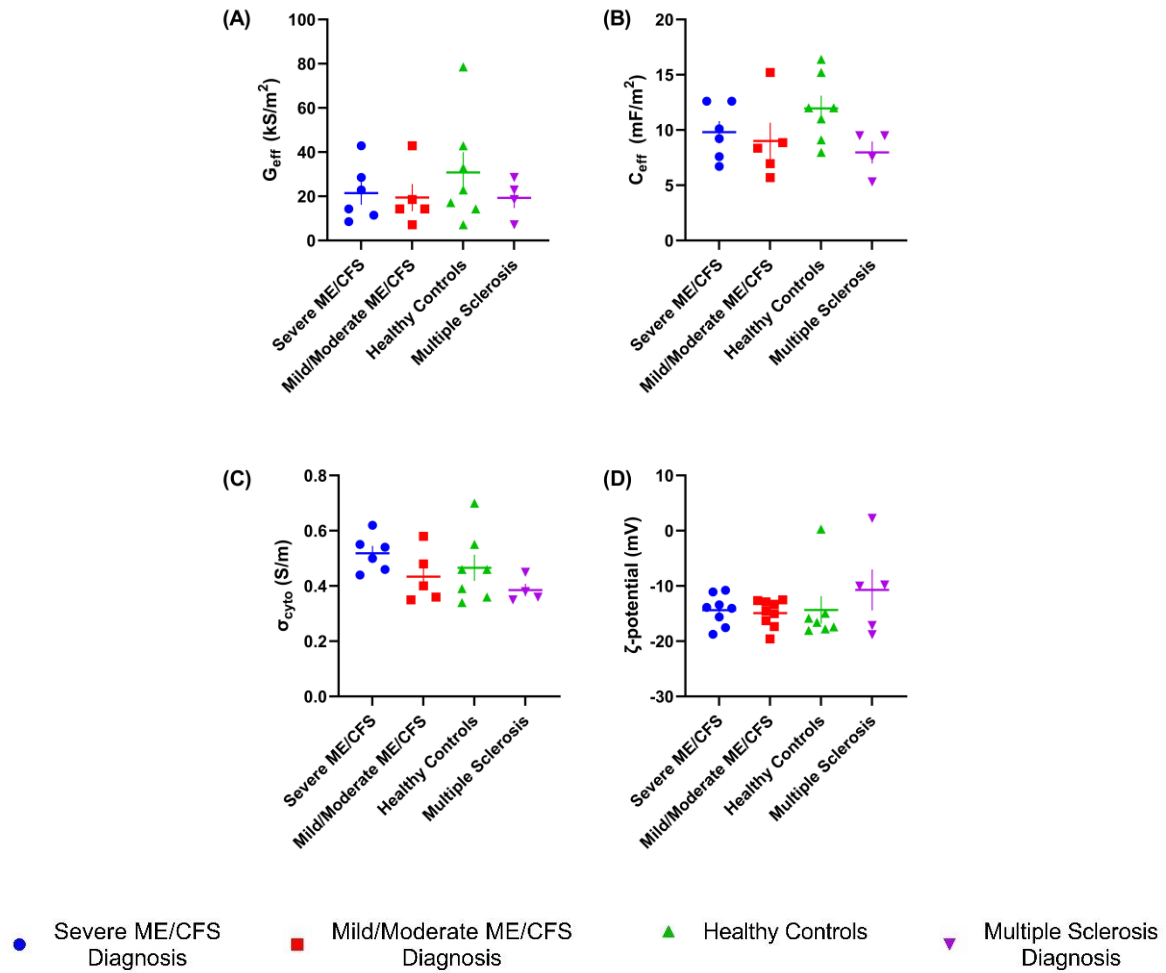


Figure 19 | Comparing initial values of **[A] G_{eff}** **[B] C_{eff}** **[C] σ_{cyto}** of unchallenged PBMCs in severe ME/CFS diagnosis (blue circles, n = 6), mild/moderate ME/CFS diagnosis (red squares, n = 5), healthy controls (green triangles, n = 7), and MS diagnosis (purple inverted triangles, n = 4) donors. Values were calculated from MATLAB-fitted models of DEP spectra which possessed an R^2 value of over 0.7. No statistical differences were found between donor cohorts in values of G_{eff} , C_{eff} , and σ_{cyto} using ordinary one-way ANOVAs followed by Tukey's multiple comparisons (* $p < 0.05$). **[D] ζ -potential** of unchallenged PBMCs in severe ME/CFS diagnosis (blue circles, n = 8), mild/moderate ME/CFS diagnosis (red squares, n = 9), healthy controls (green triangles, n = 7), and MS diagnosis (purple inverted triangles, n = 5) donors. No statistical differences in ζ -potential between cohorts were identified using Kruskal-Wallis test followed by Dunn's multiple comparison (* $p < 0.05$). Mean lines plotted ($\pm$ SEM).

Shapiro-Wilk tests for normality identified values of G_{eff} , C_{eff} , and σ_{cyto} were normally distributed for all donor cohorts ($p > 0.05$). In contrast, values of ζ -potential and cell radius were found

to be non-normally distributed ($p = 0.0003$). Ordinary one-way ANOVAs followed by Tukey's multiple comparisons test were used to compare unchallenged values of G_{eff} , C_{eff} , and σ_{cyto} between donor cohorts, and the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons tests were used to compare unchallenged values of ζ -potential and cell radius between cohorts. No statistical difference was found between the mean ($\pm$ SEM) values of G_{eff} (**Figure 19A**), C_{eff} (**Figure 19B**), σ_{cyto} (**Figure 19C**), or ζ -potential (**Figure 19D**) between any donor cohorts when unchallenged. Together these results suggest unchallenged PBMCs of individuals with severe ME/CFS diagnosis, mild/moderate ME/CFS diagnosis, and MS diagnosis have no statistically significant differences in their electrophysiological properties of G_{eff} , C_{eff} , σ_{cyto} , and ζ -potential than that of healthy controls. The electrical properties of unchallenged PBMCs are thus unable to distinguish between donor cohorts alone, and baseline characteristics are not a potential diagnostic biomarker. Furthermore, it infers that any pathophysiological changes to PBMCs in ME/CFS and MS do not significantly affect their dielectric properties or ζ -potential when unchallenged.

However, small differences in C_{eff} between cohorts may hint at potential differences in PBMC morphology, altered expression of glycoproteins or adhesion markers on the cell membrane. Although not significant, mean values of C_{eff} in healthy controls may potentially be slightly greater than that of severe ME/CFS diagnosis, mild/moderate ME/CFS diagnosis, and MS donors (**Figure 19B**). Membrane capacitance increases with cell surface area (Golowasch and Nadim, 2013), and is proportional to cell surface area for a membrane of constant thickness. However, there were no statistically significant differences between donor cohorts in PBMC radius (μm) as determined using the Kruskal-Wallis test followed by Dunn's multiple comparison ($*p < 0.05$), as shown in **Figure 20**.

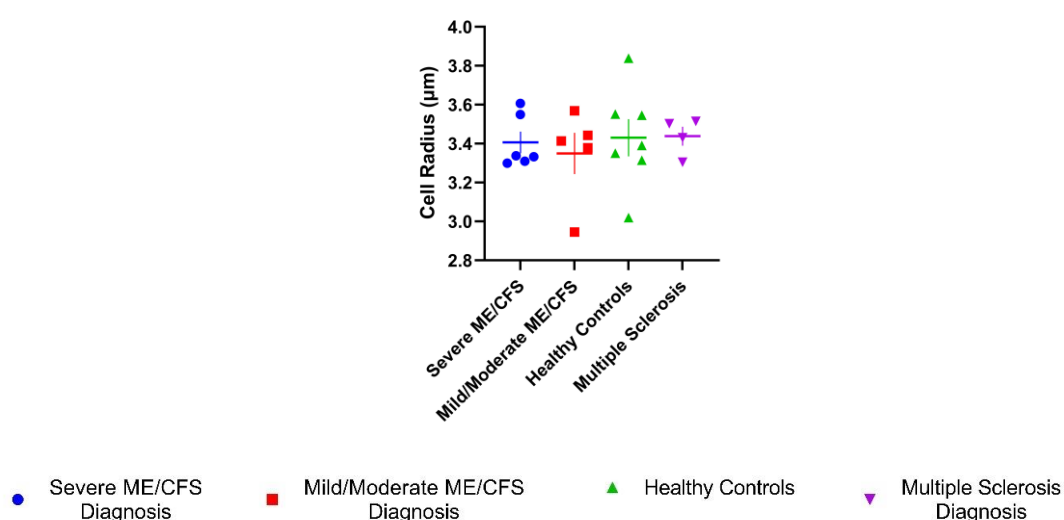


Figure 20 | Cell radius (μm) of unchallenged PBMCs in severe ME/CFS diagnosis (blue circles, $n = 6$), mild/moderate ME/CFS diagnosis (red squares, $n = 5$), healthy controls (green triangles, $n = 7$) and multiple sclerosis diagnosis (purple inverted triangles, $n = 4$) donors. No statistical difference using Kruskal-Wallis test followed by Dunn's multiple comparisons ($*p = 0.05$). Mean lines plotted ($\pm$ SEM).

The marginal increase in C_{eff} in healthy controls could potentially indicate there are more invaginations, microvilli, or ruffles which increase the cell membrane surface area of PBMCs in healthy donors than that of severe ME/CFS diagnosis, mild/moderate ME/CFS diagnosis, and MS donors. No differences in cell morphology between cohorts were visible using the Nikon TS100 inverted phase contrast microscope, corroborated by Esfandypour et al. (2019) who found no significant difference in PBMC size between ME/CFS patient cells and healthy control cells using live microscopy imaging (Esfandypour et al., 2019). However, future work could investigate whether there are any differences in membrane morphology using scanning electron microscopy. The composition of the lipid bilayer also affects C_{eff} , and previous observations have found decreased levels of phosphatidylcholines and sphingomyelins in ME/CFS (Che et al., 2021) and significantly decreased levels of membrane phospholipids PE and PS for PBMCs in MS (Hon et al., 2009). The composition of lipids in PBMC membranes in ME/CFS and MS could be analysed further by methods such as flow cytometry (Waddington et al., 2019). Differences in C_{eff} could also be due to cell-surface modifications, for example increased N-glycosylation significantly increases membrane capacitance in neural stem cells and progenitor cells (Yale et al., 2018). Alterations in the expression of cell surface receptors and adhesion markers have been shown in NK cells, CD4⁺ T-cells, and CD8⁺ T-cells in ME/CFS, such as dysregulated CD24 expression in B cells in ME/CFS patients (Mensah et al., 2018) and decreased expression of CD57 in T-cells in ME/CFS (Espinosa and Urra, 2019).

Recent studies have identified polymorphisms in TRP channels (Marshall-Gradisnik et al., 2015b, Marshall-Gradisnik et al., 2016c), and differences in TRP channel expression (Balinas et al., 2019) and function (Cabanas et al., 2018, Cabanas et al., 2019a) in ME/CFS (previously discussed in **Section 3.2.3.3.**). Similarly, differences in TRP channel expression have been reported in literature in MS (Çakır et al., 2021). The non-significant differences in G_{eff} between donor cohorts suggest TRP channel dysfunction in ME/CFS and MS does not significantly impact values of G_{eff} in PBMCs from that of healthy controls when unchallenged. Similarly, the results suggest dysfunction in Ca²⁺ mobilisation due to TRP dysfunction documented in ME/CFS (Nguyen et al., 2017, Du Preez et al., 2023) does not correspond to significant differences in σ_{cyto} . However, values of σ_{cyto} were marginally higher in severe ME/CFS than mild/moderate ME/CFS, healthy controls, and MS donors. Quantification of cytoplasmic ion concentrations such as Ca²⁺ using methods such as the fluorescent indicator dye fura-2 in future study is required to determine whether this is the case.

No significant differences in ζ -potential were identified in severe ME/CFS diagnosis and mild/moderate ME/CFS diagnosis donors compared with healthy controls. This evidence supports the fact that no studies have reported increased PBMC aggregation in ME/CFS. Although insignificant, the mean value of ζ -potential in donors with an MS diagnosis had a smaller magnitude than healthy

controls. This suggests that PBMCs from MS donors may possibly be more unstable and more prone to aggregation than healthy controls.

Current literature values of some studies reporting G_{eff} , C_{eff} , σ_{cyto} , and ζ -potential in unchallenged freshly isolated PBMCs donated from human healthy controls are detailed in **Table 5**. No papers currently report the specific dielectric properties of PBMCs in either ME/CFS or MS, and very limited on the ζ -potential of PBMCs across all donor cohorts.

Table 5 | Values of dielectric parameters and ζ -potential of unchallenged fresh PBMCs and PBMC sub-populations from healthy control donors reported in current literature. Mean values $\pm$ SEM. (Becker et al., 1995, Yang et al., 1999, Chan et al., 2000, Wang et al., 2017, Mohamed et al., 2019).

Study	Method	Cell Type	Membrane Conductance ($\mu S/m$)	Membrane Capacitance ($mF m^{-2}$)	σ_{cyto} (S/m)	ζ -Potential (mV)
(Chan et al., 2000)	ROT (Single-shell model)	PBMCs	-	11.6 ± 4.2	-	-
(Mohamed et al., 2019)	DEP (DEP medium: 280 mM D-mannitol solution)	Monocytes	0.001	-	0.011	-
(Yang et al., 1999)	ROT (Tens of cells)	B-cells	-	12.6 ± 3.5	0.73 ± 0.18	-
		T-cells	-	10.5 ± 3.1	0.65 ± 0.15	-
		Monocytes	-	15.3 ± 4.3	0.56 ± 0.10	-
		Granulocytes	-	11.0 ± 3.2	0.60 ± 0.13	-
(Wang et al., 2017)	Microfluidic Device	Lymphocytes	-	23.9 ± 3.9	-	-
		Granulocytes	-	19.5 ± 2.2	-	-
(Becker et al., 1995)	ROT	T-cells	-	11 ± 1.1	0.62 ± 0.073	-
(Chakraborty et al., 2020)	Zetasizer	Monocytes	-	-	-	-20.9 ± 0.7
		Unstimulated macrophages	-	-	-	-21.8 ± 0.5
(Bondar et al., 2012)	Zetasizer	Mononuclear cells	-	-	-	-21.9 ± 0.2

On analysis of the dielectric properties of PBMCs in healthy controls in **Table 4**, values of C_{eff} compare favourably to that of literature values. However, σ_{cyto} is lower than that of PBMC sub-populations reported in literature (Becker et al., 1995, Yang et al., 1999). Differences in the dielectric values quoted from literature compared with unchallenged healthy controls reported here could be

due to differences in methodologies; the devices and electrokinetic techniques used to measure dielectric properties were different as ROT was used in both studies, in comparison with the 3DEP which uses conventional DEP. Measurements recorded by Becker et al. (1995) and Yang et al. (1999) were also from single-cell populations isolated from freshly donated whole-blood samples, whereas the values in **Table 4** are from frozen mixed PBMC population samples. Freezing cells can induce injuries resulting in changes in cell morphology, metabolic activity, function, and cell death due to changes in extracellular osmolarity as the extracellular freezing medium crystallises, imposing severe osmotic stress, and the formation of intracellular ice crystals (Meneghel et al., 2020). Crystallisation can cause a decrease in membrane integrity (Marquez-Curtis et al., 2022), and a difference in dielectric properties which has been seen in bovine tissue (Matković and Šarolić, 2022).

The average ζ -potential of healthy control biological repeats (**Table 4**) has a slightly smaller magnitude than literature cited healthy PBMC values (**Table 5**). These differences could be due to changes in the cell surface during recovery post-thawing, or due to differences in the PBMC suspension media. The most important factor which affects ζ -potential is pH. The pH of the DEP medium used in this study was $7.53 (\pm 0.15)$, compared with pH 7.4 media reported in literature (Bondar et al., 2012, Chakraborty et al., 2020). σ_{med} also affects ζ -potential, as both an increased ionic strength and an increase in the presence of trivalent ions compresses the electric double layer (Malvern Instruments 2015). The low conductivity of DEP medium in comparison with highly conductive PBS (Bondar et al., 2012) and HEPES buffer (Chakraborty et al., 2020) may contribute to differences. As V_m depends on differences in the intracellular and extracellular ion concentrations, differences in the ionic composition of media used in literature may have changed the average PBMC V_m . In a recent study, changes in the V_m of RBCs incubated in isosmotic media of different ionic strengths was proportional to changes in ζ -potential, with a best fit achieved when a coefficient of proportionality independent of double layer thickness was added (Ξ) (Hughes et al., 2021). As such, changes in V_m may have resulted in the change in ζ -potential. Parallel patch-clamp or voltage-sensitive fluorescent dyes (such as DiSBAC₂(3)) to measure V_m would be of great interest, but limited time, resources, and the number of cells available prevented this from occurring within this chapter. Particle concentration was greater in both literature studies at approximately 5×10^5 cells/mL compared with 1.5×10^5 cells/mL due to limited sample availability, which can affect the measured ζ -potential as analytical signal is a function of particle concentration (PTL, 2022).

As the mean specific membrane capacitance values of B-cells, T-cells, monocytes, and granulocytes are different (Yang et al., 1999), and the dielectric properties of PBMCs measured are the aggregate sum of the dielectric properties of all subpopulations within the sample, differences in proportions of PBMC subpopulations between cohorts may also contribute to dielectric differences.

For example, whereas T-lymphocytes have smooth surfaces with only few projections, B-lymphocytes have lots of microvilli contributing to a slightly higher mean specific membrane capacitance. The mean specific membrane capacitance as measured by Yang et al. (1999) using electrorotation is $10.5 (\pm 3.1)$ mF/m² in T-lymphocytes compared with $12.6 (\pm 3.5)$ mF/m² in B-lymphocytes. Monocytes have a larger diameter of 8 – 14 μ m and their cell surface contains few microvilli but are covered in well-developed broad-based ruffles and ridges. Their specific membrane capacitance is higher at $15.3 (\pm 4.3)$ mF/m². Increased proportions of effector memory CD8⁺ T-cells and decreased terminally differentiated effector CD8⁺ cells have been reported in ME/CFS, particularly severely affected ME/CFS (Cliff et al., 2019).

To summarise, baseline measurements of the electrical properties of PBMCs from the four donor cohorts show no statistically significant differences. This indicates any pathological changes in ME/CFS or MS do not significantly affect their dielectric properties or ζ -potential when unchallenged and are not potential diagnostic biomarkers. However, small differences in C_{eff} between cohorts may hint at differences in PBMC morphology, altered expression of glycoproteins or adhesion markers on the cell membrane in ME/CFS.

3.4.2. Changes in the Electrical Properties of PBMCs During Hyperosmotic Challenge

As previously stated, Esfandyarpour et al. (2019) reported the overall electrical impedance response of PBMCs hyperosmotically challenged over 1.5 hours in autologous plasma supplemented with 200 mM NaCl in ME/CFS patients was significantly different to healthy controls. These results provided insights into distinct electrical differences that occur in ME/CFS during hyperosmotic challenge, paving the way towards a potential diagnostic biomarker as well as validating ME/CFS as a pathological condition – reducing the stigma surrounding ME/CFS. However, the aggregate measures of impedance did not identify where the electrical differences lie, such as whether the values were specific to the donors' PBMCs, associated with something within the patient's own plasma, or other cellular interactions at the interface of their nanoneedle device. Additionally, Esfandyarpour et al. (2019) did not assess the specificity of their findings by testing other diseases which share similar characteristics.

To investigate the effects of hyperosmotic challenge on the electrical properties of PBMCs from individuals with a severe ME/CFS diagnosis, mild/moderate ME/CFS diagnosis, healthy controls, and MS diagnosis (disease control), PBMCs were incubated in control media (unchallenged), hyperosmotic NaCl medium, and hyperosmotic mannitol medium for up to 2 hours. Values of G_{eff} , C_{eff} ,

σ_{cyto} , and ζ -potential were measured every 30 minutes using the 3DEP machine and zetasizer to assess more detailed electrical changes. The nature of the 3DEP and zetasizer data collection methods restricted the time-resolution of the data to 30-minute intervals as opposed to the real-time measurements collected by the nanoneedle assay by Esfandypour et al. (2019). Hyperosmotic media was used instead of hyperosmotic donor plasma to minimise the influence of plasma-associated factors contributing to impedance changes, and because COVID-19 related measures at the time of testing restricted access to fresh whole-blood samples. Responses to both hyperosmotic NaCl and hyperosmotic mannitol media were evaluated to determine whether any changes are due to generic osmotic pressures, or due to increases in NaCl concentrations.

Changes in Electrophysiological Properties Over Time

Values of G_{eff} , C_{eff} , and σ_{cyto} of PBMCs were measured from individuals with a severe ME/CFS diagnosis ($n = 2$), mild/moderate ME/CFS diagnosis ($n = 2$), healthy controls ($n = 2$), and MS diagnosis ($n = 3$) every 30 minutes up to 1.5-hours during hyperosmotic NaCl media challenge, shown in **Appendix A.2. Figure 62**. In contrast with the significant increase in impedance unique to ME/CFS samples reported by Esfandypour et al. (2019), no clear trend could be seen in any dielectric parameter against time for donors in any cohort during incubation in hyperosmotic NaCl media in this small sample size.

Measurements of ζ -potential were taken every 30 minutes up to 2-hours during incubation in hyperosmotic NaCl media in severe ME/CFS ($n = 8$) **Figure 21(A)**, mild/moderate ($n = 9$) **Figure 21(C)**, healthy controls ($n = 7$) (**Figure 21(E)**), and MS donors ($n = 5$) **Figure 21(G)**. To determine the effect of the hyperosmotic stressor, ζ -potential was also measured every 30 minutes up to 2-hours during hyperosmotic mannitol incubation in severe ME/CFS ($n = 5$) (**Figure 21(B)**), mild/moderate ($n = 6$) (**Figure 21(D)**), healthy controls ($n = 5$) (**Figure 21(F)**), and MS donors ($n = 2$) (**Figure 21(H)**). No statistically significant differences in ζ -potential were detected using three-way ANOVAs followed by Tukey's multiple comparisons ($*p < 0.05$), to compare the effect of time, treatment, and whether diagnosed with ME/CFS or not.

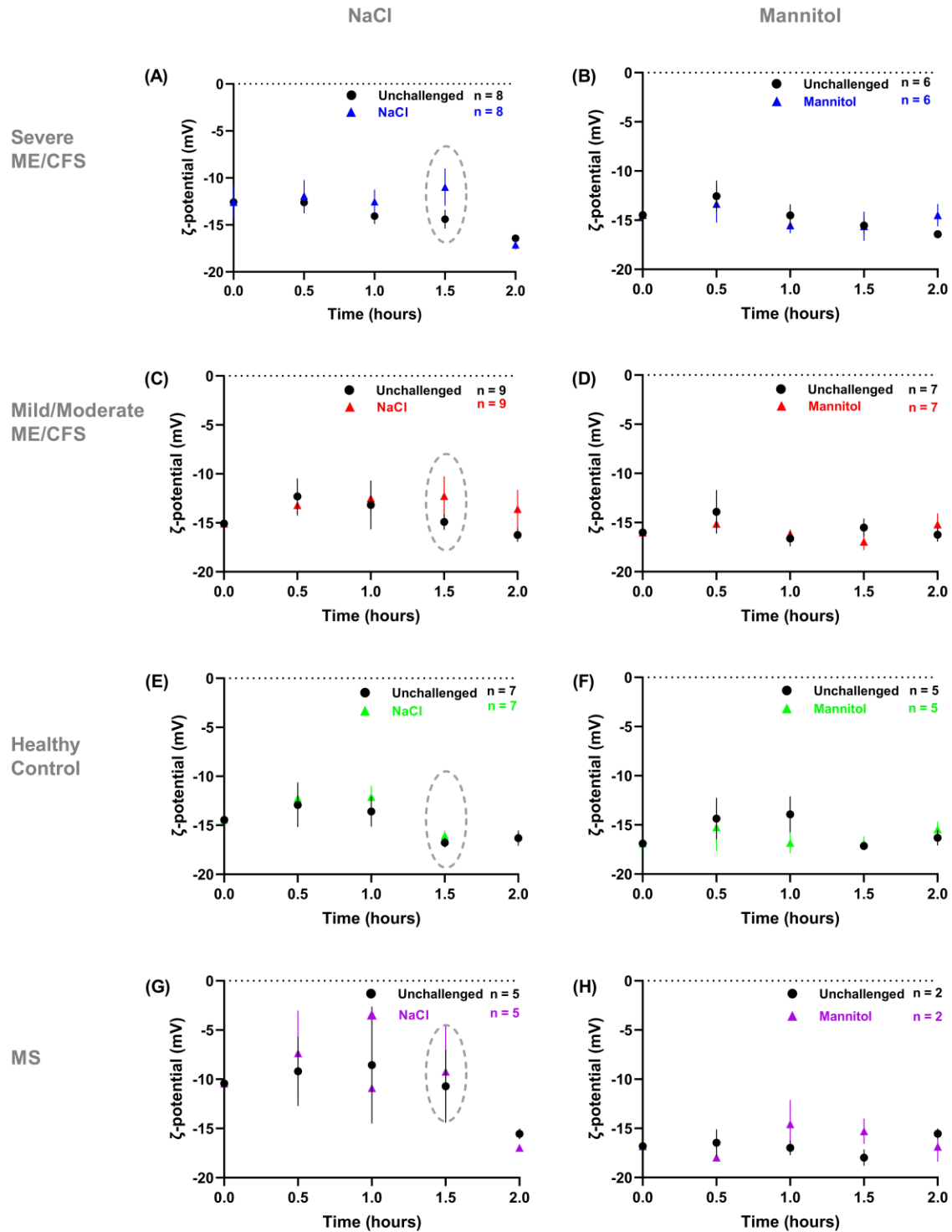


Figure 21 | ζ -potential (mV) of PBMCs versus time during incubation in hyperosmotic NaCl medium in **|A|** severe ME/CFS diagnosis donors (unchallenged black circles, NaCl blue triangles; n = 8) **|C|** mild/moderate ME/CFS diagnosis (unchallenged black circles, NaCl red triangles; n = 9) **|E|** healthy controls (unchallenged black circles, NaCl green triangles; n = 7) **|G|** MS diagnosis (unchallenged black circles, NaCl purple triangles; n = 5). ζ -potential (mV) of PBMCs versus time during incubation in hyperosmotic mannitol medium in **|B|** severe ME/CFS diagnosis donors (unchallenged black circles, mannitol blue triangles; n = 6) **|D|** mild/moderate ME/CFS diagnosis (unchallenged black circles, mannitol red triangles; n = 7) **|F|** healthy controls (unchallenged black circles, mannitol green triangles; n = 5) **|H|** MS diagnosis (unchallenged black circles, mannitol purple triangles; n = 2). Mean datapoints plotted $\pm$ SEM. No statistical significance found using three-way ANOVA followed by Tukey's multiple comparisons (*p<0.05).

The greatest differential response in ζ -potential to hyperosmotic challenge between donor cohorts occurred at time 1.5-hours. Mean values of ζ -potential in severe ME/CFS diagnosis donors (**Figure 21A**) and mild/moderate ME/CFS diagnosis donors (**Figure 21C**) increased following 1.5-hours NaCl hyperosmotic challenge (circled in grey dashed lines) compared with isosmotic incubation. The magnitude of ζ -potential decreased from -14.39 mV in unchallenged PBMCs to -10.97 mV in NaCl challenged PBMCs in severe ME/CFS donors. This change is statistically significant using a paired two-tailed t-test ($p = 0.0496$), although t-tests are not favoured for this mode of analysis given there are multiple groups. In contrast, the mean ζ -potential of unchallenged PBMCs in healthy controls was -16.77 mV compared with -16.07 mV in hyperosmotic NaCl challenged.

As the change in ζ -potential following 1.5-hour hyperosmotic challenge best differentiated donor cohorts, the data collection protocol was amended to prioritise measuring G_{eff} , C_{eff} , σ_{cyto} , and ζ -potential solely after 1.5-hour incubation. This was decided based on limited number of PBMCs available in frozen aliquots for each donor, to streamline data collection, and to maximise data collection from a greater sample size in the time given. 1.5-hour hyperosmotic challenge was also the time at which there was the greatest difference in impedance between ME/CFS and healthy controls in the study by Esfandyarpour et al. (2019).

Changes in Electrical Properties After 1.5-Hour Hyperosmotic Challenge

Values of G_{eff} , C_{eff} , σ_{cyto} , and ζ -potential of PBMCs from all cohorts following 1.5-hour incubation in control media (unchallenged), hyperosmotic NaCl media, and hyperosmotic mannitol media is plotted in **Figure 22**. Values of G_{eff} , C_{eff} , and σ_{cyto} were obtained from MATLAB-fitted models of DEP spectra which possessed an R^2 value of over 0.7. The Shapiro-Wilk test for normality deemed values of C_{eff} and σ_{cyto} were normally distributed, whereas G_{eff} and ζ -potential were non-normally distributed ($p > 0.05$). A mixed effects model with Geisser-Greenhouse correction was performed followed by Tukey's multiple comparisons to determine whether any of the electrical parameters significantly changed from unchallenged baseline values following hyperosmotic NaCl or hyperosmotic mannitol challenge, within or between cohorts.

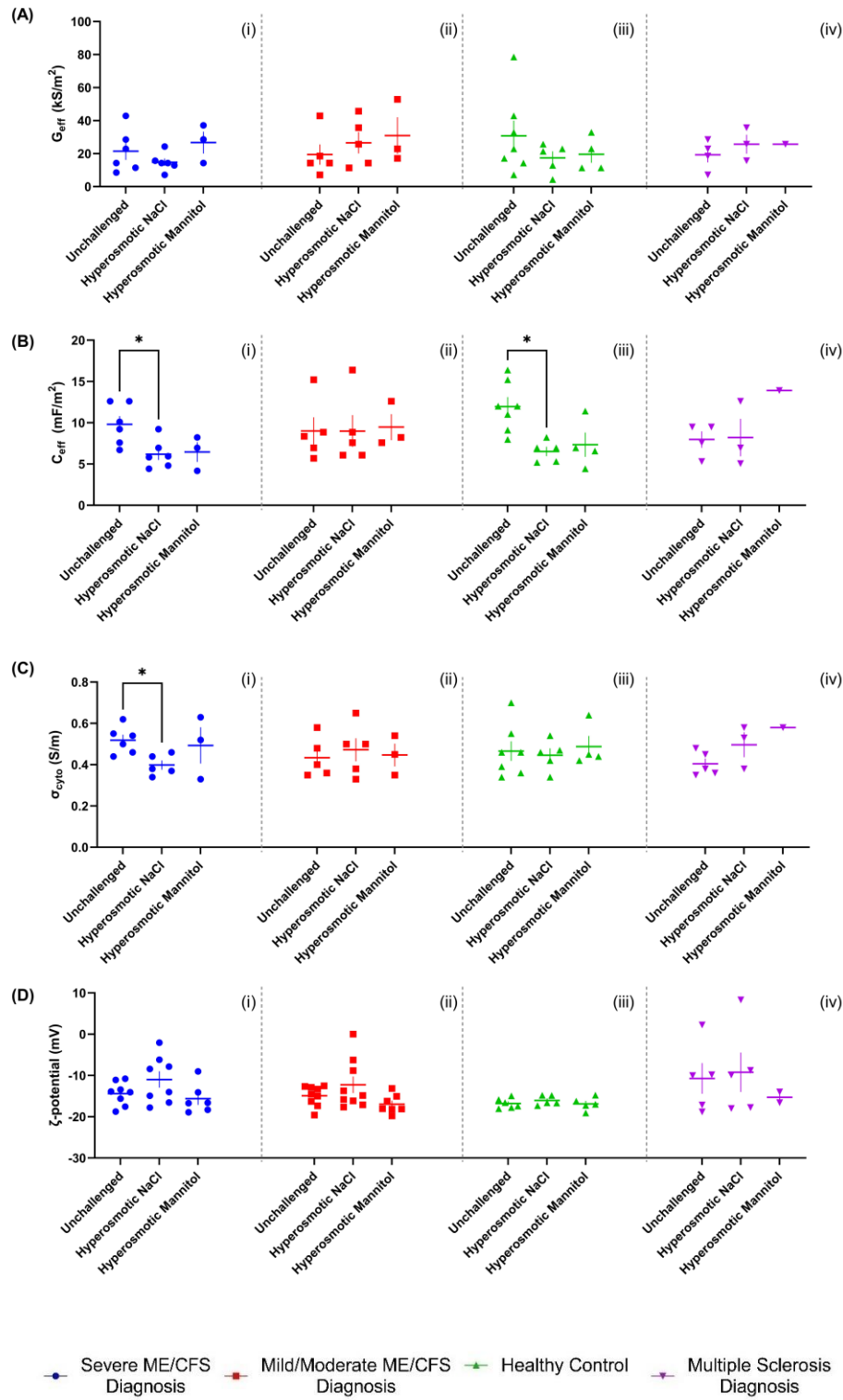


Figure 22 | Values of **|A|** G_{eff} **|B|** C_{eff} **|C|** σ_{cyto} obtained from MATLAB-fitted models of DEP spectra which possessed an R^2 value of over 0.7. (i) severe ME/CFS diagnosis (blue circles: unchallenged $n = 6$; NaCl $n = 6$; mannitol $n = 3$) (ii) mild/moderate ME/CFS diagnosis (red squares: unchallenged $n = 6$; NaCl $n = 6$; mannitol $n = 3$) (iii) healthy controls (green triangles: unchallenged $n = 7$; NaCl $n = 5$; mannitol $n = 4$) (iv) MS diagnosis donors (purple inverted triangles: unchallenged $n = 4$; NaCl $n = 3$; mannitol $n = 1$). **|D|** ζ -potential in (i) severe ME/CFS diagnosis (blue circles: unchallenged $n = 8$; NaCl $n = 8$; mannitol $n = 6$) (ii) mild/moderate ME/CFS diagnosis (red squares: unchallenged $n = 9$; NaCl $n = 9$; mannitol $n = 7$) (iii) healthy controls (green triangles: unchallenged $n = 6$; NaCl $n = 5$; mannitol $n = 5$) (iv) MS diagnosis donors (purple inverted triangles: unchallenged $n = 5$; NaCl $n = 5$; mannitol $n = 2$). Significant differences were determined using mixed effects model performed with Geisser-Greenhouse correction followed by Tukey's multiple comparisons (* $p < 0.05$). Mean lines plotted ($\pm$ SEM).

No significant differences in any electrical properties were found in unchallenged, hyperosmotic NaCl, or hyperosmotic mannitol incubated PBMCs between donor cohorts. No significant differences were found in either G_{eff} or ζ -potential between unchallenged and hyperosmotic NaCl challenged or hyperosmotic mannitol challenged PBMCs in any donor cohort. However, significant differences in C_{eff} were identified between unchallenged and hyperosmotic NaCl challenged PBMCs in severe ME/CFS donors (* p = 0.0266) (**Figure 22B(i)**) and healthy controls (* p = 0.0149) (**Figure 22B(iii)**). A significant difference in σ_{cyto} was identified between unchallenged and hyperosmotic NaCl challenged PBMCs in severe ME/CFS donors (* p = 0.0388) (**Figure 22C(i)**), which was not observed in any other cohort.

Changes in G_{eff} , C_{eff} , σ_{cyto} , and ζ -potential following hyperosmotic challenge were subsequently normalised by donor to ensure significant or null electrical changes in one donor were not skewing the mean value of all biological repeats, thus ensuring any electrical changes occur in each donor individually. Normalisation is particularly important when considering potential diagnostic applications. Normalisation of values also helps to eliminate systematic errors. To normalise changes in G_{eff} , C_{eff} , and σ_{cyto} for each donor individually, values measured at T1.5-hour control were subtracted from T1.5-hour hyperosmotic NaCl treatment, plotted in **Figure 23(i) A-C**. Values of T1.5-hour control subtracted from T1.5-hour hyperosmotic mannitol treatment are plotted in **Figure 23(ii) A-C**. Although this method of normalisation was chosen, other methods could have been used. Normalised values of G_{eff} , C_{eff} , σ_{cyto} , and ζ -potential were determined to be normally distributed using a Shapiro-Wilk test for normality ($p > 0.05$).

Noise in the 3DEP spectra data of PBMCs incubated in all three media (RPMI control media, hyperosmotic NaCl, and hyperosmotic mannitol) resulted in the R^2 value of multiple 3DEP spectra across different donors to be less than 0.7. The lower R^2 value could be due to noise caused by the relatively high conductivity DEP medium used (191 mS/m), although 3DEP spectra of platelets suspended in DEP media as high as 800 mS/m have been acquired with relatively noise-free spectra using the 3DEP (Hoettges et al., 2019). Of consequence to the noise of the 3DEP spectra, the threshold of $R^2 = 0.7$ for MATLAB-modelled DEP spectra resulted in automatic exclusion of data from multiple donors. For example, the threshold of $R^2 = 0.7$ removed data for normalised hyperosmotic mannitol incubation from five donors within the mild/moderate ME/CFS diagnosis and MS diagnosis donor cohorts, with only one donor in each cohort satisfying the criteria of possessing MATLAB-models of $R^2 = 0.7$ for both unchallenged and hyperosmotic mannitol. Consequently, no statistical tests including mild/moderate ME/CFS and MS donors could be performed for hyperosmotic mannitol challenge. Therefore, to determine whether the normalised parameters differed between donor cohorts and treatment, two different statistical tests were conducted. First, mixed effect models followed by

Šidák's multiple comparisons (* $p < 0.05$) were performed to determine how normalised parameters were affected by severe ME/CFS diagnosis or healthy control, and NaCl and mannitol treatment. Second, a one-way ANOVA was used to determine the effect of all donor cohorts on normalised parameters following NaCl incubation followed by Tukey's multiple comparisons (* $p < 0.05$). No statistically significant differences were identified in G_{eff} for either hyperosmotic challenge. However, statistical differences between healthy controls and both mild/moderate ME/CFS diagnosis (* $p = 0.0342$) and MS diagnosis (* $p = 0.0145$) donors were seen in normalised changes in C_{eff} during hyperosmotic NaCl treatment (**Figure 23B(i)**). The change in σ_{cyto} was significant between severe ME/CFS and MS donors (* $p = 0.0202$), and between severe ME/CFS and healthy controls (# $p = 0.0229$) (**Figure 23C(i)**).

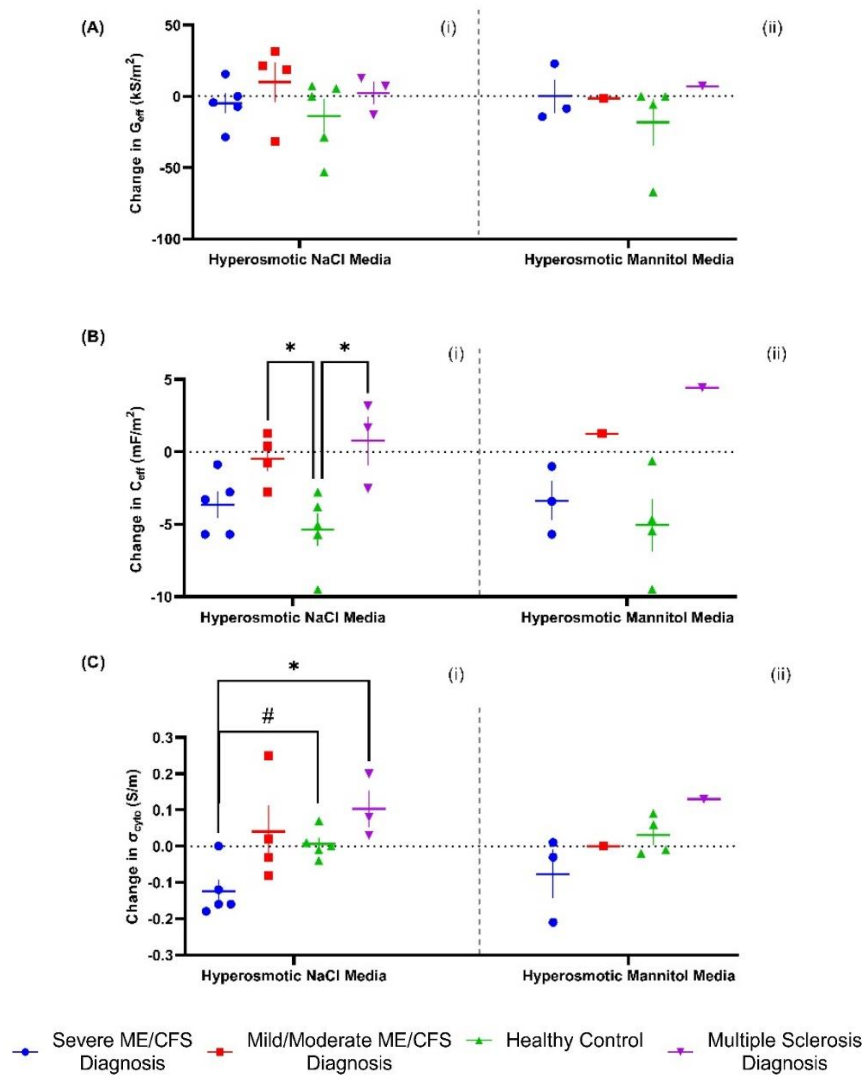


Figure 23 | Donor normalised values of **|A|** G_{eff} **|B|** C_{eff} **|C|** σ_{cyto} calculated by T1.5-hour hyperosmotic challenge – T1.5-hour control incubation using MATLAB-fitted models of DEP spectra which possessed an R^2 value of over 0.7. Normalised values challenge in **(i)** hyperosmotic NaCl media **(ii)** hyperosmotic mannitol media. Severe ME/CFS diagnosis (blue circles: NaCl $n = 5$; mannitol $n = 3$). Mild/moderate ME/CFS diagnosis (red squares: NaCl $n = 4$; mannitol $n = 1$). Healthy controls (green triangles: NaCl $n = 5$; mannitol $n = 4$). MS diagnosis donors (purple inverted triangles: NaCl $n = 3$; mannitol $n = 1$). Statistical significance calculated using a one-way ANOVA followed by Tukey's multiple comparisons (* $p < 0.05$), and mixed effects model followed by Šidák's multiple comparisons (# $p < 0.05$). Mean lines plotted ($\pm$ SEM).

Normalised values of G_{eff} , C_{eff} , and σ_{cyto} from MATLAB-fitted models of DEP spectra irrespective of R^2 value are plotted in **Figure 24**. Mixed effects models followed by Tukey's multiple comparisons were used to compare the normalised changes in electrical parameters between cohorts and between hyperosmotic treatments. No statistical significance was present in G_{eff} or σ_{cyto} between cohorts for either hyperosmotic treatment, or between treatments within the same cohorts. However, normalised values of C_{eff} were statistically different between mild/moderate and healthy controls following hyperosmotic NaCl treatment (** $p = 0.0042$) (**Figure 24B(i)**). There was also a significant difference in normalised C_{eff} in MS diagnosis donors between hyperosmotic NaCl challenge and hyperosmotic mannitol challenge (* $p = 0.0153$) (**Figure 24B**).

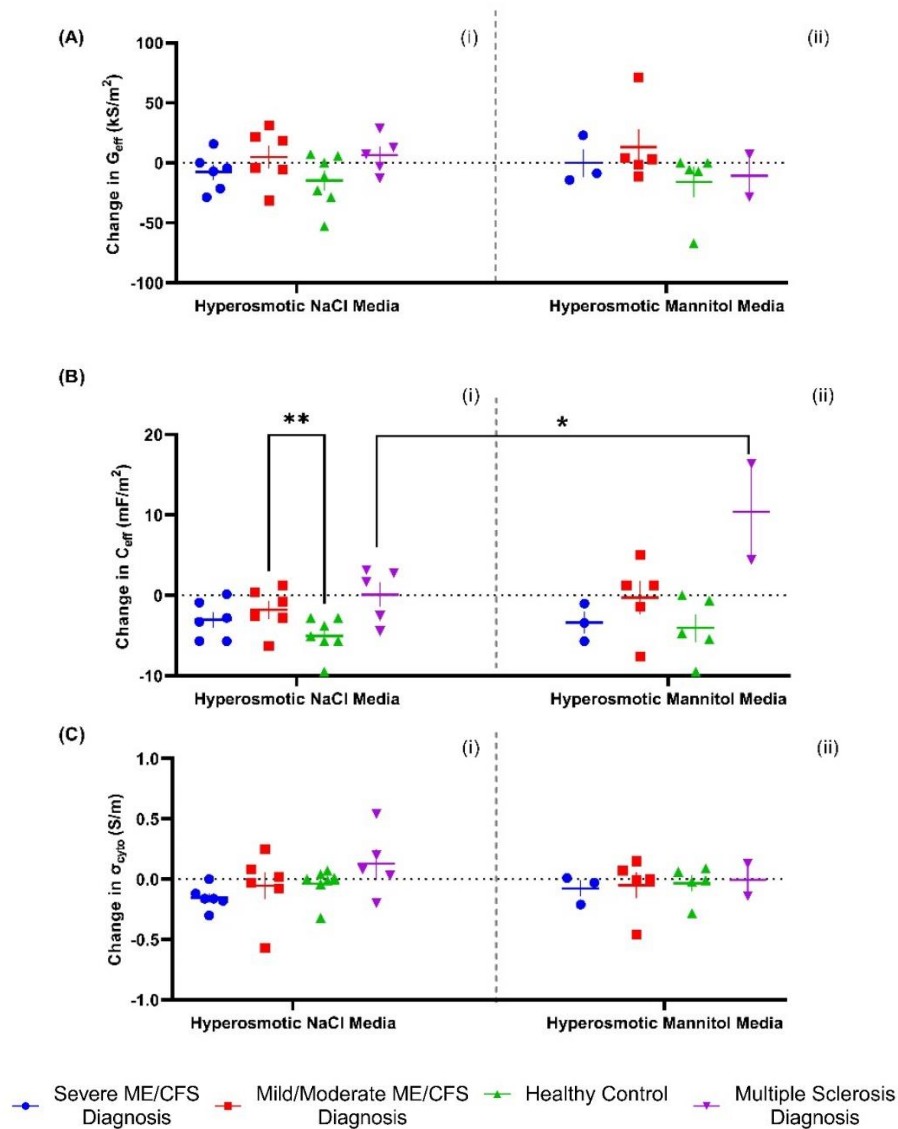


Figure 24 | Donor normalised values of **[A] G_{eff}** **[B] C_{eff}** **[C] σ_{cyto}** calculated by T1.5-hour hyperosmotic challenge – T1.5-hour control incubation using MATLAB-fitted models of DEP spectra irrespective of R^2 value. Normalised values challenge in (i) hyperosmotic NaCl media (ii) hyperosmotic mannitol media. Severe ME/CFS diagnosis (blue circles: NaCl $n = 6$; mannitol $n = 3$). Mild/moderate ME/CFS diagnosis (red squares: NaCl $n = 6$; mannitol $n = 5$). Healthy controls (green triangles: NaCl $n = 7$; mannitol $n = 5$). MS diagnosis donors (purple inverted triangles: NaCl $n = 5$; mannitol $n = 2$). Statistical significance calculated using two-way matched mixed effects model followed by Tukey's multiple comparisons (* $p < 0.05$). Mean lines plotted ($\pm$ SEM).

Paired comparisons between normalised changes in G_{eff} , C_{eff} , and σ_{cyto} following hyperosmotic NaCl versus mannitol treatment are plotted in **Appendix A.2 Figure 63**. Paired mixed-effects analysis matched models followed by Tukey's multiple comparisons were used to compare normalised values for hyperosmotic NaCl vs. hyperosmotic mannitol treatment for each donor cohort. No statistical differences between treatment were found in any dielectric parameter in severe ME/CFS diagnosis, mild/moderate ME/CFS diagnosis, and healthy controls – indicating PBMCs respond to both osmotic stimuli in the same way electrically. In contrast, in MS diagnosis donors there was a statistically significant difference between hyperosmotic treatments in normalised values of C_{eff} (* $p = 0.0153$) (**Appendix A.2 Figure 63B(iv)**). However, the sample size is small with a $n = 2$.

Normalised values of ζ -potential calculated by subtracting T1.5-hour control from T1.5-hour hyperosmotic NaCl treatment and T1.5-hour hyperosmotic mannitol challenge are plotted in **Figure 25**. The mean normalised value of ζ -potential increased in all donor cohorts following hyperosmotic NaCl incubation, although the $\pm$ SEM was large between different donors in the same cohort for all four cohorts. In contrast, the mean normalised value of ζ -potential was close to zero following hyperosmotic mannitol incubation. Two-way ANOVA paired mixed-effects analysis matched models followed by Tukey's multiple comparisons found no statistical significance (* $p < 0.05$) in the normalised changes in ζ -potential between donor cohorts, or between different hyperosmotic treatments.

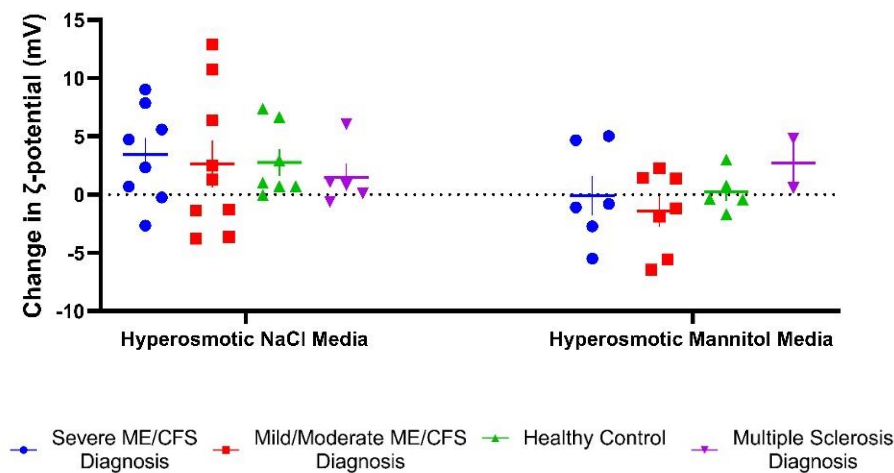


Figure 25 | Donor normalised values of ζ -potential following challenge in hyperosmotic NaCl media (left) and hyperosmotic mannitol media (right) for individuals with severe ME/CFS diagnosis (blue circles: NaCl $n = 8$; mannitol $n = 6$), mild/moderate ME/CFS diagnosis (red squares: NaCl $n = 9$; mannitol $n = 7$), healthy controls (green triangles: NaCl $n = 7$; mannitol $n = 5$), and multiple sclerosis diagnosis (purple inverted triangles: NaCl $n = 5$; mannitol $n = 2$). Normalised values calculated by T1.5-hour hyperosmotic challenge – T1.5-hour control incubation from the average of all technical repeats. Statistical significance calculated using two-way matched mixed effects model followed by Tukey's multiple comparisons (* $p < 0.05$, ** $p < 0.01$). Mean lines plotted ($\pm$ SEM).

Differences in Osmolarity

Osmolarity and osmolality refers to the number of solute molecules in a volume of solvent or solution weight respectively. The osmolality of plasma is tightly regulated between 285-295 mOsm/kg and is the same as the osmolality in most tissues. The separation of the two distinct intracellular and extracellular microenvironments via the impermeable cell plasma membrane acts to maintain cellular homeostasis. When the osmolarity of the extracellular fluid is equal to intracellular osmolarity the fluid is termed isotonic. However, when it is greater than that of the intracellular osmolarity it is termed hyperosmotic, and the imbalance in osmolarity imparts an osmotic pressure on the cell causing hyperosmotic stress. Hyperosmolarity promotes an osmotic efflux of water from the cell and an increase in intracellular ionic strength, resulting in cell shrinkage and intracellular dehydration. Other damaging effects include oxidative stress, mitochondrial depolarisation, DNA damage, protein carbonylation, and cell cycle arrest, increasing a cells' susceptibility to apoptosis (Brocker et al., 2012). Trypan-blue staining was used to ensure cell viability immediately post-thawing, at time 0 post-recovery incubation and at time 1.5-hours of control or hyperosmotic challenge to ensure electrical parameters measured by the 3DEP and zetasizer were not influenced by PBMC death. The viability of PBMCs as measured using trypan blue staining did not change during hyperosmotic challenge, with cell viability remaining above 95 % at time 1.5-hours for all donor cohorts.

The effect of hyperosmotic stress on the electrical properties of PBMCs was explored in this chapter using both NaCl and mannitol. The concentrations of five key ions Na^+ , K^+ , Cl^- , Ca^{2+} , and Mg^{2+} in the physiological media, hyperosmotic mannitol media, hyperosmotic NaCl media, and DEP media is written in **Table 6**, to compare with typical concentrations in plasma and PBMC cytoplasm. Values of serum Na^+ above 145 mM is classified as hyponatremia, with severe symptoms evident above 158-160 mM (Kim, 2006). 200 mM NaCl was chosen to keep to the same concentration as Esfandarypour et al. (2019). 200 mM NaCl was the first concentration Esfandarypour et al. (2019) trialled during their study and was not altered based on the success of their first experiments.

Table 6 | Concentrations of Na⁺, K⁺, Cl⁻, Ca²⁺, and Mg²⁺ ions in the physiological incubation media, hyperosmotic mannitol media, hyperosmotic NaCl media and DEP medium, in comparison with the concentration of these ions typically in plasma and PBMCs cited from literature (Pilas and Durack, 1997, Goldstein, 1990, Baylor and Hollingworth, 2000, Murphy, 2000, Gillham and Sidebotham, 2007, Bisello and Friedman, 2008, Pfortmueller et al., 2018, Esfandyarpour et al., 2019).

Ion	Physiological Media and Hyperosmotic mannitol media (mM)	Hyperosmotic NaCl media (mM)	DEP media (mM)	Plasma (mM)	Cytoplasm (mM)
Na ⁺	132.9	200	17.8	135 (Esfandyarpour et al., 2019)	10 (Esfandyarpour et al., 2019)
K ⁺	5.3	5.3	0.5	5 (Esfandyarpour et al., 2019)	140 (Esfandyarpour et al., 2019)
Cl ⁻	108.8	175.9	17.8	97-107 (Pfortmueller et al., 2018)	~ 58 (Pilas and Durack, 1997)
Ca ²⁺	0.4	0.4	0.09	2.2-2.7 (Goldstein, 1990)	~0.0001 (Baylor and Hollingworth, 2000, Bisello and Friedman, 2008)
Mg ²⁺	0.4	0.4	0.2	0.75 – 1.0 (Gillham and Sidebotham, 2007)	0.5 – 1 (Murphy, 2000)

Changes in G_{eff} , C_{eff} , σ_{cyto} , and ζ -potential of PBMCs incubated in hyperosmotic NaCl and hyperosmotic mannitol media in donors with a severe ME/CFS diagnosis, mild/moderate ME/CFS diagnosis, healthy controls and MS diagnosis were investigated. The NaCl and mannitol hyperosmotic stressor both elicited the same change in electrical properties, which could suggest that both osmotic stressors trigger the same pathways. Alternatively, the PBMCs may be acting by completely different mechanisms which lead to the same electrical changes.

During hyperosmotic stress, osmotic efflux of water results in cell shrinkage in most mammalian cells. It was thus expected that a decrease in cell radius would be observed. However, as shown in **Figure 26**, no change in cell radius was observed following 1.5-hour hyperosmotic incubation with either NaCl or mannitol stressor in healthy controls, severe ME/CFS diagnosis, mild/moderate ME/CFS diagnosis, or MS diagnosis individuals using a matched mixed effects model followed by Tukey's multiple comparisons (* $p < 0.05$). Additionally, Esfandyarpour et al. (2019) found no detectable size change using standard live microscopy imaging. Paired values of normalised changes in cell radius between 1.5-hour NaCl vs. 1.5-hour mannitol treatments for donors in each cohort are shown in **Appendix A.2 Figure 64**.

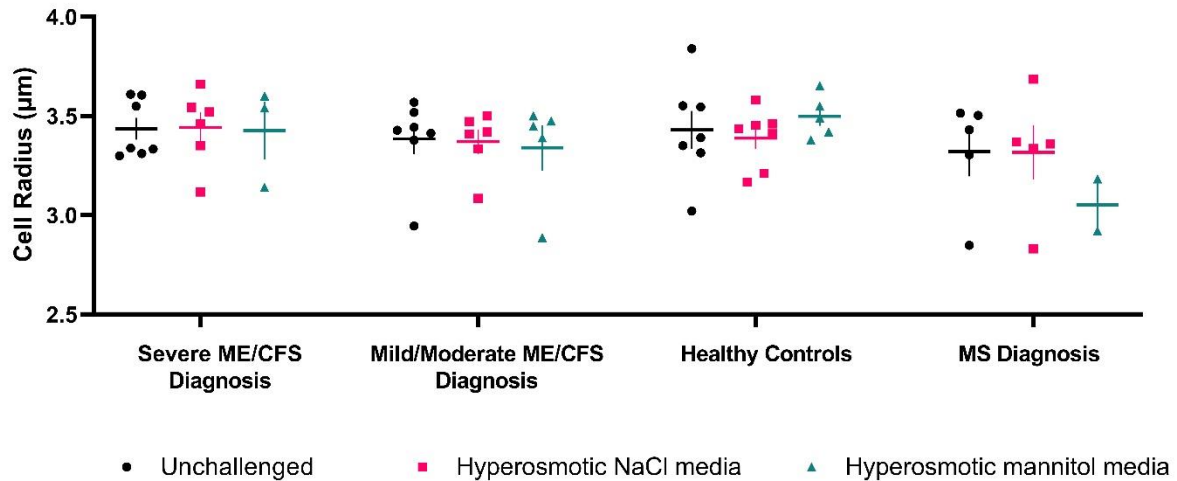


Figure 26 | The radius of PBMCs after 1.5-hour incubation in physiological media (black dots: severe ME/CFS $n = 7$; mild/moderate ME/CFS $n = 7$; healthy controls $n = 7$; MS diagnosis $n = 5$), 1.5-hour incubation in hyperosmotic NaCl media (pink squares: severe ME/CFS $n = 6$; mild/moderate ME/CFS $n = 6$; healthy controls $n = 7$; MS diagnosis $n = 5$), and hyperosmotic mannitol media (green triangles: severe ME/CFS $n = 3$; mild/moderate ME/CFS $n = 5$; healthy controls $n = 5$; MS diagnosis $n = 2$). Statistical significance calculated using two-way matched mixed effects model followed by Tukey's multiple comparisons (* $p < 0.05$, ** $p < 0.01$). Mean lines plotted ($\pm$ SEM).

It was hypothesised that C_{eff} would decrease as the cells shrink due to the osmotic efflux of water under hyperosmotic stress. Values of C_{eff} decreased in healthy controls and severe ME/CFS diagnosis donors. Decreased values of C_{eff} could have been due to changes in cell morphology better visualised using high resolution transmission electron microscopy than the inverted microscope used in this experiment. However, normalised changes in C_{eff} were close to zero in mild/moderate ME/CFS diagnosis and MS diagnosis donors. This could be due to glycoprotein modifications on the surface of the mild/moderate ME/CFS plasma membrane buffering the accumulation of ions at the cell surface.

σ_{cyto} is a measure of the number of free ions within the cell cytoplasm up to the cell membrane. In healthy controls, no change in σ_{cyto} was observed following incubation in hyperosmotic NaCl media or hyperosmotic mannitol media. To speculate, the lack of change in σ_{cyto} could be due to normal regulatory volume increases in healthy cells. Decreases in cell volume during hyperosmotic stress results in increased σ_{cyto} as ion transport systems such Na^+/H^+ exchanger increase intracellular ion concentrations and result in water influx. Synthesis and uptake of neutral amino acids or their derivatives, such as sorbitol, myo-inositol, and glycerol substitute the inorganic ions to counteract the ionic increase, protect against apoptosis, and ensure cellular biochemical function is protected (Docampo et al., 2013). In mild/moderate ME/CFS and to a greater extent severe ME/CFS, this mechanism may over-compensate leading to a decrease in σ_{cyto} . Trypan blue was used to measure the

viability of cells. However, it is possible in the case of MS cells that the hyperosmotic environment has triggered apoptosis, which has been shown to lead to an increase in σ_{cyto} (Labeed et al., 2006).

As discussed in **Section 3.2.3.**, ME/CFS pathophysiology is currently hypothesized by some researchers to be contributed by cellular metabolic, mitochondrial dysfunction and a deficiency in ATP synthesis (Maksoud et al., 2023). A metabolomic analysis reported decreased levels of the purines uric acid and plasma adenosine in the blood of individuals diagnosed with ME/CFS, indicating a decrease in the synthesis of ADP and ATP (Naviaux et al., 2016). The addition of a NaCl as a hyperosmotic stressor was thus specifically chosen by Esfandyarpour et al. (2019) to challenge PBMCs in ME/CFS because large quantities of ATP would be required to actively transport Na^+ out of the cell via the Na/K ATPase pump to maintain intracellular Na^+ concentrations at ~ 10 mM (shown in **Table 6**). As mitochondrial dysfunction in individuals with ME/CFS is postulated to result in cellular ATP deficiency, it would be hypothesised that the PBMCs are unable to export Na^+ that may have diffused into the cells via leak channels, depending on the permeability of PBMCs to this ion. However, this contradicts the decrease in σ_{cyto} that was observed, suggesting ME/CFS may not be due to a specific Na^+ and Cl^- ion channelopathy.

3.4.3. Diagnostic Applications

No reliable, quantitative, repeatable, and validated biomarker for ME/CFS diagnosis currently exists. A diagnostic tool is urgently required to accelerate a correct ME/CFS diagnosis and prevent misdiagnosis, ensuring the correct treatment plan is followed to optimise patient outcome as well as stopping the stigma that ME/CFS is a psychiatric condition and not real. As previously stated, a good biomarker would effectively and objectively differentiate those with ME/CFS from the rest of the population using a low-cost, highly sensitive, and specific parameter measured using a technique which is easily accessible and adoptable (Califf, 2018, Maksoud et al., 2023). The dielectric properties of PBMCs during hyperosmotic stress reported in **Section 3.4.2.** are promising candidates of a ME/CFS diagnostic biomarker; similar to the observations detected by Esfandyarpour et al. (2019).

Optimising 3DEP Analysis for Diagnosis – ABCD Method

The 3DEP machine is a commercially available DEP cytometry platform that is relatively low cost, and can quickly measure the dielectric properties of thousands of cells in 30 seconds. The differential responses in the dielectric properties of PBMCs to hyperosmotic stress in individuals with

a severe ME/CFS diagnosis and mild/moderate ME/CFS diagnosis, compared with healthy controls and MS diagnosis measured in **Section 3.4.2.** shows promise towards a diagnostic biomarker. Of the dielectric properties measured, the best candidate for a diagnostic biomarker is the relative change in σ_{cyto} following 1.5-hour hyperosmotic challenge, which showed a statistical difference between severe ME/CFS diagnosis and both healthy controls using a mixed effects model followed by mixed effect models followed by Šidák's multiple comparisons (* $p < 0.05$), and between severe ME/CFS diagnosis and MS diagnosis using an ordinary one-way ANOVA (* $p < 0.05$). However, limitations of using σ_{cyto} as a diagnostic marker (as well as other dielectric properties derived from modelling 3DEP spectra) is the increase in time and expertise required to analyse the 3DEP data. A diagnostic method of analysis which takes the information gathered from a 3DEP spectra without MATLAB modelling is preferential as it reduces the expertise and complexity required, as well as eliminating the time of data processing and the necessity to measure cell radius. Obtaining the average PBMC radius manually (as described in **Section 3.3.7.**) is time-consuming and potentially subjective/operator bias when deciding what cells are chosen to measure. Alternatively, automated codes which measure cell radius increase the risk of mistaking debris for cells, ghosting, or the purchase of machines dedicated to measuring cell radius increases experimental costs and logistics of equipment required.

A validated technique of analysing raw 3DEP spectra datapoints is the Mean Difference Value (MDV) approach, which has been used to analyse urine sample data for bladder cancer diagnosis (Hoque et al., 2020). By simply averaging the relative DEP force of the highest nine frequencies and subtracting from the average of the lowest nine frequencies, MDV compares the overall difference in spectra between disease and control whilst removing noise, ensuring no subjectivity and does not require DEP modelling or cell radius measurements. Adopting the MDV method of analysis to the ME/CFS data showed no statistical difference between any donor cohorts, but a similar concept exists called the ABCD method (described in **Section 3.3.7.**). As shown in **Figure 27**, the ABCD method is an easy way to quickly gauge differences in the dielectric properties of disease vs. control, as G_{eff} , C_{eff} , and σ_{cyto} relate to different regions of the DEP spectrum. As each of A, B, C, and D is the average of 5 datapoints, it reduces the noise of individual outliers. To minimise the time taken to analyse data (crucial for clinical applications), ensure there was consistency, no subjectivity, or complexity to data processing and minimal operator input, no datapoints or technical repeats were excluded from ABCD data analysis. This method thus includes all donors and does not take into account whether the spectra is noisy or whether MATLAB modelling would give an $R^2 > 0.7$, thus including data from donors who were excluded during specific MATLAB modelling in **Figure 23**.

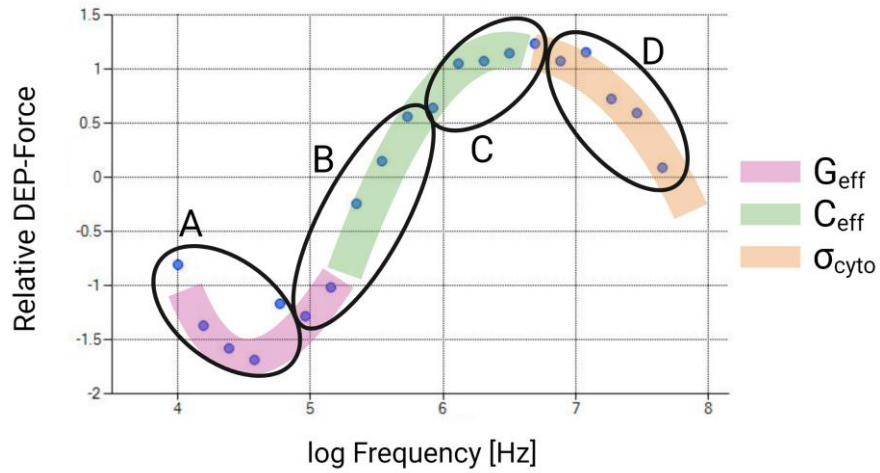


Figure 27 | A diagram to explain how the ABCD method relates to the dielectric parameters obtained by modelling DEP spectra obtained by the 3DEP. Figure adapted from (Kruczek, 2022).

Normalised ABCD Values for 1.5-hour Hyperosmotic NaCl Media Incubation

Subtracting the A, B, C, or D value of 1.5-hour physiological incubation from the respective A, B, C, or D value of 1.5-hour hyperosmotic NaCl media incubation to normalise the data is shown in **Figure 28**.

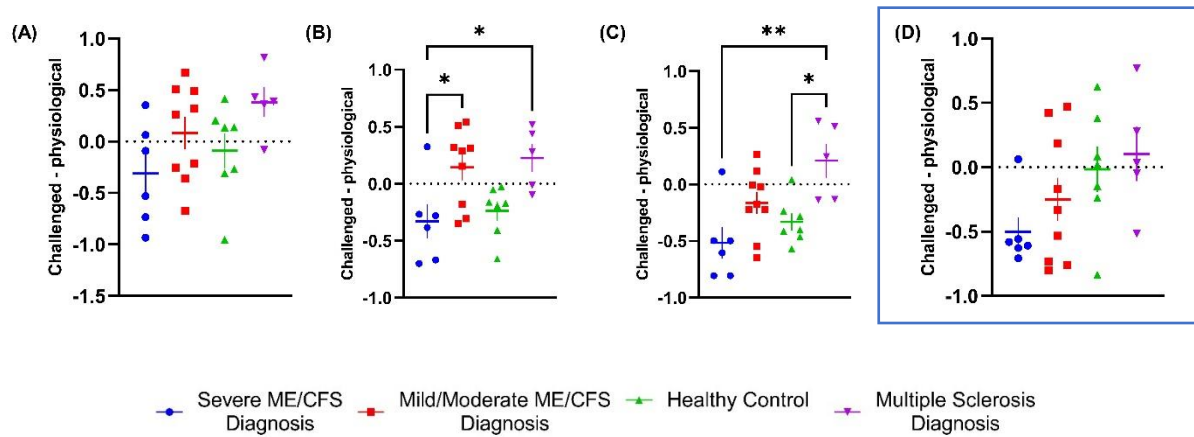


Figure 28 | ABCD data analysis technique for 1.5-hour NaCl incubation – 1.5-hour physiological incubation in PBMC samples from severe ME/CFS (blue circles: $n = 6$), mild/moderate ME/CFS (red squares: $n = 9$), healthy controls (green triangles: $n = 7$), and MS (purple inverted triangles: $n = 5$) donors. **[A]** Normalised A Values **[B]** Normalised B Values **[C]** Normalised C Values **[D]** Normalised D Values. Each datapoint is a different donor. Lines plots mean of biological repeats $\pm$ SEM. Statistical significance denoted by * $p < 0.05$; ** $p < 0.01$ using ordinary one-way ANOVA.

Ordinary one-way ANOVA of A data analysis demonstrated no statistical difference between donor cohorts, and the data had a large $\pm$ SEM. No significant difference is reflective of the non-statistically significant change in G_{eff} in **Figure 23A(i)** and could be attributable to noise and abnormal

DEP behaviour commonly found at low frequencies as seen in other studies (Hughes et al., 2021). However, as reflected by the significant differences in C_{eff} (in **Figure 23B(i)**), B data analysis (**Figure 28B**) showed statistical significance ($*p < 0.05$) using post-hoc Tukey's multiple comparisons between severe ME/CFS diagnosis and mild/moderate ME/CFS diagnosis ($*p = 0.0377$), and between severe ME/CFS diagnosis and MS diagnosis donors ($*p = 0.0330$). Statistical significance using ordinary one-way ANOVA is also achieved between MS donors and both severe ME/CFS ($**p = 0.0023$) and healthy controls ($*p = 0.0208$) using post-hoc Tukey's multiple comparisons (**Figure 28C**). No statistically significant differences were found between donor cohorts in D data analysis (**Figure 28D**) using an ordinary one-way ANOVA, though the same trend is seen as in normalised values of σ_{cyto} (**Figure 23C(i)**). Normalised D values of severe ME/CFS are less than that of mild/moderate, which are less than healthy controls, which are less than MS donors. There is a statistically significant difference between severe ME/CFS and healthy controls using an unpaired two-tailed t-test ($*p = 0.0486$), and between severe ME/CFS and MS donors ($*p = 0.0263$), although multiple t-tests are not favoured for this mode of analysis (where multiple groups are compared) because it increases the risk of false positives (Laerd Statistics, 2018). The normalised D values show the most promise as a diagnostic test because both the severe and mild/moderate ME/CFS donors follow the same trend, which is different to that of healthy controls and MS donors.

In fact, when grouping together both severe ME/CFS and mild/moderate ME/CFS cohorts as ME/CFS Diagnosis, and both healthy controls and the disease control MS as No ME/CFS Diagnosis, there was a statistical difference in normalised D values using an unpaired two-tailed t-test ($*p = 0.0466$), plotted in **Figure 29A**. To evaluate the diagnostic accuracy of the D value, a ROC curve was used. A ROC curve plotting the true positive rate (sensitivity) against the false positive rate (100-specificity) is often used to evaluate the performance of a binary classification diagnostic biomarker depending on the discrimination threshold set, providing unbiased estimates on sensitivity and specificity (Fletcher et al., 2010, Hsu et al., 2014). A non-parametric ROC curve differentiating ME/CFS diagnosis from No ME/CFS diagnosis is shown in **Figure 29B**. The Area Under the Curve (AUC) was 0.7111 with a p value of 0.0637. A p value of less than 0.05 disproves the null hypothesis that the test does distinguish between the two categories and indicates that the test discriminates ME/CFS diagnosis donors from No ME/CFS diagnosis donors better than 'flipping a coin'. For a 'best' threshold of -0.1581 (chosen as it is closest to 0.0, 100.0 on the ROC curve), the sensitivity is 73.33% and specificity is 75.00%. This shows promise for normalised D values as a diagnostic marker of ME/CFS.

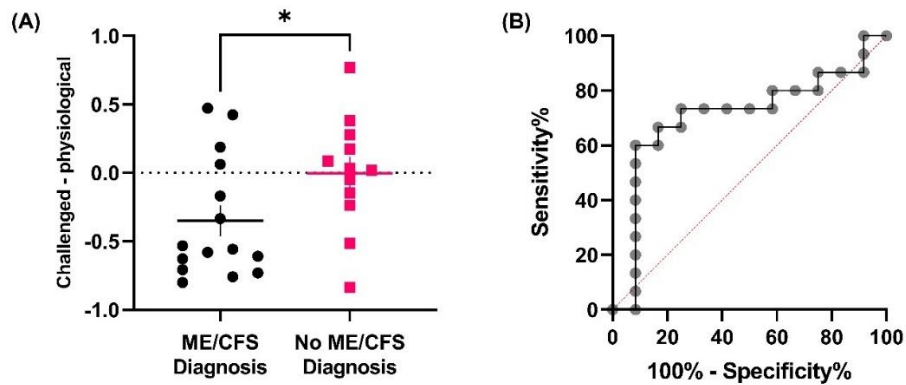


Figure 29 | **A** | Normalised D values of 1.5-hour NaCl incubation – 1.5-hour physiological incubation for ME/CFS donors (black circles: n = 15) and no ME/CFS donors (pink squares: n = 12). Each datapoint is a different donor. Lines plots mean of biological repeats $\pm$ SEM. Statistical significance denoted by * $p < 0.05$ using unpaired two-tailed t-test. **B** | The non-parametric ROC curve (grey dots connected by black line) plots sensitivity% (true positive rate) against 100-specificity% (proportion of true negatives). The 45° line (red) denotes the theoretical plot of no discrimination between ME/CFS and no ME/CFS diagnosis donors.

It is important to remember that without a current diagnostic test to confidently diagnose ME/CFS, it is possible that some donors recruited to this study have been misdiagnosed and do not have ME/CFS despite the donation criteria used by the UK ME/CFS Biobank. Due to the reduced severity, prevalence, and frequency of symptoms, misdiagnosis is more likely to have occurred in mild/moderate ME/CFS donors. Out of interest, excluding datapoints from mild/moderate ME/CFS donors from the graph and ROC curve in **Figure 29** is shown in **Figure 30**. Again, there was statistical significance using an unpaired two-tailed t-test between the normalised D values of severe ME/CFS diagnosis and No ME/CFS donors (* $p = 0.0179$) (**Figure 30A**). The ROC analysis calculated an AUC of 0.8333 with a p value of 0.0246, with a threshold of -0.5363 resulting in a sensitivity of 83.33% and specificity of 91.67% (**Figure 30B**).

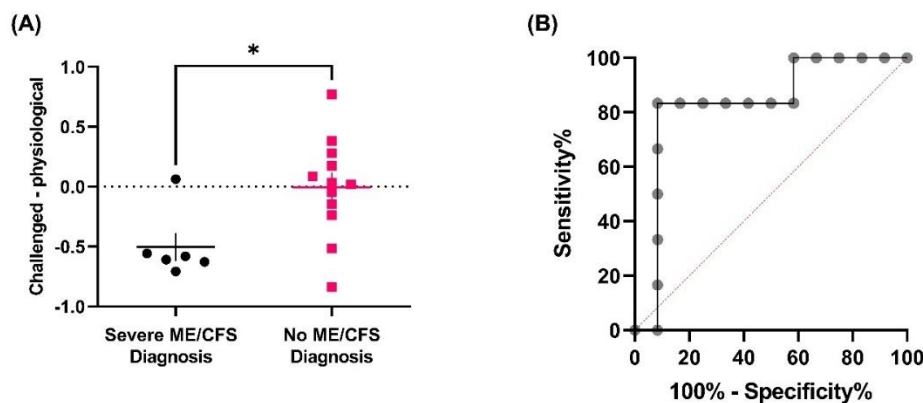


Figure 30 | **A** | D values of 1.5-hour NaCl incubation – 1.5-hour physiological incubation for severe ME/CFS diagnosis donors (black circles: n = 6) and no ME/CFS diagnosis donors (pink squares: n = 12). Each datapoint is a different donor. Lines plots mean of biological repeats $\pm$ SEM. Statistical significance denoted by * $p < 0.05$ using unpaired two-tailed t-test. **B** | The non-parametric ROC curve (grey dots connected by black line) plotting sensitivity% (true positive rate) against 100-specificity% (proportion of true negatives). The 45° line (red) denotes the theoretical plot of no discrimination between ME/CFS and no ME/CFS.

The sensitivity and specificity of these results are very promising for this pilot study but require a greater n number to improve reliability, validity, and understand the repeatability of this biomarker. The ideal diagnostic biomarker would differentiate both severe ME/CFS and mild/moderate ME/CFS from the No ME/CFS cohort, thus the following sample size calculations assume mild/moderate ME/CFS donors were correctly diagnosed. The required sample size to differentiate all ME/CFS from No ME/CFS using normalised D values with a power of 0.95, was thus calculated using G*Power (version 3.1.9.7.) software using the data from **Figure 29A**. A two-tailed t-test using the difference between the two independent means with A priori power analysis, effect size $d = 0.8135$, α err prob = 0.05 and power ($1 - \beta$ error probability) = 0.95 calculated the sample size required to be a total sample size of 82 donors consisting of 41 donors within the ME/CFS diagnosis cohort and 41 donors in the No ME/CFS diagnosis cohort.

Normalised ABCD Values for 1.5-hour Hyperosmotic Mannitol Media Incubation

Subtracting the A, B, C, or D value of 1.5-hour physiological media incubation from the respective A, B, C, or D value of 1.5-hour hyperosmotic mannitol incubation to normalise the data is shown in **Figure 31**.

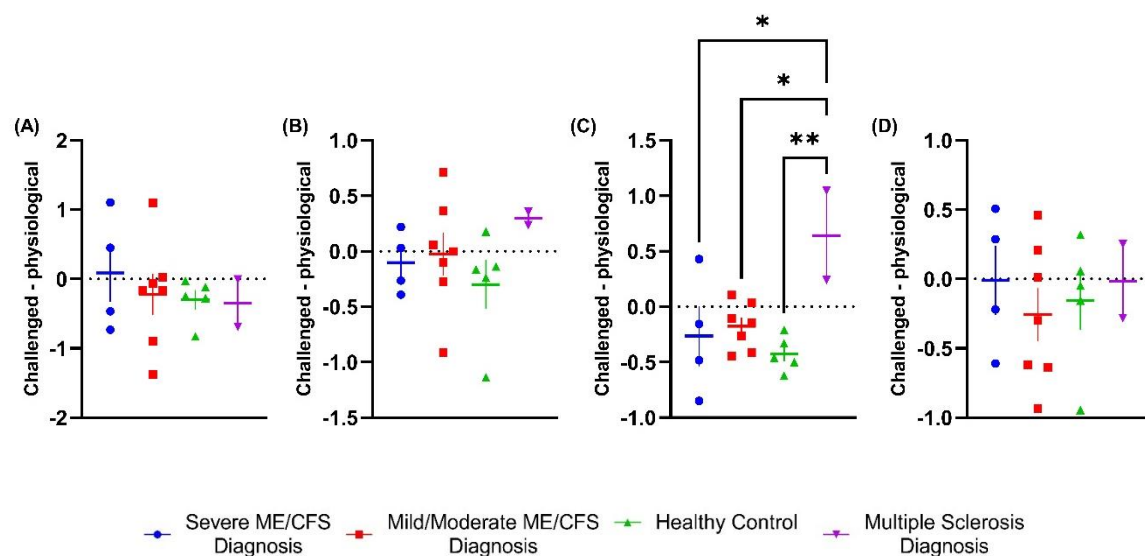


Figure 31 | ABCD data analysis technique for 1.5-hour mannitol incubation – 1.5-hour physiological incubation in PBMC samples from severe ME/CFS (blue circles: $n = 4$), mild/moderate ME/CFS (red squares: $n = 7$), healthy controls (green triangles: $n = 5$), and MS (purple inverted triangles: $n = 2$) donors. **[A]** Normalised A Values **[B]** Normalised B Values **[C]** Normalised C Values **[D]** Normalised D Values. Each datapoint is a different donor. Lines plots mean of biological repeats $\pm$ SEM. Statistical significance denoted by * $p < 0.05$; ** $p < 0.01$ using ordinary one-way ANOVA.

No statistical difference was seen in normalised A values (**Figure 31A**) between any of the cohorts, similar to the difference in normalised values of G_{eff} in **Figure 23A(ii)** where no difference could be seen. Interestingly, no trend could also be seen in normalised B values or normalised D values

in **Figure 31B** and **Figure 31D** respectively. Statistical significance was achieved in normalised C values (**Figure 31C**) between MS donors with severe ME/CFS (*p = 0.0333), mild/moderate ME/CFS (*p = 0.0383), and healthy controls (**p = 0.0092) using ordinary one-way ANOVA post-hoc Tukey's multiple comparisons. This suggests that calculating normalised C values following 1.5-hour hyperosmotic mannitol challenge could hold promise as a potential biomarker for MS, though data from more MS donors are required to increase the reliability of this finding.

It is difficult to compare ABCD results to normalised values of C_{eff} and σ_{cyto} in **Figure 23(ii-iii)** due to the fact that the number of MATLAB models with $R^2 < 0.7$ excluded derived values of C_{eff} and σ_{cyto} for many donors from **Figure 23**, which are now included in **Figure 31**.

Normalised Values of ζ -Potential for Diagnosis

The Lab Blue Label zetasizer (Malvern Panalytical) is a commercially available device which measures ζ -potential quickly (three technical repeats can be measured in approximately 5 minutes) and requires a low cell count with 120 000 PBMCs loaded into the cuvette for each biological repeat. The relatively low cost of the zetasizer and the speed of data collection from isolated and treated PBMCs lends itself to be a good candidate for a diagnostic device.

Normalised ζ -Potentials for 1.5-hour Hyperosmotic NaCl

Normalised changes in ζ -potential using hyperosmotic NaCl 1.5hr – physiological 1.5 hr (**Figure 25**) did not show any statistical differences between donor cohorts. However, the percentage change in ζ -potential (%) between 1.5-hour hyperosmotic NaCl incubation and 1.5-hour physiological incubation in severe ME/CFS diagnosis, mild/moderate ME/CFS diagnosis, healthy controls, and MS diagnosis donors is shown in **Figure 32A**. There is clearly no trend in the mean percentage change of biological repeats between different cohorts. However, there is an obvious difference in the $\pm$ SEM between different cohorts. Both severe ME/CFS diagnosis and mild/moderate ME/CFS diagnosis have much larger $\pm$ SEM in comparison with both healthy controls and MS donors which are clustered closer to zero. As such, plotting the magnitude change in ζ -potential (%) (**Figure 32B**) shows the mean magnitude change in ζ -potential (%) to be markedly greater in both severe ME/CFS and mild/moderate ME/CFS compared with both healthy controls and MS diagnosis donors (not significant using an ordinary one-way ANOVA). As was performed with the D value 3DEP data, values of the magnitude change in ζ -potential (%) of severe ME/CFS and mild/moderate ME/CFS donors are grouped together into ME/CFS Diagnosis, and healthy controls and MS donors into No ME/CFS Diagnosis, and is plotted

in **Figure 32C**. A significant difference was observed between ME/CFS diagnosis and No ME/CFS diagnosis using an unpaired two-tailed t-test (*p = 0.0229). Non-parametric ROC analysis differentiating ME/CFS diagnosis and No ME/CFS diagnosis (**Figure 32D**) calculated an AUC of 0.8294 with a p value of 0.0049 and a threshold of 12.73 differentiating both cohorts with a sensitivity of 80.00% and specificity of 70.59%. The required sample size to differentiate all ME/CFS diagnosis from No ME/CFS diagnosis using the magnitude of the percentage change in ζ -potential following 1.5-hour hyperosmotic NaCl incubation was calculated again using G*Power (version 3.1.9.7.) software using the data from **Figure 32**. A two-tailed t-test using the difference between the two independent means with A priori power analysis, effect size d = 1.0486532, α err prob = 0.05, and power (1 – β err prob) = 0.95 calculates the sample size required to be a total sample size of 50 donors consisting of 25 donors within the ME/CFS Diagnosis cohort and 25 donors in the No ME/CFS Diagnosis cohort.

A significant difference was observed between severe ME/CFS diagnosis and No ME/CFS diagnosis using an unpaired two-tailed t-test (*p = 0.0393) (**Figure 32E**). Non-parametric ROC analysis differentiating Severe ME/CFS diagnosis and No ME/CFS diagnosis (**Figure 32F**) calculated an AUC of 0.800 with a p value of 0.033 and a threshold of 17.80, differentiating both cohorts with a sensitivity of 90.0% and specificity of 75.0%. ζ -potential shows promise as a diagnostic technique with better combined sensitivity and specificity to normalised D value ME/CFS diagnosis vs. No ME/CFS diagnosis 3DEP analysis.

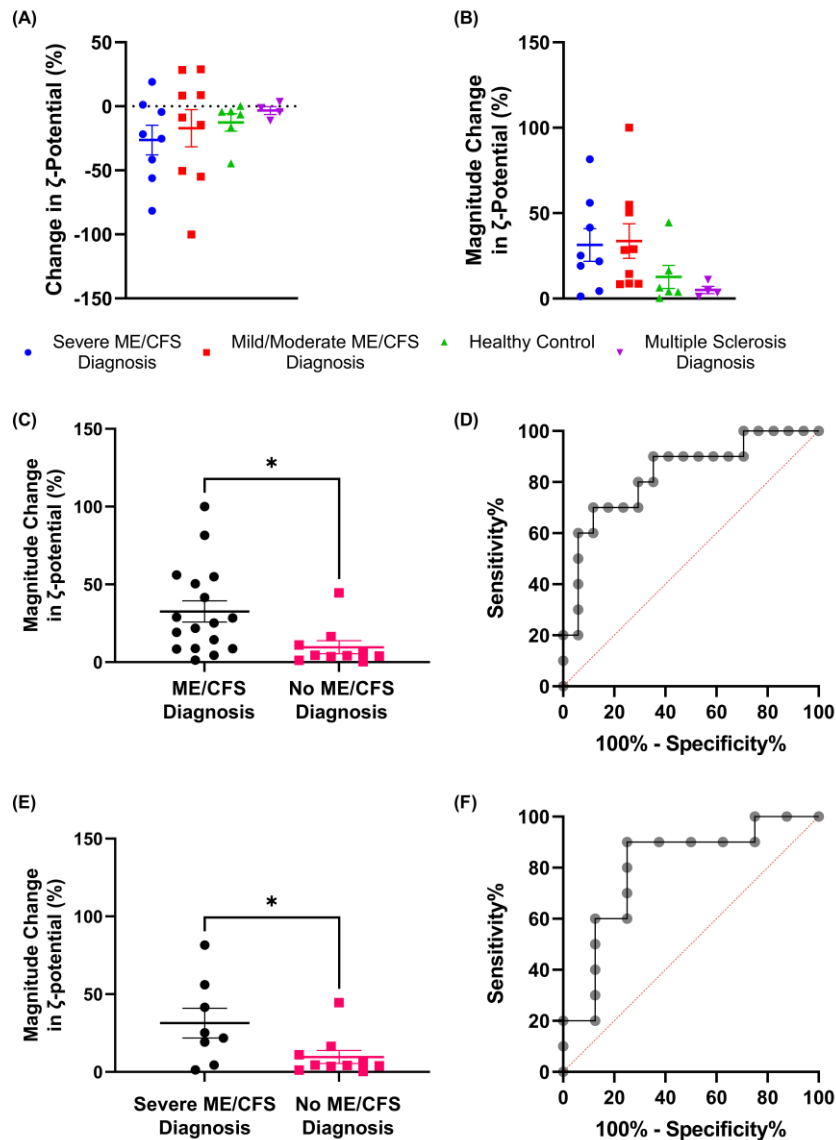


Figure 32 | Normalised ζ -potential data for PBMCs incubated in hyperosmotic NaCl media for 1.5 hours in severe ME/CFS (blue circles: $n = 8$), mild/moderate ME/CFS (red squares: $n = 9$), healthy controls (green triangles: $n = 6$) and MS (purple inverted triangles: $n = 4$). |A| The change in ζ -potential (%) |B| The magnitude changes in ζ -potential (%). |C| The magnitude changes in ζ -potential of ME/CFS diagnosis (black circles: $n = 17$) and No ME/CFS diagnosis (pink squares: $n = 10$). Each datapoint is a different donor. Lines plot mean of biological repeats $\pm$ SEM. Statistical significance denoted by * $p < 0.05$ using unpaired two-tailed t-test. |D| The non-parametric ROC curve (grey dots connected by black line) plotting sensitivity% (true positive rate) against 100-specificity% (proportion of true negatives). The 45° line (red) denotes the theoretical plot of no discrimination between ME/CFS and no ME/CFS. |E| The magnitude change in ζ -potential of severe ME/CFS (black circles: $n = 8$) and No ME/CFS diagnosis (pink squares: $n = 10$). Each datapoint is a different donor. Lines plots mean of biological repeats $\pm$ SEM. Statistical significance denoted by * $p < 0.05$ using unpaired two-tailed t-test. |F| ROC curve (grey dots connected by black line) plotting sensitivity% (true positive rate) against 100-specificity% (proportion of true negatives). The 45° line (red) denotes the theoretical plot of no discrimination between severe ME/CFS and no ME/CFS.

Normalised ζ -Potentials for 1.5-hour Hyperosmotic Mannitol

Similar trends in the percentage change in ζ -potential (%) during hyperosmotic NaCl incubation are seen during hyperosmotic mannitol incubation (**Figure 33**). As before, no trend is

obvious in the percentage change between any of the donor cohorts, but there is a clear difference in the $\pm$ SEM of severe ME/CFS and mild/moderate ME/CFS compared with healthy controls and MS donors (**Figure 33A**). Similarly, there are no significant differences in the mean magnitudes of the percentage change between donor cohorts, but the severe ME/CFS and mild/moderate ME/CFS have markedly greater values than both healthy controls and MS donors which are closer to zero (**Figure 33B**). Values of the magnitude change in ζ -potential (%) of ME/CFS Diagnosis and No ME/CFS Diagnosis (plotted in **Figure 33C**) are significantly different (* $p = 0.0205$) using an unpaired two-tailed t-test. Non-parametric ROC analysis identifies an AUC of 0.8681 with a p-value of 0.0079, and for a threshold of 5.758 there is a sensitivity of 92.31% and specificity of 71.43% (**Figure 33D**) - again showing good promise as a diagnostic biomarker.

The required sample size to differentiate all ME/CFS diagnosis from No ME/CFS diagnosis using the magnitude of the % change in ζ -potential following 1.5-hour hyperosmotic mannitol incubation was calculated again using G*Power (version 3.1.9.7.) software using the data from **Figure 33**. A two-tailed t-test using the difference between the two independent means with A priori power analysis, effect size $d = 1.330646$, α err prob = 0.05 and power ($1 - \beta$ err prob) = 0.95 calculated the sample size required to be a total sample size of 32 donors consisting of 16 donors within the ME/CFS diagnosis cohort and 16 donors in the No ME/CFS diagnosis cohort.

A significant difference is observed between severe ME/CFS diagnosis and No ME/CFS diagnosis using an unpaired two-tailed t-test (* $p = 0.0175$) (**Figure 33E**). Non-parametric ROC analysis differentiating Severe ME/CFS diagnosis and No ME/CFS diagnosis (**Figure 33F**) calculated an AUC of 0.9048 with a p value of 0.0152 and a threshold of 4.447 differentiates both cohorts with a sensitivity of 71.4% and specificity of 100.0%.

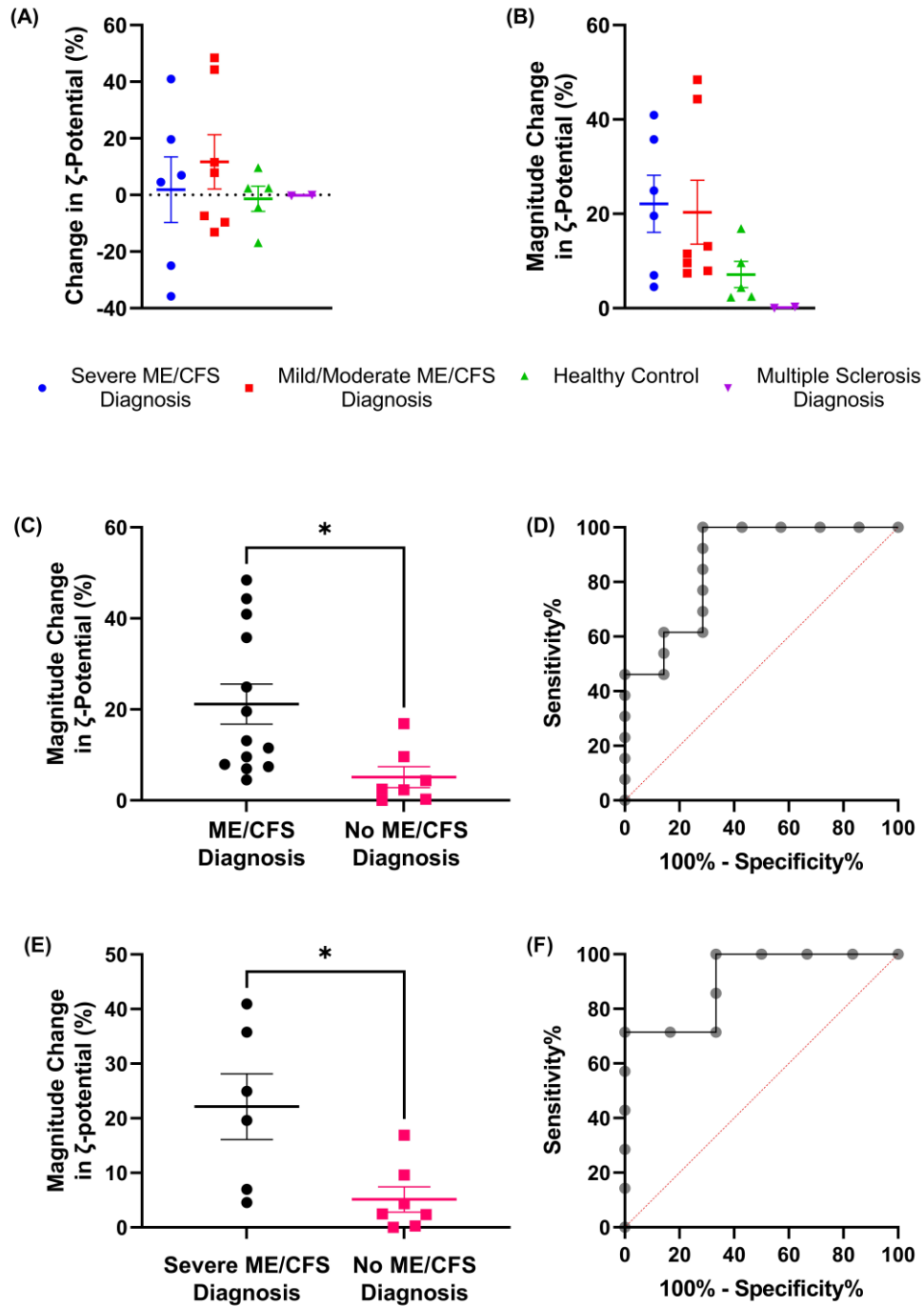


Figure 33| Normalised ζ -potential data for PBMCs incubated in hyperosmotic mannitol media for 1.5 hours in severe ME/CFS (n = 6), mild/moderate ME/CFS (n = 7), healthy controls (n = 5), and MS (n = 2). **|A|** The percentage change in ζ -potential (%) **|B|** The magnitude of the percentage change in ζ -potential (%). **|C|** The magnitude change in ζ -potential of ME/CFS (black circles: n = 13) and No ME/CFS diagnosis (pink squares: n = 7). Each datapoint is a different donor. Lines plots mean of biological repeats $\pm$ SEM. Statistical significance denoted by * p < 0.05 using unpaired two-tailed t-test. **|D|** ROC curve (grey dots connected by black line) plotting sensitivity% (true positive rate) against 100-specificity% (proportion of true negatives). The 45° line (red) denotes the theoretical plot of no discrimination between ME/CFS and no ME/CFS. **|E|** The magnitude change in ζ -potential of severe ME/CFS (black circles: n = 6) and No ME/CFS diagnosis (pink squares: n = 7). Each datapoint is a different donor. Lines plots mean of biological repeats $\pm$ SEM. Statistical significance denoted by * p < 0.05 using unpaired two-tailed t-test. **|F|** ROC curve (grey dots connected by black line) plotting sensitivity% (true positive rate) against 100-specificity% (proportion of true negatives). The 45° line (red) denotes the theoretical plot of no discrimination between severe ME/CFS and no ME/CFS.

Combining Normalised D Value and Magnitude Change in ζ -Potential (%) into One Diagnostic Biomarker

Although the three biomarkers described are good biomarkers for ME/CFS, none of them perfectly discriminate between ME/CFS Diagnosis and No ME/CFS Diagnosis. To attempt to find a better discriminator, multiple logistic regression was applied using GraphPad Prism 9 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com) to combine both the normalised D values and magnitude of the percentage change in ζ -potential following 1.5-hour NaCl incubation. Combining these two biomarkers was tested initially as opposed to the hyperosmotic mannitol incubation results to streamline the protocol by minimising the number of PBMC treatment conditions.

Figure 34A plots the ROC curve generated when multiple logistic regression was used to differentiate ME/CFS diagnosis donors from No ME/CFS diagnosis using both normalised D values and the magnitude of the percentage change in ζ -potential following 1.5-hour NaCl incubation. The percentage correctly predicted was 76.0%. The AUC of 0.833 is statistically different from 0.5 (p-value = 0.0055) disproving the null hypothesis that the diagnostic test has no discriminatory ability. The ROC curve shows together both biomarkers provide a sensitivity of 80.0% and a specificity of 80.0%.

The ROC curve generated when multiple logistic regression was used to differentiate severe ME/CFS diagnosis donors from No ME/CFS diagnosis using both normalised D values and the magnitude of the percentage change in ζ -potential following 1.5-hour NaCl incubation is plotted in **Figure 34B**. The AUC of is 0.8667, with a p-value of 0.0170. The sensitivity and specificity of differentiating severe ME/CFS diagnosis donors from No ME/CFS diagnosis donors is 100.0% and 70.0% sensitivity and specificity.

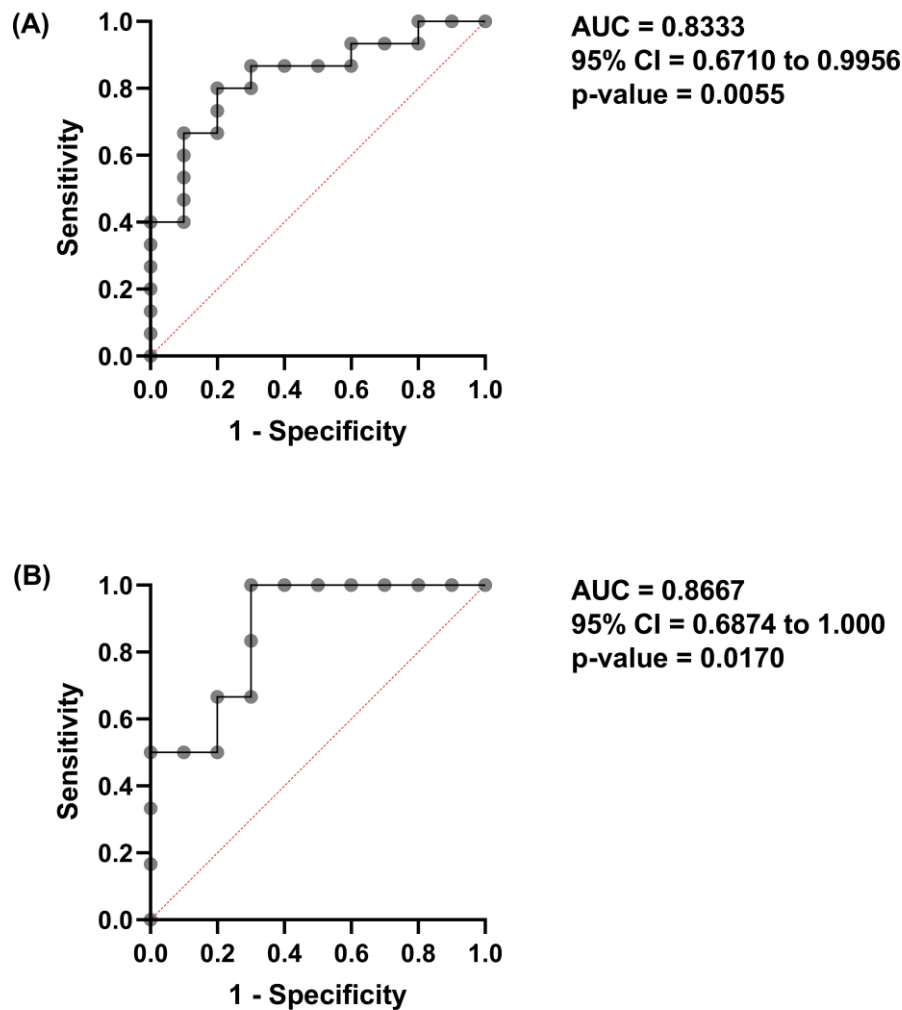


Figure 34 | Combining the 3DEP normalised D values from 1.5-hour incubation in hyperosmotic NaCl media, and the Zetasizer magnitude of the percentage change in ζ -potential after 1.5-hour incubation in hyperosmotic NaCl media into one diagnostic biomarker. Non-parametric ROC curve (grey line) plotting sensitivity (true positive rate) against 1-specificity (proportion of true negatives) for **[A]** ME/CFS diagnosis (n = 15) vs. No ME/CFS diagnosis (n = 10) **[B]** Severe ME/CFS diagnosis (n = 8) vs. No ME/CFS diagnosis (n = 10). The 45° line (red) denotes the theoretical plot of no discrimination between ME/CFS and no ME/CFS. (AUC denotes the Area Under Curve, 95% CI is of the AUC, and a p-value <0.05 disproves the null hypothesis that the test does distinguish between the two categories).

Binary logistic regression was next used to assess combining the normalised D values following 1.5-hour NaCl incubation with the magnitude of the percentage change in ζ -potential following 1.5-hour mannitol incubation. Binary logistic regression correctly predicted 77.8% of donors into the correct cohort using these two biomarkers in combination, with a ROC curve (**Figure 35A**) possessing an AUC of 0.8831, and a p-value of 0.0075. A 'best' threshold has a sensitivity of 81.8% and a specificity of 71.4%.

The normalised D value following 1.5-hour NaCl incubation with the magnitude of the percentage change in ζ -potential following 1.5-hour mannitol incubation was then used to classify

severe ME/CFS donors and No ME/CFS donors. 81.82% of were correctly classified, with an ROC curve (Figure 35B) possessing an AUC of 0.9286, and a p-value of 0.0233. A 'best' threshold of 0.5379 has a sensitivity of 81.8% and a specificity of 71.4%. The 'optimum' threshold has a sensitivity of 100.0% and a specificity of 71.4%.

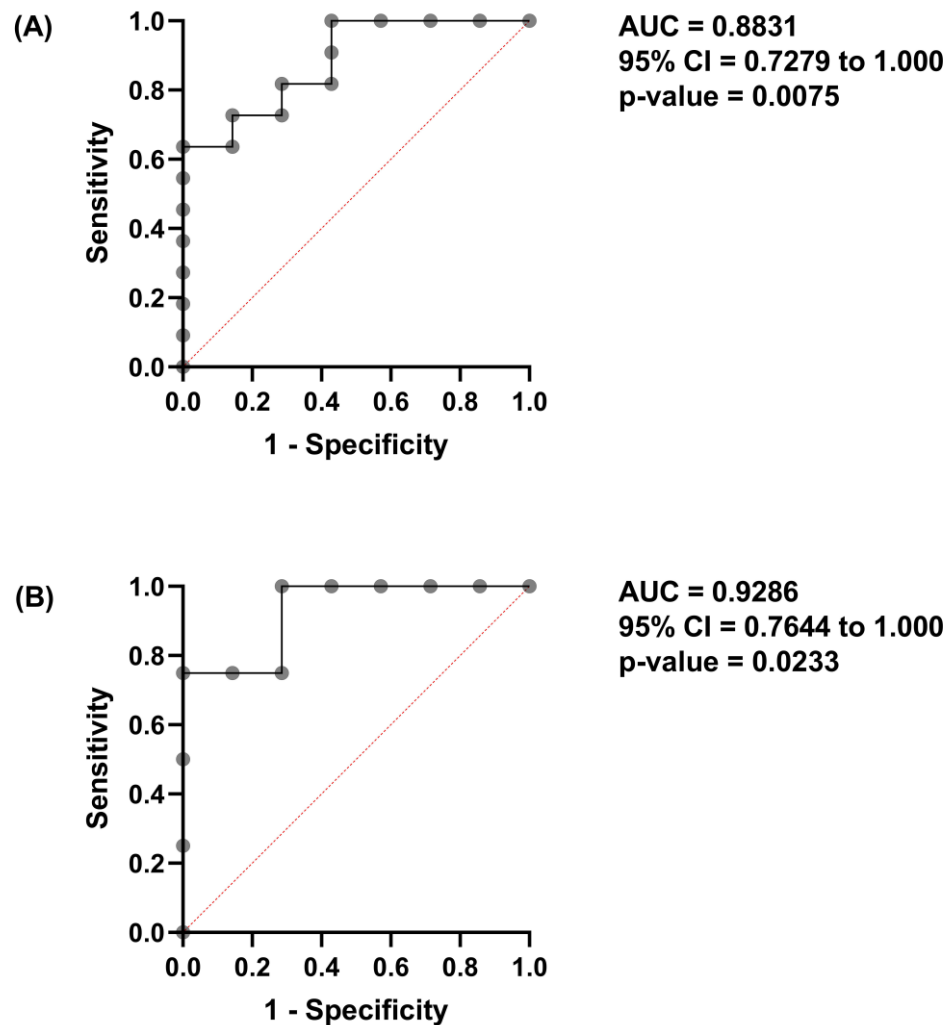


Figure 35|Combining the 3DEP normalised D values from 1.5-hour incubation in hyperosmotic NaCl media, and the zetasizer magnitude of the percentage change in ζ -potential after 1.5-hour incubation in hyperosmotic mannitol media into one diagnostic biomarker. Non-parametric ROC curve (grey line) plotting sensitivity (true positive rate) against 1-specificity (proportion of true negatives) for **|A|** ME/CFS diagnosis (n = 15) vs. No ME/CFS diagnosis (n = 10) **|B|** Severe ME/CFS diagnosis (n = 8) vs. No ME/CFS diagnosis (n = 10). The 45° line (red) denotes the theoretical plot of no discrimination between ME/CFS and no ME/CFS. (AUC denotes the Area Under Curve, 95% CI is of the AUC, and a p-value <0.05 disproves the null hypothesis that the test does distinguish between the two categories).

Combining these three possible biomarkers: the normalised D value following 1.5-hour NaCl incubation, the magnitude of the percentage change in ζ -potential following 1.5-hour NaCl incubation, and the magnitude of the percentage change in ζ -potential following 1.5-hour mannitol incubation was explored next. Binary logistic regression again correctly predicted 77.8% of donors into the correct

cohort, with a ROC curve (**Figure 36A**) possessing an AUC of 0.8831 and p-value of 0.0075. For severe ME/CFS donors vs. No ME/CFS donors, combining all three biomarkers correctly predicts 81.8% of participants. The ROC curve (**Figure 36B**) has an AUC of 0.9286, and a p-value of 0.0233, and demonstrates a sensitivity of 100.0% and a specificity of 71.4%.

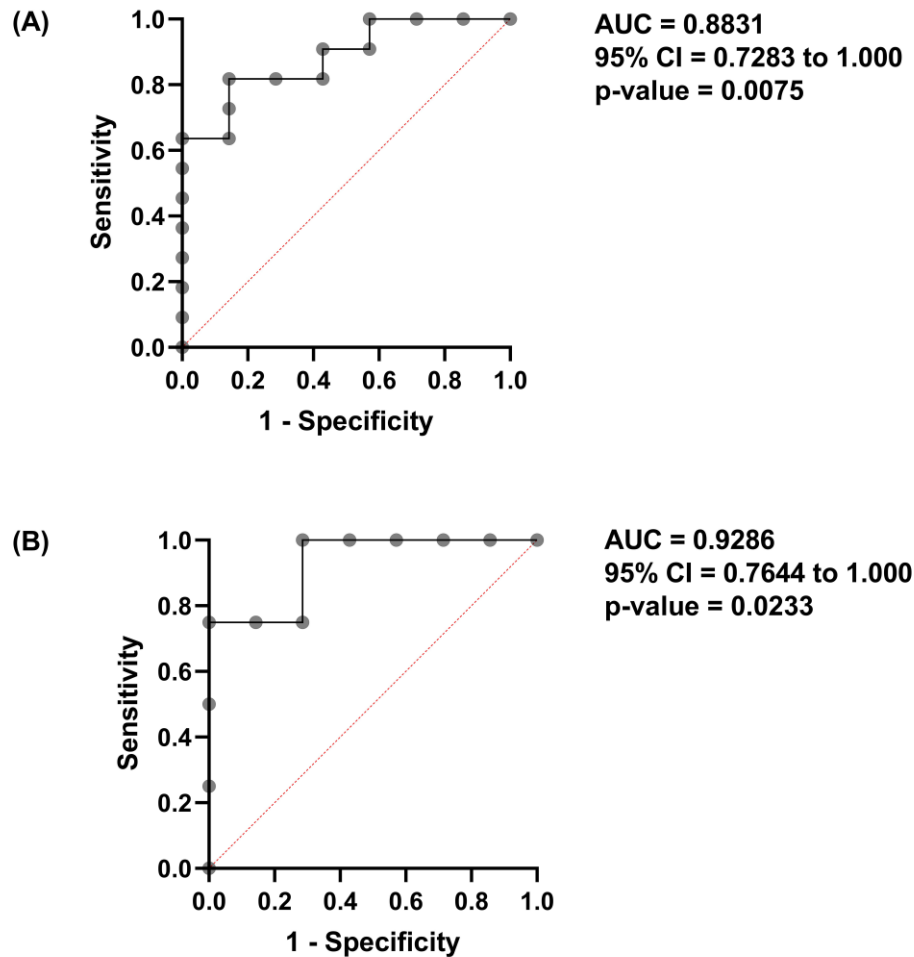


Figure 36| Combining the 3DEP normalised D values from 1.5-hour incubation in hyperosmotic NaCl media, the zetasizer magnitude of the percentage change in ζ -potential after 1.5-hour incubation in hyperosmotic NaCl media, and the zetasizer magnitude of the percentage change in ζ -potential after 1.5-hour incubation in hyperosmotic mannitol media into one diagnostic biomarker. Non-parametric ROC curve (grey line) plotting sensitivity (true positive rate) against 1-specificity (proportion of true negatives) for **|A|** ME/CFS diagnosis (n = 15) vs. No ME/CFS diagnosis (n = 10) **|B|** Severe ME/CFS diagnosis (n = 8) vs. No ME/CFS diagnosis (n = 10). The 45° line (red) denotes the theoretical plot of no discrimination between ME/CFS and no ME/CFS. (AUC denotes the Area Under Curve, 95% CI is of the AUC, and a p-value <0.05 disproves the null hypothesis that the test does distinguish between the two categories).

Summary of Results

Performance of Biomarkers

To evaluate the performance of all five biomarkers proposed in this chapter, ROC analysis was used as it is recommended for evaluating binary classifiers as it is considered the most objective method of evaluating classification performance (Berrar and Flach, 2011). Parameters to consider when evaluating performance include the AUC. The AUC is a measure of reliability (Missailidis et al., 2020b), with a value of 0.5 suggesting no discrimination or ability to differentiate a disease and control cohort and that the ROC curve will be a 45-degree line. A value of 0.7 to 0.8 is considered acceptable, 0.8 to 0.9 is excellent, and more than 0.9 is outstanding (Mandrekar, 2010). A p-value against the null hypothesis of $*p < 0.05$ for the AUC indicates the test discriminates between disease and control, whereas above that indicates the AUC may equal 0.50 and is not a discriminatory biomarker.

Sensitivity is a percentage defining the number of individuals who are correctly diagnosed with the disease (true positives) out of the total subjects who have the disease (**Equation 15**); it is the probability of getting a correct positive diagnosis.

$$Sensitivity = \frac{True\ Positive}{True\ Positive + False\ Negative} \quad \text{Equation 15}$$

True positive refers to individuals correctly diagnosed with the disease. False negative refers to individuals who have the disease but were diagnosed as not having the disease. Specificity is a percentage defining the number of individuals who have a correct negative test result out of the total subjects who do not have the disease (**Equation 16**); the probability of getting a correct negative test result (Šimundić, 2009).

$$Specificity = \frac{True\ Negative}{True\ Negative + False\ Positive} \quad \text{Equation 16}$$

True negative refers to individuals correctly diagnosed as not having the disease. False positive refers to individuals who were diagnosed to have disease but do not.

The threshold of each biomarker chosen to differentiate ME/CFS diagnosis and no ME/CFS diagnosis was the ‘best’ threshold value which gave a combined sensitivity and specificity closest to (0.0, 100.0) on the ROC curves. A table quoting the AUC for non-parametric ROC curves, sensitivities, and specificities of the different biomarkers at differentiating all ME/CFS donors compared with no ME/CFS diagnosis explored in this section are outlined in **Table 7**.

Table 7 | Comparing the performance of each diagnostic biomarker at differentiating individuals with an ME/CFS diagnosis against those who have No ME/CFS Diagnosis. Sensitivity and specificity were chosen against the ‘best’ threshold value from the ROC curve, chosen as it is closest to 0.0, 100.0 on the ROC curve. AUC refers to area under the non-parametric ROC curve; p-value is against the null hypothesis that the AUC equals 0.50 and is not a discriminatory biomarker; 95% CI of the true AUC; sensitivity is the true positive rate; specificity is the proportion of true negatives.

Biomarker	AUC	95 % CI	p-value	Sensitivity (%)	Specificity (%)
Normalised D value	0.7111	0.5007 to 0.9216	0.0637	73.33	75.00
Magnitude of Change in ζ -Potential (%) T1.5-hour NaCl Incubation	0.8294	0.6639 to 0.9949	0.049*	80.00	70.59
Magnitude of Change in ζ -Potential (%) T1.5-hour Mannitol Incubation	0.8681	0.6891 to 1.000	0.0079*	92.31	71.43
Combining Normalised D value and Magnitude of Change in ζ -Potential (%) T1.5-hour NaCl Incubation	0.8333	0.6710 to 0.9956	0.0055*	80.00	80.00
Combining Normalised D value and Magnitude of Change in ζ -Potential (%) T1.5-hour Mannitol Incubation	0.883	0.7279 to 0.9956	0.008*	81.80	71.40
Combining Normalised D value, Magnitude of Change in ζ -Potential (%) T1.5-hour NaCl Incubation and Magnitude of Change in ζ -Potential (%) T1.5-hour Mannitol Incubation	0.883	0.728 to 1.000	0.008*	81.80	85.70

Table 8 displays the AUC for non-parametric ROC curves, sensitivities, and specificities of the different biomarkers at differentiating severe ME/CFS donors compared with no ME/CFS diagnosis.

Table 8 | Comparing the performance of each diagnostic biomarker at differentiating individuals with a Severe ME/CFS diagnosis against those who have No ME/CFS Diagnosis. Sensitivity and specificity were chosen against the 'best' threshold value from the ROC curve, chosen as it is closest to 0.0, 100.0 on the ROC curve. AUC refers to area under the non-parametric ROC curve; p-value is against the null hypothesis that the AUC equals 0.50 and is not a discriminatory biomarker; 95% CI of the true AUC; sensitivity is the true positive rate; specificity is the proportion of true negatives.

Biomarker	AUC	95 % CI	p-value	Sensitivity (%)	Specificity (%)
Normalised D value	0.8333	0.6181 to 1.000	0.0246*	83.33	91.67
Magnitude of Change in ζ -Potential (%) T1.5-hour NaCl Incubation	0.8000	0.5772 to 1.000	0.0330*	90.00	75.00
Magnitude of Change in ζ -Potential (%) T1.5-hour Mannitol Incubation	0.9048	0.7368 to 1.000	0.0152*	71.43	100.00
Combining Normalised D value and Magnitude of Change in ζ -Potential (%) T1.5-hour NaCl Incubation	0.8667	0.6874 to 1.000	0.0170*	100.00	70.00
Combining Normalised D value and Magnitude of Change in ζ -Potential (%) T1.5-hour Mannitol Incubation	0.929	0.764 to 1.000	0.0233*	100.00	71.40
Combining Normalised D value, Magnitude of Change in ζ -Potential (%) T1.5-hour NaCl Incubation and Magnitude of Change in ζ -Potential (%) T1.5-hour Mannitol Incubation	0.929	0.764 to 1.000	0.0233*	100.00	71.40

The normalised D value biomarker had the lowest AUC and a p value of greater than 0.05, sensitivity and specificity of the five biomarkers. The other four biomarkers had an AUC greater than 0.8, sensitivities of 80% or more, and specificities of more than 70%. The best performing individual

biomarker was the magnitude of change in ζ -Potential (%) T1.5-hour mannitol Incubation, with an AUC of 0.8681, sensitivity of 92.31%, and specificity of 71.4%. However, combining all three individual biomarkers into one yielded better result, with an AUC of 0.883, sensitivity of 81.80% and specificity of 85.70%. Deciding the optimum biomarker is a balance between whether sensitivity or specificity is more important – that is to say whether it is more important to ensure all individuals who have it are diagnosed, or whether it is more important to ensure those who do not have it are not incorrectly diagnosed with it. In the case of ME/CFS, specificity is important to ensure individuals are following the correct treatment plan. There is no cure for ME/CFS and treatment currently consists of alleviation of symptoms and co-morbidities. It is important that someone with similar symptom presentation of ME/CFS but a different illness receives the correct treatment and medication where available.

Specificity of Electrical Changes During Hyperosmotic Stress to ME/CFS

Within this chapter, MS was used to determine the specificity of the diagnostic biomarker to ME/CFS, as a disease control with similar symptom presentation. MS is a chronic, inflammatory autoimmune disease characterised by demyelination of the central nervous system for which there is no cure. Although the precise aetiology of MS is unknown (Hundehege et al., 2017, Böttcher et al., 2019, De Rasmio et al., 2020), it is thought to be triggered by an autoimmune reaction following an infection in genetically predisposed individuals (Çakır et al., 2021, Domínguez-Mozo et al., 2022). Essential pathogenic steps include infiltration of activated macrophages and auto-reactive T cells into the central nervous system, mediating demyelination of neuronal axons, loss of oligodendrocytes, destruction of neurons, and loss of the blood-brain barrier (Black et al., 2013, Hundehege et al., 2017, Çakır et al., 2021). It has a highly variable disease course (Böttcher et al., 2019), leading to motor, sensory, and cognitive symptoms (Meyer-Arndt et al., 2023) such as blurred vision, muscle weakness, balance problems, and fatigue (Çakır et al., 2021).

Although they are each distinct illnesses, there are overlaps in disease characteristics between MS and ME/CFS. MS is most prevalent in women (Harbo et al., 2013), the specific aetiology is unknown, and like in ME/CFS there is evidence of differential TRP channel expression (Çakır et al., 2021) including TRPM3 expression in PBMCs compared to healthy controls (Clarke, 2018), increased activation (Canto et al., 2018), mitochondrial dysfunction, and oxidative stress (De Rasmio et al., 2020, Domínguez-Mozo et al., 2022) in PBMCs. Most importantly, 70% of patients with MS report fatigue – 38% of which describe fatigue to be their most disabling symptom (Kaya Aygünoğlu et al., 2015). Based on these similarities it was chosen to be a disease-control to assess whether the differences in electrical characteristics in ME/CFS are specific, or also occur in diseases sharing a similar phenotype

– decreasing the specificity of these parameters for diagnostic biomarker applications. To best-match phenotypes of ME/CFS and MS patients within the study, four of the five MS donors recruited disclosed fatigue was their main symptom. It is promising that with the inclusion of MS as a disease control, the specificity of all five biomarkers in this study are high. However, it is important that other disease controls are assessed in future experiments. Other diseases with similar symptom presentation include Gulf-War disease (Whistler et al., 2009), pernicious anaemia, and fibromyalgia (Regland et al., 2015, Groven et al., 2020).

Comparability to Other Diagnostic Biomarkers Proposed

Performance of these biomarkers favourably compare to diagnostic biomarkers published in other studies. One of the highest performing diagnostic biomarkers was published by Missailidis et al. (2020b) who combined assays measuring lymphocyte death rate, mitochondrial respiratory function and TORC1 activity to a single diagnostic biomarker with both sensitivities and specificities of approximately 95%. Individually, the three assays each had sensitivities and specificities both ranging from around 76% to 89%. However, both individually and collectively all three techniques are difficult to implement in a clinical setting, requiring expertise, time and laboratory intensive protocols including two to three-day cell culture. Other biomarker studies include metabolomic analytes in plasma assay yielding an AUC of 0.794 (Che et al., 2021) and a natural killer cell cytotoxicity assay resulting in an AUC of 0.776 (Fletcher et al., 2010). Combined analysis on the expression of two mitochondrial proteins ATP synthase subunit beta and aconitase resulted in a sensitivity of 93% and specificity of 70% (Ciregia et al., 2016).

However, the biomarkers of this study are not as consistent at differentiating ME/CFS and healthy controls than the changes in impedance measured by the nanoneedle array reported by Esfandyarpour et al. (2019). 100% of ME/CFS donors in their study had a markedly different impedance response to hyperosmotic stress than healthy controls. However, their device has not yet been tested on disease controls. Additionally, although their experimental procedure has the potential to act as a low-cost, real-time ultrasensitive assay capable of measuring impedance patterns for point-of-care diagnostics of ME/CFS, the nanoelectronic assay is not used routinely and requires further instrumental development to be deployed. The 3DEP device (DEPtech) and Blue Label Lab Zetasizer (Malvern Panalytical) are not certified medical devices, but they are commercially available, easy to set up, and adopt in a laboratory environment. Both the 3DEP (DEPtech) and Zetasizer (Malvern Panalytical) machines are simple, rapid, non-contact, high-throughput, low-cost, non-destructive methods of measuring the dielectric properties of PBMCs. Furthermore, both machines

only require small numbers of PBMCs. For 3DEP analysis, a cell concentration of 1×10^6 cells/mL was chosen. This has been determined the optimum cell concentration for use with the 3DEP as it is the combination of the lowest number of cells required with the lowest DEP spectra noise. At lower concentrations there are less cells to interfere with the collimated light beam in the 3DEP resulting in an increase in noise. At higher concentrations above 1×10^7 cells/mL the light beam is restricted, increasing noise and also increasing the number of cells required, which is disadvantageous for diagnostic applications (Hoettges et al., 2019). For three technical repeats, this equates to approximately 300 000 cells. Likewise, the number of cells required for each zetasizer sample was 120000 cells. Limitations to the 3DEP and zetasizer device include that although they are commercially available, they are not certified for medical use. There is also some skill required to operate both machines – as small microfluidic devices they are susceptible to bubbles which increases the signal-to-noise ratio.

Sample Preparation Time

Use of a blood-based biomarker to diagnose ME/CFS is attractive as blood-based biomarkers are accessible, relatively non-invasive, pose minimal risks to patients and direct (Maksoud et al., 2023). For these qualities they are in development for the diagnosis of many diseases including breast cancer (Loke and Lee, 2018), Parkinson's disease (Klatt et al., 2021), and Alzheimer's disease (Padala and Newhouse, 2023). An ideal blood-based biomarker would necessitate minimal preparation time, such as a finger-prick to provide point-of-care diagnosis. However, the protocol of the diagnostic test proposed in this chapter requires the isolation of PBMCs; a multi-step process which adds significantly to protocol duration if it were to be adopted for clinical practice. Esfandyarpour et al. (2019) first attempted to measure the impedance of whole blood as opposed to PBMCs but found there were no reliable or repeatable patterns. PBMCs were thus chosen specifically for these experiments to assess the repeatability of the experiments by Esfandyarpour et al. (2019). Furthermore, PBMCs such as NK cells have been strongly associated with ME/CFS pathophysiology (Strayer et al., 2015, Luis Rivas et al., 2018), and have been shown to possess changes in the expression and function of TRP ion (discussed in **Section 3.2.3.3.**) (Marshall-Gradisnik et al., 2016b, Cabanas et al., 2018, Cabanas et al., 2019a).

Once the time-limiting sample preparation steps (frozen vials have thawed, the cells have recovered post-thawing and been hyperosmotically treated) are complete, triplicate 3DEP measurements and triplicate ζ -potential measurements can be run in parallel with one operator in approximately 15-minutes. Eliminating the necessity to take c-chip images, measure cell radius and

model DEP spectra in MATLAB by simply calculating the normalised D value significantly decreases the time, complexity, and expertise of data analysis. Together, the logistics of the 3DEP and zetasizer experiments facilitate a protocol whereby data collection and data analysis on frozen PBMC samples from suspected ME/CFS donors can be performed in one day **Figure 37**.

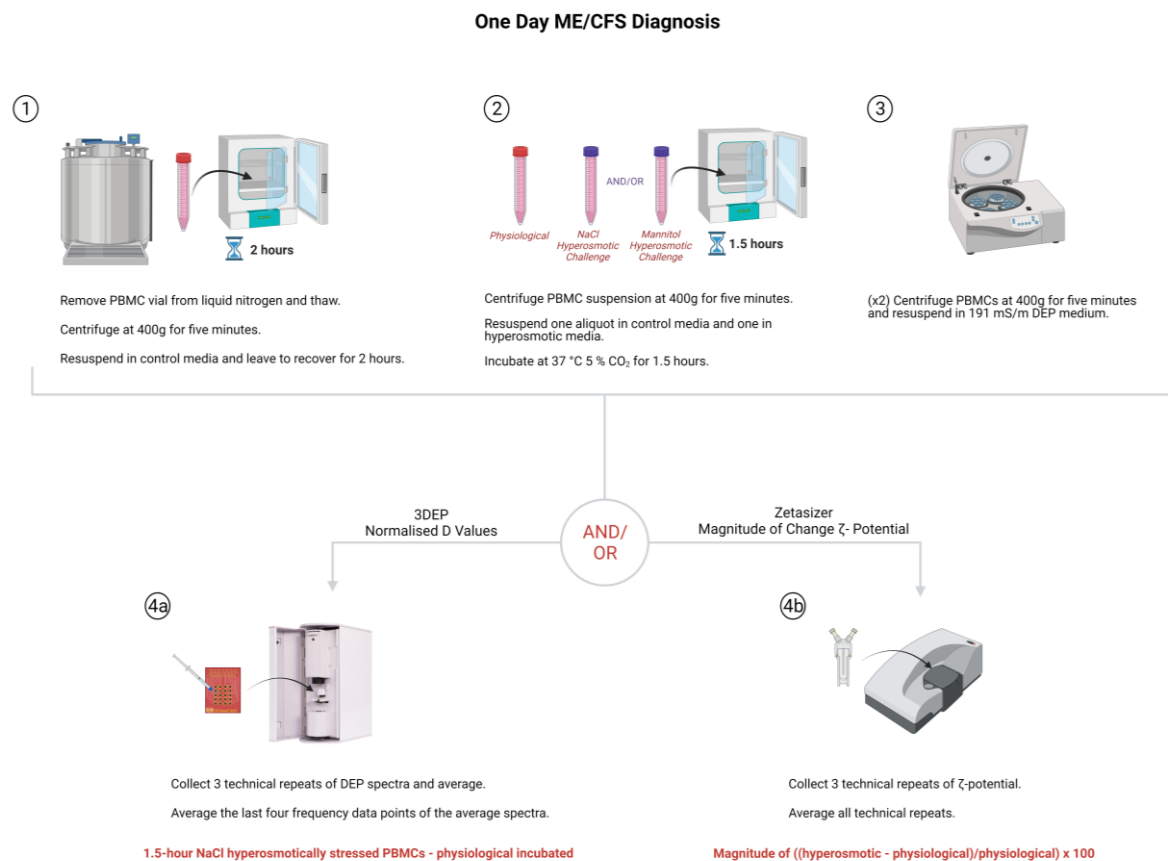


Figure 37 | A summary of the streamlined protocol optimised for analysing the electrical properties of frozen PBMCs for one-day diagnosis. (Created with BioRender.com)

Frozen vs Fresh PBMC Samples

The initial plan for this study was to use fresh PBMC samples, by collecting whole blood from ME/CFS donors and performing same-day PBMC isolation and 3DEP experiments at the UK ME/CFS Biobank situated at the Royal Free Hospital in London. However, the commencement of these experiments planned for April 2020 were disrupted by the COVID-19 counter-measures which prohibited inter-county travel, restricted blood donations due to social distancing measures (further enforced by the vulnerability of ME/CFS patients), and limited laboratory access. To circumvent these complications and proceed with experiments, contingency measures included purchase of frozen PBMC aliquots.

Advantages of using fresh PBMC samples as opposed to frozen include eliminating potential changes in cellular electrical properties caused by damage to cell morphology during freezing (such as ice crystals puncturing cell membranes) or damage via the freezing medium – providing more accurate measurements of the hyperosmotic response of PBMCs in physiology. As per the UK ME/CFS Biobank standard operating procedures, the freezing medium used to freeze samples included 20% DMSO. DMSO has been shown to be toxic to cells at doses of 10% (Galvao et al., 2014). To avoid associated toxicities, thawed samples were immediately resuspended in control medium to dilute the DMSO. Following the advice of immunologists working at the UK ME/CFS Biobank, cells were incubated for approximately two-hours to recover post-thawing before starting hyperosmotic challenge. Although a longer recovery time would have been advantageous, two-hour recovery time was used to reduce the time of sample preparation for same-day diagnostic application. Trypan-blue staining demonstrated cell viability remained above 95% between time 0 post-recovery incubation and at time 1.5-hours of control or hyperosmotic challenge. Trypan-blue staining was used to ensure electrical parameters measured by the 3DEP and zetasizer were not influenced by PBMC death. Although increased lymphocyte-death has been identified as a potential biomarker of ME/CFS in itself (Missailidis et al., 2020b), comparing differences in PBMC death and recovery between cohorts were outside the scope of this particular study. Inclusion of dead lymphocytes into 3DEP analysis would have also generated plateau stages within the spectrum, which was not observed. As a consequence of using frozen PBMC samples, consideration of these factors has to be made when evaluating the accuracy of raw values of G_{eff} , C_{eff} , σ_{cyto} and ζ -potential.

As the thawing and hyperosmotic challenge protocol was consistent among all samples, any impact on cell function or phenotype caused by freezing did not impact the diagnostic ability of this device. Differences in cell recovery between donor cohorts, although not quantified, would have been incorporated within the differences between cohorts and have supplemented the diagnostic technique.

A benefit of using frozen samples as opposed to fresh PBMCs is the adoptability of this diagnostic protocol with respect to sample collection and storage. Being able to freeze PBMC samples for analysis at a later time and transport them across the country with less time-sensitivity increases the flexibility and practicality of sample processing in clinical laboratories. Whilst optimising and assessing whether their device could be used on patient samples collected globally, Esfandyarpour et al. (2019) used their assay on patient samples stored in different storage conditions including 4 °C, room temperature, -20 °C frozen, and liquid nitrogen storage. After fresh samples, 1-week liquid nitrogen storage and 24-hour storage at room temperature were the next most successful techniques; they both preserved the same impedance pattern expressed in fresh samples but were attenuated.

This suggests that the difference between ME/CFS and controls seen using the 3DEP and zetasizer may have been more pronounced in fresh samples, perhaps contributing to the less pronounced changes observed between healthy controls and ME/CFS donors compared to those identified by Esfandyarpour et al. (2019). Nonetheless, using frozen PBMC samples demonstrated high specificity and sensitivity as a diagnostic technique in this chapter.

Population Recruitment

As highlighted in other studies, the heterogeneity of symptoms within ME/CFS donors, unknown aetiologies, and fluctuations in symptom presentation and severity over time increases the difficulties in identifying a specific biomarker (Querec et al., 2023). Furthermore, with no current biomarker, recruitment of donors to the study may result in including individuals who may not have ME/CFS – impacting the sensitivity and specificity of this study. To minimise this, donors were recruited using the CCC criteria as it is stricter and requires the presence of more symptoms to achieve a diagnosis.

A limitation of this study is the small donor numbers and representation of biological sex and ethnicities. The vast majority of donors within this study were female, and all were aged 35 – 65. It would have been better to have used age and gender matched participants as Esfandyarpour et al. (2019) did to account for variances in PBMC electrophysiology due to age and gender. As the incidence of ME/CFS is 3 times greater in females than males, it is not uncommon for ME/CFS studies to only recruit female donors (Fletcher et al., 2009), or for the majority of participants to be female (Huth et al., 2016, Polo et al., 2019). Future work is needed to investigate the effect of age and gender on the electrical hyperosmotic response of PBMCs. It is very important that future experiments recruit donors of more ethnicities and racial groups. One study found the incidence of ME/CFS to be more prevalent in Pakistani, Indian, Bangladeshi, and Black Caribbean individuals than white individuals (Bhui et al., 2011), thus it is even more important that donors other than white British are recruited.

3.5. Conclusion

No quantitative test or effective diagnostic marker currently exists to diagnose ME/CFS; a debilitating disease of unknown pathophysiology and no cure. There is a necessity to further research the pathological mechanisms driving this disease and identify and validate a reliable, easily adoptable biomarker sufficiently sensitive and specific at identifying individuals with ME/CFS. A diagnostic

biomarker would replace the current subjective and unreliable diagnostic criteria guidelines, accelerate correct diagnosis, ensure correct treatment plans are followed from disease onset, and remove the stigma surrounding this low-quality-of-life condition and validate patients' symptoms.

The pilot study described in this chapter investigated the baseline electrical properties of PBMCs collected from individuals with severe ME/CFS, mild/moderate ME/CFS, healthy controls and MS donors using the 3DEP and zetasizer machines. Although slight variations were observed, no statistically significant differences were found in the baseline values of G_{eff} , C_{eff} , σ_{cyto} , and ζ -potential in unchallenged PBMCs from severe ME/CFS, mild/moderate ME/CFS, healthy controls, and MS donors. The lack of significance implies that any pathological changes in ME/CFS or MS do not significantly affect the dielectric properties or ζ -potential of PBMCs when unchallenged and therefore are not potential diagnostic biomarkers. However, after 1.5-hour hyperosmotic challenge, significant differential changes in C_{eff} and σ_{cyto} could be seen between donor cohorts. Interestingly, there were no statistical differences in the electrical response when using NaCl or mannitol as the hyperosmotic stressor, implying that the electrophysiological responses may be attributable to a common mechanism to osmotic stress. To determine whether differential electrical changes between donor cohorts are due to ion channelopathies affecting Na^+ or Cl^- transport, ion channel blockers and medium withdrawals are required in future experiments.

Of most relevance, σ_{cyto} decreased in severe ME/CFS donors and mild/moderate ME/CFS donors but remained unchanged in healthy controls and increased in MS donors. Additionally, the changes in ζ -potential in both ME/CFS donor cohorts were much more varied than that of no ME/CFS diagnosis cohorts. Three candidates of diagnostic biomarkers were identified including 'normalised D value' following hyperosmotic NaCl incubation, the magnitude of the percentage change in ζ -potential following hyperosmotic NaCl incubation, and the percentage changes in ζ -potential following hyperosmotic mannitol incubation. The latter biomarker performed the best, with a sensitivity and specificity of 92.31 and 71.43 respectively. Combining all three together produces an optimum performing biomarker with a sensitivity of 81.80% and a specificity of 85.70%. These results compare favourably with other biomarkers for ME/CFS.

Advantages of using the 3DEP and zetasizer machines over the nanoneedle array include they are easier to set up, low-cost, high-throughput, and readily available. In comparison the nanoneedle array is not used routinely, requires further instrumental development to be deployed and is difficult. The biomarkers found in this study ensure absolutely no operator-bias or subjectivity, as they do not exclude outliers, technical repeats or in the case of the 3DEP, noisy DEP spectra. Furthermore, the biomarkers differentiate from MS disease controls, which has thus far been untested in impedance

measurements using the nanoneedle assay. However, further experiments are required to increase the small sample size of this study to bring the statistical power to 0.95, and to recruit donors of other ethnicities and more male donors.

4. THE ELECTROPHYSIOLOGICAL PROPERTIES OF WHITE BLOOD CELLS FROM COVID-19 NAÏVE VS. COVID-19 EXPOSED DONORS

This second research chapter examines the electrical properties of PBMCs donated from individuals who have never had COVID-19 compared with individuals who have recovered from COVID-19, or received one, two, or three doses of a SARS-CoV-2 vaccine.

4.1. Chapter Overview, Aims, and Objectives

Both previous infection with SARS-CoV-2 (Chandrashekar et al., 2020, Deng et al., 2020, Chemaitelly et al., 2022) and vaccination (Polack et al., 2020, Amit et al., 2021, Petter et al., 2021, Voysey et al., 2021) have proven to be highly effective in providing protection against reinfection and symptomatic disease. However, a number of studies have shown that both the humoral and cellular immune response to SARS-CoV-2 declines over time and across variants (Ibarrondo et al., 2020, Patel et al., 2020, Self et al., 2020, Seow et al., 2020, Gaebler et al., 2021, Morales-Núñez et al., 2021, Tseng et al., 2022, O'Shea et al., 2023). As such, there is a necessity to identify a quantifiable, sensitive, and specific immune marker which statistically correlates with protection from SARS-CoV-2 infection and symptomatic disease; known as a correlate of protection (CoP). An easily measurable and adoptable CoP, which identifies whether an individual is susceptible to disease, is important for evaluating the efficacy of vaccines, accurately ascertaining population-level immunity, informing immunisation programmes, and developing recommendations on public health and social measures (Gilbert et al., 2022a, Misra and Theel, 2022, Mcvernon and Liberman, 2023). The majority of research studies of CoP have focussed on humoral immune responses (Misra and Theel, 2022, Kalimuddin et al., 2023). But growing research has demonstrated that SARS-CoV-2 specific T-cell responses are important in SARS-CoV-2 protection and clearance, demonstrated in antibody-seronegative individuals who have sufficient protection from infection (Seow et al., 2020, GeurtsvanKessel et al., 2022).

Given that the electrical properties of PBMCs change following antigen stimulation and TCR activation, it was hypothesised that differential changes in the electrical properties of PBMCs following stimulation with the SARS-COV-2 spike protein, as measured using DEP, could pave the way towards a new CoP to SARS-CoV-2 and other challenges. In collaboration with University of Surrey researchers in the Faculty of Health and Medical Sciences, the primary objective was to investigate in a pilot study whether PBMCs from donors who had never had COVID-19 (COVID-19 naïve) and donors who had recovered from COVID-19, or had received their first or second vaccine doses (COVID-exposed) donor cohorts, possess differential electrophysiological responses after incubation with the receptor binding domain (RBD) of the SARS-CoV-2 spike protein.

4.2. Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2)

4.2.1. Background

Overview of SARS-CoV-2

Coronavirus disease-2019 (COVID-19) is a severe pneumonia-associated respiratory syndrome caused by the novel beta coronavirus strain, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) (Yang et al., 2020b, Zhou et al., 2021). From the first outbreak reported in December 2019 in Wuhan, China, SARS-CoV-2 quickly spread and became prevalent worldwide, posing a great threat globally to public health, global economies, increased poverty, and social cohesion. The World Health Organisation (WHO) declared COVID-19 to be a global pandemic on March 11, 2020 (Ciotti et al., 2020, Schijns and Lavelle, 2020, Zhou et al., 2021, Yuan et al., 2023). By April 2021, 219 countries and territories had reported cases of COVID-19, and there was a global death toll of 3,076,021 (Worldometer, 2021). In August 2022, the global death toll had increased to more than 6 million (Yuan et al., 2023).

Structure of SARS-CoV-2

Belonging to the *Coronaviridae* family and the subgenus *Sarbecovirus*, SARS-CoV-2 is an enveloped single-stranded positive-sense RNA virus [(+)gRNA] (Ciotti et al., 2020, Yang et al., 2020b, Yamada and Takaoka, 2023). The virus's (+)gRNA is complexed with nucleocapsid (N) protein, and is surrounded by a lipid bilayer envelope expressing three viral structural proteins; membrane (M) protein, spike (S) protein, and envelope (E) protein (Redondo et al., 2021, Yamada and Takaoka, 2023). The structure of SARS-CoV-2 is shown in **Figure 38**. The S protein consists of two subunits (S1 and S2), which are separated by a polybasic furin cleavage site which is not found in its relative SARS-CoV-1 (Coutard et al., 2020). The furin site has been proposed to allow pH-independent viral entry, promoting replication and transmission, thus could be the cause of increased transmissibility and infectivity compared to other coronaviruses (Winstone et al., 2021).

The S protein contains the receptor binding domain (RBD), which specifically recognises and facilitates high-affinity binding of SARS-CoV-2 to the angiotensin-converting enzyme 2 (ACE2) receptor on host cells (Hoffmann et al., 2020, Isho et al., 2020, Walls et al., 2020). The RBD contains a core and receptor-binding motif (RBM). The RBM is the main functional motif of the RBD as it mediates contact and forms the interface between the S-protein and ACE2 on host cells (Shang et al., 2020, Yi et al., 2020). Once bound, SARS-CoV-2 fuses with host cell membranes via the S2 subunit (Pang et al., 2021, Gupta and Jaiswal, 2022). Binding of the S protein to its receptor facilitates genomic RNA to enter the

cytoplasm (Assadiasl et al., 2020). Other factors which facilitate host cell entry include neuropilin-1 and the transmembrane serine protease TMPRSS2 (Lee et al., 2020). TMPRSS2 cleaves ACE2 and activates the S protein, promoting viral uptake. Once in the host cell, SARS-CoV-2 replicates and is then released (Assadiasl et al., 2020).

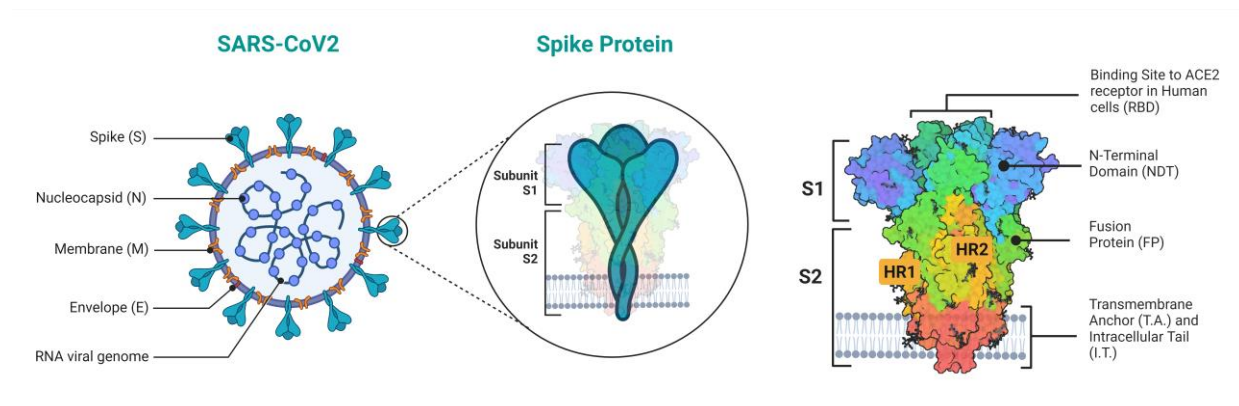


Figure 38| Structure of the SARS-CoV-2 virus, including a diagram of the SARS-CoV-2 spike protein under electron microscopy. Adapted from “Human Coronavirus Structure” and “An In-depth Look into the Structure of the SARS-CoV-2 Spike Glycoprotein”, by BioRender.com (2023). Retrieved from <https://app.biorender.com/biorender-templates>

SARS-CoV-2 Transmission and Infection

Transmission of SARS-CoV-2 is highly efficacious by the respiratory route, primarily via airborne respiratory droplets produced during coughing, sneezing, and talking. Other modes of transmission include direct contact, saliva, oral-faecal, and urine (Ciotti et al., 2020, Zhou et al., 2021). SARS-CoV-2 primarily enters through the respiratory system, but also enters via the mucosal membranes of the mouth, nose, and eyes (Zawilska et al., 2021).

Once in the host, SARS-CoV-2 primarily infects the nasal and respiratory tract. Single-cell RNA-seq analysis identified ACE2 to be highly expressed on type II alveolar epithelial cells, and expressed to a lesser extent on the surface of oral and nasal mucosa epithelial cells (Ni et al., 2020b). Other cells which express ACE2 includes bronchial epithelial cells, vascular endothelial cells (Yamada and Takaoka, 2023), and ciliated epithelial cells which shed their cilia once infected, disabling mucociliary clearance (James et al., 2022). Moreover, SARS-CoV-2 can spread from the lungs through blood circulation to other organs which express ACE2, including the heart, small intestine, kidneys, and testis (Charles et al., 2023). Critically ill COVID-19 patients also develop multiple organ damage, including cardiac injury, liver dysfunction, and acute kidney injury (Ni et al., 2020b).

Clinical Manifestation of SARS-CoV-2

The presentation and severity of symptoms across individuals is highly varied, and quickly captured global headlines. A proportion of individuals are asymptomatic, experiencing no symptoms of SARS-CoV-2 infection despite a confirmed validated laboratory/self-test (Berlin et al., 2020, Gao et al., 2021). Initial studies on small cohorts reported that asymptomatic cases accounted for 81% (Ing et al., 2020) and 78% of cases (Day, 2020), but a meta-analysis published in October 2020 of 13 studies across seven countries found asymptomatic cases to comprise of only 4 – 41%, with the proportion higher in older individuals (Byambasuren et al., 2020). In symptomatic individuals, commonly reported clinical symptoms of SARS-CoV-2 infection include influenza-like symptoms such as a persistent dry cough, fever or chills, and shortness of breath, myalgia/fatigue and headache, runny nose, and sore throat (Ciotti et al., 2020, Wang et al., 2020, Li et al., 2022a, Whitaker et al., 2022, Yuan et al., 2023). Frequently reported neurological clinical symptoms of COVID-19 include olfactory and taste dysfunctions such as anosmia and ageusia (loss of sense of smell and taste respectively) (Algahtani et al., 2022, Singhal et al., 2023). However, the Real-time Assessment of Community Transmission-1 study reported symptom profiles changed between May 2020 and March 2022 with variants, for example the Omicron variant is more associated with influenza-like symptoms and less associated with ageusia (Whitaker et al., 2022) and anosmia (Butowt et al., 2022).

In 2020 it was reported that whereas 40% of patients develop mild symptoms of COVID-19 without evidence of viral pneumonia or hypoxia, 40% experience moderate illness with evidence of lower respiratory disease, and approximately 15% of individuals develop severe disease of which a further 5% required admission into the intensive care unit (The Novel Coronavirus Pneumonia Emergency Response Epidemiology, 2020, World Health Organisation, 2020, Zawilska et al., 2021, Zhou et al., 2021, National Institutes of Health, 2023). Severely affected COVID-19 patients present with a marked innate immune response, including substantially elevated serum levels of pro-inflammatory cytokines such as IL-6, IL-1 β , IL-8, IL-17 (Cao, 2020), IL-2 IL-7, monocyte chemoattractant protein-1, and macrophage inflammatory protein-1 α (Huang et al., 2020), characterised as a cytokine storm. High levels of pro-inflammatory cytokines cause cell death and damage to heart, liver, and kidney tissue, leading to multiple organ failure. Moreover, the infiltration of neutrophils and macrophages causes diffuse alveolar damage, and the formation of hyaline membranes (Cao, 2020). Alveolar oedema and an extremely compromised oxygen-carbon dioxide exchange result in acute respiratory distress syndrome (ARDS) associated with respiratory failure (James et al., 2022, Paludan and Mogensen, 2022), sepsis and shock, thromboembolism, and multi-organ failure (World Health

Organisation, 2020). ARDS is the leading cause of death in critically ill patients and quickly progresses approximately one week from symptom onset (Olajide et al., 2021).

To reduce the rates of COVID-related hospitalisation and mortality, vaccination development was expedited globally with over 172 different vaccines clinically developed, and several authorised by the UK Department of Health and Social Care and the Medicines and Healthcare products Regulatory Agency for use in the United Kingdom (Yang et al., 2023). As of November 2022, more than 4 billion doses of a SARS-CoV-2 vaccine have been administered globally (WHO, 2022). Implementation of COVID-19 vaccines possessing approximately 90% efficacy against symptomatic disease have significantly diminished cases of ARDS (Prato et al., 2021, Swan et al., 2021, Tande et al., 2021, Tande et al., 2022, Looi, 2023).

4.2.2. SARS-CoV-2 Vaccination

The urgent necessity of a safe and effective prophylactic vaccine to contain and minimise the medical, social, and economic consequences of the COVID-19 pandemic drove the international collaboration of researchers for a SARS-CoV-2 vaccine. This resulted in record-breaking vaccine research and development, yielding effective COVID-19 vaccines at unprecedented speed which protect against SARS-CoV-2 infection, hospitalisation, and death (Chemaitelly et al., 2022). One of the vaccines which was developed was the Pfizer-BioNTech vaccine, which progressed from pre-clinical trial to Emergency Use Authorisation all in under a year (Yamey et al., 2022, Yang et al., 2023).

Pfizer/BioNTech – Comirnaty (mRNA BNT162b2)

The Pfizer/BioNTech Comirnaty (BNT162b2) vaccine is a nucleoside-modified mRNA formulated in lipid nanoparticles that encode the full-length SARS-CoV-2 S protein, modified by two proline mutations to stabilise in its prefusion conformation (Polack et al., 2020, Sahin et al., 2021, Patel et al., 2022). The lipid-nanoparticle coat is mainly composed of phospholipids, cholesterol, and ionisable cationic lipids, which ensure the mRNA is efficiently delivered into host cells (Rijkers et al., 2021, Patel et al., 2022, Zhang et al., 2023). After the lipid nanoparticle is injected intramuscularly, it attaches to host cells and undergoes endocytosis, whereby the mRNA is inserted into the cytoplasm and is delivered to ribosomes. Ribosomes translate the mRNA to synthesise the viral S-protein, which is either released from the cell (Route 1) or degraded by proteasomes to generate antigenic peptide epitopes (Route 2), as described:

(Route 1) The secreted exogenous proteins are endocytosed by professional APCs such as DCs, which process and present the antigens using MHC class II. (Rijkers et al., 2021). The TCR of T-helper (Th) cells bind to the presented S-protein antigens, and their CD4 protein interacts with the MHC-II complex, resulting in the production of cytokines including IL-2, IL-4, and IL-5. These cytokines can drive the differentiation of B-cells into plasma-cells which produce antibodies to the viral S-protein – neutralizing and destroying the virus. The interleukins also stimulate Th-cells to proliferate and differentiate into effector and memory T-cells (Mascellino et al., 2021). Clinical studies have found successful inducement of antibody responses to both the full-length S2-protein and RBD (Patel et al., 2022).

(Route 2) Antigenic peptide epitopes generated are exported to the ER and loaded onto MHC class I molecules, to be expressed on the cell surface (Wadhwa et al., 2020). The TCR of cytotoxic CD8⁺ T-cells interact with the MHC-1 complex and, with appropriate co-stimulation, they become activated, characterised by IFN γ , IL-2, and TNF production (Wadhwa et al., 2020, Bettini and Locci, 2021, Rijkers et al., 2021). A SARS-CoV-2 specific CD8⁺ T-cell response is elicited following BNT162b1 vaccination in humans (Sahin et al., 2020) and BNT162b2 in macaques (Vogel et al., 2020, Vogel et al., 2021), and mice (Lu et al., 2020, Vogel et al., 2021).

Oxford/AstraZeneca – Vaxzevira (ChAdOx1-S [recombinant])

The AstraZeneca vaccine is a viral vector vaccine, in which a modified replication deficient chimpanzee DNA adenovirus was genetically engineered to encode the SARS-CoV-2 S-protein, which evokes an immune response solely to the S-protein and not to the adenovirus itself. The adenovirus is injected intramuscularly, and latches onto host cells. The DNA is released into the host cell cytoplasm and migrates to the nucleus, where host enzymes convert the DNA into mRNA. The mRNA migrates to free and ER-bound ribosomes, which translate the mRNA to the S-protein. The S-protein is expressed on the cell membrane and is presented by MHC-I and MHC-II complexes, again leading to T-cell and B-cell activation and the production of antibodies (Mascellino et al., 2021).

Effectiveness of Vaccination

In December 2020, a randomized placebo-controlled, observer-blinded, pivotal efficacy trial consisting of 43 548 participants across 152 clinical sites within the United States, Argentina, Brazil, Germany, Turkey, and South Africa reported a two-dose regimen of BNT162b2 conferred 95% protection in preventing COVID-19 (Polack et al., 2020). However, limitations of this landmark phase 2/3 clinical trial included little ethnic diversity within cohorts – of the recruited donors only 10% were black, 5% Asian and 26% Hispanic/Latinx. Additionally, most donors were aged over 18, with 25 427

aged between 18 – 55, and 17 879 aged over 56 (Pfizer, 2021). The integrity of the data collected during the trial has also been brought into question with accusations of mislabelled laboratory samples, inappropriate storage of vaccines at the wrong temperature and deviation in protocols (Thacker, 2021). Following this clinical trial, early observational real-world data demonstrated that BNT162b2 is highly effective against SARS-CoV-2 infection related hospitalization and death (Amit et al., 2021) and reducing the viral loads in infected individuals (Petter et al., 2021). Studies have reported 70% effectiveness in preventing symptomatic and asymptomatic infection 21 days after a single dose in health-care workers (Hall et al., 2021), 91% effective against COVID-related hospital admissions 28 days after the first dose in Scotland (Vasileiou et al., 2021), and 92% effective in protecting against symptomatic COVID following two-doses in Israel (Dagan et al., 2021). Although immune protection wanes over time, booster vaccinations restore protection (Andrews et al., 2022b, Grewal et al., 2023).

Similarly, the ChAdOx1 vaccine is highly effective, with an efficacy of 70% at preventing symptomatic COVID, 14 days after the second dose (Voysey et al., 2021). A test negative case-control study of over 150 000 adults found a single dose of either vaccine has a real-world effectiveness of approximately 80% in adults aged 70 years and older (Lopez Bernal et al., 2021b) and 62% in long-term care facility residents (Shrotri et al., 2021).

To determine whether and for how long an individual is protected from SARS-CoV-2 following either natural infection or vaccination, there is a necessity to identify an easily measurable immune marker which correlates with a high level of protection.

4.2.3. Correlates of Protection to SARS-CoV-2

Previous infection with SARS-CoV-2 has been shown to provide protection against reinfection in rhesus macaques (Chandrashekar et al., 2020, Deng et al., 2020), and both BNT162b2 and ChAdOx1-S vaccines have been shown to be highly effective at reducing the risk of hospitalisation, severe disease, and death due to COVID-19 (Polack et al., 2020, Amit et al., 2021, Hall et al., 2021, Petter et al., 2021). However, there is substantial evidence that immune protection against COVID-19 declines over time. The number of antibodies (Ibarrondo et al., 2020, Patel et al., 2020, Self et al., 2020, Seow et al., 2020, Gaebler et al., 2021, Morales-Núñez et al., 2021, Tut et al., 2023), antibody neutralising activity, and T-cell immune response (O'Shea et al., 2023) tends to decrease over time, and the risk of COVID-19 reinfection increases (Vega-Magaña et al., 2022). There is a necessity to identify a traceable marker which determines the immune status of an individual to SARS-CoV-2.

From the start of the pandemic, national newspaper headlines raised the questions of the world's population, such as "How long does Covid immunity really last?" – The Telegraph, and "Covid reinfections in the UK: how likely are you to catch coronavirus again?" – The Guardian (Gallagher, 2021, Hardy, 2021, Geddes, 2022). To answer these questions, scientists continue to investigate immune markers which statistically correlate with a level of protection that prevents a clinically relevant outcome, known as correlates of protection (CoP) (Gilbert et al., 2022a, Misra and Theel, 2022, Escudero-Pérez et al., 2023). Identifying easily testable CoPs for SARS-CoV-2 would be invaluable in assessing whether an individual is at risk of symptomatic disease and requires vaccination or revaccination, accurately ascertaining population-level immunity, informing immunisation programmes, and developing recommendations on public health and social measures (Misra and Theel, 2022, Mcvernon and Liberman, 2023). A validated CoP would also prove invaluable in the approval and clinical validation of new vaccines to SARS-CoV-2, assessing efficacy of existing vaccines in new populations and against other variants of concern (VOC), in addition to evaluating vaccine regimen modifications (Gilbert et al., 2022b).

4.2.3.1. Antibodies as Correlates of Protection

Humoral Immune Response to SARS-CoV-2

During SARS-CoV-2 infection and vaccination, most individuals mount a robust humoral response and generate IgM, IgG, and IgA binding antibodies and neutralising antibodies (NAb) (Lau et al., 2021, Goldblatt et al., 2022). Infection and vaccine-elicited highly potent monoclonal antibodies (Seow et al., 2020) and NAb predominantly recognise epitopes of the S protein (including the S1 subunit and RBD) (Amanat et al., 2020, Brouwer et al., 2020, Suthar et al., 2020, Wajnberg et al., 2020, Pang et al., 2021) and the N protein (Okba et al., 2020), as they are the most immunogenic of the four SARS-CoV-2 structural proteins (Luchsinger et al., 2020, Okba et al., 2020).

The humoral response to infection or vaccination can be divided into two phases, including the extrafollicular (EF) response and the germinal centre (GC) phase (Lau et al., 2021). The EF responses occur within a few days, are T-cell independent and refer to rapid proliferation and differentiation of B-cells to short-lived plasma cells which secrete low-affinity antibodies (Chen et al., 2022a). IgM NAb to SARS-CoV-2 are produced during the EF B-cell response (Lau et al., 2021); the titre of IgM antibodies increases first (Assadiasl et al., 2020). The GC phase occurs days to a week later within secondary lymphoid organ such as lymph nodes or the spleen, and can last for months (Qi et al., 2022). The GC phase refers to antigen-primed B and T-cell dependent B-cell clonal expansion,

somatic hypermutation and class switch recombination to generate long-lived plasma cells or memory cells which produce high-affinity antigen-specific antibodies (Choi and Morel, 2020, Morales-Núñez et al., 2021, Gupta and Jaiswal, 2022). High affinity IgG and IgA-switched NAb to SARS-CoV-2 are produced during the GC reaction (Assadiasl et al., 2020, Lau et al., 2021).

Within 2-3 weeks post-symptom onset (PSO), seroconversion occurs in over 90% of individuals and virus-specific IgM, IgG (Isho et al., 2020, Long et al., 2020, Prévost et al., 2020, Self et al., 2020, Zhao et al., 2020), and IgA (Seow et al., 2020) have been generated. The time of seroconversion varies between individuals (Seow et al., 2020) as RBD-specific IgG responses have been shown to occur in some individuals as early as six days (Suthar et al., 2020), and nine days (Premkumar et al., 2020) after PCR-test confirmed SARS-CoV-2 infection. Serum NAb titres peak around day 23 PSO/post-vaccination (Abani et al., 2021, Qi et al., 2022). IgG and IgM titres have been shown to plateau within six days of seroconversion (Long et al., 2020) and then decline (Ibarrondo et al., 2020, Patel et al., 2020, Self et al., 2020, Seow et al., 2020, Gaebler et al., 2021, Morales-Núñez et al., 2021, Rubio-Acero et al., 2021). Whereas IgM antibody levels decrease and become undetectable more quickly, IgG antibodies last longer (Assadiasl et al., 2020) and IgA levels are less affected (Gaebler et al., 2021).

The measured rate of antibody titre decline following infection or vaccination varies amongst studies. Dispinseri et al. (2021) found NAb titres in symptomatic patients drop after 5-8 weeks but are still present in most patients after 8 months regardless of ages and co-morbidities (Dispinseri et al., 2021). This drop is supported by Wajnberg et al. (2020) who found only a modest decline in anti-S protein titres after five months (Wajnberg et al., 2020). Dan et al. (2020) reported NAb titres decline 6 – 8 months after infection, and estimate that NAb are lost in approximately 24% of convalescent plasma donors 6 – 8 months PSO (Dan et al., 2020). In contrast, Crawford et al. (2021) reported NAb titres decline 4-fold between one to four months PSO (Crawford et al., 2021), and a small study in health-care workers found systemic neutralising IgA antibody titres may even be lost two months PSO (Marot et al., 2021). Despite anti-spike antibodies decreasing following vaccination, levels of memory B-cells have been reported to increase (Terrerri et al., 2022).

Antibody Titres as Correlates of Protection to SARS-CoV-2

Antibodies play an important role in clearing SARS-CoV-2 from the host (Wu et al., 2020a). NABs act to block ACE2-dependent viral entry into host cells mediated by the S-protein, via steric hindrance and directly competing for interface residues (Shi et al., 2020a, Morales-Núñez et al., 2021). In addition to interfering with viral uptake into host cells, NABs prevent viral genomes from uncoating

in endosomes, and cause viral particles to aggregate (Pang et al., 2021). IgG antibody titres to the S-protein (Wajnberg et al., 2020) and to the RBD (Luchsinger et al., 2020, Dispinseri et al., 2021, Nayak et al., 2021) have been shown to significantly correlate with neutralisation of the SARS-CoV-2 virus, as measured using neutralisation assays. Antibodies may also indirectly modulate acute SARS-CoV-2 disease by facilitating phagocytosis, facilitating the complement cascade, and cellular cytotoxicity (Abani et al., 2021, Morales-Núñez et al., 2021).

Strong evidence supports the hypothesis that increased serum antibody and NAb titres elicited by infection or S-protein-based vaccination correlate with a decreased risk of both re-infection and symptomatic disease (Khoury et al., 2021, Cromer et al., 2022, Fong et al., 2023, Hertz et al., 2023, Khoury et al., 2023), functioning as a CoP (Gilbert et al., 2022a, Misra and Theel, 2022, Perry et al., 2022). Many studies have reported the incidence of PCR-test confirmed SARS-CoV-2 infection is lower in seropositive individuals than seronegative individuals (Addetia et al., 2020, Harvey et al., 2021, Letizia et al., 2021, Lumley et al., 2021, Atti et al., 2022), and the presence of anti-S and anti-N IgG antibodies substantially reduces the risk of reinfection (Lumley et al., 2021, Atti et al., 2022). The risk of re-infection in seropositive young adults has been proposed to be one-fifth that of seronegative individuals (Letizia et al., 2021). The coronavirus efficacy phase 3 clinical trial supports these findings, reporting increased titres of NAb and binding antibodies following vaccination were inversely associated with COVID-19 risk, and the risk of symptomatic COVID-19 to be ten times greater in individuals with undetectable or negative antibody levels (Gilbert et al., 2022b). This study was corroborated by a small study on a fishing vessel which found that pre-existing NAb titres protected against re-infection (Addetia et al., 2020). Khoury et al. (2021) estimated that a neutralisation level of 20.2% of mean convalescent level is required for 50% protection from symptomatic SARS-CoV-2 infection (Khoury et al., 2021).

Additional evidence clearly supporting the protective role antibodies provide against SARS-CoV-2 includes anti-spike NAb have been shown *in vitro* to block infection of human cells and protect against *in vivo* viral replication (Dispinseri et al., 2021). NAb titres within the first few weeks of infection correlate with the time to viral clearance (negative RT-PCR result), whereas an absence of NAb correlates with mortality (Dispinseri et al., 2021). Furthermore, passive transfer of potent neutralising antibodies to antigens such as the RBD and non-RBD epitopes of the S-protein provides protection to high-dose SARS-CoV-2 challenge in many animal models including naïve rhesus macaques (Zost et al., 2020, McMahan et al., 2021), cynomolgus macaques (Cobb et al., 2022), and Syrian hamsters (Rogers et al., 2020, Corbett et al., 2021, Tostanoski et al., 2022). As such, use of convalescent sera therapy received emergency use authorisation from the FDA in August 2020 (Gupta

and Jaiswal, 2022). Furthermore, monoclonal NAb which bind the S-protein RBD to neutralise the virus have been approved for therapeutic interventions such as REGN-COV-2 (Gupta and Jaiswal, 2022).

However, the efficacy of convalescent plasma antibodies has been inconsistent. Early use of convalescent plasma containing higher anti-SARS-CoV-2 IgG antibody titres has been shown to improve clinical status (Shen et al., 2020, Libster et al., 2021) and be associated with reduced mortality risk than transfusion of low antibody titre plasma in hospitalised COVID-19 patients (Joyner et al., 2021). In contrast, a large randomised controlled, open-label trial which recruited over 16 000 patients found the mortality rate ratio was not improved following treatment with high-titre convalescent plasma (Abani et al., 2021). This finding is corroborated by a systematic review and meta-analysis of 19 randomised clinical trials which found no statistical benefit of using convalescent plasma on all-cause mortality (Mihalek et al., 2023). Some studies suggest that patients with a higher IgG antibody titre is associated with a more severe clinical classification (Zhang et al., 2020, Zhao et al., 2020), possibly due to antibody-dependent enhancement of SARS-CoV-2 infection (Cao, 2020) by pro-inflammatory mechanisms such as Fcγ stimulation (Lee et al., 2020).

Measuring Antibody Titres as Correlates of Protection and Their Limitations

To determine the seroprevalence of individuals in a population, a large number of tests, platforms and methodologies are used to measure antibody titres, though the majority measure the level of IgM and IgG antibodies against S and N proteins (Assadiasl et al., 2020). Serological assays include enzyme-linked immunosorbent assays (ELISA), rapid antibody assays, (Luchsinger et al., 2020), chemiluminescence assays (Zhang et al., 2020, Camacho et al., 2023), western blotting (Ciotti et al., 2020), high-throughput serological assays (Luchsinger et al., 2020) and virus neutralisation assays (Assadiasl et al., 2020). ELISA tests are considered the gold-standard method of measuring antibody titres, as they are widely flexible and can measure any antigen of interest, thus have been used to quantify antibody titres to SARS-CoV-2 in a multitude of studies (Isho et al., 2020, Luchsinger et al., 2020, Okba et al., 2020, Premkumar et al., 2020, Shen et al., 2020, Zhao et al., 2020, Crawford et al., 2021).

High sensitivities and low false-positive rates have been reported for commercially available antibody assays; different immunoassays report sensitivities and specificities between 60 – 100%, depending on the assay manufacturer and the SARS-CoV-2 variant (Meschi et al., 2020, Pickering et al., 2020, Valdivia et al., 2021, Walker et al., 2021, Camacho et al., 2023). However, there are limitations of antibody assays. For example, the vast majority of serological assays determine the

quantity of serum RBD-Abs and S-Abs and do not determine their functionality or whether they provide protection against infection (Gupta and Jaiswal, 2022). ELISAs are also labour-intensive and require expertise (Sakamoto et al., 2018, Cerda-Kipper et al., 2019). Assays targeting the RBD and N-proteins have a greater sensitivity and correlation with neutralisation titres (Walker et al., 2021), but the gold-standard method of evaluating the neutralisation capacity of antibodies to SARS-CoV-2 is the plaque reduction neutralisation test (PRNT). PRNTs quantify levels of NAbs during viral attachment and host cell entry, but they are challenging to perform, have long turnaround times, and necessitate live viruses – requiring highly skilled and experienced researchers to use a biosafety level 3 laboratory (Morales-Núñez et al., 2021). Lastly, there is no agreed upon ‘threshold’ which correlate with a reduced risk of symptomatic disease (Misra and Theel, 2022). Peak antibody concentrations reached vary between individuals, with reports of 25-fold differences in levels elicited by vaccination (Cromer et al., 2022), and titres also depend on the time since infection, disease severity (Seow et al., 2020, Wu et al., 2020a) and variant (Yadav et al., 2021). It is very important to also consider the cellular immune response, as SARS-CoV-2 specific T-cell responses have been shown to provide sufficient protection in antibody-seronegative individuals (Seow et al., 2020, GeurtsvanKessel et al., 2022).

4.2.3.2. Cellular Immune Responses as Correlates of Protection

Although the vast majority of research into CoP focus on antibody-mediated protection, growing evidence demonstrates that cellular immune responses provide fundamental protection from SARS-CoV-2, and clearance of SARS-CoV-2 infection relies on CD4⁺ and CD8⁺ T-cell responses as well as NAb action (Kent et al., 2022).

T-Cell Immune Response to SARS-CoV-2

Strong evidence shows asymptomatic, mild, or severe SARS-COV-2 infection induces robust CD4⁺ T-cell and CD8⁺ T-cell responses (Grifoni et al., 2020, Ni et al., 2020a, Sekine et al., 2020, Rodda et al., 2021). Studies have shown 93% of convalescent patients possess detectable circulating CD4⁺ T cells specific to SARS-CoV-2 S-protein, N-protein, and M-protein (Dan et al., 2020, Grifoni et al., 2020, Rydzynski Moderbacher et al., 2020), and 50% of acute COVID-19 cases (Rydzynski Moderbacher et al., 2020). Virus specific follicular T-cells, required for IgG and high quality NAb responses, make up a significant proportion of SARS-CoV-2 specific CD4⁺ cells in acute and convalescent COVID-19 (Rydzynski Moderbacher et al., 2020). In addition to serum antibodies, memory T and B cell responses

are important in the protection of SARS-CoV-2 infection and modulate SARS-CoV-2 severity (Grifoni et al., 2020, Khoury et al., 2021, Sette and Crotty, 2021, Kent et al., 2022). Memory T-cell responses have been shown to occur following mild infection, as evident by the formation of T-dependent IgG RBD-specific memory B-cells (Rodda et al., 2021). Memory T-cell responses present within a few weeks PSO (Law et al., 2022), with SARS-CoV-2 specific CD4⁺ T-cells detected as early as day four PSO (Rydzynski Moderbacher et al., 2020) and are still present in more than 90% of subjects more than 6 months PSO (Dan et al., 2020, GeurtsvanKessel et al., 2022). Robust CD8⁺ T-cell responses also occur, and 70% of recovered COVID-19 patients establish CD8⁺ memory responses (Grifoni et al., 2020) within one-month PSO, decreasing to 50% 6 – 8 months PSO (Dan et al., 2020). Longitudinal data published by Gangaev et al. (2021) support this, reporting that SARS-CoV-2 specific CD8⁺ T-cell responses in severe COVID patients remain five months following discharge from hospital, and a transition to functional memory T-cells (Gangaev et al., 2021).

Robust CD4⁺ and CD8⁺ T-cell responses are also elicited by vaccination (Sadarangani et al., 2021, Vogel et al., 2022). Sahin et al. (2020) demonstrated that donors vaccinated with BNT162b1 possess strong CD4⁺ and CD8⁺ T cell responses to peptides encoding the SARS-CoV-2 RBD, with peptide stimulation triggering IFN γ and IL-2 secretion in both RBD-specific CD4⁺ and CD8⁺ fractions (Sahin et al., 2020). IFN- γ is a cytokine produced and secreted by activated CD4⁺ and CD8⁺ T-cells, that acts to activate macrophages, up-regulate MHC I and MHC II, and induce IgG production in activated B-cells (Yang et al., 2022). In healthy adults, Sahin et al. (2021) found robust expansion of SARS-CoV-2 S-specific CD4⁺ T-cells in all participants, with strong IFN γ ⁺ or IL-2⁺ indicative of CD4⁺ T-helper type 1 cell phenotype. Moreover, 90% participants mounted de novo CD8⁺ T-cell responses following BNT162b2 booster vaccination, and both CD4⁺ and CD8⁺ cells were still detectable nine weeks after (Sahin et al., 2021). Polyfunctional CD4⁺ T-cells have been shown to persist 141-210 days following the second SARS-CoV-2 vaccination (Vogel et al., 2022).

The major CD4⁺ and CD8⁺ T-cell epitopes include S-protein, N-protein, M-protein, and open reading frame 3 (Dan et al., 2020) (Le Bert et al., 2020, Law et al., 2022), though Thieme et al. (2020) identified the M-protein induces the highest frequencies of CD4⁺ T-cells. Peng et al. (2020) identified 41 peptides containing CD4⁺ and CD8⁺ epitopes, and Gangaev et al. (2021) identified 18 epitopes recognised by CD8⁺ T-cells, including eight specific for SARS-CoV-2 and 10 shared with SARS-CoV-1. Vaccination elicits T-cell recognition to SARS-CoV-2 epitopes across the length of the S-protein (Sahin et al., 2021).

Cellular Immunity Correlate of Protection

Many studies indicate T-cell responses correlate with protection against SARS-CoV-2 infection (Escudero-Pérez et al., 2023), with SARS-CoV-2 specific CD4⁺ and CD8⁺ T-cells being associated with decreased COVID-19 severity (Rydzynski Moderbacher et al., 2020). The contribution of T-cell responses during infection or vaccination means despite lower levels of NAb titres, an individual may have sufficient protection from COVID-19 (Seow et al., 2020), as shown in seronegative individuals who possess SARS-CoV-2 specific T-cells and experience asymptomatic or mild COVID-19 (GeurtsvanKessel et al., 2022). Early induction of IFN- γ secreting SARS-CoV-2 specific T-cell responses and increased titres of airway T-cells have also been shown to correlate with earlier viral control, decreased disease severity and improved clinical outcomes (Tan et al., 2021, Law et al., 2022). Absolute numbers of CD4⁺ and CD8⁺ T-cells have been reported to be lower in patients with moderate or severe COVID-19, and predict to a high sensitivity and specificity COVID-19 severity (He et al., 2020, Liu et al., 2020, Sekine et al., 2020).

Evidence suggests CD8⁺ T-cells are important effector cells which function in clearing SARS-CoV-2 infections. Vaccine-induced CD8⁺ T-cells have been proposed to mediate early protection, as protection against SARS-CoV-2 has been shown to occur as early as ten days after mRNA vaccination, at which point NAb titres are very low. Oberhardt et al. (2021) supported this hypothesis and demonstrated robust expansion and mobilisation of stable and fully functional CD8⁺ T-cells within one week of BNT162b2 vaccination when NAb and circulating CD4⁺ T-cells were barely detectable (Oberhardt et al., 2021). In addition, McMahan et al. (2021) found depletion of CD8⁺ T-cells in rhesus macaques resulted in greater viral loads and virus breakthrough during SARS-CoV-2 challenge (McMahan et al., 2021). Furthermore, studies have shown higher proportions of CD8⁺ T-cells observed in mild disease (Peng et al., 2020), with the magnitude of the CD8⁺ response inversely associated with the severity of COVID-19 (Peng et al., 2020, Sekine et al., 2020, Cohen et al., 2021, Peng et al., 2022). However, this was not repeated in a study by Thieme et al. (2020), who found the S-, M-, and N-proteins induce CD4⁺ dominant T-cell responses, and that the T-cell response of non-critical patients was equal or even less than that of robust responses measured in critical COVID-19 patients (Thieme et al., 2020).

CD4⁺ T-cell responses are more commonly detected than CD8⁺ (Law et al., 2022). The success of vaccination relies on CD4⁺ T-cell responses, which promote durable antibody responses, NAb responses, and affinity-matured B-cell memory (Rydzynski Moderbacher et al., 2020). CD4⁺ T-cells also contribute to antibody-independent responses to promote antiviral immunity such as IFN- γ production, which has been demonstrated to protect mice against lethal SARS-CoV challenge (Zhao et

al., 2016a). NAb responses are dependent on CD4⁺ T-cells (Grifoni et al., 2020), demonstrated by NAb titres correlating with N-protein specific T-cells (Ni et al., 2020a). In PBMCs, Peng et al. (2020) identified significant correlations between overall T-cell responses and S-specific T-cell responses with S-specific antibody titres, RBD-specific antibody titres, and NP-specific antibody titres (Peng et al., 2020). Levels of circulating follicular helper T-cells (cTFH) elicited after SARS-CoV-2 infection also correlate with S-specific B-cell and antibody responses (Juno et al., 2020). Patients with the highest neutralising activity have increased frequencies of certain cTFH subsets, suggesting frequencies of specific B-cell and S-specific cTFH phenotypes could be used as biomarkers (Juno et al., 2020).

Measuring Cellular Immune Responses

Use of functional T-cell responses as CoP in large-scale applications has been hindered by a lack of standardized methods (da Silva Antunes et al., 2023). Multiple techniques using SARS-CoV-2 derived peptides, or epitopes can be used to measure antigen-specific T-cell activation and function to SARS-CoV-2, including enzyme-linked immunosorbent spot (ELISpot) assays (Sahin et al., 2021, Vogel et al., 2022), intracellular cytokine staining (ICS) (Sahin et al., 2020, Vogel et al., 2022), IFN- γ release assays (IGRAs) (Peng et al., 2020, Vogel et al., 2022) and activation induced marker (AIM) assays (Kent et al., 2022) among others. However, these methodologies each harbour limitations with regards to their technical complexity, affordability, throughput, or depth of analysis (da Silva Antunes et al., 2023, Gondré-Lewis et al., 2023). ELISpot assays are one of the most commonly used assays and are a robust and highly quantitative method of measuring cytokine secretion at the single-cell level (Sloata et al., 2011). Although ELISpots are relatively high throughput and cost-effective, their sensitivity is limited and does not inform the phenotype of the responding CD4⁺ and/or CD8⁺ T-cells (Negri et al., 2020, Gondré-Lewis et al., 2023). Considering the incubation durations, ELISpot assays are also time-intensive and take days to perform, thus are less easily adoptable on a widescale (Ansari and Strom, 2010). The AIM assay detects early responding T-cells which are independent of cytokine production (Peng et al., 2020), whereas ICS assays measure functional responses as they measure cytokine production at the single-cell level (Gong et al., 2022). However, both methods are relatively laborious and require expensive cell-cytometry equipment (da Silva Antunes et al., 2023).

4.2.3.3. Electrical Changes in PBMCs as a Potential Correlate of Protection

As increased T-cell functionality, memory cell populations, and NAb action each correlate with a reduced risk of catching SARS-CoV-2, earlier viral control, decreased disease severity and improved clinical outcomes, other methods which quantify these immune functions could serve as a CoP. Given

that the changes in the electrical properties of T-cells and B-cells following TCR and BCR activation, such as increased intracellular Ca^{2+} ion concentrations (described in detail in **Section 2.2.7.**), it was hypothesised that the electrical response of PBMCs to stimulation with SARS-COV-2 would be different in individuals who possess immune memory. As DEP is a fast, low-cost, and easily adoptable technique, I investigated whether differences in the electrical properties of PBMCs following stimulation with SARS-CoV-2 specific antigens could pave the way towards a new CoP to SARS-CoV-2. The zetasizer machine was unavailable for the duration of these experiments, so ζ -potential data was unfortunately not collected in parallel.

4.3. Methods

4.3.1. Ethical Approval

A favourable ethical opinion was given to this study by the University of Surrey Ethics Committee (UEC/2017/052/FHMS, FHMS20/21 192 EGA).

4.3.2. Overview of Experiments

An overview and timeline of the experiments conducted to determine whether changes in the electrical properties of PBMCs following SARS-COV-2 stimulation could be used as a potential CoP is displayed below in **Figure 39**.

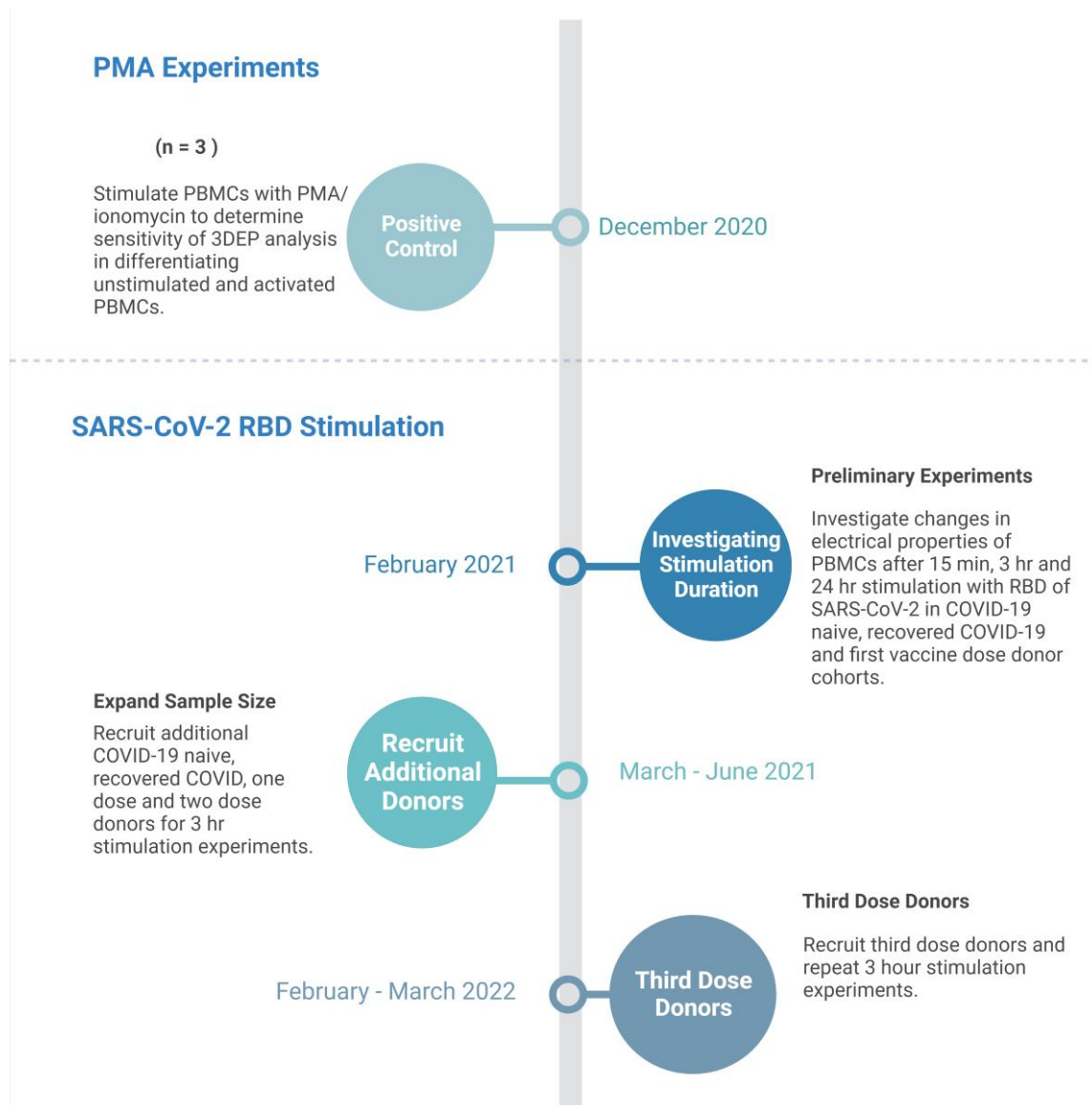


Figure 39| Timeline and overview of experiments conducted to achieve chapter aims and objectives. The dashed line separates the preliminary experiments exploring proof of concept and the start of the *in vitro* SARS-CoV-2 experiments. (Created with BioRender.com).

4.3.3. Subjects

Phorbol 12-myristate 13-acetate /Ionomycin Experiments

Three donors were recruited for *in vitro* phorbol 12-myristate 13-acetate/ ionomycin (PMA/Ionomycin) PBMC stimulation experiments. Donor characteristics of study participants are detailed below in **Table 9**. All donors were screened via a self-reported health issues questionnaire and were considered healthy and taking no medication known to affect their immune system.

Table 9 | Baseline characteristics of n = 3 donors whose PBMCs were isolated and stimulated for three hours with PMA/ionomycin. Continuous variables are expressed as mean ($\pm$ SD) and categorical variables as frequency (fractions of the donor cohort).

	n = 3
Age (years)	32 ($\pm$ 5)
Gender	Female (2/3)
Ethnicity	White (3/3)

SARS-CoV-2 Stimulation Experiments

Changes in the electrical properties of PBMCs following incubation with the SARS-CoV-2 spike protein was investigated in a total of n = 18 different donors. Again, all donors were screened via a self-reported health issues questionnaire and considered healthy and taking no medication known to affect their immune system. Participants were categorised into four donor cohorts, including COVID-19 Naïve, Recovered COVID-19, First Dose, Second Dose, and Third Dose donors. COVID-19 Naïve donors had not experienced symptoms of COVID-19 since the outbreak of the pandemic and had a negative rapid antibody test (SureScreen Diagnostics COVID-19 Antibody Rapid Test) for IgM and IgG antibodies on the day prior to blood donation. Recovered COVID-19 donors were participants who had previously had COVID-19, confirmed by a positive PCR test, with symptoms lasting 3-4 weeks. All had negative lateral flow tests for COVID-19 on the day prior to blood donation and were not experiencing symptoms of long COVID. First Dose donors were participants who had received the first dose of either the Pfizer/BioNTech or AstraZeneca vaccine within the last 14 days, Second Dose participants had received the second dose of a Pfizer/BioNTech or AstraZeneca vaccine, and Third Dose participants had received the third dose of the Pfizer vaccine. Of these 18 donors, nine donated multiple times in different donor cohorts; for example, one donor donated in the COVID-19 Naïve, First Dose, Second Dose, and Third Dose donor cohorts. Characteristics of participants recruited for each experiment including age, gender, time between natural infection/vaccination and experiment, and which COVID-19 vaccine the donor received is outlined in **Table 10**. Unfortunately, due to difficulties in donor recruitment, laboratory access, and availability of recruiting donors from a specific donor cohort, the number of donors in each cohort is very limited, especially first dose donors.

Table 10 | Baseline characteristics of COVID-19 naïve (n = 8), recovered COVID-19 (n = 3), first dose (n = 2), second dose (n = 5), and third dose (n = 8) donor cohorts whose PBMCs were isolated and stimulated for three hours with the RBD of the COVID-19 spike protein. Continuous variables are expressed as mean ($\pm$ SD) and categorical variables as frequency (fractions of the donor cohort).

	COVID-19 Naïve (n = 8)	Recovered COVID-19 (n = 3)	First Dose (n = 2)	Second Dose (n = 5)	Third Dose (n = 8)
Age (years)	37 ($\pm$ 11)	28 ($\pm$ 1)	26 ($\pm$ 2)	31 ($\pm$ 14)	29 ($\pm$ 8)
Gender	Female (6/8)	Male (2/3)	Female (2/2)	Female (4/5)	Female (4/8)
Ethnicity	White (7/8)	White (3/3)	White (2/2)	White (4/5)	White (6/8)
Time Since Symptom Onset (days)	-	58 ($\pm$ 12)	-	-	-
Time Since Latest Vaccination (days)	-	-	12 ($\pm$ 2)	38 ($\pm$ 21)	65 ($\pm$ 20)

4.3.4. Isolation of Peripheral Blood Mononuclear Cells from Whole Blood

Isolation of PBMCs from whole blood (**Section 4.3.4.**) and PBMC stimulation (**Section 4.3.5.**) was performed by Dr Alexander Stewart and Emma Sinclair following aseptic techniques. Peripheral whole blood was collected from all donors by a qualified phlebotomist via venepuncture using BD-Bioscience lithium heparin vacutainers. Complete media RPMI-1640 medium with L-glutamine (ThermoFisher, UK, 11875093) supplemented with 10% foetal calf serum (Sigma-Aldrich, UK, F9665) and 1% penicillin-streptomycin (Sigma-Aldrich, UK, #11074440001; 50 000 U/mL penicillin, 50 mg/mL streptomycin solution) was mixed with freshly collected whole blood 1:1. The diluted blood sample was carefully layered on top of Ficoll-paque (GE-healthcare, UK) and centrifuged 400 g, 30 mins, acceleration/deceleration = 2, to isolate PBMCs from the whole blood via standard density centrifugation. A P1000 Gilson pipette was used to remove and aliquot the buffy layer into falcon tubes, which were topped up with complete media and spun at 400 g, 15 mins, acceleration/deceleration = 8. The supernatant was discarded, resuspended in complete media, and 10 μ L removed for cell counting using the C-Chip Disposable Haemocytometer, DHC-N01 (Labtech, Uckfield, UK), following the C-Chip data collection method outlined in **Section 3.3.6.** and cell concentration analysis method outlined in **Section 3.3.7.** PBMC concentration was subsequently amended to 10×10^6 cells/ mL suspended in complete media, and 100 μ L containing 1×10^6 cells were aliquoted into the required number of wells in a 96-well plate for PBMC stimulation.

4.3.5. PBMC Stimulation

Control Incubation: PBS (Sigma-Aldrich, UK, D8537) was added as a vehicle control, after which the cells were incubated at 37 °C, 5% CO₂ for 15 minutes, 3 hours, or 24 hours.

Stimulation with PMA/Ionomycin: PBMCs were stimulated with 200 ng/mL PMA and 1 µM ionomycin (PMA/ionomycin; Sigma-Aldrich, UK) in complete media for three hours at 37 °C, 5% CO₂.

SARS-CoV-2 Spike Glycoprotein Receptor Binding Domain: PBMCs were stimulated with 500 ng/mL recombinant human coronavirus SARS-CoV-2 Spike Glycoprotein RBD (Active) (Abcam, UK, ab273065) in complete media at 37 °C, 5% CO₂ for 15 minutes, 3 hours, or 24 hours at 37 °C, 5% CO₂.

SARS-CoV-2 Buffer Control: PBMCs were treated with 1.02% imidazole (Sigma-Aldrich, UK, I2399), 0.82% Na₃PO₄ (Sigma-Aldrich, UK, 342483), and 1.74% NaCl (Sigma-Aldrich, UK, S9888) – the same concentrations as within the SARS-CoV-2 Spike Glycoprotein RBD stimulant buffer (Abcam, UK, ab273065) in complete media for 3 hours at 37 °C, 5% CO₂.

4.3.6. Data Collection

Following 15-minute, 3-hour, or 24-hour stimulation, cell suspensions were transferred from the cell plates to Eppendorf® Safe-Lock microcentrifuge tubes and spun at 400 g, 5 mins, acceleration/deceleration = 8. The supernatant was discarded, and the cell pellet resuspended in 1 mL DEP medium (prepared following the protocol in **Section 3.3.3.**, supplemented with PBS to a conductivity of 102 mS/m). The cell suspension was spun again at 400 g for 5 mins, and the cell pellet resuspended in DEP medium at a volume required for a cell concentration of 1 x 10⁶ cells/ mL. (When trialling the exclusion of the wash step to streamline the protocol for clinical applications, after the first centrifugation the cell pellet was immediately resuspended in DEP medium at a volume required for 1 x 10⁶ cells/ mL).

3DEP Data Collection

The electrophysiological parameters of PBMCs were subsequently measured using the 3DEP (DEPtech, UK) following the 3DEP Data Collection methods outlined in **Section 3.3.6.** A minimum of three technical repeats were measured for each sample.

C-Chip Data Collection

Simultaneously to 3DEP data collection, PBMCs injected into the C-Chip Disposable Haemocytometer, DHC-N01 (Labtech, Uckfield, UK) were analysed under the Nikon TS100 inverted phase contrast microscope and photographed under x10 and x20 magnification using Vimba Viewer (V2.4.0, Allied Vision) for cell radius and cell concentration data analysis.

4.3.7. Data Analysis and Statistical Methods

3DEP Data Analysis

Raw data collected by the 3DEP was analysed using the 3DEP Data method outlined in **Section 3.3.7.** to obtain values of G_{eff} , C_{eff} , and σ_{cyto} from DEP spectra modelled with an R squared value of above 0.8.

Cell Radius Analysis

The average cell radius of 100 PBMCs per sample was analysed using ImageJ (National Institutes of Health, V1.54d), as described in **Section 3.3.7.**

Data Analysis and Statistical Analysis

All statistical analysis was conducted in GraphPad Prism 9 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com).

Comparing the Baseline Electrical Properties Between Donor Cohorts

To assess whether baseline values of G_{eff} , C_{eff} , σ_{cyto} , and cell radius were normally distributed, the D'Agostino & Pearson ("omnibus K2") test for normality ($p > 0.05$) was used, because it a versatile and powerful test which computes how far from Gaussian distribution values are based on skewness and kurtosis (Motulsky, No Date). For non-normally distributed data, the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test was used. For normally distributed data, a one-way ANOVA test followed by Tukey's multiple comparisons were used to assess the differences in the baseline electrical properties between donor cohorts because it is a powerful post-hoc analysis.

Simple linear regression models fitted using least squares regression were used to assess the trend in the baseline electrical properties of PBMCs against duration of time between the date of the experiment and the date of symptom onset or date of most recent vaccination. The coefficient of the

slope, the 95% CI, and the p-value against the null hypothesis that the overall slope is equal to zero (*<0.05, **<0.01) were considered. The goodness-of-fit of the regression model was calculated using the sum of squares of the vertical distances between the best-fit line and the datapoints (SS_{reg}), and the vertical distance between the null-hypothesis horizontal line and the datapoints (SS_{tot}).

$$r^2 = 1 - \frac{SS_{reg}}{SS_{tot}} \quad \text{Equation 17}$$

The r^2 value indicates the goodness-of-fit of the regression model and was used to determine the variance in G_{eff} , C_{eff} , σ_{cyto} , and cell radius attributable to the number of days between the date of the experiment and the date of COVID-exposure.

Comparing the Differences in the Electrical Response to SARS-CoV-2 Stimulation Between Donor Cohorts

To determine which stimulation duration to use, the MDVs were calculated by averaging the relative DEP force of the highest nine frequencies and subtracting from the average of the lowest nine frequencies. The stimulation duration which had the biggest difference in the MDVs of unchallenged and challenged PBMCs between donor cohorts was chosen for subsequent experiments.

Repeated measures two-way ANOVAs followed by Tukey's multiple comparisons were used to statistically compare the differences in G_{eff} , C_{eff} , σ_{cyto} , and cell radius in matched values of unchallenged and challenged PBMCs, and between unmatched donor groups. Whether the electrical properties of PBMCs incubated in control media were statistically different to incubation in SARS-CoV-2 buffer control media or SARS-CoV-2 S-protein RBD stimulation media was assessed using an ordinary one-way ANOVA followed by Tukey's multiple comparison (*p<0.05). MDVs and ABCD values (ABCD data analysis previously described in **Section 3.3.7.**) were also calculated to compare donor cohorts.

To determine whether the electrical response to RBD-stimulation correlated with the duration of time between the date of the experiment and the date of symptom onset or the date of vaccination, simple linear regression was used. The coefficient of the slope, the 95% CI, and the p-value against the null hypothesis that the overall slope is equal to zero (*<0.05, **<0.01) were considered. The goodness-of-fit of the regression model was calculated using **Equation 17** as above.

To determine whether differences in the electrical properties of PBMCs between donor cohorts performed in line with or better than the CoP proposed in published studies, a ROC curve was plotted in GraphPad Prism 9. The 'best' threshold of optimal sensitivity and specificity at differentiating COVID-19 naïve individuals to third dose donors was the threshold closest to (0.0,

100.0) on the ROC curve. The AUC for the ROC curve, the p-value of the AUC, the sensitivity, and specificity of the CoP at the 'best threshold' was recorded.

4.4. Results and Discussion

4.4.1. Electrophysiological Characterisation of PBMCs After Three-Hour Stimulation with PMA

Before investigating how the electrical properties of PBMCs change in COVID-19 naïve vs. COVID-19 exposed donors following stimulation with the SARS-CoV-2 spike protein, changes in the dielectric properties of PBMCs following activation with a known activating agent was investigated as a positive control. This was to validate the 3DEP device for detecting PBMC activation and changes in electrical properties following stimulation. PBMCs isolated from three healthy donors were treated with phorbol 12-myristate 13-acetate (PMA)/ionomycin for three hours, after which the changes in G_{eff} , C_{eff} , and σ_{cyto} were analysed using the 3DEP.

Values of G_{eff} , C_{eff} , and σ_{cyto} for unstimulated and PMA/ionomycin stimulated PBMCs were obtained from MATLAB-modelled DEP spectra with a goodness-of-fit r^2 greater than 0.9 and are recorded in **Table 11**.

Table 11 | Electrophysiological parameters of PBMCs from healthy donors (n = 3) in control vs. PMA/ionomycin stimulated cells as measured by the 3DEP. (Mean $\pm$ SEM).

	Unstimulated	PMA/Ionomycin Stimulated	Paired two-tailed t-test
G_{eff} (kS/m ²)	0.62 $\pm$ 0.58	5.29 $\pm$ 1.41	* $p = 0.0159$
C_{eff} (mF/m ²)	7.63 $\pm$ 0.26	7.38 $\pm$ 0.21	ns
σ_{cyto} (S/m)	0.36 $\pm$ 0.043	0.25 $\pm$ 0.014	ns
Cell radius (μ m)	3.13 $\pm$ 0.021	3.21 $\pm$ 0.1	ns

Average values of G_{eff} , C_{eff} , and σ_{cyto} for unstimulated and PMA/ionomycin stimulated PBMCs of each biological repeat are plotted in **Figure 40**. G_{eff} significantly increased following PMA/ionomycin stimulation from 0.62 $\pm$ 0.58 kS/m² to 5.29 kS/m² $\pm$ 1.41 (paired two-tailed t-test, $p = 0.0159$), whereas both C_{eff} and σ_{cyto} decreased though not significantly ($p = 0.5050$ and $p = 0.1009$ respectively). Together these results demonstrate PBMCs stimulated with PMA/ionomycin can be differentiated from unstimulated PBMCs based on the changes in their electrical properties, measured by the 3DEP.

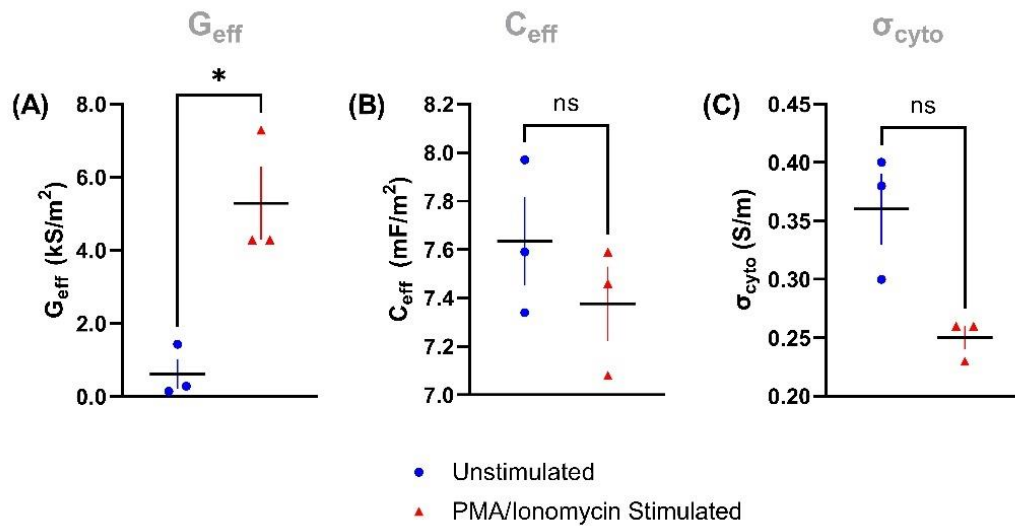


Figure 40 | Scatter plots of the electrophysiological properties of PBMCs when unstimulated (incubated in control media) vs. PMA/ionomycin stimulated for three-hours. **[A]** G_{eff} **[B]** C_{eff} **[C]** σ_{cyto} . Black lines and error bars plot mean $\pm$ SEM. Paired two-tailed t-test, G_{eff} : $p = 0.0159$ (* $p < 0.05$).

PMA/ionomycin is a potent stimulating agent of white blood cells. PMA is a phorbol diester which activates PKC, bypassing the T-cell membrane receptor complex to trigger T-cell activation and production of cytokines such as IL-2 (Ai et al., 2013). PMA is also used to stimulate differentiation of THP-1 cells into macrophages (Takashiba et al., 1999). Ionomycin is a carboxylic acid antibiotic produced by the bacterium *Streptomyces globatus* (Lomora et al., 2015). Ionomycin is a potent calcium ionophore and acts to increase the cytoplasmic concentration of Ca^{2+} in cells (Qiu et al., 2020). As a lipid soluble molecule, ionomycin binds Ca^{2+} in a 1:1 ratio, thus increasing the permeability and facilitating the transport of Ca^{2+} across the plasma membrane (Durham and Russo, 2000). Ionomycin also releases Ca^{2+} from intracellular stores without involving G proteins, resulting in the activation of calcium-activated Cl^- channels. Furthermore, ionomycin acts to release intracellular Ca^{2+} stores by the same mechanism as inositol-phosphate-3 pathway (Yoshida and Plant, 1992). Ionomycin also binds and transports other divalent cations such as Mg^{2+} , Pb^{2+} , and some lanthanide series trivalent cations more efficiently than Ca^{2+} (Erdahl et al., 2000).

The increase in G_{eff} following PMA/ionomycin stimulation could be attributable to transmembrane K^+ and Ca^{2+} conductance across the cell membrane. It is possible that an increase in Ca^{2+} ion concentration following PMA/ionomycin treatment resulted in the activation of K_{Ca} 3.1 ion channels, resulting in K^+ efflux and cell hyperpolarisation. The efflux of K^+ may have been marginally greater than the increase in cytoplasmic Ca^{2+} from influx and release from intracellular stores, resulting in a non-significant reduction in σ_{cyto} . The hypothesis of membrane hyperpolarisation is

further supported by a study by Brent *et al.* (1996) which demonstrated using the transmembrane potential sensitive dye diSBAC2-(3) that HL-60 derived monocytes treated with ionomycin exhibit membrane hyperpolarisation. Furthermore, when using the $K_v1.2$, $K_v1.3$, and Ca^{2+} -activated K^+ channel ion channel blocker charybdotoxin, the ionomycin-induced membrane hyperpolarisation was inhibited (Brent *et al.*, 1996).

The change in G_{eff} indicates that there are differences in the DEP spectra which differentiate unstimulated and PMA/ionomycin stimulated PBMCs. However, detecting differences in DEP spectra which do not require DEP spectra modelling or cell radius measurements would be advantageous for a diagnostic biomarker/ clinical device, and better suited for widespread adoptability. The DEP spectra plotting relative DEP force (arbitrary units) against log Frequency (Hz) (mean $\pm$ SEM) in control treated (blue circles, blue line) versus PMA/ionomycin treated (red triangles, red line) PBMCs is shown in **Figure 41A**. Qualitatively, the greatest difference between the two average DEP spectra can be seen at low frequencies between 15 kHz to 91 kHz, and at high frequencies between 4.92 MHz to 11.9 MHz. Given the significant increase in G_{eff} and the marked decrease in σ_{cyto} following PMA/ionomycin stimulation (**Figure 40A&C**), it is not surprising given low frequencies correspond to G_{eff} and high frequencies correspond to σ_{cyto} . The cross-over value between positive and negative DEP remained the same.

One method used to quickly gauge differences in the DEP spectra after PMA/ionomycin stimulation vs. control was by calculating the MDVs. This approach was previously explored in **Section 3.4.3.** in the ME/CFS Chapter, but to recap it is a technique whereby the average relative DEP force of the highest nine frequencies is subtracted from the lowest nine frequencies. MDVs have been used to identify absolute differences in DEP spectra, and was first outlined by Graham *et al.* (2015). More recently MDVs were used to differentiate DEP spectra of cells in urine samples from individuals with bladder cancer versus healthy controls (Graham *et al.*, 2015, Hoque *et al.*, 2020). Averaging groups of raw DEP spectra data points from all five technical repeats reduces noise, and removes the step of measuring cell size which is needed for DEP spectra MATLAB-modelling to obtain electrophysiological parameters such as G_{eff} . The MDV method is a quick method of differentiating DEP spectra for cells of arbitrary size, important for clinical assays (Hoque *et al.*, 2020). Calculated MDVs of unstimulated PBMCs were more negative than PMA/ionomycin stimulated PBMCs in all three donors (**Figure 41B**), with a differentiating value of 1.57. A two-tailed paired t-test found no statistical significance between unstimulated and stimulated PBMCs ($p = 0.1295$). Although the differences in MDVs and relative DEP force at 4.92 MHz + 11.9 MHz is not significant by paired two-tailed t-test, the respective threshold values separating all unstimulated and stimulated PBMCs signify that electrophysiological changes occurring in PBMCs following three-hour PMA/ionomycin stimulation are detectable by the 3DEP.

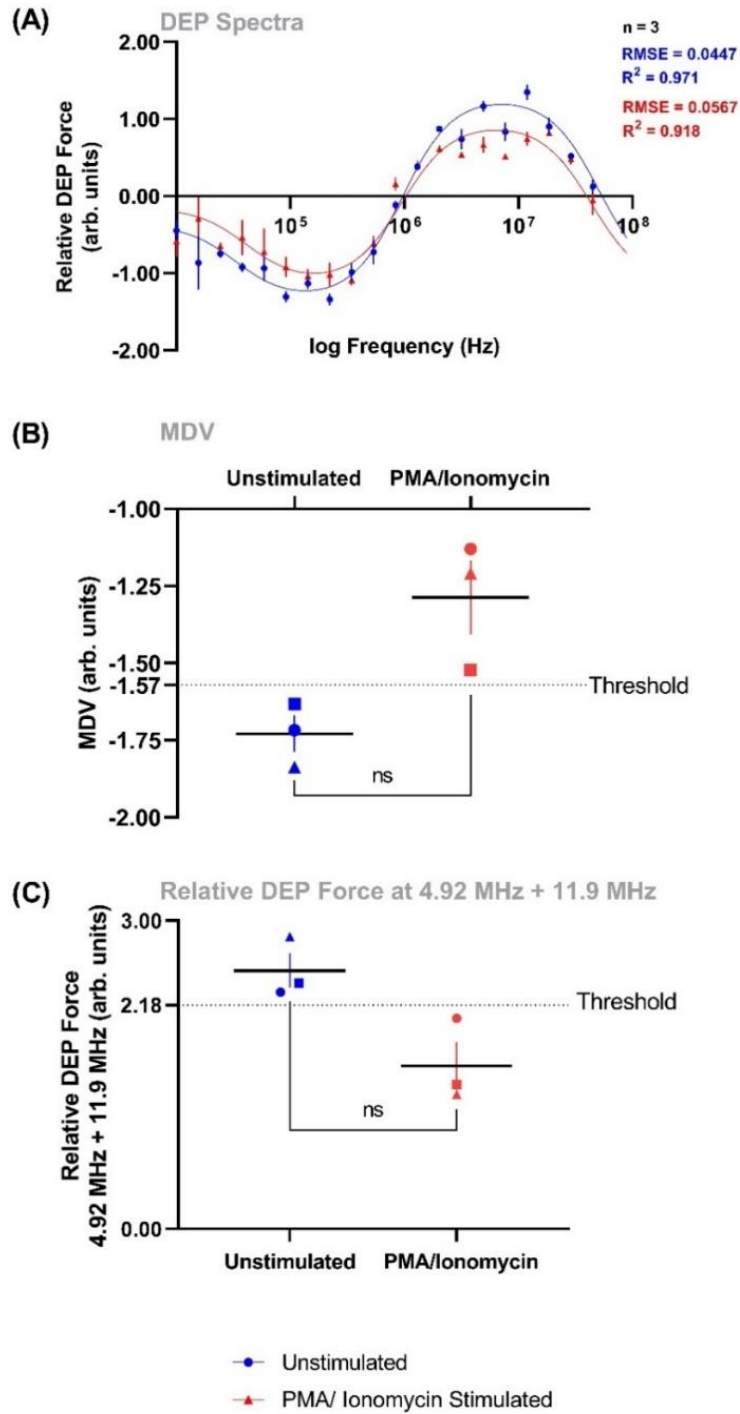


Figure 41 | Electrophysiological parameters of PBMCs after 3-hour stimulation with PMA/ionomycin.

[A] DEP spectra plotting relative DEP force against log frequency (Hz) (MEAN $\pm$ SEM). R^2 : goodness of fit of the model. RMSE: difference between predicted and actual values in the DEP spectra model. **[B]** The MDV of unstimulated PBMCs vs. PMA/ionomycin three-hour stimulated PBMCs. A discriminator of -1.57 separates control and stimulated PBMCs. **[C]** The addition of the relative DEP force taken from datapoints 15 and 17 from the DEP spectra of each donor. A discriminator of 2.18 separates all control and stimulated values. (Black lines and error bars plot mean $\pm$ SEM. Paired two-tailed t-test: ns > 0.05. Donor 1: circle, donor 2: triangle, donor 3: square).

Based on the aforementioned difference in DEP spectra at high frequencies (**Figure 41A**), another method investigated to quickly differentiate stimulated and unstimulated PBMCs for each donor was by adding the relative DEP force at 4.92 MHz (DEP spectra datapoint 15) to the relative DEP force at 11.9 MHz (DEP spectra datapoint 17). As shown in **Figure 41C**, a threshold of 2.18 separates unstimulated and PMA/ionomycin treated cells, but a paired two-tailed t-test ($p = 0.1295$) determined this was not significant.

We can see from the clear difference in G_{eff} between PMA/ionomycin and control treated PBMCs that changes in the electrophysiological properties of PBMCs following stimulation can be quantified using the 3DEP. One limitation of these preliminary PMA/ionomycin experiments was the small sample size, with recruitment of only $n = 3$ donors. The objective of these experiments was solely proof of concept – to determine whether there are electrophysiological differences detectable by the 3DEP, and by extension DEP, when PBMCs are stimulated. The difference detected in G_{eff} ($*p < 0.05$) following PMA/ionomycin stimulation, the marked difference in DEP spectra, and the differentiating thresholds separating arbitrary values of MDVs and relative DEP force (although insignificant), was determined sufficient to proceed with the SARS-CoV-2 experiments. Experiments to determine whether stimulation of PBMCs with the RBD of the SARS-CoV-2 spike protein translates to changes in cellular electrical properties detectable by the 3DEP, and whether these changes differ between individuals who have been exposed to SARS-CoV-2 (either through natural infection or vaccination) or not, thus proceeded.

4.4.2. Baseline Electrophysiological Properties of PBMCs in COVID-19 Naïve Individuals Compared with Donors who Have Recovered from COVID-19 or Been Vaccinated

Before investigating how the electrical properties of PBMCs change following activation with the SARS-CoV-2 spike protein, the baseline values of G_{eff} , C_{eff} , and σ_{cyto} of unchallenged PBMCs from individuals who were COVID-19 naïve, had recovered from COVID-19, or had received one, two, or three doses of a SARS-CoV-2 vaccine were measured. Values of G_{eff} , C_{eff} , σ_{cyto} , and cell radius obtained from MATLAB-fitted models of DEP spectra with an R^2 value greater than 0.85 for donors unmatched across cohorts are written in **Table 12** and plotted in **Figure 42**.

Table 12 | Values of G_{eff} , C_{eff} , σ_{cyto} and cell radius of unchallenged PBMCs from unmatched COVID-19 naïve ($n = 5$), recovered COVID-19 ($n = 4$), first dose ($n = 2$), second dose ($n = 5$), and third dose ($n = 5$) donors. Mean values $\pm$ SEM.

	G_{eff} (kS/m ²)	C_{eff} (mF/m ²)	σ_{cyto} (S/m)	Cell Radius (μ m)
COVID-19 Naïve	4.2 ± 0.7	10.9 ± 0.9	0.39 ± 0.02	3.48 ± 0.09
Recovered COVID-19	3.3 ± 2.1	11.7 ± 0.9	0.36 ± 0.02	3.41 ± 0.07
First Dose	8.2 ± 6.1	12.0 ± 0.6	0.39 ± 0.01	3.36 ± 0.16
Second Dose	4.1 ± 1.0	11.8 ± 0.9	0.32 ± 0.01	3.43 ± 0.07
Third Dose	10.7 ± 2.7	12.7 ± 0.4	0.28 ± 0.02	3.41 ± 0.07

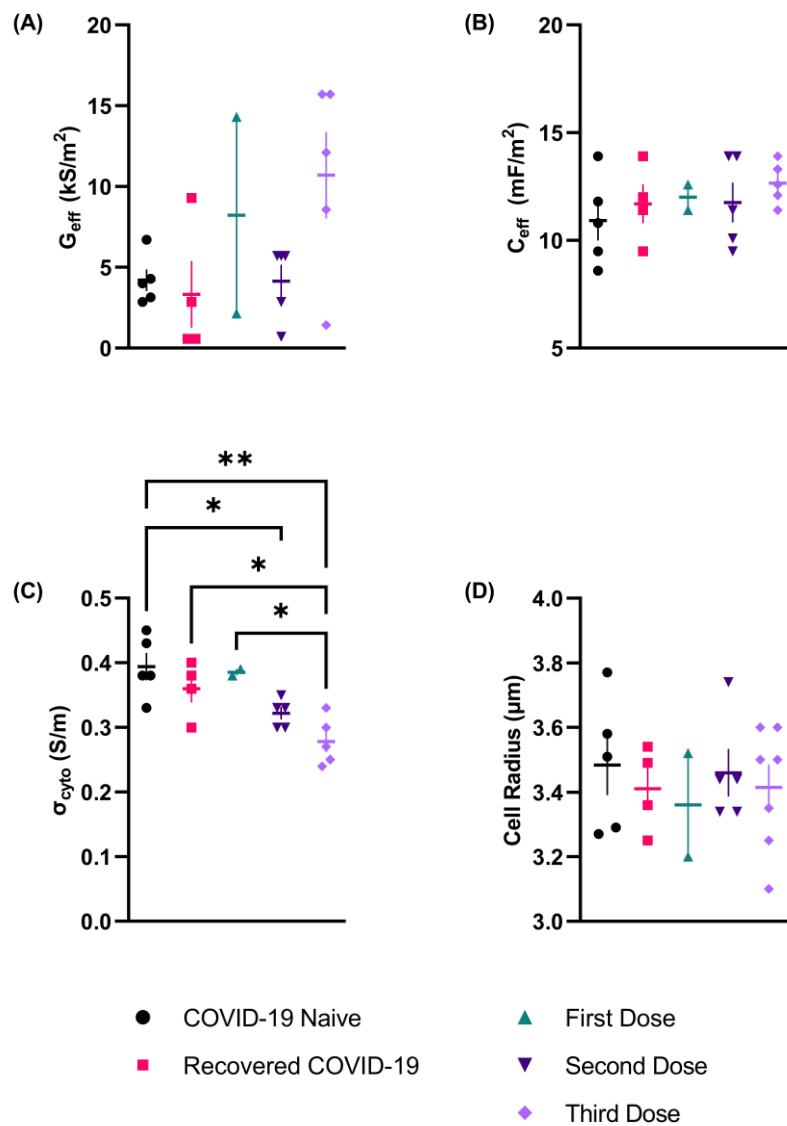


Figure 42 | Baseline values of **[A]** G_{eff} **[B]** C_{eff} **[C]** σ_{cyto} in unchallenged freshly isolated PBMCs from unmatched donors who are COVID-19 naïve (black circles: $n = 5$), have recovered COVID-19 (pink squares: $n = 4$), received their first dose (teal triangle: $n = 2$), second dose (dark purple inverted triangle: $n = 5$) and third dose (lilac diamonds: $n = 5$) of a SARS-CoV-2 vaccine. Values are calculated from MATLAB-fitted models of DEP spectra which possessed an R^2 value of over 0.85. Statistical significance calculated using a Brown-Forsythe and Welch ANOVA followed by multiple comparisons (* $p < 0.05$). Mean lines plotted ($\pm$ SEM).

Values of G_{eff} were deemed not normally distributed ($p = 0.0317$) using the D'Agostino & Pearson ("omnibus K2") test for normality ($p > 0.05$), whereas C_{eff} , σ_{cyto} , and cell radius were found to be normally distributed ($p = 0.2809$, $p = 0.3735$, and $p = 0.9003$ respectively). The non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test was thus used to compare baseline values of G_{eff} between donor cohorts, and a one-way ANOVA followed by Tukey's multiple comparisons test were used to compare the baseline values of C_{eff} , σ_{cyto} , and cell radius between donor cohorts.

The mean value of G_{eff} (**Figure 42A**) tended to be greater in unmatched donors from third dose vaccinated cohorts at $10.7 \pm 2.7 \text{ kS/m}^2$ than COVID-19 naïve donors at $4.2 \pm 0.7 \text{ kS/m}^2$, but this was not statistically significant (>0.9999). There was no statistically significant difference identified in baseline values of cell radius (**Figure 42D**) and C_{eff} (**Figure 42B**) between cohorts, although the values of C_{eff} in third dose donors were marginally greater at $12.7 \pm 0.4 \text{ mF/m}^2$ than COVID-19 naïve donors at $10.9 \pm 2.1 \text{ mF/m}^2$. In contrast, the mean values of σ_{cyto} (**Figure 42C**) decreased as the number of vaccinations increased, with a significant decrease from $0.39 \pm 0.02 \text{ S/m}$ in COVID-19 naïve to $0.32 \pm 0.01 \text{ S/m}$ in second donors ($*p = 0.0489$) and $0.28 \pm 0.02 \text{ S/m}$ in third dose donors ($**p = 0.0012$). There was also a significant decrease between recovered and third dose ($*p = 0.0316$) and between first dose and third dose ($*p = 0.0234$).

These trends in G_{eff} , C_{eff} , σ_{cyto} , and cell radius hold true when analysing matched donor data from individuals who were recruited to both COVID-19 naïve and at least one other cohort post-vaccination, shown in **Figure 43**. Values of G_{eff} increase in all matched donors as their vaccination status increases (**Figure 43A**), but the increase is not statistically significant comparing between COVID-19 naïve vs. following their third dose of a SARS-CoV-2 vaccine using a paired two-tailed t-test ($p = 0.1176$) (**Figure 43B**). The mean values of both C_{eff} (**Figure 43C&D**) and cell radius (**Figure 43G&H**) again showed no difference in donors before and after vaccination. The mean values of σ_{cyto} decreased as the number of vaccination doses increased (**Figure 43E**), although a paired two-tailed t-test showed this was not significant between matched COVID-19 naïve and third dose (**Figure 43F**).

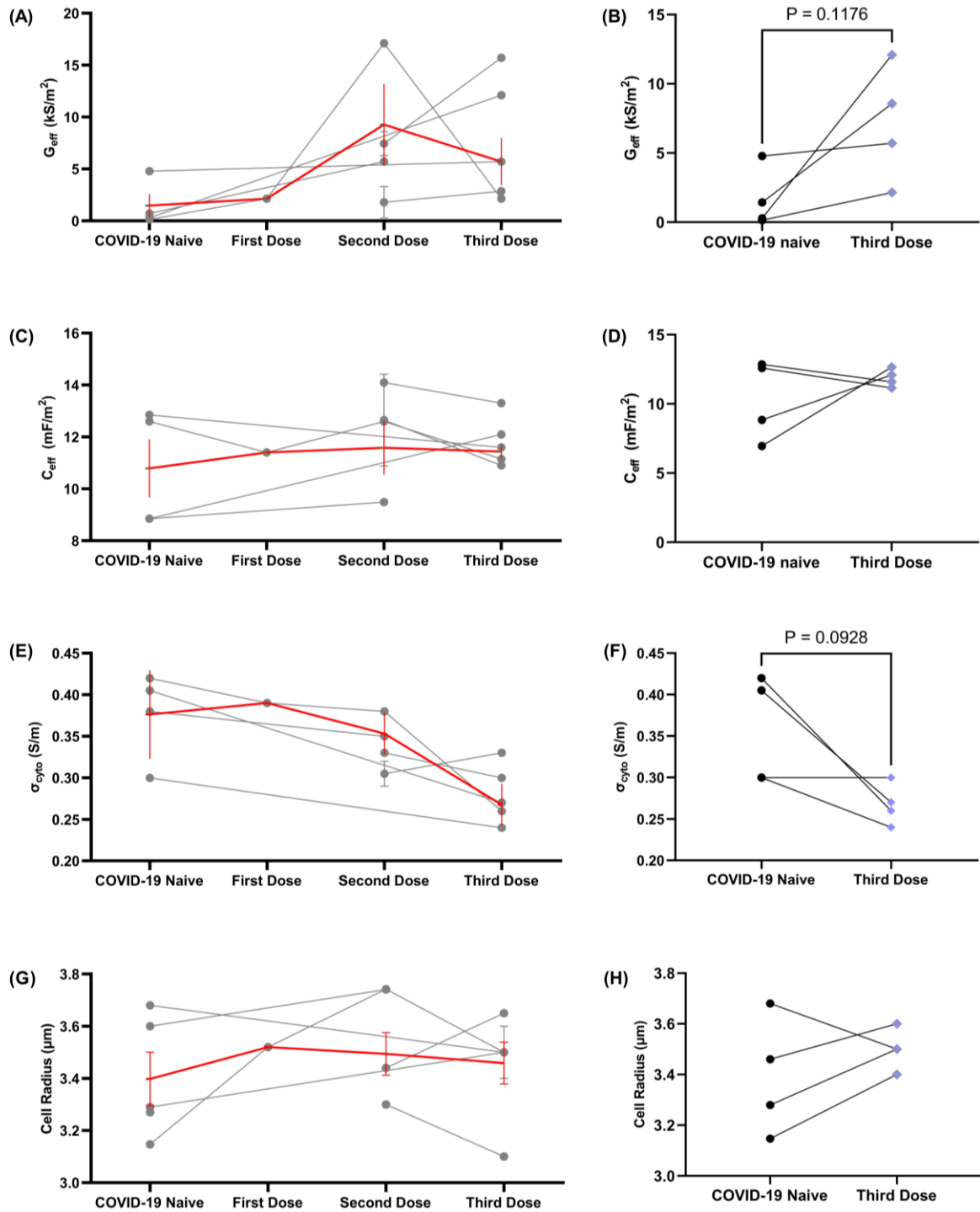


Figure 43 | Matched baseline values in **[A]** G_{eff} **[C]** C_{eff} **[E]** σ_{cyto} **[G]** cell radius for donors recruited to more than one donor cohort ($n = 6$). Each line connects datapoints from the same donor. The red line connects the mean values of these biological repeats ($\pm$ SEM). Matched values in **[B]** G_{eff} **[D]** C_{eff} **[F]** σ_{cyto} **[H]** cell radius in donors recruited to both the COVID-19 naïve cohort and third dose donor cohort including the p-value obtained on application of a two-tailed paired t-test between baseline values of σ_{cyto} in donors when they were COVID-19 naïve vs. following third vaccine dose ($n = 4$).

In summary, some evidence suggests that the baseline electrical properties of PBMCs may change following SARS-CoV-2 vaccination, as demonstrated by significant differences in σ_{cyto} between COVID-19 naïve donors and unmatched second and third dose donors. Moreover, these changes

amplify with the number of vaccinations. However, these changes are not observed in the recovered COVID-19 cohort. Insignificant but consistent increases in G_{eff} and decreases in σ_{cyto} were apparent in matched COVID-19 naïve donors following their third vaccination.

Why the baseline electrophysiological properties of PBMCs change following multiple vaccinations is unknown. As these experiments took place during times of national lockdowns, social distancing, and isolation, it is likely that either SARS-CoV-2 infection or vaccination was the main challenge. Cases of the common cold, flu, and other respiratory diseases were substantially less during the time of these experiments than in previous years (Iacobucci, 2020, Kadambari et al., 2022), and remained low during the time of third dose donor experiments (ECDC, 2022, UK Health Security Agency, 2023). Furthermore, experiments in COVID-19 naïve and third dose donors both took place around the same month (February/March of consecutive years), accounting for other seasonal changes. However, given the widespread impact of the COVID-19 pandemic on mental health and stress levels of the population (Manchia et al., 2022), it is worth noting that psychological stress causes systemic inflammation (Barrett et al., 2021) and significantly higher plasma levels of cytokines such as IL-6 in individuals with higher cortisol levels (Kim and Maes, 2003, Reale et al., 2012).

One hypothesis as to why the electrical properties of PBMCs changed following SARS-CoV-2 vaccination is that PBMCs are more activated or have a greater reactivity. The time of vaccination also coincided with the ending of lockdown restrictions, and as individuals socialised more there was a greater immune challenge from the environment, regardless of symptomatic illnesses. The decrease in σ_{cyto} follows the same trend as the electrical changes seen in the activated PBMCs in the positive control PMA/ionomycin stimulation experiments (**Section 4.4.1**). Again future work including parallel experiments to quantify PBMC activation such as flow cytometry of PBMC activation markers (Abcam, No Date-a, Gerasimova et al., 2022), cytokine secretion assay with flow cytometry (Rezk et al., 2020), or ICS (Sahin et al., 2020, Vogel et al., 2022) would be invaluable. Langgartner et al. (2023) reported that during *ex vivo* culture, PBMCs isolated from individuals who received the BNT162b1 vaccine secrete 600 times more pro-inflammatory IL-6 and two times more anti-inflammatory IL-10 in basal conditions than non-vaccinated individuals (Langgartner et al., 2023). There is also growing evidence of significant changes in PBMC gene expression after vaccination, including genes involved in TNF- α signalling via NF- κ B, IL6-JAK STAT3 signalling, and inflammatory responses (Liu et al., 2021). This is supported by Bergamaschi et al. (2021) who found the first BNT162b2 vaccination causes systemic inflammation including increases in IL-15 and IFN- γ , and increases in TNF- α and IL-6 following the second vaccination (Bergamaschi et al., 2021). Additionally, as previously mentioned, vaccination relies on the long-term production of NAb by long-lived plasma cells or memory cells during the GC reaction in a secondary lymphoid organ such as lymph nodes or the spleen (Gupta and Jaiswal, 2022).

As such, memory B-cell activation and differentiation in GCs following vaccination has been documented to last for months (Qi et al., 2022), although how many of these activated memory B-cells enter peripheral circulation is unknown, or whether there would be sufficient number to affect the congregate DEP spectrum. Increased PBMC reactivity following COVID-19 vaccinations may also have meant they were more sensitive and easily activated during cell preparation. For example, the high centrifugal force applied during density gradient centrifugation can artifactually activate PBMCs (Sun and Sethu, 2018).

Another hypothesis is that this is due to a change in the proportions of different PBMC subpopulations between donor cohorts, which contribute to derived electrical values. All DEP spectra were modelled as a single population as peaks from multiple population were not evident. Nonetheless, different PBMC populations in healthy individuals have been documented to have different dielectric properties. For example, the σ_{cyto} of B-cells in healthy donors has been measured at 0.73 ± 0.18 S/m using ROT, compared with 0.56 ± 0.10 in monocytes (Yang et al., 1999). As a DEP spectrum is an aggregate measure of all PBMCs within the cell sample, it is possible that a change in the contribution of a subpopulation (such as T-cells) due to altered cell counts or size following vaccination results in a different overall DEP spectrum. Sahin et al. (2021) found BNT162b2 vaccination (consisting of a priming dose and a booster dose) induces epitope specific CD8⁺ T-cells, which were still detectable eight weeks later. This finding was supported by Langgartner et al. (2023) who reported that several per cent of total peripheral blood CD8⁺ T cells are RBD-specific IFN γ ⁺ CD8⁺ T cells following vaccination. Other studies have also found changes in lymphocyte subpopulations following vaccination. During the clinical trial of BNT162b2, lymphopenia after two doses was not reported (Polack et al., 2020), but a real-world study in a much larger cohort reported increased risk of both leukopenia and neutropenia following the second dose of the BNT162b2 vaccine (Sing et al., 2022). Liu et al. (2021) reported no significant change in overall lymphocyte counts in healthy controls after vaccination, although there was a reduction CD8⁺ T cells, proliferating CD8⁺ T cells, $\gamma\delta$ -T-cells, and an increase in CD14⁺ monocytes (Liu et al., 2021). Additionally, Manunta et al. (2021) found PBMC fractions recovered using density gradient centrifugation from early COVID-19 patients had reduced circulating lymphocytes and monocytes in addition to markedly greater numbers of low-density neutrophils with altered cell size than healthy controls (Manunta et al., 2021). However, no difference in average cell radius was measured between donors (**Figure 42D**). To determine whether the concentration of PBMC subpopulations differ between donor cohorts, PBMC immunophenotyping by flow cytometry to measure the expression of ‘cluster of differentiation’ CD markers could have been used to differentiate and characterise sub-populations.

Changes in gene expression in PBMCs following SARS-CoV-2 infection has also been documented, but not identified following vaccination. Network-based data analysis of gene expression from PBMCs from a small cohort of COVID-19 patients compared with healthy controls highlighted differential expression of ion channel genes such as up-regulation of the TRP channels TRPM1, TRPM3, TRPV5, and the potassium calcium-activated channel KCNN3, and down-regulation of TRPC1 and KCNA5 (Zhang and Feng, 2023). TRP ion channels have been proposed to be involved in COVID-19 pathology (Bousquet et al., 2021, Sukocheva et al., 2022), and impaired TRPM3 ion channel function has been linked to long COVID-19 – resembling the phenotype of ME/CFS. Impaired TRPM3 ion channel activity indicates there is impaired Ca^{2+} mobilisation. Use of the TRPM3 agonist pregnenolone sulphate (Preg-S) and the antagonist ononetin to assess TRPM3 function could be used to compare TRPM3 ion channel function between cohorts (Sasso et al., 2022).

Although limited by a small cohort size, no significant changes were identified between COVID-19 naïve donors and first dose donors. Therefore, it is possible that changes in the electrical properties of PBMCs occur with increasing number of exposures, as similarly no significant differences were observed between the electrical properties of COVID-19 naïve and recovered COVID-19 donors. A reduced level of lymphocytes (lymphopenia) has been reported post-COVID, with significant decreases in the numbers of CD8^+ cells in mild and severe patients compared with healthy controls (Zheng et al., 2020), and further reductions in CD4^+ , B-cells, NK cells (Huang et al., 2020, Shi et al., 2020b, Zheng et al., 2020), monocytes, eosinophils, and basophils (Qin et al., 2020) during severe COVID-19 infection. A review by Zhang et al. (2021) found forty studies which reported peripheral T-cell lymphopenia is worse in severe than mild COVID-19, with significant decreases in CD3, CD4^+ , and CD8^+ T-cell counts in severe disease (Zhang et al., 2021b). Other changes in PBMC subsets reported in literature include an increased count and percentage of CD19^+ lymphocytes in recovered COVID-19 paediatric patients (Budziło et al., 2023). Different studies have reported lymphopenia to persist in some individuals for 80-102 days (Varghese et al., 2021), 16 weeks after hospital discharge (Wang et al., 2021b). However, as the recovered COVID-19 individuals recruited to the experiments of this chapter were mild COVID-19 patients and lymphopenia is correlated with disease severity (Toori et al., 2021), it is possible any changes to their immune system recovered by the time of the experiment.

Given the changes in the immune system over time following natural infection and vaccination, such as increased SARS-CoV-2 IgG specific memory B-cells (Rodda et al., 2021) and decreased anti-SARS-CoV-2 T-cells (Ni et al., 2020a), it is important to consider whether any differences in the number of days between the experiment and either vaccination or natural infection with SARS-CoV-2 affects the electrical changes observed. To determine whether the values of G_{eff} , C_{eff} , and σ_{cyto} were affected by the time since COVID-19 exposure, baseline values in recovered COVID-19,

first dose, second dose and third dose donor cohorts were plotted against the duration of time (days) that had passed between either testing positive for COVID-19 or receiving the vaccine, and the date of the experiment (**Figure 44**). Simple linear regression was used to model how the electrical properties of PBMCs for each donor cohort against the number of days since either natural or artificial exposure to COVID-19, under the assumption that trends followed a linear pattern. The coefficient and the 95% CI of the slope is quoted in **Table 13**, in addition to the P-value which determines whether the gradient of the slope is significantly greater than zero (* $p < 0.05$) and the r^2 value denoting the goodness-of-fit of the linear regression line. Unfortunately, the difficulties in recruiting first dose donors meant only two data points could be collected, from which no trend can be inferred without further data collection.

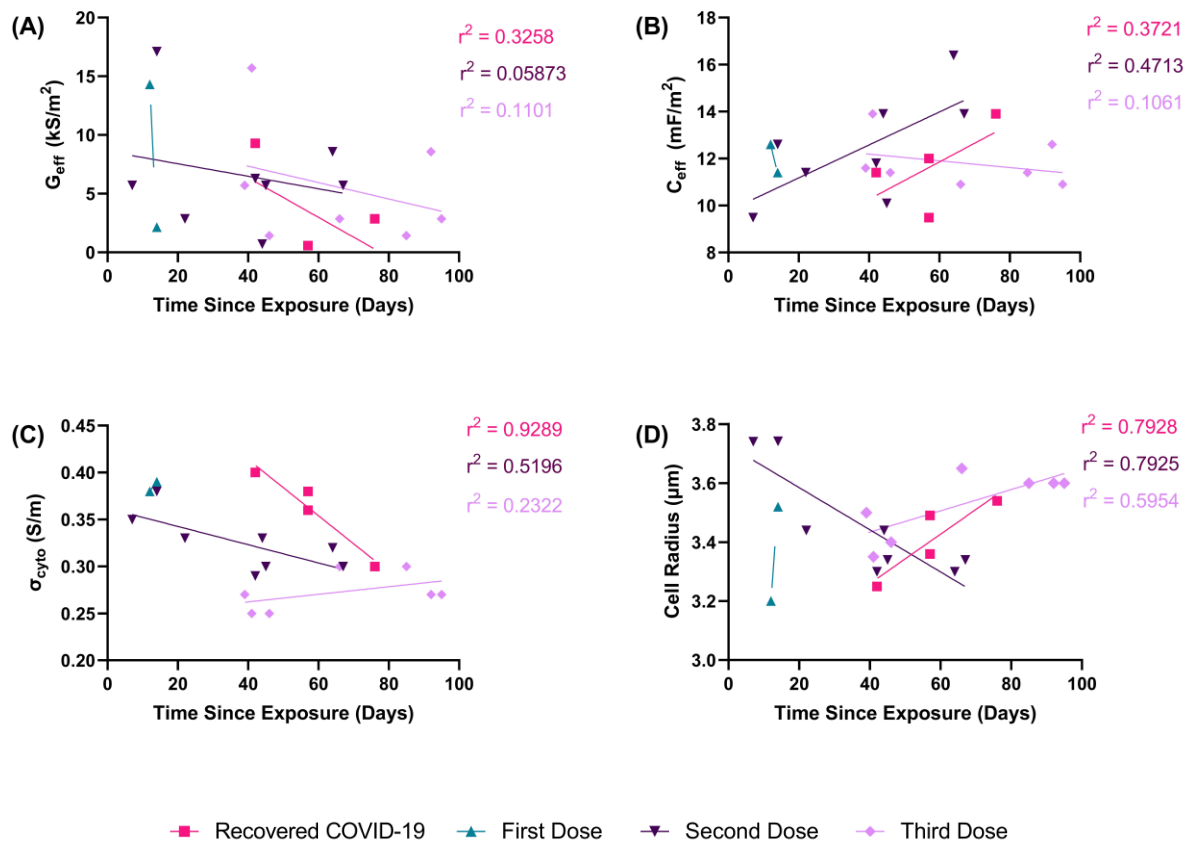


Figure 44 | Baseline values of **[A]** G_{eff} **[B]** C_{eff} **[C]** σ_{cyto} **[D]** cell radius against the time since exposure (days) to COVID-19 either through natural immunity or vaccination and the date of the experiment in recovered COVID-19 donors (pink squares, $n = 4$ donors), first dose donors (teal triangles, $n = 2$ donors), second dose (purple inverted triangles, $n = 5$ donors) and third dose (lilac diamonds, $n = 7$ donors). Lines plot simple linear regression models, and corresponding r^2 values indicate the goodness-of-fit of the regression model by determining the variance attributable to the number of days between the date of the experiment and the date of COVID-exposure.

Table 13 Simple linear regression models were fitted to indicate the trend in the electrical parameters between date of experiment and date of exposure, either by natural infection or vaccination, in unmatched donors. Coefficient denotes the gradient of the slope, and the 95% CI. P-value determines whether the slope is significantly greater than zero (*<0.05, **<0.01). r^2 values indicate the goodness-of-fit of the regression model by determining the variance attributable to the number of days between the date of the experiment and the date of COVID-exposure.

	Donor Cohort	Coefficient	95% CI	P-value	r^2 Value
G_{eff}	Recovered COVID-19	-0.169	-0.908 to 0.570	0.429	0.3258
	Second Dose	-0.0533	-0.267 to 0.160	0.563	0.05873
	Third Dose	-0.0698	-0.298 to 0.158	0.467	0.1101
C_{eff}	Recovered COVID-19	0.0796	-0.235 to 0.394	0.390	0.3721
	Second Dose	0.0702	-0.00407 to 0.144	0.060	0.4713
	Third Dose	-0.0143	-0.0622 to 0.0335	0.476	0.1061
σ_{cyto}	Recovered COVID-19	-0.00299	-0.00551 to -0.000474	0.0362 *	0.9289
	Second Dose	-0.000972	-0.00191 to -3.845e-005	0.0436 *	0.5196
	Third Dose	0.000403	-0.000440 to 0.00125	0.274	0.2322
Cell Radius	Recovered COVID-19	0.00837	-0.00465 to 0.0214	0.110	0.7928
	Second Dose	-0.00714	-0.0115 to -0.00280	0.0069 **	0.7295
	Third Dose	0.00361	0.000189 to 0.00702	0.0422 *	0.5954

As shown in **Figure 44C** and **Table 13**, σ_{cyto} significantly decreases with time since exposure to COVID-19 in recovered COVID-19 donors (* $p = 0.0362$), significantly decreases but to a lesser extent in second dose donors (* $p = 0.0436$), and does not significantly change in third dose donors. Moreover, both the coefficient and intercept of the linear regression model decrease with the number of exposures (successive exposure reduces the gradient). The linear regression models of σ_{cyto} for recovered COVID-19, second dose, and third dose donors appear to trend to the same value, but that value is not the baseline of COVID-19 naïve. Although the humoral and cellular immune responses to SARS-CoV-2 have been shown to wane over time, this is estimated to occur after 6 – 8 months (Dan et al., 2020) which is outside of the timeframe of these experiments. It is possible that the decrease in σ_{cyto} may indirectly correlate with immune protection. Following natural infection, memory T-cell responses present within a few weeks PSO (Law et al., 2022) and have been shown to persist, as well as memory B cell responses, for at least three months (Rodda et al., 2021). Moreover, levels of memory B-cells have been reported to increase months following vaccination (Terreri et al., 2022) and natural infection, including a prominent population of IgG⁺CD27⁺CD21⁺ RBD-specific memory B-cells which increased between 1 to 3 months (Rodda et al., 2021). One hypothesis to this trend in σ_{cyto} is that the proportion of PBMCs of different subtypes continually change following vaccination, including

an increase in PBMCs over time with a lower σ_{cyto} . Again, PBMC immunophenotyping using flow cytometry is required to investigate this.

Interestingly, cell radius (**Figure 44D**) was shown to change with duration since COVID-19 exposure, correlating with a significant decrease in second dose donors (** $p=0.0436$) and a significant increase in third dose donors (* $p=0.0422$). No significant trends were observed in G_{eff} (**Figure 44A**) or C_{eff} (**Figure 44B**) over time; as the coefficients of the linear regression model were not statistically greater than zero ($p>0.05$) for any donor cohort (**Table 13**). Additionally, the r^2 values are very low indicating the number of days since exposure to COVID-19 can not be used to predict values of G_{eff} or C_{eff} . However, negative coefficients of the linear regression models indicate values of G_{eff} decreases with time since exposure to COVID-19 in all donor cohorts, contrasting with values of C_{eff} which increase over time in both recovered COVID-19 and second dose cohorts.

Whether these changes in electrical properties following vaccination are indicative of immune protection requires a SARS-CoV-2 specific challenge to ensure the electrical changes observed between cohorts are specific to COVID-19 protection and there is not a confounding factor.

4.4.3. Electrical Changes in PBMCs Following *In Vitro* Stimulation with the Receptor Binding Domain of the SARS-CoV-2 Spike Protein

To investigate whether the electrophysiological response of PBMCs to SARS-CoV-2 stimulation could potentially function as a CoP, changes in the values of G_{eff} , C_{eff} , and σ_{cyto} in PBMCs donated from COVID-19 naïve, recovered COVID-19, first dose, second dose and third dose donors following *in vitro* stimulation with SARS-CoV-2 S-protein RBD was assessed. The RBD antigen was chosen as the stimulant as it is responsible for binding to the host cell ACE2 receptors and is highly immunogenic – donors vaccinated with BNT162b1 possess strong CD4⁺ and CD8⁺ T cell responses to peptides encoding the SARS-CoV-2 RBD (Sahin et al., 2020), T-dependent IgG RBD-specific memory B-cells are generated following mild infection (Rodda et al., 2021), 90% of serum antibodies target the RBD (Goldblatt et al., 2022), and the RBD is the main target of NAb (Prévost et al., 2020), with NAb titres to the RBD significantly correlating with neutralisation of the SARS-CoV-2 virus (Luchsinger et al., 2020, Dispinseri et al., 2021, Nayak et al., 2021). The SARS-CoV-2 Spike Glycoprotein RBD (Active) (Abcam, ab273065) chosen to stimulate the PBMCs has been used in previous publications (Tichauer et al., 2021, Gerasimenko et al., 2022, Tsilioni and Theoharides, 2023).

Deciding the In Vitro Stimulation Duration

To determine the optimal duration to stimulate the PBMCs for, changes in the electrophysiological properties of PBMCs after 15-minute, 3-hour, and 24-hour stimulation with the RBD of the SARS-CoV-2 spike protein were preliminarily assessed in small cohorts using the 3DEP machine. Initial donor recruitment to the 15-minute ($n = 3$), 3-hour ($n = 6$), and 24-hour ($n = 4$) stimulation experiments was very limited (donor characteristic tables are included in **Appendix A.3 Tables 21 and 23**). These experiments were solely used to quickly preliminary assess whether there are great differences in the electrophysiological response of PBMCs between donor cohorts following any of the RBD-stimulation durations to decide on a protocol moving forward.

A 15-minute stimulation duration was investigated because activation and transcription of immediate-early genes (IEG) occurs within minutes of stimulation (Akins et al., 1996, Bahrami and Drabløs, 2016, Gallo et al., 2018), and coronavirus infection has been shown to activate IEGs including Activator Protein-1 and EGR1 (Yuan et al., 2022). As the innate immune response occurs within minutes (Marshall et al., 2018), differences in the PBMC electrical response to a 15-minute stimulation between donor cohorts compares differences in the innate immune response. In contrast, a three-hour stimulation was chosen because during COVID-19 re-infection, circulating memory B-cells are activated and quickly differentiate to antibody-secreting cells (Chen et al., 2022a), which is expected to occur within 3-hours. Finally, 24-hour stimulation duration was chosen based on the results of previous studies which have found the expression of T-B cell receptor ligand pairs, such as ICOS and CD40L in CD4⁺ cells increase following 20-hour stimulation with the S-protein in mild COVID-19 individuals (Rodda et al., 2021). Furthermore, Olajide et al. (2021) found frozen human PBMCs treated with the spike glycoprotein S1 (100 ng/mL) for 24-hours resulted in production of TNF α , IL-6, IL-1 β , and IL-8 (Olajide et al., 2021).

The stimulation duration which best differentiated the donor cohorts was 3-hours. Values of G_{eff} , C_{eff} , and σ_{cyto} following 15-minute and 24-hour RBD-stimulation are described in **Appendix A.3. Table 22 and Table 24** respectively. No marked differences in control/stimulated normalised values of G_{eff} , C_{eff} , or σ_{cyto} (**Appendix A.3 Figure 67A**) were observed between donor cohorts following 15-minute RBD-stimulation. Greater differences in normalised values of G_{eff} , C_{eff} , and σ_{cyto} between cohorts were observed following 3-hour and 24-hour stimulation (**Appendix A.3 Figure 67B**). The 3-hour stimulation period was chosen because a shorter protocol duration is most desirable and adoptable for CoP methodologies and overcomes limitations of current CoP methodologies such as the gold-standard ELISA assays.

The parameter which best differentiated donor cohorts were the MDVs calculated for the three-hour stimulation. Given that the primary objective was to investigate a quick and easily adoptive technique of measuring a CoP, MDVs (previously described during analysis of the ME/CFS data in **Section 3.4.3.**) were calculated for each stimulation duration (plotted in **Figure 45**). MDVs do not require cell radius measurements or modelling of DEP spectra, saving time and removing all possibility of operator-bias. Moreover, the MDV method is a validated technique which uses multiple raw datapoints to remove noise and has no subjectivity. Whereas the MDVs of both 15-minute and 24-hour stimulated PBMCs are close to 1.0 showing little difference between donor cohorts, there is a statistically significant difference in MDVs of the 3-hour stimulated PBMCs using an unpaired two-tailed t-test (* $p < 0.05$). As the pandemic limited donor recruitment and there was an urgent necessity to find a quick and easily adoptive CoP, a decision to focus solely on 3-hour stimulation was decided. To reliably evaluate the specific electrophysiological changes that occur following 15-minute and 24-hour stimulation many more biological repeats are required.

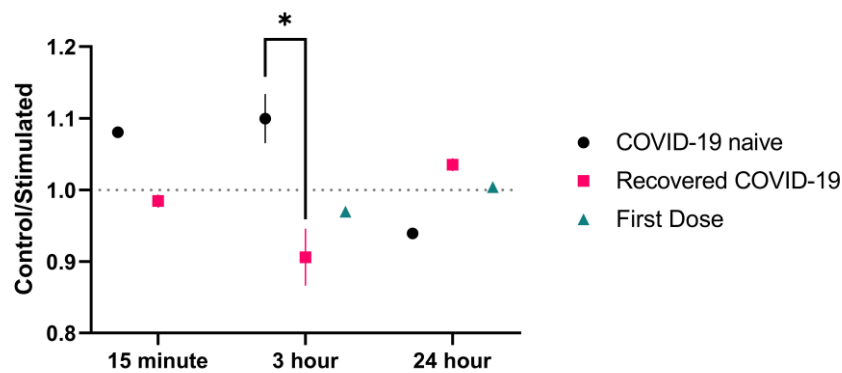


Figure 45 | Control/stimulated MDVs of COVID-19 naïve (blue circle), recovered COVID-19 (red triangle) and first dose (green diamond) donors after 15-minute, 3 hour and 24-hour RBD-stimulation. MEAN $\pm$ SEM. 15-minute: COVID-19 naïve $n = 1$, recovered COVID-19 $n = 2$. Three-hour: COVID-19 naïve ($n = 2$), recovered COVID-19 ($n = 3$), first dose ($n = 1$). 24-hour: COVID-19 naïve ($n = 1$), recovered COVID-19 ($n = 2$), first dose ($n = 1$). Unpaired two-tailed t-test * $p < 0.05$.

Normalised values of G_{eff} , C_{eff} , or σ_{cyto} in the same donor following 15-minute, 3-hour and 24-hour incubation with the RBD can be seen in a COVID-19 naïve ($n = 1$), recovered COVID-19 ($n = 2$) and first dose donor ($n = 1$) in **Appendix A.3 Figures 68-70**. Given the small sample size, no clear trends can be seen.

Three-Hour In Vitro RBD-Stimulation

Changes in the electrophysiological properties of PBMCs following 3-hour RBD-stimulation were subsequently investigated in a larger donor cohort to determine whether a differential dielectric

response to stimulation differentiates unmatched COVID-19 naïve, recovered COVID-19, first dose, second dose, and third dose donor cohorts and could be used as a CoP. Values of G_{eff} , C_{eff} , and σ_{cyto} in unchallenged and SARS-CoV-2 stimulated PBMCs for each donor cohort were calculated from MATLAB-fitted models of DEP spectra which possessed an r^2 value of over 0.85, and are written in **Table 14** and plotted in **Figure 46**. The average cell radius of unchallenged compared with SARS-CoV-2 RBD-stimulated samples for each donor cohort are plotted in **Figure 47**. For each parameter, repeated measures two-way ANOVAs followed by Tukey's multiple comparisons (* $p < 0.05$) were used to determine whether there were any statistically significant differences between unchallenged and SARS-CoV-2 RBD stimulated PBMCs, or between donor cohorts.

Table 14 | Values of G_{eff} , C_{eff} , and σ_{cyto} of unchallenged PBMCs from unmatched COVID-19 naïve ($n = 5$), recovered COVID-19 ($n = 4$), first dose ($n = 2$), second dose ($n = 5$), and third dose ($n = 5$) donors. Mean values $\pm$ SEM.

	G_{eff} (kS/m ²)		C_{eff} (mF/m ²)		σ_{cyto} (S/m)	
	<i>Unstim.</i>	<i>Stim.</i>	<i>Unstim.</i>	<i>Stim.</i>	<i>Unstim.</i>	<i>Stim.</i>
COVID-19 Naïve	4.2 $\pm$ 0.7	5.6 $\pm$ 1.3	10.9 $\pm$ 0.9	9.6 $\pm$ 0.9	0.39 $\pm$ 0.02	0.37 $\pm$ 0.02
Recovered COVID-19	3.3 $\pm$ 2.1	1.8 $\pm$ 0.9	11.7 $\pm$ 0.9	10.1 $\pm$ 0.5	0.36 $\pm$ 0.02	0.36 $\pm$ 0.02
First Dose	8.2 $\pm$ 6.1	7.9 $\pm$ 6.4	12.0 $\pm$ 0.6	11.7 $\pm$ 2.2	0.39 $\pm$ 0.01	0.37 $\pm$ 0.01
Second Dose	4.1 $\pm$ 1.0	6.4 $\pm$ 1.3	11.8 $\pm$ 0.9	10.8 $\pm$ 1.0	0.32 $\pm$ 0.01	0.30 $\pm$ 0.01
Third Dose	10.7 $\pm$ 2.7	3.8 $\pm$ 0.8	12.7 $\pm$ 0.4	13.0 $\pm$ 1.0	0.28 $\pm$ 0.02	0.28 $\pm$ 0.02

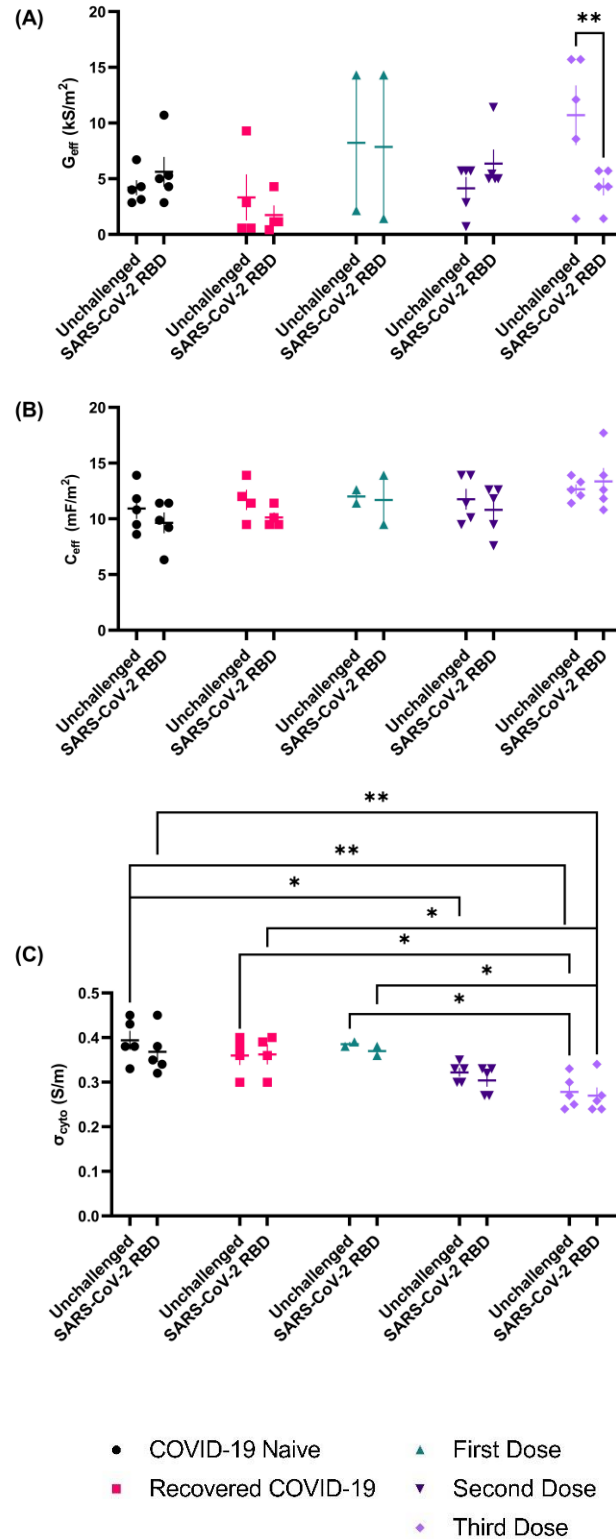


Figure 46 | Values of **[A]** G_{eff} **[B]** C_{eff} **[C]** σ_{cyto} in unchallenged and 3-hour SARS-CoV-2 RBD stimulated PBMCs from unmatched individuals who are COVID-19 naïve (black circles: $n = 5$), have recovered COVID-19 (pink squares: $n = 4$) and received their first dose (teal triangle: $n = 5$), second dose (dark purple inverted triangle: $n = 5$), and third dose (lilac diamonds: $n = 5$) of a SARS-CoV-2 vaccine. Values are calculated from MATLAB-fitted models of DEP spectra which possessed an R^2 value of over 0.8. Statistical significance calculated using a repeated measures two-way ANOVA followed by Tukey's multiple comparisons (* $p < 0.05$, ** $p < 0.01$). Mean lines plotted ($\pm$ SEM).

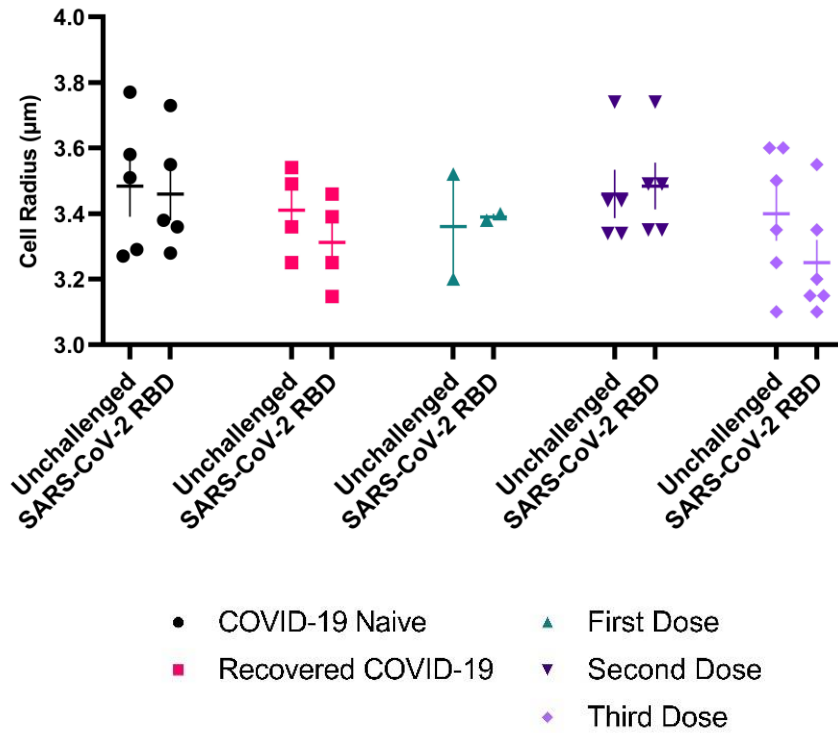


Figure 47 | Values of cell radius in unchallenged and 3-hour SARS-CoV-2 RBD stimulated PBMCs from unmatched individuals who are COVID-19 naïve (black circles: $n = 5$), have recovered COVID-19 (pink squares: $n = 4$) and received their first dose (teal triangle: $n = 5$), second dose (dark purple inverted triangle: $n = 5$), and third dose (lilac diamonds: $n = 9$) of a SARS-CoV-2 vaccine. Statistical significance calculated using a repeated-measures two-way ANOVA followed by Tukey's multiple comparisons (* $p < 0.05$). Mean lines plotted ($\pm$ SEM).

The only electrical parameter which significantly changed from its baseline unchallenged value following SARS-CoV-2 RBD stimulation was a significant decrease in G_{eff} (** $p = 0.0039$) (**Figure 46A**) in third dose donors from $10.7 \pm 2.7 \text{ kS/m}^2$ to $3.8 \pm 0.8 \text{ kS/m}^2$, which was not seen in the other donor cohorts. No significant differences were seen in either C_{eff} (**Figure 46B**), σ_{cyto} (**Figure 46C**), or cell radius (**Figure 47**) following RBD stimulation in any cohort. However, as was seen in baseline values, statistically significant differences in σ_{cyto} were observed between RBD-stimulated PBMCs in COVID-19 naïve and third dose donors (** $p = 0.0033$), recovered COVID-19 and third dose donors (* $p = 0.0105$), and first dose and third dose donors (* $p = 0.0337$).

To normalise changes in G_{eff} , C_{eff} , σ_{cyto} , and cell radius for each donor individually, unchallenged values were subtracted from RBD-stimulated values; the same normalisation method which was performed in **Chapter 3**. The changes in each parameter for unmatched donors across each cohort are plotted in **Figure 48**. To determine whether the normalised values of each electrical parameter were statistically different from each other, ordinary one-way ANOVAs followed by Tukey's multiple comparison were used (* $p < 0.05$). As expected, the normalised values of G_{eff} were significantly

different between COVID-19 naïve and third dose donors (* $p = 0.0229$) and second dose and third dose donors (* $p = 0.0119$). No statistically significant differences were observed in normalised values of C_{eff} (Figure 48B), σ_{cyto} (Figure 48C), or cell radius (Figure 48D).

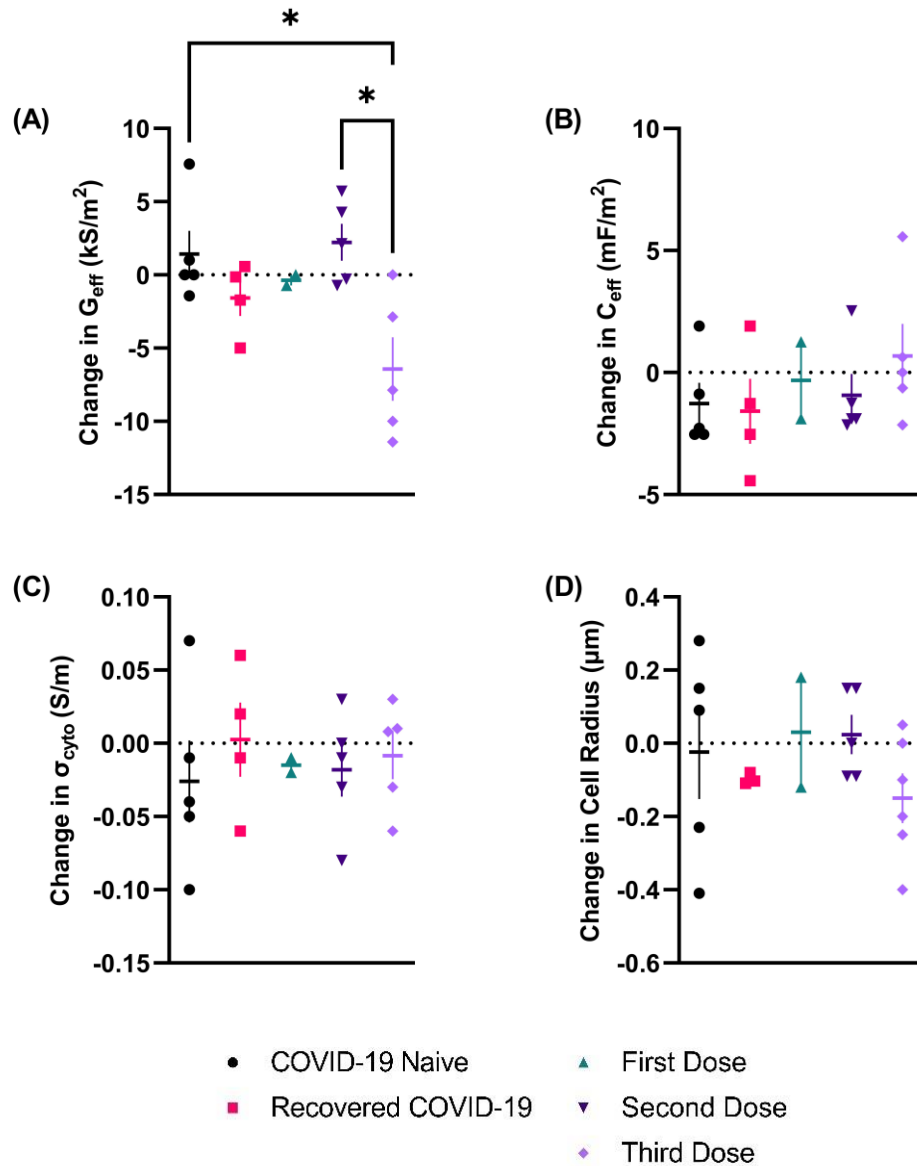


Figure 48 | Normalised values of **[A]** G_{eff} **[B]** C_{eff} **[C]** σ_{cyto} **[D]** cell radius following 3-hour SARS-CoV-2 RBD stimulated PBMCs from unmatched individuals who are COVID-19 naïve (black circles: $n = 5$), have recovered COVID-19 (pink squares: $n = 4$) and received their first dose (teal triangle: $n = 2$), second dose (dark purple inverted triangle: $n = 5$) and third dose (lilac diamonds: $n = 5$) of a SARS-CoV-2 vaccine. Values are calculated from MATLAB-fitted models of DEP spectra which possessed an r^2 value of over 0.8. Statistical significance calculated using a one-way ANOVA followed by Tukey's multiple comparisons. Mean lines plotted ($\pm$ SEM).

Normalised values of G_{eff} (Figure 49A-B), C_{eff} (Figure 49C-D), σ_{cyto} (Figure 49E-F), and cell radius (Figure 49G-H) in matched donors recruited to both COVID-19 naïve and at least one other cohort post-vaccination are shown in Figure 49.

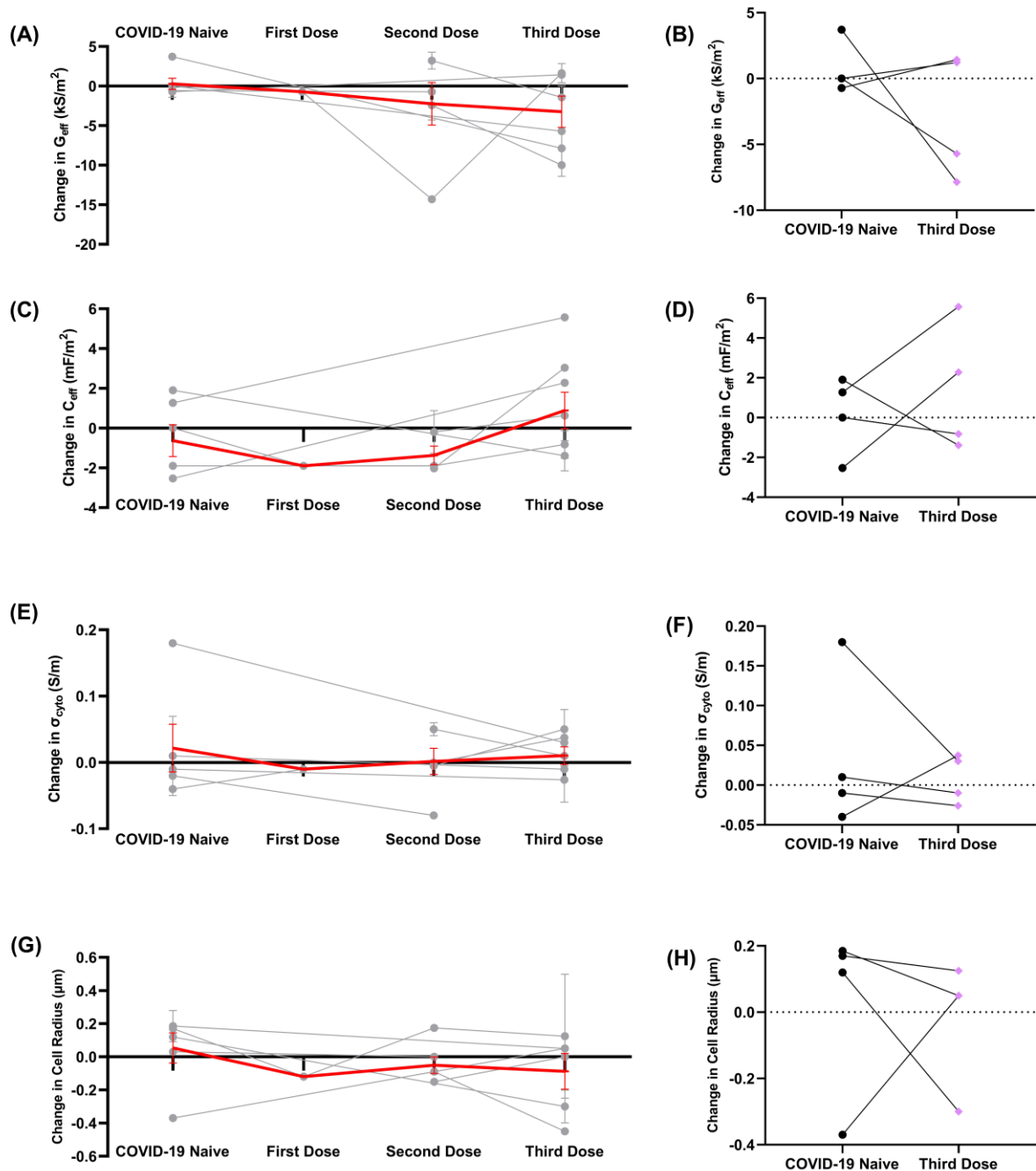


Figure 49 | Normalised values of **[A]** G_{eff} **[C]** C_{eff} **[E]** σ_{cyto} **[G]** cell radius for matched donors recruited to more than one donor cohort ($n = 6$). Each line connects datapoints from the same donor. The red line connects the mean values of these biological repeats ($\pm$ SEM). Normalised values of **[B]** G_{eff} **[D]** C_{eff} **[F]** σ_{cyto} **[H]** cell radius for matched donors recruited to both the COVID-19 naïve cohort and third dose donor cohort. Statistical significance investigated using a two-tailed paired t-test between baseline values of σ_{cyto} in donors when they were COVID-19 naïve vs. following third vaccine dose ($n = 4$).

As was the case in unmatched donors, no trends in C_{eff} , σ_{cyto} , or cell radius are seen between COVID-19 naïve and third dose donors. However, in matched donors there was also no significant difference in normalised values of G_{eff} between COVID-19 naïve and third dose donors (paired two-tailed t-test $p = 0.3587$), although there is a steep decrease in normalised values of G_{eff} in two of the

four donors. One reason as to why a statistical difference in G_{eff} was observed in unmatched donors and not matched donors could be because the sample size was smaller, or the time between vaccination and the date of experiment affected the electrical response to RBD-stimulation. The time since natural infection or vaccination to SARS-CoV-2 and the date of the experiment for each donor varied between 7 – 95 days, thus the number, functionality, and response of SARS-CoV-2 specific CD4⁺ and CD8⁺ T-cells to the RBD-stimulation may have resulted in different electrical responses across this period. However, simple linear regression found no correlation between the electrical response to RBD-stimulation and the number of days since either natural or artificial exposure to COVID-19 (**Appendix A.3 Figure 71**). For each electrical parameter, the gradient of the slope was not significantly greater than zero, with a p value greater than 0.05 for each donor cohort. Furthermore, the r^2 values denoting the goodness-of-fit of the linear regression line were all less than 0.3. Altogether, this indicates the duration of time between the experiment and COVID-19 exposure does not predict the RBD-stimulation response to SARS-CoV-2 in any donor cohort. The coefficient of the linear regression model, the 95% CI of the slope, the p-value, and the r^2 value is quoted in **Appendix A.3 Table 25**.

Studies have shown that SARS-CoV-2 specific CD4⁺ and CD8⁺ T-cell immune responses are established and maintained in naturally infected and vaccinated donors between 7-95 days post-exposure. In BNT162b2 vaccinated donors, robust expansion of fully functional SARS-CoV-2 specific CD8⁺ T-cells occurs within one week of vaccination (Oberhardt et al., 2021), and expansion of SARS-CoV-2 specific CD4⁺ T-cells occurs within 29 days of vaccination (Sahin et al., 2021). In most donors, the immune memory is also maintained more than 95 days post-vaccination. Strong IFN γ ⁺ or IL-2⁺ CD8⁺ and CD4⁺ T-helper type 1 cell responses are maintained nine weeks after booster dose (Sahin et al., 2021), and polyfunctional CD4⁺ T-cells persist 141-210 days following the second vaccination (Vogel et al., 2022). Similarly, SARS-CoV-2 specific CD4⁺ T-cells have been detected as early as day four PSO in recovered COVID-19 donors (Rydzynski Moderbacher et al., 2020) and memory T-cell responses within a few weeks PSO (Law et al., 2022). Longitudinal assessments show SARS-CoV-2-specific memory B and T-cells persist for a minimum of three months PSO – including a prominent population of IgG⁺CD27⁺CD21⁺ RBD-specific memory B-cells which increase between 1 to 3 months (Rodda et al., 2021). Another study found T-cell responses in recovered COVID-19 donors persist up to 10 months PSO (Law et al., 2022). On this basis, it was hypothesised that SARS-CoV-2 specific adaptive immune responses would be observed following 3-hour RBD-stimulation. However, in parallel experiments to the 3DEP, Dr Alexander Stewart and Emma Sinclair investigated changes in the gene expression of PBMCs donated from three second dose donors at a single-cell level following 3-hour RBD-stimulation using the Cell Ranger (10x Genomics). Their data analysis did not identify any changes in gene transcription in memory B-cells or memory T-cells related to BCR or TCR signalling in response to

three-hour RBD-stimulation. This indicates that the adaptive immune response was not activated by RBD-stimulation, although transcriptomic changes do not always correlate with phenotypic changes. Cells of the innate immune system were activated at this timepoint, as innate cells including classical monocytes and DCs had upregulated gene expression of pro-inflammatory chemokines and cytokines including TNF, IL1A, and IL-6.

The SARS-CoV-2 Spike Glycoprotein RBD (Active) (Abcam, ab273065) used in this study has been used in previous publications (Tichauer et al., 2021, Gerasimenko et al., 2022, Tsilioni and Theoharides, 2023); the buffer of which contains 1.02% imidazole, a potent TLR7/8 agonist (Hemmi et al., 2002, Yang et al., 2021). Although imidazole was hypothesised to cause the observed activation of the innate immune cells, values of G_{eff} in the vehicle control (PBMCs incubated in the buffer without the RBD-protein) were comparable to matched unchallenged PBMCs, and were statistically different from matched SARS-CoV-2 RBD treated PBMCs analysed using a repeated measures one-way ANOVA followed by Tukey's multiple comparisons (* $p = 0.0426$). Values of G_{eff} are plotted in **Figure 50**.

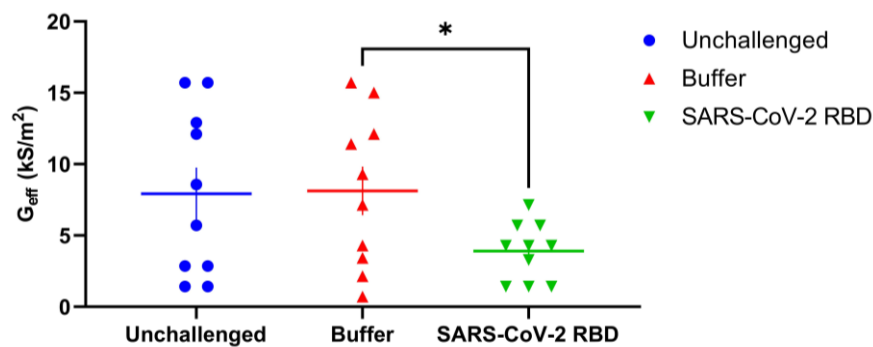


Figure 50 | Values of G_{eff} ($n = 10$ donors) measured in unchallenged PBMCs (blue circles) compared with three-hour incubation in the buffer minus the SARS-CoV-2 RBD (red triangle), and incubation in the buffer with the SARS-CoV-2 RBD (green inverted triangles). Statistical significance assessed using a matched one-way ANOVA followed by Tukey's multiple comparison (* $p < 0.05$). Mean lines plotted ($\pm$ SEM).

The significant decrease in G_{eff} between COVID-19 naïve and third dose donors may therefore be due to vaccine-induced changes in the innate immune response. Fohse et al. (2021) reported that cytokine production following SARS-CoV-2 specific and non-specific stimulation of innate immune cells changed following BNT162b2 vaccination (Föhse et al., 2021). Other changes in the innate immune system following three-doses of SARS-CoV-2 mRNA vaccinations includes significantly increased classical and intermediate inflammatory monocytes, INF γ production, and the perforin and granzyme content of NK cells (Saresella et al., 2022). The decrease in G_{eff} following SARS-CoV-2 stimulation may thus be

associated with changes in the proportions of innate immune cells following vaccination, or changes in their activation.

4.4.4. Streamlining Data Analysis

Although the change in G_{eff} following three-hour RBD-stimulation statistically differentiates individuals who are COVID-19 naïve from unmatched third dose vaccinated donors (**Section 4.4.3.**), a method of data analysis which takes the information gathered from a 3DEP spectrum without MATLAB modelling would be preferential, by reducing the expertise and complexity required, eliminating the time of data processing, and the necessity to measure cell radius. As shown in **Figure 51**, there are big differences in the DEP spectra of unstimulated (**Figure 51A**) and RBD-stimulated (**Figure 51B**) PBMCs between different cohorts at low frequencies (circle in orange), as expected as the low-frequencies correspond to values of G_{eff} .

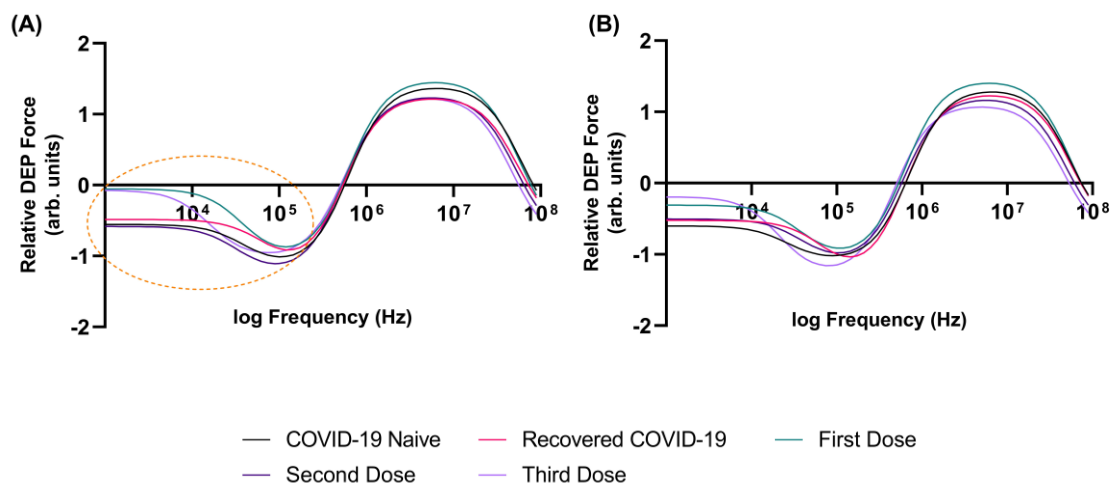


Figure 51 | Modelled DEP spectra of fresh PBMCs after 3-hour stimulation with the receptor binding domain of the SARS-CoV-2 spike protein in COVID-19 naïve (blue line; $n = 9$), recovered COVID-19 (red line; $n = 5$), first dose (green line; $n = 2$) and second dose (purple line; $n = 8$) donors. **A** | Unstimulated PBMCs **B** | RBD-stimulated samples. Mean relative DEP force of donor cohort plotted against log frequency (Hz).

Using the same validated methods of 3DEP spectra analysis as used in **Section 3.4.3**, MDV and ABCD data analysis methods were applied. MDVs were normalised for each donor by subtracting the unchallenged values from the RBD-stimulated values, plotted in **Figure 52A**. Although the mean MDVs increased between COVID-19 naïve (0.017 ± 0.04) and both second dose (0.14 ± 0.10) and third dose (0.010 ± 0.06) donors, ordinary one-way ANOVA followed by Tukey's multiple comparison demonstrated these changes were not significant ($p = 0.7458$ and $p = 0.9056$ respectively). Normalised MDVs in recovered COVID-19, first dose, second dose, and third dose donor cohorts were plotted

against the duration of time (days) that had passed between either testing positive for COVID-19 or receiving the vaccine, and the date of the experiment (**Figure 52B**). Although simple linear regression models demonstrated normalised MDVs increase in recovered COVID-19 ($r^2 = 0.8338$) and decrease in second dose donors ($r^2 = 0.4653$) over time since exposure to SARS-CoV-2, the gradient of these models were not significantly different from zero ($p > 0.05$). The coefficient and the 95% CI of the simple linear regression models are quoted in **Table 15**, in addition to the P-value which determines whether the gradient of the slope is significantly greater than zero ($*p < 0.05$) and the r^2 value denoting the goodness-of-fit of the linear regression line.

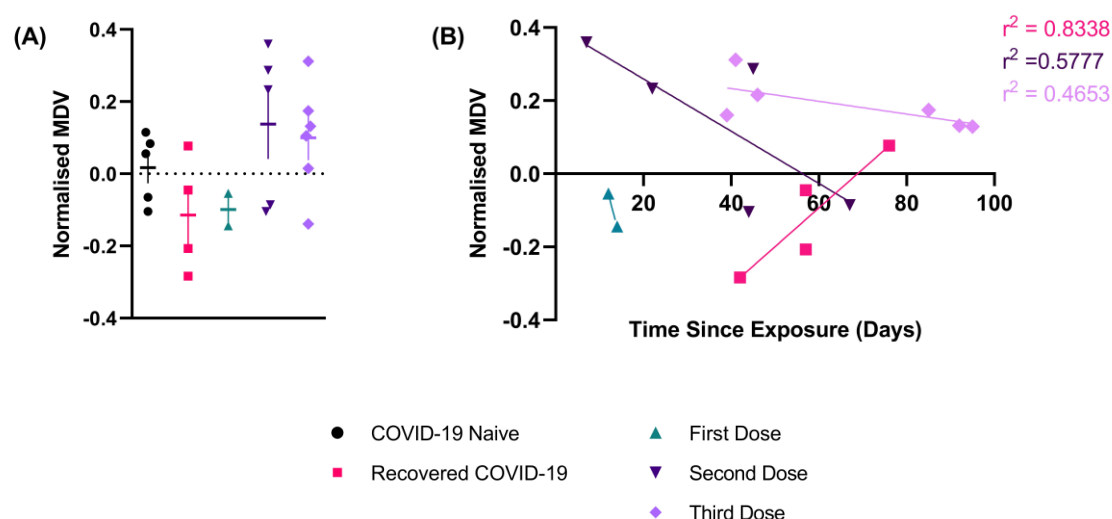


Figure 52 | A Normalised MDVs following 3-hour RBD-stimulation in COVID-19 naïve (black circles: $n = 5$), recovered COVID-19 (pink squares: $n = 4$), first dose (teal triangles: $n = 2$), second dose (dark purple inverted triangles: $n = 5$), and third dose (lilac diamonds: $n = 6$) donors. Black lines plots mean of biological repeats $\pm$ SEM. Statistical significance denoted by $*p < 0.05$ using ordinary one-way ANOVA. **| B** Normalised MDV over time in recovered COVID-19, first dose, second dose, and third dose donors. Fitted lines plot simple linear regression models, and corresponding r^2 values indicate the goodness-of-fit of the regression model by determining the variance attributable to the number of days between the date of the experiment and the date of COVID-exposure.

Table 15 | Simple linear regression models were fitted to indicate the trend in the normalised MDVs between date of experiment and date of exposure, either by natural infection or vaccination, in unmatched donors. Coefficient denotes the gradient of the slope, and the 95% CI. P-value determines whether the slope is significantly greater than zero ($*p < 0.05$, $**p < 0.01$). r^2 value indicates the goodness-of-fit of the regression model by determining the variance attributable to the number of days between the date of the experiment and the date of COVID-exposure.

	Donor Cohort	Coefficient	95% CI	P-value	r^2 Value
MDV	Recovered COVID-19	0.0106	-0.00380 to 0.0250	0.0869	0.8338
	Second Dose	-0.00715	-0.0184 to 0.00408	0.136	0.5777
	Third Dose	-0.00175	-0.00434 to 0.000852	0.136	0.4653

Similarly, normalised A, B, C, or D values were not statistically different between donor cohorts using ordinary one-way ANOVAs followed by Tukey's multiple comparisons. Normalised A values and normalised values of datapoint 2 for each donor cohort, and how the values change over time since most recent exposure is plotted in **Appendix A.3 Figure 72**.

Therefore, changes in the value of G_{eff} following three-hour RBD-stimulation best differentiates COVID-19 naïve and third dose donors, remaining the best candidate for CoP when using DEP. Changes in G_{eff} following RBD-stimulation for both cohorts are plotted in **Figure 53A**, and are statistically different using an unpaired two-tailed t-test (* $p = 0.0229$). To evaluate the diagnostic accuracy of the change in G_{eff} , a ROC curve plotting the true positive rate (sensitivity) against the false positive rate (100-specificity) was used, as shown in **Figure 53B**. The AUC was 0.7278 with a p value of 0.0942. For a 'best' threshold of -1.001 kS/m^2 (chosen as it is closest to 0.0, 100.0 on the ROC curve), the change in G_{eff} following RBD-stimulation differentiates COVID-19 naïve and third dose donors with a sensitivity of 60.00% and specificity of 88.89%.



Figure 53 **[A]** Change in G_{eff} in COVID-19 naïve (black circles: $n = 9$) vs. third dose donors (lilac diamonds: $n = 10$). Black lines plot the mean of all biological repeats $\pm$ SEM. Statistical significance denoted by * $p < 0.05$ using unpaired two-tailed t-test. **[B]** The non-parametric ROC curve (grey dots connected by black line) plotting sensitivity% (true positive rate) against 100-specificity% (proportion of true negatives). The 45° line (red) denotes the theoretical plot of no discrimination between COVID-19 naïve and third dose donors.

The sensitivity of this method at distinguishing COVID-19 naïve and third dose donors is low at 60.00%, but still comparable to other CoP immunoassays which have quoted sensitivities and specificities between 60 – 100%, depending on the assay manufacturer and the SARS-CoV-2 variant (Meschi et al., 2020, Pickering et al., 2020, Valdivia et al., 2021, Walker et al., 2021, Camacho et al., 2023). Moreover, to become a validated CoP, actual protection from disease correlating with the candidate metric is required. Although the time of data collection and analysis is quick, given the sensitivity and specificity

is markedly lower than that of the gold-standard CoP ELISAs, further optimisation and investigation into understanding why G_{eff} decreases is needed before it can be considered a competing CoP.

4.4.5. Limitations

Donor Recruitment

The main limitation of this pilot study was the limited number of participants, particularly in the first dose donor cohort, due to difficulties in recruiting donors who fulfilled the criteria, laboratory access, and social distancing measures during the pandemic. A higher sample size is required to increase the validity of the statistically significant differences observed in σ_{cyto} in unchallenged PBMCs between donor cohorts, and in G_{eff} following RBD stimulation. Furthermore, in donor cohorts of this size it is not possible to reliably evaluate age, gender, ethnicity, or vaccine-related differences in the electrical response to PBMC stimulation. Increased age (Romero Starke et al., 2021, Zimmermann and Curtis, 2022), male gender (Jin et al., 2020), and ethnic minorities (Irizar et al., 2023) have been identified as risk-factors to COVID-19 and correlate with the severity of infection. However, as the mean age of all donors was 31.5 ± 11 years, a gender imbalance persisted in each donor cohort, and 75% of all participants were white, additional donor recruitment is required to investigate this further. Additionally, within the vaccinated cohorts, the majority of donors received the BNT162b2 mRNA vaccine and only two received the Oxford/AstraZeneca vaccine, thus a larger cohort is also required to identify vaccine-related differences. It would also be interesting in future experiments to explore whether the time between vaccine doses affects the PBMC electrical properties.

As previously mentioned in **Section 4.3.3.**, an individual was assigned COVID-19 naïve if they had not experienced symptoms to suspect COVID-19 since the outbreak of the pandemic. However, it is possible that some of the COVID-19 naïve donors had previously experienced asymptomatic SARS-CoV-2 infections, given the similarities of symptom presentation to the common cold and flu viruses and mixed reports stating 4 - 41% of individuals present with asymptomatic SARS-CoV-2 infection (Byambasuren et al., 2020). Particularly before the launch of NHS Test and Trace on the 28th May 2020, preceding the widespread availability of COVID-19 PCR test centre sites, implementation of mass screening and availability of free rapid lateral flow self-tests in April 2021, it was uncertain whether someone had asymptomatic COVID-19 early in the pandemic. The analytical sensitivity of lateral flow tests also vary between kits between approximately 76 - 92%, varies according to COVID-19 variant, and is reduced in individuals with a lower viral load (UK Health Security Agency, 2021, Mackenzie et al., 2022). Therefore, negative rapid antibody tests (SureScreen Diagnostics COVID-19 Antibody Rapid

Test) for virus-specific IgM and IgG antibodies were required on the day prior to blood donation to increase the certainty that COVID-19 naïve individuals had not had a previous SARS-CoV-2 infection, but it can take time for seroconversion to occur, and as previously reviewed in **Section 4.2.3.1.**, antibodies decline over time. IgG antibodies are detectable in approximately 90% of individuals 10-11 days following infection (Isho et al., 2020), but can take 19 – 22 days (Long et al., 2020).

Assessing Immune Status

When assessing whether the statistically significant difference in the change in G_{eff} to SARS-CoV-2 stimulation between COVID-19 naïve and third dose donors could function as a CoP, it was assumed that vaccinated individuals are immune. However, to investigate whether the electrical response of PBMCs to SARS-CoV-2 stimulation could function as a CoP, future experiments necessitate validation against other CoP such as NAb function, antibody titres, and functional T-cell response to be quantified in parallel using methods such as ELISpot assays (Sahin et al., 2021, Vogel et al., 2022), ICS (Sahin et al., 2020, Vogel et al., 2022), or IGRAs (Peng et al., 2020, Vogel et al., 2022). Furthermore, it requires continual real-time observation and follow-up of the donors to identify whether they acquire SARS-CoV-2 infection or symptomatic infection, in addition to whether this potential CoP withstands against different VOC.

Variants of Concern (VOC)

The mutation rate of SARS-CoV-2 is very high because there is no mismatch repair system during SARS-CoV-2 replication. Thousands of SARS-CoV-2 variants have emerged since the pandemic began, but VOC such as alpha, beta, gamma, delta, and omicron BA.1 and BA.2 are those which pose a greater threat to global public health because they are more transmissible and/or more virulent and/or public health and social measures such as treatments, vaccines and diagnostics are less effective against them (European Centre for Disease Prevention and Control, 2021, Zawilska et al., 2021). It is important to consider whether a CoP is effective across different VOC. For example, the level of neutralisation activity from two doses of COVID-19 vaccine is less in Delta and Omicron variants than the alpha variant (Shen et al., 2022). The effectiveness of either the BNT162b2 or ChAdOx1 vaccine in protecting against symptomatic disease is also lower for the Delta variant (Lopez Bernal et al., 2021a) and much lower for the Omicron variant (Andrews et al., 2022a) than for the alpha variant. As the technique explored in this chapter investigates the electrical response to the RBD, it is important to consider whether the change in G_{eff} in third dose donors correlates to protection

against VOC which have a mutated RBD. Future experiments which explore the electrical effect of other SARS-CoV-2 epitopes which are conserved across variants requires exploration.

Dielectrophoresis of a Heterogenous Population

As PBMCs are a heterogenous population, values of G_{eff} , C_{eff} , and σ_{cyto} obtained from MATLAB-fitted models of the combined DEP spectra constitute an aggregate contribution of the different subpopulations. The dielectric properties of PBMCs differ between leukocyte subpopulations, and as such can be separated using DEP (Yang et al. 1999). Huang et al. (2014) identified different cross-over frequencies of monocytes, NK cells, B-lymphocytes, and T-lymphocytes. Therefore, it is not possible in studies which use heterogenous populations to determine whether changes in the electrical properties are due to specific changes in a certain cell type – for example, whether SARS-CoV-2 stimulation resulted in CD4⁺ or CD8⁺ activation. Additional experiments are required to better correlate electrical changes to PBMC action, such as using flow cytometry to assess the composition of different PBMC subpopulations between donor cohorts using CD markers (Abcam, No Date-b), and quantify well-established markers of PBMC activation, such as expression of CD25, CD69 and CD19 for lymphocyte activation (Abcam, No Date-a, Haller et al., 2000, Dieterlen et al., 2014, Gerasimova et al., 2022). Alternatively, 3DEP analysis of single cell populations isolated using methods such as magnetic-activated cell sorting (MACS), fluorescence-activated cell sorting (FACS), microfluidic cell sorting, or dielectrophoretic cell sorters. However, disadvantages to these techniques include machinery costs, large sample volumes are required, the rapid flow and fluorescent labelling of cells in FACS or conjugated magnetic beads in MACS can damage the viability of sorted cells (Hu et al., 2016). As the primary objective of this study was to identify a potential CoP to SARS-CoV-2, investigating any differences in the overall combined PBMC electrical response to SARS-CoV-2 between donor cohorts using a fast, easily adoptive, relatively inexpensive, and quick tool was favoured (similarly to **Chapter 3**).

Streamlining the Experimental Protocol

As shown in **Figure 54**, the protocol for 3DEP data collection used in this study is quick and has a fast-turnaround compared with other methods used to measure CoP to SARS-CoV-2. However, to consider reducing the data collection protocol further, the major time limiting steps include PBMC isolation (Step 2) and the three-hour RBD-stimulation (Step 4). In comparison, sample washing, 3DEP data collection and cell microscopy collectively only takes 15 – 20 minutes per sample. To reduce the

time of sample preparation and increase the flexibility of data collection in a clinical setting, it would be beneficial to determine whether the differences in normalised values of G_{eff} withstand when frozen PBMCs are used instead of freshly isolated PBMCs. Furthermore, whether removing the wash step before PBMC analysis (Step 5) affects the sensitivity or specificity of normalised G_{eff} , as this would act to decrease the sample preparation time and shorten the experimental protocol – improving its adoptability in clinical practice.

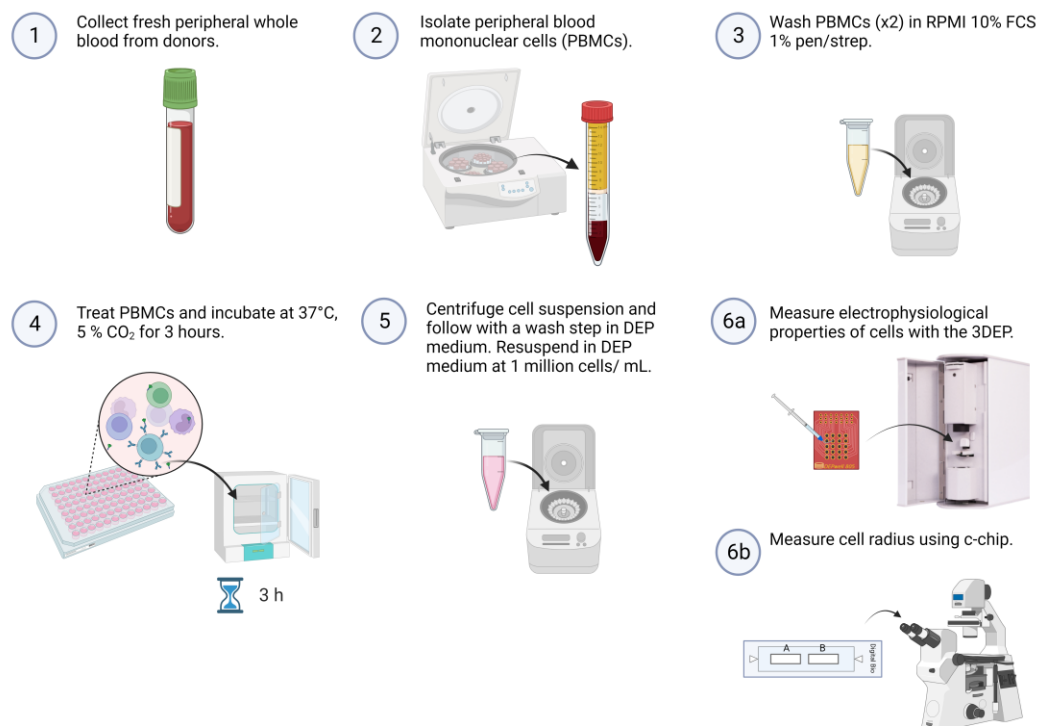


Figure 54| Optimised experimental protocol for isolating PBMCs from fresh whole blood and 3-hour stimulation for evaluating immune status. Created with BioRender.com.

4.4.6. Conclusion

There is a great necessary to find a validated CoP that can determine whether an individual is protected against SARS-CoV-2 infection and severe symptomatic presentation. A low-cost, easily adoptable method of measuring a reliable immune marker would be invaluable in evaluating the efficacy of vaccines, accurately ascertaining population-level immunity, informing immunisation programmes, and developing recommendations on public health and social measures. Current methods used to measure CoP including antibody titres, NAb function, and functional T-cell response assays have limitations including they lack an ‘absolute threshold’ to SARS-CoV-2 infection/symptomatic presentation, and they are either time intensive, technically complex, require

expensive machinery, or low-throughput. In comparison, DEP is a quick, low-cost, and high-throughput technique. Given that the electrical properties of PBMCs change when activated, as shown during the positive control PMA/ionomycin stimulation experiments, it was hypothesised that DEP may be able to distinguish differences in cellular immune activation to the SARS-CoV-2 RBD between individuals who are protected against COVID-19 and those who have no adaptive immune response. Based on published literature quoting the decreased rate of re-infection post-COVID, high efficacies of vaccination, and protection persisting for more than 6 months, donors who were recovered COVID-19 or had received one, two, or three doses of either the mRNA Pfizer-BioNTech (BNT162b2) or the recombinant replication-deficient viral vector Oxford-AstraZeneca (ChAdOx1) vaccine within 7 – 92 days were considered to have an adaptive immune memory to SARS-CoV-2.

Following 3-hour RBD-stimulation, G_{eff} significantly decreased in third dose donors (** $p = 0.0039$), which was not observed in any other cohort. This change in G_{eff} differentiated unmatched COVID-19 naïve and third dose donors with a sensitivity of 60.00% and specificity is 88.89%. Although DEP is advantageous in that data collection is quick and shows promise as a potential CoP, given the sensitivity and specificity is markedly lower than that of the gold-standard CoP ELISAs, further optimisation and investigation into understanding why G_{eff} decreases, and only in third dose donors, is needed for protocol optimisation before it can be considered a competing CoP. Interesting insights into differences in the baseline electrical properties of unchallenged PBMCs were reported in this chapter between donor cohorts, with an increase in G_{eff} and significant decrease in σ_{cyto} between COVID-19 naïve and both second dose and third dose donors.

5. INVESTIGATING THE DIELECTRIC PROPERTIES OF PRIMARY CHONDROCYTES DURING CELL CULTURE AND IN DIFFERENT MEDIUM CONDUCTIVITIES

This final research chapter investigates how the electrophysiological properties of chondrocytes change over time in cell culture, and during acute suspension in media of different ionic conductivities.

5.1. Chapter Overview, Aims, and Objectives

During monolayer cell culture, chondrocytes rapidly dedifferentiate and adopt a more fibroblastic phenotype (Mao et al., 2019). Chondrocyte dedifferentiation greatly impacts both research and clinical applications, such as cell-based cartilage repair therapies which require significant *in vitro* expansion to obtain sufficient chondrocyte numbers for reimplantation. As chondrocytes adopt a more fibroblastic phenotype, the efficacy and success of reimplantation surgery decreases, with up to 70% of patients developing fibrocartilaginous fill (Bartlett et al., 2005, Huang et al., 2016, Mao et al., 2019). Therefore, the first aim of this chapter was to investigate whether the dielectric properties of primary chondrocytes change over time in cell culture, which could pave the way towards a new tool which evaluates electrophysiological changes over time to identify changes in chondrocyte phenotype.

The second aim of the study presented in this chapter was to investigate how the dielectric properties of chondrocytes change during acute suspension in media of different ionic conductivities. Current research provides evidence that V_m , membrane conductance (both transmembrane and membrane surface), membrane capacitance, σ_{cyto} , and ζ -potential are electrostatically interconnected within biological cells. This electrostatic interconnectivity is termed the cellular electrome (Hughes et al., 2021). To investigate the cellular electrome of healthy cells in more detail, the experiments in this chapter investigate how the dielectric properties of chondrocytes change during acute suspension in media of different ionic conductivities, and over time in cell culture. This chapter examines how dielectric parameters are interconnected in an adherent healthy cell model, and the mechanism by which this may occur – expanding on the theory of the CM-model applied during DEP. The data collected contributed to ongoing research within the DEP research team at the University of Surrey to evaluate what parameters can be inferred from the CM-model – distinguishing a method of measuring V_m using DEP. Using DEP to characterise the electrical properties of cells during health and disease is very valuable, as provides more detailed electrophysiological characterisation of cells and may infer changes in cell function.

5.2. Literature Review

5.2.1. Chondrocyte Dedifferentiation During Cell Culture

Over 250 million people worldwide are affected by cartilage injury and degradation (Chen et al., 2022b). Due to the avascularity of cartilage, the absence of mesenchymal cells, and the reduced migration ability of chondrocytes to travel to the site of injury, articular cartilage has very limited regenerative capabilities (Costa et al., 2018, Mao et al., 2019). Cartilage tissue-engineering based on autologous chondrocytes can be used to repair cartilage lesions. To perform autologous chondrocyte implantation (ACI), a biopsy is taken from a small non-bearing site of a joint, the ECM is enzymatically digested, and chondrocytes are isolated (Costa et al., 2018). Significant *in vitro* expansion of isolated chondrocytes is required to obtain a sufficient cell number before re-implantation (Mao et al., 2019) – a workable number of chondrocytes requires at least 2-3 weeks of chondrocyte expansion (Chen et al., 2022b).

However, during standard monolayer cell culture protocols, chondrocytes rapidly dedifferentiate and change phenotype to become more fibroblastic (Layman et al., 1972, Lin et al., 2008, Schulze-Tanzil, 2009, Mao et al., 2019, Ghosh et al., 2021), and senescent (Piñeiro-Ramil et al., 2023). Dedifferentiation of chondrocytes during the required *in vitro* expansion for ACI contributes to suboptimal clinical outcomes (Mao et al., 2019) as cells adopt a fibroblast-like morphology and an abnormal ECM. Only 15-30% of defects show hyaline-like repair tissue following treatment, with the remaining cohort developing fibrocartilaginous fill (Bartlett et al., 2005, Huang et al., 2016). As the hyaline chondrocyte phenotype is lost, the efficacy of ACI is much reduced (Duan et al., 2015) as the response of chondrocytes to stretch and mechanical loading is altered *in vivo* after reimplantation (Das et al., 2008).

To overcome chondrocyte dedifferentiation, three-dimensional culture aggregate systems (Wolf et al., 2008, Caron et al., 2012, Huang et al., 2016), high-density culture (Schulze-Tanzil et al., 2002), use of different media supplementations (Jakob et al., 2001), chemical inhibitors (Chen et al., 2022b), and sustained hypoxia (Mennan et al., 2019) have been assessed. However, phenotypic changes in chondrocytes following passage three are still recognised to limit their applications in research and clinical use (Kwon et al., 2021). Characterised chondrocyte implantation (CCI) is licensed in Europe to assess the chondrocyte phenotype prior to reimplantation to optimise patient outcomes (Saris et al., 2008, Vanlauwe et al., 2011). Other methods which can be used to monitor chondrocyte dedifferentiation include changes in gene expression (Ma et al., 2013), although Stenberg et al. (2014) found no significant differences in gene expression between patients with a successful outcome and

failures (Stenberg et al., 2014). Chen et al. (2022) investigated the use of single-cell RNA sequencing, live-cell metabolic assays, transpose-accessible chromatin with high-throughput sequencing (ATAC-seq) and developed an image-based approach of predicting chondrocyte plasticity (Chen et al., 2022b). Additional methods of assessing changes in chondrocytes during cell culture would prove very beneficial for research and clinical applications.

Research on the electrophysiological properties of chondrocytes over time in cell culture is limited, although some evidence has demonstrated that the electrical properties of chondrocytes are affected by the duration in cell culture and passage number. Hdud et al. (2012) quantified the expression of TRPV4, TRPV5, and TRPV6 ion channels using western blotting in passage 0 to passage 3 monolayer-cultured chondrocytes. Expression of TRPV5 and TRPV6 increased with passage number and time, suggesting changes in phenotype alter the expression of these channels (Hdud et al., 2012). However, changes in the dielectric properties of chondrocytes over the duration in cell culture remains uncharacterised. The first aim of this chapter was thus to investigate whether there are any changes in the dielectric properties of chondrocytes during monolayer cell culture over time; with a goal of providing deeper insight into chondrocyte dedifferentiation at the electrical level. This work acts to provide a foundation for future experiments which can relate changes in electrical properties to changes in chondrocyte phenotype, in the hope of identifying a low-cost, easily adoptable, and high throughput technique which could assess chondrocyte quality control for research and clinical applications.

5.2.2. Investigating the Chondrocyte Cellular Electrome Model

5.2.2.1. *The Cellular Electrome Model*

In parallel with the aforementioned experiments, the second aim of this chapter was to investigate how the dielectric properties of chondrocytes are affected by acute suspension in media of different ionic conductivities to provide greater insights into the developing theory of the cellular electrome model.

The cellular electrome (described in **Section 2.2.**) is shown again and summarised in **Figure 55**. Recent studies in suspension cell types including RBCs (Hughes et al., 2021, Hughes et al., 2022a, Hughes, 2023) and platelets (Hughes et al., 2022b, Hughes, 2023) have shown relationships between V_m , membrane conductance (both transmembrane and membrane surface), membrane capacitance, σ_{cyto} , and ζ -potential, establishing an interconnected cellular electrome model. For example, strong pairwise correlations between ζ -potential and G_{eff} , and between V_m and σ_{cyto} have been observed in

platelets (Hughes et al., 2022b). Significant linear relationships between V_m and σ_{cyto} have also been found in RBCs (Hughes et al., 2021). Furthermore, strong negative correlations have been observed between σ_{cyto} and G_{eff} in RBCs (Hughes et al., 2021), consolidated by reported antiphase circadian rhythms in G_{eff} and σ_{cyto} in RBCs (Henslee et al., 2017a) – suggesting that both parameters share a common origin.

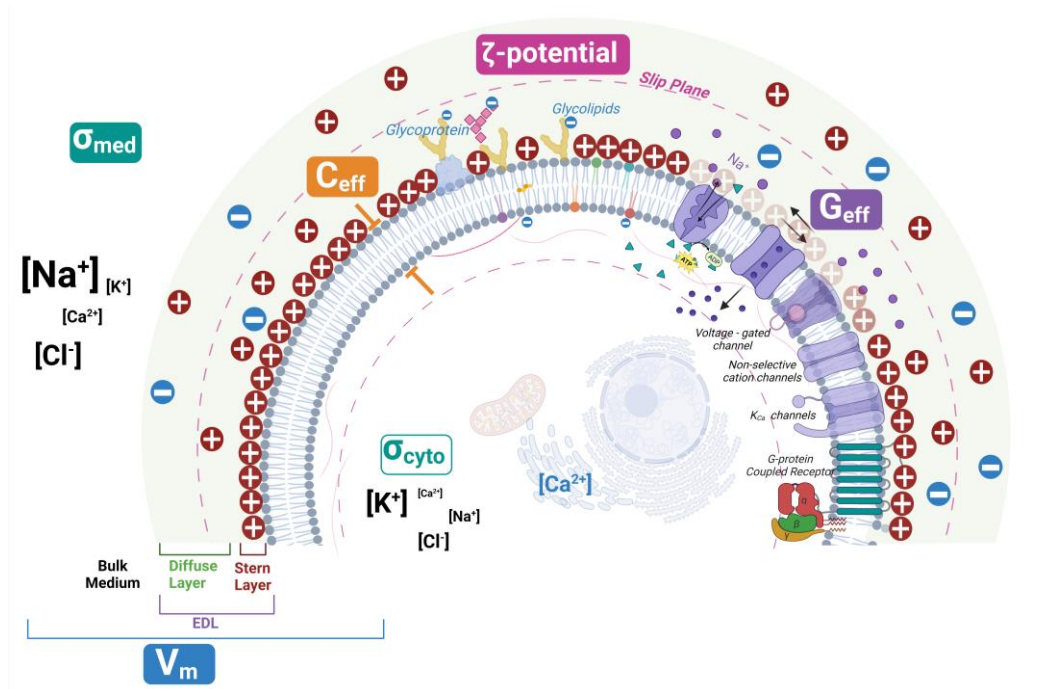


Figure 55 | Overview of the cellular electromechanical model. Font size is relative to ion concentrations, denoted by [ion]. (Created with BioRender.com).

Moreover, modulation of V_m has been shown to alter ζ -potential in both RBCs (Miyake and Kurihara, 1983, Hughes et al., 2021) and platelets (Hughes et al., 2022b). The effect of V_m on ζ -potential was investigated by incubating RBCs in isosmotic media of different ionic strengths, as lowering the ionic strength of extracellular media is known to change V_m and the thickness of the EDL in RBCs (Moersdorf et al., 2013). The change in V_m was found to be proportional to changes in ζ -potential, with a best fit achieved when a coefficient of proportionality independent of double layer thickness was added (Ξ) (Hughes et al. 2021). Traditionally, cellular V_m has been considered to depend purely on the differences in the intracellular and extracellular concentrations of ions such as K^+ , Na^+ , Cl^- , and Ca^{2+} , and their transmembrane conduction across the cell membrane (described in **Section 2.2.3.**) (Yang and Brackenbury, 2013, Hughes et al., 2021). Potential differences have not been considered to extend past the membrane, and the electric field past the membrane assumed to equal zero (Hughes et al., 2022a). To calculate V_m , the concentration of intracellular and extracellular ions used in the GHK equation (**Equation 2**) uses that of the bulk medium in the Gouy-Chapman model as opposed to the concentration of ions at the cell surface within the EDL. The concentration of ions at the cell surface

is defined by the Poisson-Boltzmann equation (**Equation 18**), with counterion concentrations greatest at the cell surface due to electrostatic attraction, and co-ion concentration decreasing with distance to the cell surface (Hughes et al., 2021).

$$c_i(0) = c_{oi} \exp\left(\frac{-z_i e \psi}{kT}\right) \quad \text{Equation 18}$$

In **Equation 18**, $c_i(0)$ is the concentration of ion i at the Stern layer, c_{oi} is the concentration of ion i in the bulk medium, z is the valency, e is electron charge, k is Boltzmann's constant, and T is temperature. Although the variation in ion concentration surrounding a cell is accounted for in the permeability coefficients of the GHK equation, evidence suggests there is an endogenous electric field which extends beyond the cell membrane to at least the plane of shear, with V_m effects beyond the membrane into the intracellular and extracellular spaces (Hughes et al., 2021, Hughes et al., 2022a). The measurable changes in ζ -potential with V_m in RBCs (Hughes et al., 2021) and platelets (Hughes et al., 2022b) suggests that as opposed to a one-way causal relationship of extracellular ion concentration affecting V_m ; V_m itself also affects extracellular ion concentrations within the EDL (Hughes et al., 2022a).

The impact of V_m on changing extracellular ion concentrations may need to be incorporated into the GHK equation for a more accurate V_m model (Hughes et al., 2021). Ingolf Bernhardt et al. (1984) previously altered the GHK equation to consider ion concentrations with respect to inner and outer membrane surface charges (Bernhardt et al., 1984), but was dismissed with the assumption that surface charges are too small to significantly affect V_m in studies where low ionic strength media is not used (Hughes et al., 2022a). However, this assumption may not be the case as when platelets are activated by storage at 4 °C, Hughes et al. (2022b) found both V_m and ζ -potential were connected to the amount of binding of the surface adhesion molecule P-selectin. This effect observed in platelets is further indicative of the effect V_m itself has on surface charge and consequently the availability of anion and cationic molecules at the cell surface, resulting in differences in the number of ions available for membrane transport. Moreover, it can change how charged macromolecules such as antibodies, approach, and bind to the cell surface – thus altering protein-cell interactions (Hughes et al., 2022a, Hughes et al., 2022b). Understanding the cellular electrome model in more detail would ultimately provide greater insight into how changes in the electrical properties affect cell function and cell interactions with their extracellular environment – which is important in assessing changes in function during health and disease states.

5.2.2.2. The Influence of Medium Conductivity on the Cellular Electrome

To understand the mechanism by which the properties in the cellular electrome are related, the effects of σ_{med} on V_m , G_{eff} , C_{eff} , and σ_{cyto} have previously been explored in suspension cells including RBCs (Hughes et al., 2022a) and platelets (Hoettges et al., 2019). Results of these experiments have suggested that ion imbalances within the inner and outer EDLs form a component of the resting V_m , and form a capacitor across the cell membrane which interconnects electrical parameters (Hughes et al., 2022a, Hughes, 2023).

One assumption of the CM-model is that σ_{cyto} is largely electrically independent of the extracellular environment (Hughes et al., 2022a). However, Hoettges et al. (2019) demonstrated that this is not the case, recording values of σ_{cyto} in platelets to increase from 150 mS/m in media of 200 mS/m, to 400 mS/m in media of 800 mS/m using the 3DEP. Hughes et al. (2022a) further investigated the relationship of σ_{med} and σ_{cyto} in RBCs, by suspending cells in different conductivities of an isotonic medium (145 mM NaCl, 7.5 mM KCl) diluted with an isotonic sucrose/glucose solution. Hughes et al. also investigated the effects of different treatments including DMSO, neuraminidase (cleaves sialic acids to remove surface charge on the extracellular leaflet of the cell membrane bilayer), valinomycin (increases membrane K^+ permeability), or neuraminidase + valinomycin. A linear relationship between σ_{cyto} and σ_{med} ($r^2 > 0.99$) was identified irrespective of RBC treatment conditions, with best fit lines plotted using **Equation 19**:

$$\sigma_{cyto} = A \cdot \sigma_{med} + B \quad \text{Equation 19}$$

Where A and B are constants. The constant B did not change with σ_{med} and drug-treatment, suggesting a residual value related to cytoplasmic components, unrelated to membrane diffusion such as proteins with low membrane permeability. However, the gradient of the line (A) varied with drug treatment, suggesting σ_{cyto} is a combination of outer and inner membrane surface charges (Hughes et al., 2022a).

Similarly to σ_{cyto} , Hughes et al. (2022a) found a linear relationship between V_m and σ_{med} , again consisting of a component dependent on σ_{med} and a value independent of σ_{med} (V_x). The relationship between V_m and σ_{med} has been fitted by **Equation 20**, derived by Hughes et al. (2022a):

$$V_m = \frac{(1/\kappa) \sigma_{med} \Lambda e^{(2-\frac{1}{A})}}{2C} + V_x \quad \text{Equation 20}$$

whereby $1/\kappa$ is Debye length; Λ is the number of ions per m^3 for 1 S m^{-1} medium conductivity; A is the ratio of free anions and cations in bulk medium and double layer, considering the action of Poisson-Boltzmann redistribution; V_x is constant. The linear relationship between the σ_{med} -dependent value of V_m suggested it is independent of diffusion and is instead electrostatic in origin; an imbalance of charge

at the inner membrane surface of the EDL which acts across the membrane capacitance to produce a voltage. In contrast, the σ_{med} -independent value of V_m (V_x) differed between control-treated RBCs and RBCs treated with drugs that affect membrane ion permeability drugs (e.g., valinomycin affects K^+ membrane permeability) suggesting this contribution to V_m is due to diffusion. The consistency of V_x in untreated RBCs incubated in different σ_{med} with different extracellular ion concentrations suggests this component arises from diffusion, with ion permeability remaining the same for different intracellular and extracellular ion concentrations, as calculated using the GHK equation.

G_{eff} and C_{eff} have also been shown to be dependent on σ_{med} in RBCs (Gascoyne et al., 1997, Hoettges et al., 2019, Hughes et al., 2021). However, this dependence not been observed in platelets or Jurkat cells (Hoettges et al., 2019). The effects of σ_{med} on σ_{cyto} , V_m , G_{eff} , C_{eff} , and ζ -potential using DEP has not been explored in any adherent cell types, such as chondrocytes. In parallel with investigating the changes in the electrical properties of chondrocytes over time, the experiments of this chapter investigate how the electrical properties of chondrocytes change with σ_{med} as a healthy cell model of an adherent cell type.

5.3. Methods

5.3.1. Primary Bovine Chondrocyte Cell Culture

Primary bovine chondrocytes were cultured in Gibco Dulbecco's Modified Eagle Medium (DMEM) (low glucose, 4 mM L-glutamine, sodium bicarbonate; Sigma-Aldrich, UK, #D6046) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Sigma-Aldrich, UK, #F2442), 2 % penicillin-streptomycin (Sigma-Aldrich, UK, #P4333), 1 % amphotericin B solution (Sigma-Aldrich, UK, #A2942) and 80 mM sucrose (Sigma-Aldrich, UK, #S9378). Cells were cultured in vented T75 flasks at 37 °C, 5 % CO₂, 95 % humidity, and passaged when 70-80 % confluent. To passage, culture medium was aspirated from the culture flask, and cells were washed with DMEM without FBS. To dissociate cells from the flask, 1.5 mL trypsin-EDTA (0.25 %) (ThermoFisher, UK, #25200056) was added and incubated for 5 minutes. To ensure cells were no longer adhered, the flask was gently tapped. DMEM was added to quench the trypsinization reaction, and the solution was centrifuged for 10 minutes at 285 g. The supernatant was aspirated, and the pellet resuspended in cell culture media. Cells were passaged at a split ratio of approximately 1:3, with the remaining cells used for DEP and/or ζ -potential measurements. The number of passages conducted for each biological repeat ($n = 5$) depended on the passage number the primary cell line was obtained at, and after what passage the primary cells

became unviable or when they were disposed of. Electrophysiological measurements were taken whilst in suspension of different percentages of saline: DEP media to adjust σ_{med} .

5.3.2. Chondrocyte Preparation

Following isolation from cell culture, aliquots of chondrocytes were each re-suspended at 1×10^6 cells/mL in a solution of different ionic conductivity for one five-minute wash step after which they were immediately characterised (acute suspension). A high-strength physiological ionic stock solution consisting of 145 mM NaCl (Sigma-Aldrich, UK, #S9625) and 7.5 mM KCl (Sigma-Aldrich, UK, #P9333) in DI H₂O was used to prepare solutions of five different conductivities (described in **Table 16**). Solutions consisted of 0 %, 1 %, 3 %, 10 %, 33 %, and 100 % stock solution, diluted in DEP medium containing 8.5 % w/v sucrose (Sigma-Aldrich, UK, #S9378), 0.3 % w/v dextrose (Sigma-Aldrich, UK, #G8270), 250 μ M MgCl₂ (ThermoFisher, UK, #447155000) and 100 μ M CaCl₂ (ThermoFisher, UK, #L13191.0I). A seventh media consisting of 10 mM tetraethylammonium chloride (TEA) (Sigma-Aldrich, UK, #T2265) supplemented to 10% saline: DEP medium was prepared to examine the effect of K⁺ channel activity on chondrocyte DEP properties over cell culture duration.

All media were filter sterilised using a 0.22 μ m filter (Millex Syringe-driven filter 0.22 μ m (Sigma-Aldrich, UK, #SLGP033RS) or Bottle Top Filtration Unit 0.22 μ m (StarLab, UK, #CC8221-5226) depending on media volume). Values of medium osmolality and conductivity were measured before commencing each experiment with mean measurements ($\pm$ SD) detailed in **Table 16**. Media osmolality was measured using the Gonotec® Osmomat Freezing Point Osmometer Model 3000 Basic (Wolflabs, UK, #32.B). σ_{med} was measured using the Hanna Instruments HI-99301 EC/TDS/Temperature Meter – High Range (Hanna Instruments, # HI-99301) and media pH measured using the Hanna Instruments 9811-51 pH/EC/TDS/°C meter (Hanna Instruments, #HI-9811-51). Measurements for cell culture medium were measured at 37 °C, and all saline: DEP medium were measured at room temperature (18-22 °C) as these were the temperatures the media were used at. All equipment was calibrated prior to measurement and three technical repeats obtained before each experiment.

Table 16 | Mean ($\pm$ SD) values of σ_{med} (mS/m) and osmolality (mOsm/kg).

Medium	Conductivity (mS/m)	Osmolality (mOsm/kg)
Cell Culture Medium	1370 $\pm$ 20	324 $\pm$ 16
0% saline: DEP medium	8 $\pm$ 0	292 $\pm$ 21
1% saline: DEP medium	21 $\pm$ 2	286 $\pm$ 19
3% saline: DEP medium	50 $\pm$ 6	275 $\pm$ 9
10% saline: DEP medium	150 $\pm$ 5	279 $\pm$ 10
10% saline: DEP medium + TEA	200 $\pm$ 18	309 $\pm$ 15
33% saline: DEP medium	447 $\pm$ 21	274 $\pm$ 7
100% saline: DEP medium	1324 $\pm$ 83	283 $\pm$ 8

5.3.3. Data Collection

Chondrocyte cell suspensions underwent one wash-step prior to data collection to wash-off remnant high conductivity cell culture media – the cell suspension aliquot underwent centrifugation at 300 g for five minutes, the supernatant was discarded, and the pellet resuspended in the same respective DEP medium at a cell concentration of 1×10^6 cells/mL. The electrophysiological properties of chondrocytes were measured using the 3DEP (DEPtech) for biological repeats 1 – 4. ζ -potential was additionally measured using the Lab Blue Label Zetasizer (Malvern Panalytical) for biological repeats 3-5. 3DEP, ζ -potential, and C-Chip data collection was performed as described previously in **Section 3.3.6**. Values of G_{eff} , C_{eff} , and σ_{cyto} were measured for chondrocytes suspended in 0%, 1%, 3%, 10%, 33%, and 100% saline: DEP medium between passage 2 – passage 6 for the first biological repeat. Based on limited absolute cell numbers, the cross-over frequencies and goodness-of-fit of the DEP spectra from these preliminary experiments, the DEP parameters of the subsequent three biological repeats were only measured at 1%, 3%, and 10%. Due to limited time and cell counts, no DEP measurements were obtained for the fifth biological repeat. ζ -potential was not measured for biological repeats 1 and 2 because access to a zetasizer was not possible.

5.3.4. Data Analysis

3DEP, ζ -Potential, and C-Chip Data Analysis

Raw data collected using the 3DEP machine and zetasizer, and C-Chip images were analysed following the methods outlined in **Section 3.3.7** to obtain values of G_{eff} , C_{eff} , σ_{cyto} , ζ -potential, cell radius (μm), cell concentration, and cell viability.

Statistical Analysis

D'Agostino-Pearson omnibus tests for normal distribution were first used to assess whether values of G_{eff} , C_{eff} , σ_{cyto} , ζ -potential, and cell radius were normally distributed, combining all measurements from different biological repeats and passage number by σ_{med} . The D'Agostino-Pearson omnibus test was used because it is a powerful and versatile test which measures the level of skewness and kurtosis of data from a normal distribution (Motulsky, No Date). Changes in the values of G_{eff} , C_{eff} , σ_{cyto} , ζ -potential, and cell radius of chondrocytes following acute incubation in different σ_{med} was analysed using repeated-measures one-way ANOVAs followed by Tukey's multiple comparisons with Geisser-Greenhouse correction (* $p < 0.05$) for normally distributed data, and the non-parametric Friedman test followed by Dunn's multiple comparisons (* $p < 0.05$) for non-normally distributed data. Matched statistical tests were chosen to be able to assess changes in the electrical parameters solely due to σ_{med} , with other variables including passage number/duration in cell culture and biological variation between bovine donors thus accounted for. Changes in G_{eff} , C_{eff} , σ_{cyto} , ζ -potential, and cell radius with duration in cell culture (as the independent variable) was subsequently assessed for each σ_{med} using linear regression.

Shapiro-Wilk normality tests were used to determine whether G_{eff} , C_{eff} , σ_{cyto} , ζ -potential, and cell radius following acute incubation in 10% saline: DEP medium and 10% saline: DEP medium + TEA were normally distributed. The sample size was too small for other normality tests to be used. Differences in these parameters following acute incubation in 10% saline: DEP medium compared with 10% saline: DEP medium + TEA were compared using a two-tailed Wilcoxon matched-pairs signed rank test for non-normally distributed properties, and a two-tailed paired t-test for normally distributed properties.

5.4. Results and Discussion

5.4.1. Assessing DEP Spectra at Different Medium Conductivities

Before assessing how the electrical parameters of chondrocytes change over time and when suspended in different σ_{med} , both the cross-over frequencies and the goodness-of-fit of MATLAB-fitted DEP models were determined for each σ_{med} . This was to evaluate whether the dielectric parameters of chondrocytes could be reliably measured when suspended in each σ_{med} . Many DEP systems require low σ_{med} as the current densities caused by high field strengths when using high ionic strength media can result in electrolysis, electrode screening, and excess joule heating at the water-electrode interface (Krishnan et al., 2009, Henriksson et al., 2022). Increases in σ_{med} flatten the DEP curve due to a decrease in the magnitude of the real part of the $K(\omega)$ factor, assuming σ_{cyto} does not change with σ_{med} (Park et al., 2019). Altogether, these limitations have meant DEP of cells in physiological media (which have conductivities of around 1400 mS/m) is difficult. However, Hoettges et al. (2019) found that the 3DEP was capable of obtaining robust data during DEP of platelets suspended in near physiological ionic strength media. As the design of the 3DEP chip incorporates relatively thick copper layers, the high charge concentrations associated with thin-film fabrication are avoided. This design allows for DEP of cell suspensions in higher-conductivity media (Hoettges *et al.* 2019).

Example DEP spectra of chondrocytes measured at passage 2 following acute suspension in DEP: saline media of conductivities 8 mS/m, 21 mS/m, 50 mS/m, 150 mS/m, and 447 mS/m are plotted in **Figure 56A-E**, and 1324 mS/m in **Figure 57**. The DEP models fitted using the MATLAB code in **Appendix B** to the DEP spectra data points are shown in **Figure 56F**, with their goodness-of-fit denoted by the r^2 values (Martin et al., 2022). In accordance with the results by Hoettges et al. (2019), the DEP spectra in **Figure 56** demonstrate that even at σ_{med} of 150 mS/m, the spectra are relatively noise-free and modellable, possessing an r^2 value of 0.92.

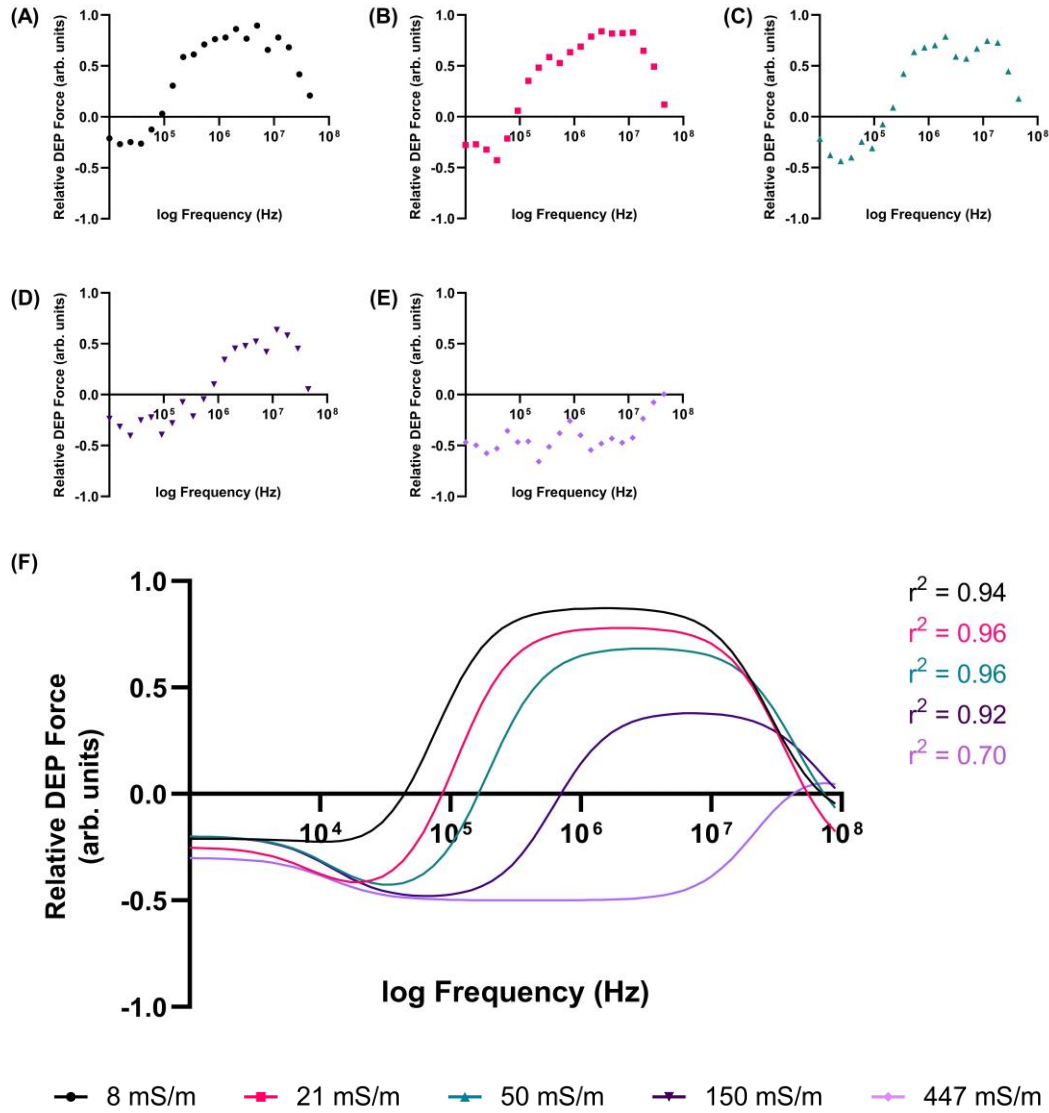


Figure 56 | Average DEP spectra of primary chondrocytes (passage 2 of the first biological repeat) in five different σ_{med} including **|A|** 8 mS/m (black circles) **|B|** 21 mS/m (pink squares) **|C|** 50 mS/m (teal triangles) **|D|** 150 mS/m (dark purple inverted triangles) **|E|** 447 mS/m (lilac line) **|F|** the corresponding MATLAB-fitted DEP models of DEP spectra in 8 mS/m (black line), 21 mS/m (pink line), 50 mS/m (teal line), 150 mS/m (dark purple line) and 447 mS/m (lilac line). r^2 values denote the goodness-of-fit of the DEP models to the DEP spectra collected by the 3DEP.

However, DEP of cells in media conductivities of 447 mS/m were more difficult to model (**Figure 56**), and in physiological media of 1324 mS/m spectra were not modellable at all and very noisy (**Figure 57**). This noise is reflected in the decrease of the r^2 values to 0.7 in spectra measured in 447 mS/m, and $r^2 = 0.0961$ at σ_{med} of 1324 mS/m. As σ_{med} increases, the signal-to-noise of DEP spectra increase as there is additional low-frequency polarisation in low-frequency bands (Hoettges et al., 2019, Hughes et al., 2021).

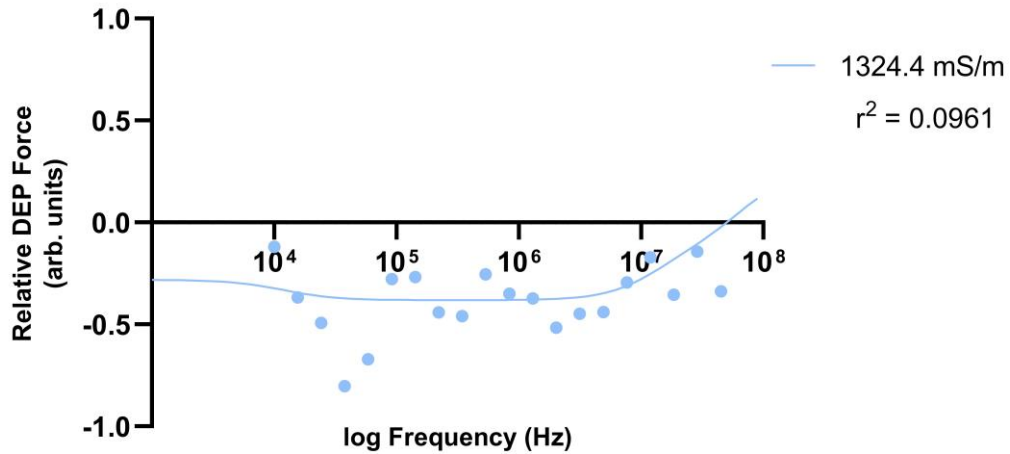


Figure 57 | Average DEP spectrum for primary chondrocytes of passage 2 of the first biological repeat suspended in DEP medium of conductivity 1324 mS/m. The MATLAB-fitted model (blue line) possessed an r^2 value of 0.0961.

As seen in other cell types (Henriksson et al., 2022), as σ_{med} increased the cross-over frequency between negative and positive DEP in chondrocytes increased, shown as an apparent shift in the DEP models (**Figure 56F**). At σ_{med} of 447 mS/m the cross-over frequency from negative to positive DEP did not occur until an AC frequency of 45 MHz – the maximum frequency of the 3DEP machine. As such, it is not possible to accurately model the DEP spectra to obtain values of σ_{cyto} . Following analysis of the DEP spectra for chondrocytes of the first biological repeat, the decision was thus made to only measure DEP properties and ζ -potential in σ_{med} of 21 mS/m, 50 mS/m, and 150 mS/m in subsequent donors.

5.4.2. The Electrophysiological Properties of Chondrocytes Change with Media Conductivity

To address the two aims of the chapter and determine how the electrical properties of chondrocytes change over time during cell culture and with σ_{med} , values of G_{eff} , C_{eff} , and σ_{cyto} (obtained from MATLAB-fitted models of DEP spectra which possessed an r^2 value of over 0.8 ($n = 4$)), ζ -potential ($n = 3$), and cell radius ($n = 5$) were measured for each cell passage following acute suspension in DEP: saline media of conductivities 21 mS/m, 50 mS/m and 150 mS/m. How the dielectric properties of chondrocytes changed with σ_{med} was assessed before investigating changes over cell culture duration. The mean values of all passages for each biological repeat (each flask) are shown in **Table 17** and plotted in **Figure 58**.

Table 17 | Electrophysiological properties of chondrocytes (n = 14) following acute suspension in DEP media of 21 mS/m, 50 mS/m, and 150 mS/m (mean $\pm$ SEM) as plotted in Figure 58.

	G_{eff} (kS/m ²)	C_{eff} (mF/m ²)	σ_{cyto} (S/m)	ζ -potential (mV)	Radius (μ m)
21 mS/m	0.465 ($\pm$ 0.09)	5.23 ($\pm$ 0.78)	0.26 ($\pm$ 0.02)	-9.33 ($\pm$ 2.44)	10.48 ($\pm$ 0.25)
50 mS/m	0.630 ($\pm$ 0.14)	6.22 ($\pm$ 1.48)	0.42 ($\pm$ 0.04)	-9.45 ($\pm$ 2.52)	10.41 ($\pm$ 0.19)
150 mS/m	1.430 ($\pm$ 0.59)	1.84 ($\pm$ 0.46)	0.66 ($\pm$ 0.05)	-8.69 ($\pm$ 2.25)	10.30 ($\pm$ 0.19)

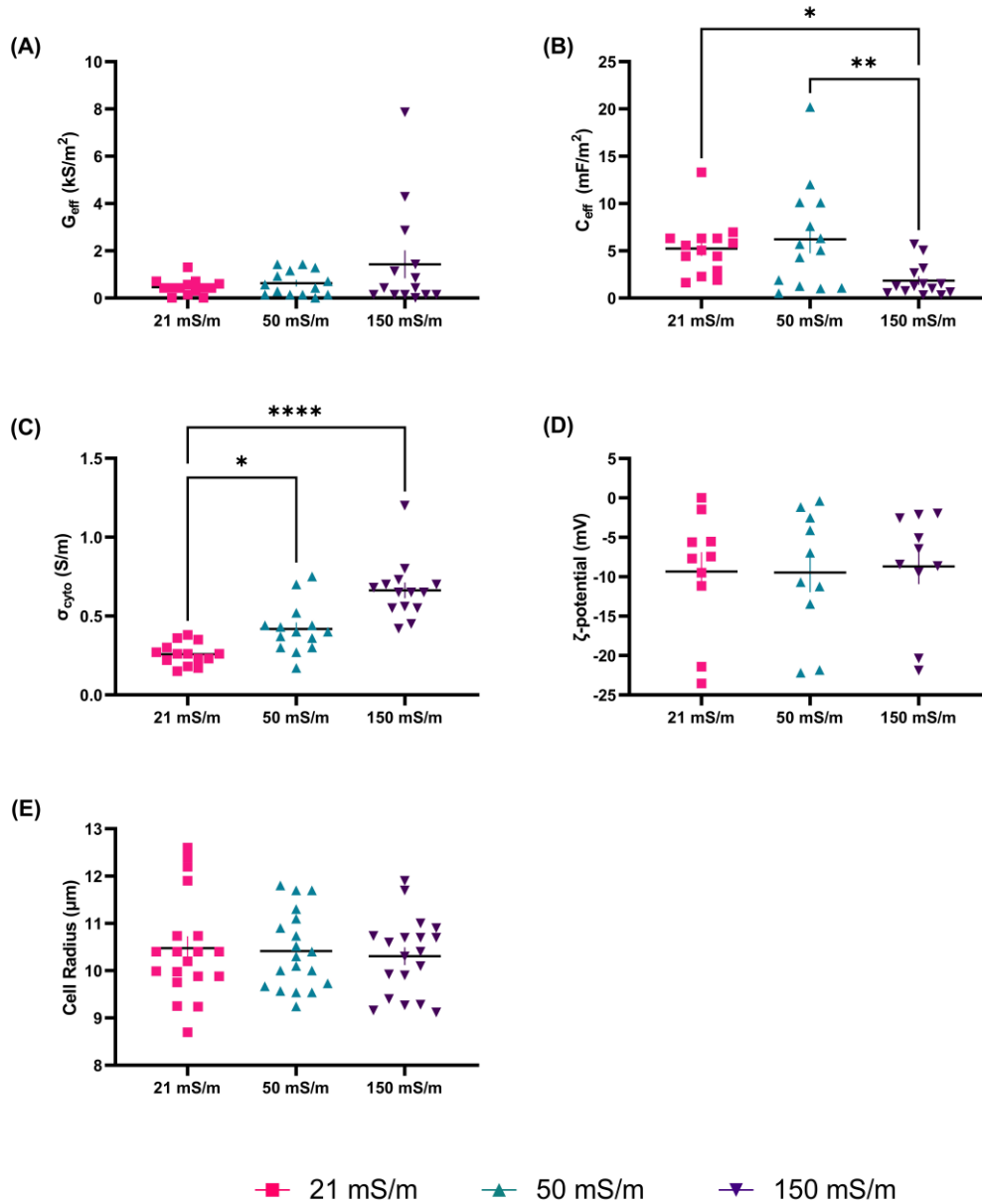


Figure 58 | Values of **[A]** G_{eff} **[B]** C_{eff} **[C]** σ_{cyto} **[D]** ζ -potential **[E]** cell radius in chondrocytes (n = 14) suspended in σ_{med} of 21 mS/m (pink squares), 50 mS/m (teal triangles), and 150 mS/m (purple inverted triangles) shown in **Table 17**. Each datapoint is a separate passage from a biological repeat (n = 4 biological repeats G_{eff} , C_{eff} , and σ_{cyto}), ζ -potential (n = 3 biological repeats), and cell radius (n = 5 biological repeats). Values of G_{eff} , C_{eff} , and σ_{cyto} were obtained from MATLAB-fitted models of DEP spectra with an r^2 value of 0.8. Changes in values of G_{eff} , C_{eff} , and σ_{cyto} with σ_{med} were compared using non-parametric Friedman tests followed by Dunn's multiple comparisons (* p <0.05, ** p <0.01, **** p <0.0001). Changes in values of ζ -potential and cell radius with σ_{med} were compared using repeated-measures one-way ANOVAs followed by Tukey's multiple comparisons with Geisser-Greenhouse correction. Mean lines plotted ($\pm$ SEM).

D'Agostino & Pearson omnibus test for normality identified measurements of G_{eff} , C_{eff} , and σ_{cyto} were not normally distributed ($p < 0.05$), whereas ζ -potential and cell radius were normally distributed ($p > 0.05$). As such, non-parametric Friedman tests followed by Dunn's multiple comparisons ($*p < 0.05$) were used to determine how G_{eff} , C_{eff} , and σ_{cyto} changed with σ_{med} (matched), and repeated-measures one-way ANOVAs followed by Tukey's multiple comparisons with Geisser-Greenhouse correction ($*p < 0.05$) were used to assess how ζ -potential and cell radius changed with σ_{med} . Repeated-measures and matched statistical tests were used to compare electrical values with σ_{med} to account for any variability between bovine donors, or due to passage number.

No statistical difference was seen in values of G_{eff} (**Figure 58A**), ζ -potential (**Figure 58D**), and cell radius (**Figure 58E**) with increasing σ_{med} (although the mean value of G_{eff} in 150 mS/m was greater than both 21 mS/m and 50 mS/m). However, mean values of C_{eff} decreased as σ_{med} increased, with values of C_{eff} significantly less in 150 mS/m ($1.84 \pm 0.46 \text{ mF/m}^2$) compared with 50 mS/m ($6.22 \pm 1.48 \text{ mF/m}^2$, adjusted $p = 0.0039^{**}$) and 21 mS/m ($5.23 \pm 0.78 \text{ mF/m}^2$, adjusted $p = 0.0421^{*}$). In contrast to C_{eff} , mean values of σ_{cyto} increased as σ_{med} increased, with σ_{cyto} significantly greater in 150 mS/m ($0.6636 \pm 0.05 \text{ S/m}$) than in 21 mS/m ($0.26 \pm 0.02 \text{ S/m}$, adjusted $p = < 0.0001^{****}$). A significant increase was also seen between acute suspension in 21 mS/m and 50 mS/m (adjusted $p = 0.0138^{*}$). Given the rapid change in the electrophysiological properties of chondrocytes following acute incubation in low σ_{med} , it is worth considering that the electrical characterisation of cells by DEP requires measurements in low conductivity media as opposed to physiological media. The low σ_{med} should be taken into consideration when characterising the electrical properties of cells in research and when considering their properties when incubated in different extracellular media.

As the osmolality and pH did not change between the different media (**Table 16**), the significant differences observed in C_{eff} and σ_{cyto} with σ_{med} must be attributable to the differences in ionic concentrations between each medium. As described in **Section 5.4.1.**, in order to reduce the noise associated with DEP spectra and maintain an r^2 value above 0.8, the three σ_{med} used had significantly lower ionic strengths than that of the ECM under physiological conditions. The concentrations of Na^+ , K^+ , Cl^- , and Ca^{2+} within each medium compared with physiological cytoplasmic and ECM concentrations are shown in **Table 18**.

Table 18 | The concentrations of Na⁺, K⁺, Cl⁻, and Ca²⁺ within each medium, compared with physiological concentrations in the chondrocyte cytoplasm and ECM (Maleckar et al., 2018).

	21 mS/m (mM)	50 mS/m (mM)	150 mS/m (mM)	Cytoplasm (mM)	ECM (mM)
Na ⁺	1.45	4.35	14.5	40	240-350
K ⁺	0.075	0.225	0.75	120-140	7-12
Cl ⁻	2.218	5.254	15.88	60-90	60-100
Ca ²⁺	0.099	0.097	0.09	1-5 x 10 ⁻⁵	6-15

As shown in **Figure 59**, in physiological conditions (right hand side) there is a concentration gradient of Na⁺, Cl⁻ and Ca²⁺ from the ECM into the cytoplasm, and K⁺ from the cytoplasm into the ECM. Whereas in low saline: DEP medium (left hand side) of σ_{med} equal to 21 mS/m, 50 mS/m, and 150 mS/m, there is an ion withdrawal and the concentration gradient reverses from the cytoplasm into the media.

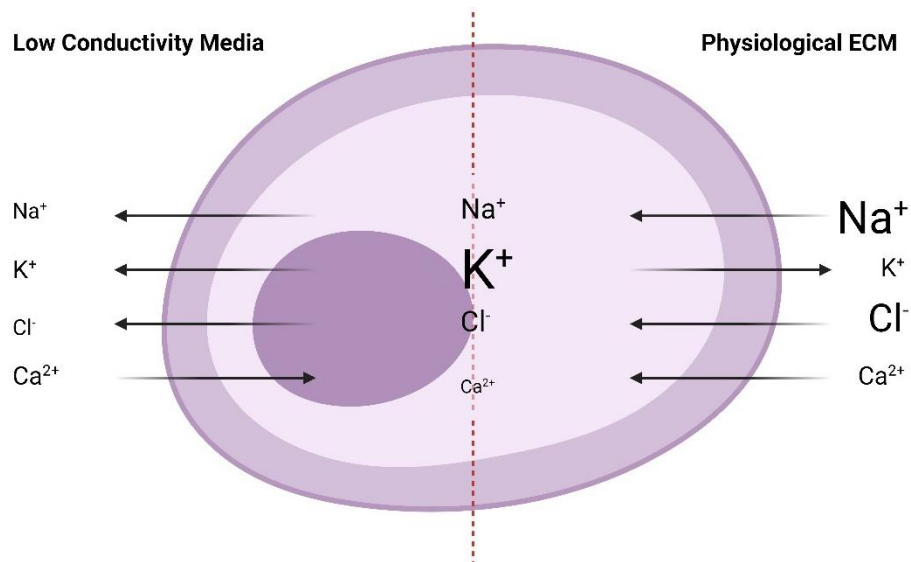


Figure 59 | Diagram of the concentration gradients in chondrocytes suspended in low conductivity DEP: saline media, (left) versus physiological extracellular matrix. (Created with BioRender.com).

It is possible that as the number of ions in the extracellular media decrease as σ_{med} decreases, the concentration gradient driving ions to leave the cytoplasm through ion channels (such as leak channels) increases, resulting in a greater reduction of σ_{cyto} . As mentioned in **Section 5.2.2.2.**, the decrease in σ_{cyto} with a reduction in σ_{med} observed here in chondrocytes has previously been reported in RBCs (Hughes et al., 2022a). The relationship between σ_{cyto} and σ_{med} consists of a σ_{med} -dependent component which refers to the ratio of intracellular and extracellular ion concentrations, as ions leak from the cytoplasm into low-conductivity media (Bernhardt et al., 1991, Richter et al., 1997), and has

been shown here to also occur in chondrocytes. The σ_{med} -independent component which refers to changes in the ion permeability of the membrane is not considered here given only the ratio of ion concentrations changed (Hughes et al., 2022a).

Changes in C_{eff} are most commonly due to changes in cell size and morphology. However, as shown in **Figure 58E**, there was no statistically significant change in cell radius observed using phase contrast microscopy. This is unsurprising as all σ_{med} used were iso-osmotic and comparable to the osmolality of the cell culture medium (**Table 16**). Normally, chondrocytes routinely change their cell volume to cope with changes in their extracellular environment. The microenvironment in which chondrocytes reside in cartilage is unique: the pH of the ECM is low and acidic at around pH 6.9 (Maleckar et al., 2018), and the extracellular osmolarity is atypically hypertonic at between 350 – 450 mOsm depending on the depth and thickness of cartilage and the joint type (Mobasheri et al., 2019). The osmotic force experienced by chondrocytes routinely changes drastically, as during physical activity significant compressive forces to cartilage decrease its volume resulting in the exudation of fluid. During these routine changes in osmotic pressure, healthy chondrocytes tightly regulate their cell volume to minimise changes in cellular osmolarity (Lewis et al., 2011). Ca^{2+} -dependent K^{+} channels are important in cell volume regulation as regulatory volume decrease is normally triggered by changes in $[\text{Ca}^{2+}]_i$. In response to membrane stretch or swell-activated intracellular signalling, non-selective cation channels are activated resulting in Ca^{2+} influx (Fernández-Fernández et al., 2008). TRPV4 is sensitive to osmotic and mechanical stress and regulates cell volume by functionally coupling to BK_{Ca} channels following chondrocyte volume increase (Fernández-Fernández et al., 2008, Lewis et al., 2013). TRPV5 is also important in regulating cell membrane potential and thus also for maintaining chondrocyte cell volume (Lewis et al., 2013). However, as all σ_{med} was iso-osmotic, stretch-activated ion channels are unlikely to have been activated. Instead of phase contrast microscopy, use of electron microscopy would have better identified membrane stretching due to changes in microvilli retraction/unfolding which could have led to a decrease in C_{eff} (Zimmermann et al., 2008, Stacey et al., 2014). Alternatively, a decrease in C_{eff} could have been due to changes in cell membrane architecture or lipid-protein interactions, such as a reduction in effective electrical polarizability or a change in membrane thickness (Pethig et al., 2002). As σ_{med} increased, the increase in the concentration of extracellular ions may have resulted in the EDL to become more condensed, thus increasing capacitance. A dependence of C_{eff} with σ_{med} has not been seen in platelets or Jurkats (Hoettges et al., 2019), but an increase in C_{eff} with σ_{med} has been seen in RBCs (Gascoyne et al., 1997).

Values of G_{eff} , C_{eff} , and σ_{cyto} of equine and canine chondrocytes suspended in σ_{med} 150 mS/m have previously been investigated in literature in a study by Abdallat et al. (2021). G_{eff} was recorded at $8.571 (\pm 1.01) \text{ kS/m}^2$, C_{eff} at $14.5 (\pm 0.01) \text{ mF/m}^2$, and σ_{cyto} (S/m) at $0.26 (\pm 0.01)$. The differences in

these values compared with the values recorded in **Table 17** could be due to differences in experimental protocol. Abdallat et al. (2019) acutely suspended cells in a solution containing 8.5% w/v sucrose, 0.5% w/v dextrose, 250 μM MgCl_2 , and 100 μM CaCl_2 adjusted to a conductivity of 150 mS/m using PBS instead of a high-strength NaCl KCl solution. Supplementation with PBS resulted in a greater overall extracellular K^+ concentration of $\sim 5\text{mM}$ compared with 0.75 mM with NaCl KCl supplementation. This supplementation increased ion concentration gradient out of the cell may have resulted in greater ion efflux causing EDL effects. Other variables contributing to the electrophysiological differences in values could be due to innate electrophysiological differences between bovine and equine/canine chondrocytes. For example, the resting V_m of canine chondrocytes have been reported at -6.7 mV (Lewis et al., 2011), compared with bovine chondrocytes at -24.26 ± 3.63 mV (Natesan, 2018). Transmembrane ion channels including TRP, BKCa (large-conductance Ca^{2+} and voltage-activated K^+ channel), SK_{Ca} (small conductance Ca^{2+} -activated K^+ channel), ENaCs (epithelial Na^+ channels), CRACs, and Cl^- channels have been reported to be expressed in both equine and bovine chondrocytes (Zhang et al., 2021a), but the expression of ion channels or permeability of the cell membrane to different ions could vary between species. Differences in the thickness, collagen, proteoglycan concentration, and chondrocyte density have been identified in bovine and equine cartilage (Nelson et al., 2021). Equine cartilage is thicker and has a greater resistance to compression to adapt to the increased pressure experienced during exercise compared with cows (Holland et al., 2014).

5.4.3. Measuring Resting V_m Using Dielectrophoresis

The results of **Section 5.4.2.** demonstrated that values of σ_{cyto} and C_{eff} in chondrocytes, a healthy adherent cell model, change with σ_{med} . This change reproduces findings of previous studies in RBCs (Hughes et al., 2022a), platelets, and Jurkats (Hoettges et al., 2019) and supports the capacitive model of the cellular electrome proposed by Hughes et al. (2022). The change in σ_{med} with σ_{cyto} thus provides greater insight into the CM-model.

Unpublished data collected by Dr Emily Kruchek, Oreoluwa Griffiths, and Rashedul Hoque within the DEP research team at Surrey have demonstrated that the relationship between σ_{cyto} and σ_{med} is also present in THP-1 (leukaemia monocytic suspension cell line) and HeLa (adherent cancer cell line) cell lines. On collating all of the collected data for all cell types, Professor Michael Hughes, Dr Rebecca Lewis, and Dr Fatima Labeed developed a theoretical capacitive model by which V_m can be calculated in non-excitable cells by DEP using **Equation 21** (submitted for publication):

$$V_m = \frac{RT}{F} \ln\left(\frac{1}{A}\right) + \Psi_{ext} \quad \text{Equation 21}$$

Whereby $A = \frac{\delta\sigma_{cyto}}{\delta\sigma_{med}}$, R is the gas constant (8.314 J/K mol), T is temperature in K, F is the Faraday constant, and Ψ_{ext} is the external surface potential of the cell. Using this equation, values of V_m can be calculated with great accuracy to values obtained using the gold-standard patch-clamp method. The gold-standard method of measuring V_m is patch-clamp. Although patch-clamp is a high-resolution, accurate electrophysiological technique, it has limitations including it is invasive, requires expensive equipment and high expertise, is time-consuming, low-throughput, and susceptible to operator bias (Mansor and Ahmad, 2015). Relative measures (not absolute) of V_m can also be obtained using optical quantification of fluorescent dyes such as DiSBAC (Bonzanni et al., 2019). In contrast, DEP is a rapid, non-contact technique which is high throughput and low cost. Furthermore, a low cell-count is required and the cells that are used can be reacquired.

A method of measuring the V_m of non-excitabile cells using DEP is thus an important contribution to furthering the level of detail that can be obtained during cellular electrical characterisation of health and disease states, providing a better understanding of the cellular electrome. Unfortunately, it was not possible to apply this technique in previous chapters to measure the V_m of PBMCs in ME/CFS and COVID-19 due to insufficient cell numbers to measure σ_{cyto} at multiple σ_{med} . However, this will be highly beneficial moving forward with future experiments.

5.4.4. Changes in Chondrocyte Electrophysiological Properties Over Time

During cell culture, primary chondrocytes lose their phenotype as they become more fibroblastic and differential gene expression occurs such as an increase in type I and type III collagen expression and a decrease in type II collagen expression (Marlovits et al., 2004, Goldring, 2005, Lin et al., 2008). By passage 7, chondrocytes are typically highly dedifferentiated (Huang et al., 2016). However, chondrocyte dedifferentiation causes problems in both research and clinical applications. For example, reimplantation of chondrocytes which have developed a more fibroblastic phenotype reduces the efficacy and success of ACI (Bartlett et al., 2005, Mao et al., 2019). Moreover, it may impact research into diseases such as osteoarthritis. Osteoarthritis is a debilitating painful disease characterized by the progressive loss and destruction of articular cartilage involving the participation of chondrocytes, matrix metalloproteinases and a disintegrin and metalloproteinase with thrombospondin repeats (ADAMTS) families of proteases (Xu et al., 2009, Chen et al., 2017). Sanchez et al. (2010) reported chondrocytes in osteoarthritic cartilage have a more depolarised membrane

potential compared with healthy cartilage (Sánchez and López-Zapata, 2010). Furthermore, osteoarthritis-induced chondrocytes have decreased values of G_{eff} and higher whole cell capacitance compared with healthy chondrocytes (Abdallat et al., 2021). However, in studies such as these which compare healthy chondrocytes with osteoarthritic chondrocytes, it is important that chondrocyte dedifferentiation has not occurred.

Currently, changes in morphological appearance (such as monitoring the transition from spherical to a more flattened cell morphology, changes in the expression of markers such as type II collagen quantified by immunocytochemistry, western blotting and RT-PCR) can be used to assess chondrocyte dedifferentiation (Marlovits et al., 2004). Until now, changes in the cellular electrome over the duration of cell culture was unexplored, but could pave the way towards an easy, quick, and adoptable tool of tracking chondrocyte dedifferentiation. For example, changes in dielectric properties such as membrane capacitance have been observed in stem cell populations as they differentiate to a certain fate (Flanagan et al., 2008, Adams et al., 2018). Changes in G_{eff} , C_{eff} , σ_{cyto} , ζ -potential, and cell radius of chondrocytes over duration in cell culture was thus investigated.

Methods of tracking the age of cells in culture include passage number, doubling time, generation number, and the number of days in cell culture. Passage number refers to the number of times a cell culture has been sub cultured into ‘daughter’ cell flasks (ECACC, 2017). However, using passage number to refer to cellular age is imprecise because it depends on the initial cell seeding densities and split ratios which can vary in different laboratories – affecting the number of times cells divide in culture (ECACC, 2017, Ahsan and Aldlerz, 2019). The population doubling number refers to the approximate number times the population has doubled since isolation, and is a better estimate of defining the age of a finite cell line (ECACC, 2017). The number of days in cell culture was chosen as a way to track the age of the cells in culture in a simple and clear way as opposed to passage number.

Figure 60 plots the values of G_{eff} , C_{eff} , σ_{cyto} , ζ -potential, and cell radius against the number of days in cell culture for cells suspended in σ_{med} of 21 mS/m, 50 mS/m, and 150 mS/m to assess how chondrocytes change as a function of time. As cells in different flasks reached confluence and required passaging at different times, the biological repeats are plotted together to show a trend over time. Simple linear regression was used to model how the electrical properties of chondrocytes change over duration in cell culture, under the assumption that trends followed a linear pattern. The coefficient and the 95% CI of the slope is quoted in **Table 19**, in addition to the P-value which determines whether the gradient of the slope is significantly greater than zero ($p < 0.05$ *) and the r^2 value denoting the goodness-of-fit of the linear regression line.

The greatest electrical difference against time was observed in ζ -potential (**Figure 60D**). The linear regression model fits ζ -potential to become less negative (more depolarised) as duration in cell culture increases with an r^2 value of 0.7924 and a p-value of 0.0002. However, it is important to note that the values of ζ -potential before day 30 (encircled in the orange circle line) are from a different biological donor than the values of ζ -potential plotted for days 30 – 75. Variability between chondrocytes obtained from different bovine donors or differences in the extracellular microenvironment of the cell culture flasks between biological repeats may account for this decrease in ζ -potential magnitude. To increase the reliability of a decrease in ζ -potential magnitude with duration in cell culture, it is important that the experiments are repeated with values of ζ -potential collected between days 0-93 in cells from the same donor, repeated for multiple donors.

Over time in cell culture, values of G_{eff} statistically increased in 50 mS/m (**Figure 60A**) and σ_{cyto} increased in 150 mS/m (**Figure 60C**), with the gradient of the linear regression model significantly non-zero ($p < 0.05$) (**Table 19**). However, the r^2 values of the linear regression models were low, at 0.2665 and 0.4749 respectively, indicating values of G_{eff} or σ_{cyto} can not be predicted reliably based on time in cell culture. There were no significant trends in C_{eff} (**Figure 60B**) or cell radius (**Figure 60E**) over time as the coefficients of the linear regression model were not statistically greater than zero ($p > 0.05$) (**Table 19**).

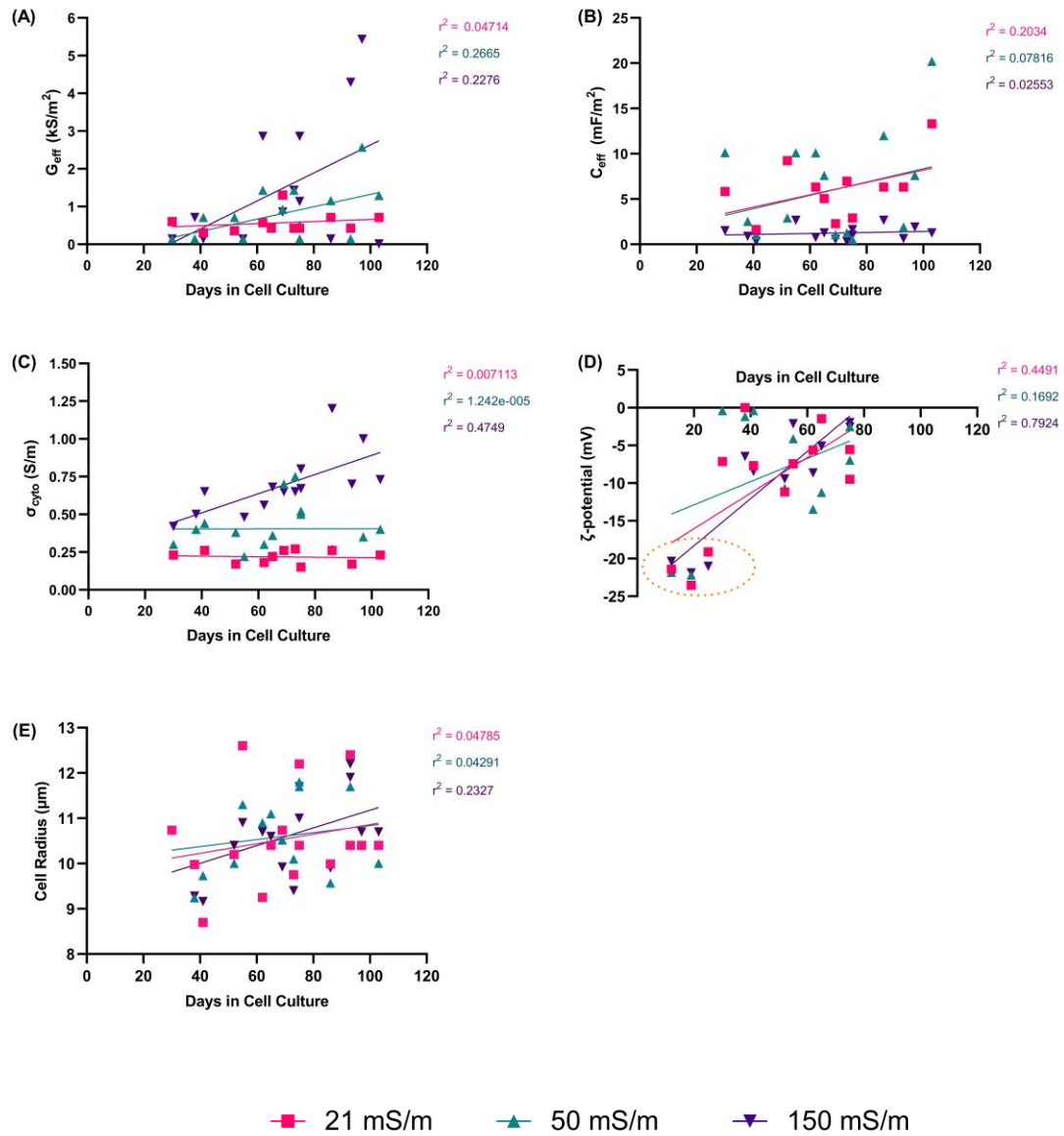


Figure 60 | Values of [A] G_{eff} [B] C_{eff} [C] σ_{cyto} of primary bovine chondrocytes suspended in media of different conductivities including 0.020 S/m (pink squares), 0.050 S/m (teal triangles) and 0.145 S/m (dark purple inverted triangles), against the number of days in cell culture. Values obtained from MATLAB-fitted models of DEP spectra which possessed an r^2 value of over 0.8. Simple linear regression models fitted to the graphs denote the goodness-of-fit of the trend line to the plotted data. r^2 values indicate the goodness-of-fit of the linear regression model by determining the variance in each parameter attributable to the number of days in cell culture.

Table 19 | Simple linear regression models fitted to indicate the trend in the change of electrical parameters over the number of days in cell culture. Coefficient denotes the gradient of the slope, and the 95% CI. P-value determines whether the slope is significantly greater than zero (*<0.05). r^2 value indicates the goodness-of-fit of the regression model by determining the variance attributable to the number of days in cell culture.

	σ_{med}	Coefficient	95% CI	P-value	r^2 Value
G_{eff}	21 mS/m	0.00278	-0.00664 to 0.0122	0.521	0.0471
	50 mS/m	0.0164	9.91e-005 to 0.0326	0.0488 *	0.267
	150 mS/m	0.0371	-0.00589 to 0.0801	0.0846	0.228
C_{eff}	21 mS/m	0.0683	-0.0336 to 0.170	0.164	0.203
	50 mS/m	0.0737	-0.0779 to 0.225	0.313	0.0782
	150 mS/m	0.00553	-0.0160 to 0.0270	0.585	0.0255
σ_{cyto}	21 mS/m	-0.000169	-0.00168 to 0.00134	0.805	0.00711
	50 mS/m	2.61e-005	-0.00440 to 0.00446	0.990	1.24e-005
	150 mS/m	0.00635	0.00215 to 0.0106	0.0064 *	0.475
ζ-potential	21 mS/m	0.236	0.0518 to 0.420	0.0171 *	0.449
	50 mS/m	0.154	-0.103 to 0.410	0.209	0.169
	150 mS/m	0.312	0.192 to 0.433	0.0002 *	0.792
Cell Radius	21 mS/m	0.0106	-0.0165 to 0.0376	0.416	0.0479
	50 mS/m	0.00776	-0.0142 to 0.0297	0.459	0.0429
	150 mS/m	0.0196	-0.000798 to 0.0399	0.0584	0.233

Interestingly, trends observed in G_{eff} , C_{eff} , and σ_{cyto} against time differed between the σ_{med} the cells were acutely suspended and measured in. The extent by which G_{eff} and σ_{cyto} increased over time (the coefficient of the linear regression model) became greater as σ_{med} increased. Similarly, the greatest coefficient in ζ -potential was greatest in the highest σ_{med} . In contrast, the extent by which C_{eff} increased reduced as σ_{med} increased.

Between 21 mS/m and 150 mS/m, concentrations of both K^+ and Na^+ increased. K^+ is important in chondrocyte signalling, with K^+ and Ca^{2+} channels linked in positive feedback loops to augment Ca^{2+} influx – regulating gene transcription and cytokine secretion (Suzuki et al., 2020). Inhibition of K^+ channels by TEA have been shown to increase V_m (Wohlrab et al., 2001) and inhibition with the K^+ channel blocker 4-aminopyridine (4-AP) suppresses chondrocyte proliferation (Wohlrab et al., 2004). Furthermore, TEA treatment has been shown to reduce K_v currents, cartilage-specific gene expression,

cartilage formation, and cell proliferation (Takács et al., 2023). Given the importance of K^+ channels in chondrocyte function, it was hypothesized that differences in the transmembrane transport of K^+ due to changes in expression or function of K^+ ion channels over cell culture duration may contribute to the trends seen in G_{eff} and σ_{cyto} following acute suspension in 150 mS/m medium. To determine the contribution that K^+ had to changes in chondrocyte DEP properties against time in cell culture, chondrocytes were subsequently treated with the non-specific K^+ ion channel blocker TEA. TEA is a small organic cation which is the same size of a hydrated K^+ ion (Khodakhah et al., 1997). Due to limited cell samples, the electrical properties of chondrocytes could only be investigated when supplemented to one σ_{med} . Supplementation to 150 mS/m was chosen because this was the conductivity which identified the greatest change in G_{eff} and σ_{cyto} over time. As shown in **Appendix A.4 Figure 74**, there was no statistically significant changes in G_{eff} , C_{eff} , σ_{cyto} , ζ -potential, or cell radius following TEA treatment (two-tailed t-tests, $*p<0.05$) and no clear trend in normalised TEA treated-control values over time (**Appendix A.4 Figure 73**). This contrasts with the study reported by Abdallat et al. (2021), which found whole cell capacitance, C_{eff} and G_{eff} significantly decreased when DEP medium adjusted to 150 mS/m using PBS was supplemented with 10 mM TEA immediately before DEP analysis. Furthermore, there was no trend in TEA treated/control normalised values over time. This indicates that any changes in G_{eff} , σ_{cyto} , and ζ -potential over time is not due to changes in K^+ efflux.

The increase in σ_{cyto} with σ_{med} over time is proposed to be related to changes in V_m . Using **Equation 21**, values of V_m were calculated for chondrocytes over duration of cell culture. Values of V_m over time is plotted in **Figure 61**, fitted with a simple linear regression model. Values of V_m were calculated using the σ_{cyto} of chondrocytes suspended in σ_{med} of 50 mS/m and σ_{med} of 150 mS/m. V_m was calculated at -16.6 mV at day 30 and decreased over time, fitting a simple linear regression model with a goodness of fit R^2 of 0.65.

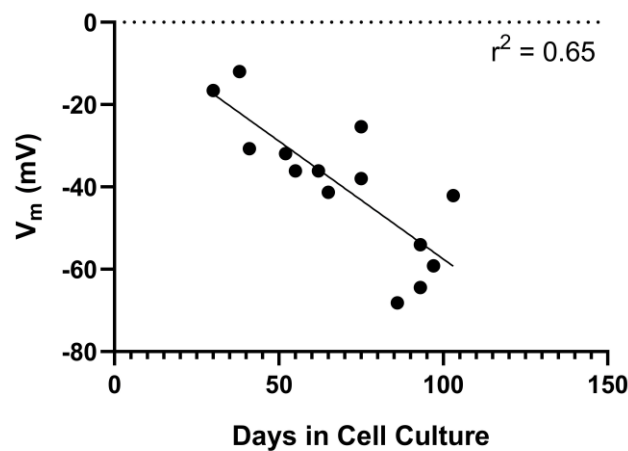


Figure 61 | Calculated values of V_m (from four separate culture flasks) plotted against the number of days in cell culture fitted by a line of simple linear regression. r^2 quotes the goodness-of-fit of the simple linear regression model fitted to the data.

The less negative resting V_m of chondrocytes compared with other cells is proposed to be a biological adaptation to withstand changes in osmotic pressures. Within their atypical environment, chondrocytes are known to tightly regulate their cell volume in response to osmotic changes, linked to changes in V_m . Osmotic pressure induced changes in cell volume have been linked to V_m , with small alterations in V_m contributing to dynamic regulation of cell volume (Barrett-Jolley et al., 2010, Lewis et al., 2011, Maleckar et al., 2018). As resting V_m is significantly higher than the equilibrium potential of K^+ , this allows rapid K^+ efflux through channels including BK_{Ca} (Lewis et al., 2011, Lewis et al., 2013). This rapid K^+ efflux minimizes increases in cell volume when ECM osmolarity is reduced due to the concomitant reduction in the osmotic drive for H_2O to enter, preventing cell swelling (Fernández-Fernández et al., 2008, Lewis et al., 2011).

Reported measurements of resting V_m in literature for chondrocytes have widely varied in literature, from values of -6.7 mV in canines (Lewis et al., 2011), -12 mV in sheep (Wright et al., 1992), to -20.2 mV in a human chondrosarcoma cell line (Funabashi et al., 2010), -39 ± 4 mV in human chondrocytes from healthy cartilage (Sánchez and López-Zapata, 2010), -41 ± 7 mV in rabbits (Sugimoto et al., 1996), and -42.7 mV in rats (Ponce, 2006), and -46 mV in mice (Clark et al., 2010). The resting V_m of bovine chondrocytes using patch-clamp is -24.26 ± 3.63 mV (Natesan, 2018), which compares to the resting V_m measured in these experiments using DEP. The mean value of V_m ($\pm$ SEM) of the biological repeats up to day 52 (passage 3) was -22.7 ± 4 mV. Current literature reports chondrocyte dedifferentiation from passage four, as the expression of collagen type II and cartilage-specific proteoglycans ceases (Schulze-Tanzil et al., 2002), and the expression of collagen type I increases (Kang et al., 2007).

Figure 61 shows the value of V_m becomes more negative as the number of days in cell culture increases. This could correspond with dedifferentiation to a fibroblastic phenotype, as proliferating fibroblasts have a resting V_m of between -20 and -30 mV (Chilton et al., 2005, Yang and Brackenbury, 2013). As the cells become more hyperpolarised over cell culture duration, this could signify that the cells are less metabolically active, more quiescent, and less mitotically active (Kadir et al., 2018). Non-proliferating fibroblasts have a V_m of -68 mV (Yang and Brackenbury, 2013). Primary cell cultures have a limited life span, following a certain number of subcultures they stop dividing and become senescent (ECACC, 2017). Measuring V_m has also been shown to be indicative of cell viability (Henslee et al., 2016, Madorran et al., 2022). Trypan blue staining has long been considered the gold-standard of cell viability analysis and did not show any indication of changes in chondrocyte viability over time. However, trypan blue staining does harbour some limitations, such as changing the shape of dead cells to a diffuse shape, resulting in an over-estimation of cell viability (Chan et al., 2015, Chan et al., 2020).

5.5. Conclusion

Chondrocyte cell culture is used extensively in both research and clinical applications, such as cell-based therapies for cartilage repair and in the characterisation and research of osteoarthritis (Piñeiro-Ramil et al., 2023). However, as chondrocyte dedifferentiation occurs during cell culture, with phenotypic changes reported as early as passage one (Darling and Athanasiou, 2005), this has significant repercussions. For example, ACI requires significant expansion *in vitro* to obtain sufficient numbers for reimplantation. Changes to a more fibroblastic phenotype during this time reduce the efficacy and success of surgery as up to 70% of patients develop fibrocartilaginous fill (Bartlett et al., 2005, Huang et al., 2016, Mao et al., 2019), which alters cartilage stretch and mechanical loading (Das et al., 2008). Re-implantation of chondrocytes which have been verified for phenotype increases the success rate of cartilage repair during ACI (Saris et al., 2008).

The experiments of this chapter thus investigated how the electrophysiological properties of chondrocytes change over the duration in cell culture to identify whether this change could lay the foundations of an additional tool for assessing changes in chondrocyte phenotype over time. The greatest difference over time was identified in ζ -potential, which became more depolarised over the duration in cell culture. However, as ζ -potential measurements before day 30 were measured in only one biological donor, the differences in time may have been skewed by biological variation. Future work necessitates more biological repeats for measurements of ζ -potential over time to increase the reliability of ζ -potential depolarisation over time. In addition to ζ -potential, significant changes in G_{eff} and σ_{cyto} were also observed over time. To correlate these changes in electrical properties with changes in chondrocyte phenotype, future experiments such as ELISA, western blotting, immunohistochemistry, and qRT-PCR to assess typical fibroblastic transitional changes such as decreased collagen I and II expression (Marlovits et al., 2004, Schulze-Tanzil et al., 2004, Kang et al., 2007) must be performed. Altogether, the electrophysiological changes in chondrocyte properties over time identified in this chapter contribute towards developing a low-cost, quick, and easily adoptable tool of assessing chondrocyte dedifferentiation prior to research and clinical applications such as ACI.

In parallel with these experiments, how the electrical properties of chondrocytes change with σ_{med} was also explored to provide greater insight into the cellular electrome model, expand on the underlying theory of the CM-model and what parameters can be derived from a DEP-spectra. Previous experiments in two suspension cell types, RBCs (Hughes et al., 2022a) and platelets (Hoettges et al., 2019) identified both V_m and σ_{cyto} change with σ_{med} , suggested to be due to both capacitive (σ_{med} -dependent) and diffusion (σ_{med} -independent) potentials (Hughes et al., 2022a). This led to a proposed

mechanism by which interconnected parameters such as V_m and ζ -potential are capacitively coupled due to ion imbalances between the inner and outer cell double layers, forming a capacitor (Hughes, 2023). However, the effect of σ_{med} on the cellular electromotive force of an adherent cell type had not been previously characterised. How the electrical properties of chondrocytes change with σ_{med} , a healthy cell model of an adherent cell type, was investigated in this chapter. Changes in both C_{eff} and σ_{cyto} were identified with σ_{med} , supporting the capacitive model proposed by Hughes et al. (2022). Understanding the cellular electromotive force model in more detail is beneficial for understanding how cells interact with their environment, such as changes in cell function, including cell-cell interactions, ion transport across the membrane, and interactions of the membrane with proteins such as antibodies (Hughes et al., 2022a). This information is invaluable when assessing the changes in the electrical properties of cells during health and disease.

Furthermore, the results of these experiments, in conjunction with changes in σ_{cyto} with σ_{med} in other cell types contributed to the development of a theory from which V_m is calculated using DEP by Professor Michael Hughes, Dr Rebecca Lewis, and Dr Fatima Labeed. Using DEP to measure V_m increases the detail of electrophysiological characterisation which can be performed, as well as being an easily adoptable, high throughput, and low-cost technique. Measuring the electrical properties of PBMCs from ME/CFS donors and donors who have had doses of the COVID-19 vaccine in two different σ_{med} could be applied in future work to characterise changes in V_m indicative of changes in function or state.

6. KEY FINDINGS, FUTURE WORK, AND CONCLUSION

This chapter summarises the key findings of research chapters 3 – 5 and suggests directions of future study.

6.1. Key Findings of the Thesis

The three studies presented in this thesis each investigated a novel application to which measuring changes in the cellular electrome of healthy and diseased cells may function as a medical biomarker. The DEPtech 3DEP machine, which utilises the phenomenon dielectrophoresis, was used to obtain values of G_{eff} , C_{eff} , and σ_{cyto} , and the Malvern Zetasizer was used to measure ζ -potential.

6.1.1. Diagnostic Biomarker for ME/CFS

Chapter 3 investigated the development of an electrical diagnostic biomarker to ME/CFS; a debilitating disease of unknown pathophysiology for which no reliable, validated, and quantitative laboratory tests currently exist. Esfandarypour et al. (2019) first demonstrated the overall impedance of PBMCs in response to hyperosmotic challenge, via incubation in autologous plasma supplemented with NaCl, is significantly different in ME/CFS donors compared with healthy controls (Esfandarypour et al., 2019). To gain a greater insight into these findings reported by Esfandarypour et al., changes in the values of G_{eff} , C_{eff} , σ_{cyto} , and ζ -potential of PBMCs donated from severe ME/CFS, mild/moderate ME/CFS, healthy controls, and MS donors were analysed over two-hour incubation in hyperosmotic NaCl media and hyperosmotic mannitol media using the 3DEP and Zetasizer.

No significant differences were found in values of G_{eff} , C_{eff} , σ_{cyto} , and ζ -potential of unchallenged PBMCs donated from individuals with ME/CFS compared with healthy controls and MS donors. This suggests any pathological changes in PBMCs in ME/CFS do not confer to changes in their cellular electrome when unchallenged. However, differential electrical responses to 1.5-hour NaCl hyperosmotic challenge were found between ME/CFS and healthy control donor cohorts, corroborating and validating the findings reported by Esfandarypour et al. (2019). The repeatability of differential electrical responses to hyperosmotic challenge strengthens the reliability of this as a diagnostic biomarker to ME/CFS, which withstands inter-laboratory variability including different electrophysiological techniques, protocols, and personnel.

As opposed to the aggregate measure of overall impedance measured by Esfandarypour et al. (2019), by using the 3DEP and Zetasizer this thesis demonstrated hyperosmotic stress induces electrical differences intrinsic to PBMCs. Following 1.5-hour hyperosmotic NaCl challenge σ_{cyto} significantly decreased in severe ME/CFS donors but did not change in mild/moderate ME/CFS, healthy controls, or MS donors; an electrical fingerprint specific to severe ME/CFS. Additionally, the magnitude of the percentage change in ζ -potential following 1.5-hour hyperosmotic challenge with NaCl and mannitol was greater in severe ME/CFS and mild/moderate ME/CFS than healthy controls

and MS donors. The increased magnitude of the percentage change in ζ -potential to hyperosmotic stress in severe ME/CFS and mild/moderate ME/CFS compared to non-ME/CFS suggests potential differences in cell-cell and cell-electrode interactions. These observations led to the development of three candidate diagnostic biomarkers for differentiating ME/CFS donors (severe and mild) from non-ME/CFS donors (healthy controls and MS), all with sensitivities and specificities of over 73%. The candidate diagnostic biomarkers included 'normalised D values' following 1.5-hour incubation in hyperosmotic NaCl, the magnitude of the percentage change in ζ -potential following hyperosmotic NaCl incubation, and the magnitude of the percentage change in ζ -potential following hyperosmotic mannitol incubation. The magnitude of the percentage change in ζ -potential following mannitol incubation demonstrated the best sensitivity and specificity at 92.3% and 71.4% respectively. Combining all three biomarkers into a single biomarker resulted in a sensitivity of 81.8% and specificity of 85.7%. The results presented in this chapter present further unbiased quantifiable proof that ME/CFS is a pathological condition, providing validation that the disease is recognised, countering the stigma faced in society by medical professionals, friends, family, and colleagues.

Comparison of NaCl and mannitol as the hyperosmotic stressor provided greater mechanistic insight into the origin of the differential electrical response. In ME/CFS donors and healthy controls, no statistical difference in values of G_{eff} , C_{eff} , σ_{cyto} , or ζ -potential were identified 1.5-hours after NaCl hyperosmotic stress compared with mannitol hyperosmotic stress. The similarities in the electrical response to both hyperosmotic stressors suggests that it may be osmotic challenge which results in differential PBMC electrical responses to challenge as opposed to increased Na^+ and Cl^- ions.

The inclusion of MS donors also provided evidence towards the specificity of electrical biomarkers to ME/CFS against a disease control, which had not previously been investigated. Interestingly, values of C_{eff} significantly decreased in both severe ME/CFS and healthy controls post 1.5-hour challenge but did not in mild/moderate ME/CFS or MS donors. Moreover, these experiments provided greater insight into the pathology of MS. Normalised changes in C_{eff} following 1.5-hour hyperosmotic NaCl incubation were significantly different in MS donors compared with healthy controls. Finally, differences between severe ME/CFS and mild/moderate ME/CFS were identified – normalised values of C_{eff} following 1.5-hour hyperosmotic challenge were significantly different between mild/moderate ME/CFS donors and healthy controls, which was not the case in severe ME/CFS donors.

In summary, a diagnostic biomarker which possesses a sensitivity of 81.8% and specificity of 85.7% has been presented. Although the sensitivity and specificity are less than the overall impedance reported by Esfandyarpour et al., the nanoneedle bioarray they used was not engineered for routine

use. In comparison, the DEPtech 3DEP and Malvern Zetasizer – commercially available, low-cost, compact, high throughput, easily adoptable techniques well-suited for diagnostic applications in clinical practice. The proposed protocol allows for ME/CFS diagnosis within one day and does not require cell radius measurement or DEP spectra modelling, saving time.

6.1.2. Correlate of Protection to SARS-CoV-2

Although Tedros Adhanom Ghebreyesus, the Director-General of the World Health Organisation, declared on the 4th of May 2023 that COVID-19 is no longer a Public Health Emergency of International Concern, it is important to remember that COVID-19 is still an established and ongoing health issue and identifying new CoP for SARS-CoV-2 remains vitally important (Wise, 2023, World Health Organisation, 2023). Despite the adoption and success of vaccination programmes, the cessation of government-imposed legal requirements to self-isolate when positive for SARS-CoV-2, and reduced recommendations to wear face coverings, hospital cases are currently increasing and new VOC are continually emerging, such as the new “Pirola” variant BA.2.86 which is sweeping the UK (Mahase, 2023). The necessity for a quick, easily adoptable, and relatively inexpensive tool to determine an individual’s immune status to SARS-CoV-2 remains a priority. The majority of CoP research focuses on the humoral immune response, though growing research has demonstrated that SARS-CoV-2 protection and clearance is greatly facilitated by specific T-cell responses. Limitations of current methods for evaluating T-cell mediated immunity as a CoP include a lack of standardised methods, and methodologies such as ELISpot are time intensive. Given the electrophysiological changes which are known to occur following PBMC activation, such as increased concentrations of cytoplasmic Ca^{2+} following TCR and BCR stimulation, and significant increases in G_{eff} in PBMCs following 3-hour PMA/ionomycin stimulation, it was hypothesised that differences in the electrophysiological properties of PBMCs following RBD-stimulation in individuals who are protected against COVID compared with COVID-19 naïve individuals could function as a CoP. The aim of the experiments in **Chapter 4** were to determine whether the electrical changes of PBMCs following RBD-stimulation in individuals who had previously been exposed to SARS-CoV-2, either through natural infection or vaccination, were different to COVID-19 naïve donors.

PBMCs of third dose donors had significantly lower baseline values of σ_{cyto} than that of recovered COVID-19 and COVID-19 naïve donors. Moreover, values of σ_{cyto} in recovered COVID-19 donors and second dose donors were found to significantly decrease with time since most recent exposure to COVID-19, reaching the values of σ_{cyto} measured in third dose donors. 3-hour *in vitro* RBD-

stimulation was demonstrated to differentiate third dose donors from healthy controls, as values of G_{eff} in third dose donors significantly decreased from baseline to equal that of COVID-19 naïve donors. However, normalised values of G_{eff} did not differentiate third dose donors from COVID-19 naïve donors with sufficient sensitivity or specificity to be determined a CoP.

6.1.3. Chondrocyte Cellular Electromotive During Cell Culture and Suspension in Different Media Conductivities

The aims of this exploratory chapter were two-fold. The first aim of **Chapter 5** was to investigate how the electrical properties of chondrocytes change during cell culture to provide greater insight into chondrocyte dedifferentiation at the electrical level. Chondrocytes rapidly dedifferentiate in monolayer cell culture and become more fibroblastic, impacting the success of cell-based cartilage repair therapies which require significant *in vitro* expansion to obtain sufficient numbers for re-implantation. Changes in the electrical properties of chondrocytes over time in cell culture were investigated to lay the foundations towards identifying an electrical biomarker which correlates with changes in chondrocyte phenotype over time to improve re-implantation success. Significant changes in G_{eff} , σ_{cyto} , and ζ -potential were identified over days in cell culture. However, there was no significant difference in G_{eff} , C_{eff} , σ_{cyto} , or ζ -potential following treatment with TEA, and no significant differences in TEA treated/control normalised values over time. This indicates differences in G_{eff} , σ_{cyto} , and ζ -potential over time were not due to changes in K^+ efflux.

The second aim was to build on the underlying theory of DEP as a method of measuring the dielectric properties of cells, by assessing how the DEP properties of primary chondrocytes change with σ_{med} . Understanding how the electrical properties of cells as measured using DEP are interconnected was previously unexplored in an adherent cell type and is important in understanding how cell function may change between health and disease. Values of C_{eff} and σ_{cyto} significantly changed with σ_{med} , supporting the capacitive model proposed by Hughes et al. (2022). These results led to Professor Mike Hughes, Dr Rebecca Lewis, and Dr Fatima Labeed developing a theory from which V_m is calculated. Use of DEP to measure cellular V_m is useful, as it overcomes limitations of other techniques such as patch-clamp and DiSBAC, as the first non-contact, label-free, and non-destructive technique of measuring V_m (which is advantageous for understanding ion channel function and cell function in more detail). Applying the developed V_m formula, values of V_m in chondrocytes were shown to hyperpolarise over time in cell culture. As the time between passages increased, indicative of a greater population doubling time, it is possible that the hyperpolarisation of V_m correlates to a decrease in metabolic activity, senescence, and dedifferentiation to non-proliferating fibroblasts

which have a V_m of -68 mV (Yang and Brackenbury, 2013). Altogether, these results suggest changes in the electrical properties of chondrocytes may have the potential to track chondrocyte dedifferentiation during cell culture, which could be further investigated in future work to apply to screening chondrocyte phenotype prior to ACL surgery.

6.2. Future Work

6.2.1. Diagnostic Biomarker for ME/CFS

To increase the reliability of this diagnostic biomarker for clinical application, future work is required to recruit more donors. Further data collection is particularly essential to the MS cohort, and for hyperosmotic mannitol challenge experiments. To differentiate ME/CFS donors from no ME/CFS donors with a power of 0.95 using normalised D values, G* power analysis calculated the total sample size for donor recruitment to consist of 82 donors. To achieve a power of 0.95, a total of 50 donors is needed for the magnitude of ζ -potential in NaCl media, and 32 donors for the magnitude of ζ -potential in mannitol media. Additional donor recruitment is needed to identify factors such as age, gender, ethnicity, duration of ME/CFS symptoms, and medication such as low-dose naltrexone. Multiple logistic regression should be implemented to determine whether these factors affect the electrical properties of PBMCs, or whether the optimum diagnostic threshold value varies between subpopulations. Given the small sample size in **Chapter 3**, this has not been possible to perform so far.

As the primary objective of this study was to initially identify a trend which could be exploited as a diagnostic biomarker, the samples were unblinded during data analysis to identify which electrical parameter best distinguished donors. However, once the diagnostic biomarker is better validated and reaches a power of 0.95, the remaining samples and future samples must remain blinded and randomised during both data collection and data analysis to ensure the biomarker is repeatable. Once the diagnostic biomarker has proven sensitivity and specificity in a controlled, blinded, randomised study, and reached a power of 0.95, the diagnostic biomarker should be taken to clinical trial. Other future work to improve the adoptability of the diagnostic biomarker in clinical practice include determining whether the use of fresh blood samples maintains the same sensitivities and specificities to facilitate point-of-care testing. Additionally, all 3DEP and ζ -potential data analysis must be automated.

Esfandypour et al. (2019) hypothesised that the differences in the impedance response between donor cohorts was attributable to something present or deficient in ME/CFS plasma, as the

electrical response to hyperosmotic stress in ME/CFS was dampened when PBMCs from ME/CFS donors were incubated in healthy control plasma. As differential electrophysiological differences were demonstrated in my experiments when cell culture medium was used as opposed to donor plasma, it is possible that the factor missing from ME/CFS plasma was also missing in the cell culture medium. To delve deeper into how the plasma of ME/CFS patients differs from healthy controls, Inductively Coupled Plasma Mass Spectrometry would prove invaluable.

Future work is also needed to understand why the hyperosmotic response in ME/CFS is different to non-ME/CFS donors. Although the electrical response to hyperosmotic NaCl and hyperosmotic mannitol was not statistically different using NaCl or mannitol as the stressor, the correlation in electrical response may not mean causation. Future experiments are necessitated to investigate the mechanistic pathway by which PBMCs electrically respond to both hyperosmotic stressors in ME/CFS. Experiments could include use of flow cytometry to identify markers of cell activation. Furthermore, use of TRP ion channel blockers such as ononetin would be insightful to determine whether ion channel dysfunction could be attributable to the decrease in σ_{cyto} identified in ME/CFS donors, given recent literature (Cabanas et al., 2019a, Cabanas et al., 2021, Eaton-Fitch et al., 2022). Quantification of σ_{cyto} ion concentrations such as Ca^{2+} using methods such as the fluorescent indicator dye fura-2 may provide clarity as to which pathways are dysregulated in ME/CFS, and which ion channels may be dysfunctional.

Although outside the scope of this study, future work should be directed towards identifying the differences in the electrical response to hyperosmotic stress detected in MS. Significant differences in C_{eff} compared to healthy controls following 1.5-hour hyperosmotic challenge may pave the way toward a low-cost, non-invasive biomarker of MS. However, increased sample size is required to determine repeatability.

6.2.2. Correlate of Protection to SARS-CoV-2

To establish whether decreased σ_{cyto} is due to increased baseline PBMC activation in third dose donors, future experiments include flow cytometry of PBMC activation markers (Abcam, No Date-a, Gerasimova et al., 2022), cytokine secretion assays with flow cytometry (Rezk et al., 2020) or ICS (Sahin et al., 2020, Vogel et al., 2022). In parallel, to determine if it is attributable to differences in the proportions of different PBMC subpopulations between donor cohorts, expression of 'cluster of differentiation' CD markers could be analysed using flow cytometry in future experiments to immunophenotype PBMC sub-populations. Furthermore, given impaired TRPM3 ion channel function

has been indicated in COVID-19 pathology, it would be insightful to determine whether it is associated with vaccination. Changes in the expression of TRPM3 channels using RT-PCR, or changes in TRPM3 function using the agonist Preg-S and antagonist ononetin could be insightful in establishing ion channel function between donor cohorts (Sasso et al., 2022).

To improve the reproducibility that G_{eff} decreases in third dose donors following RBD-stimulation, it would be valuable to recruit more donors to increase the power of the study. Additionally, increasing the number of donors would aid in establishing differences attributable to ethnicity, age, gender, which vaccine was administered and how many times COVID-19 had been contracted. Although it is not possible to recruit individuals who are COVID-19 naïve, and very difficult to recruit first dose and second dose donors given the implementation of the vaccination regimen, to investigate whether a decrease in G_{eff} following SARS-CoV-2 stimulation functions as a CoP, future experiments necessitate validation against other CoP such as NAb function, antibody titres, and functional T-cell response to be quantified in parallel using methods such as ELISpot assays, ICS, or IGRAs. Furthermore, the experimental procedure requires continual real-time observation and follow-up of the donors to identify whether they acquire SARS-CoV-2 infection or symptomatic infection, in addition to whether this potential CoP withstands against different VOC.

The mechanism by which G_{eff} decreases following 3-hour RBD-stimulation in the PBMCs of third dose donors is unknown. To investigate this, ion channel blockers such as amiodarone (a Ca^{2+} ion channel blocker shown to inhibit SARS-CoV-2 spread *in vitro*), and antagonists of $\text{K}_v1.3$ and $\text{K}_{\text{Ca}3.1}$ channels could be used. Additionally, given the relationship between G_{eff} and ζ -potential, it would be useful to use the Zetasizer to collect ζ -potential data. The use of methods to quantify changes in ion concentrations such as the fluorescent indicator dye fura-2 to measure Ca^{2+} ion concentration, which would be expected to increase on TCR or BCR stimulation, would be insightful to intra-signalling following stimulation. Additionally, it would be beneficial to investigate whether the electrical differences between donor cohorts are more pronounced when peptides with multiple different epitopes in addition to the RBD are used, to activate more TLRs and cover different VOC. Changes in the electrical properties of whole blood should also be investigated to streamline the experimental protocol, as well as frozen PBMCs to increase clinical adoptability and flexibility.

6.2.3. Chondrocyte Cellular Electrome During Cell Culture and Suspension in Different Media Conductivities

To increase the reliability that ζ -potential becomes more depolarised with the number of days in cell culture, more biological repeats are required. Additionally, more biological repeats are needed to increase the reliability that both G_{eff} and σ_{cyto} increase with time. To investigate why values of G_{eff} and σ_{cyto} change over time, changes in ion channel expression and function should be examined. TEA treatment demonstrated changes in K^+ ion conductance via K^+ channels did not contribute; therefore, future work could be performed to identify the main ion contributing to changes in chondrocyte electrophysiology over time. Hdud et al. (2012) reported the expression of TRPV5 and TRPV6 channels increase with passage number and time (Hdud et al., 2012). To determine whether changes in the expression of TRPV5 and TRPV6 ion channels contribute to the observed changes in the electrical properties of chondrocytes over time, use of ion channel blockers, qRT PCR and western blotting could be performed simultaneously with DEP experiments. Other ion channels expressed by chondrocytes which could be investigated include ENaCs, CRACs, and Cl^- channels.

To correlate changes in the electrical properties of chondrocytes over time with phenotypic changes during chondrocyte dedifferentiation, phenotypic characterisation experiments are necessitated. Given chondrocytes develop a more fibroblastic phenotype over time, DEP and ζ -potential analysis of fibroblasts is important to compare electrical fingerprints and determine the time at which the DEP parameters start to match that of fibroblasts. Additionally, quantifying changes in the expression of key proteins such as decreased collagen I and II expression, superficial zone protein, and cartilage-specific proteoglycans using methods such as ELISA, western blotting, immunohistochemistry, and qRT-PCR in parallel would identify typical fibroblastic transitional changes which could be linked to electrical changes. Together, these experiments would provide clarity in establishing the time at which chondrocytes possess a more fibroblastic phenotype, which would be very applicable for cell-based cartilage therapy. As the 3DEP and Zetasizer techniques are low-cost and easily adoptable, use of these technologies would prove invaluable as a quick tool or in parallel with current methodologies to characterise chondrocyte phenotype before ACI to avoid sub-optimal clinical outcomes. A balance between the time at which sufficient cell numbers, and minimal change to fibroblastic phenotype would be required to determine at which point the success of therapy would be compromised. If adopted in clinical practice in future, follow-up appointments would be required to determine how much fibrocartilaginous fill accumulates post-surgery to determine how reliable a technique electrical characterisation is to treatment success.

To determine whether the decrease in V_m correlates with cellular senescence, western blotting could be used to quantify the expression of phosphorylated p53 and a decrease in phosphorylated Retinoblastoma protein (pRB). Alternatively, flow cytometry assays such as the CellEvent Senescence Green Flow Cytometry Assay Kit (ThermoFisher, UK) could be used, which detects hydrolysis of β -galactosidase – a biomarker of senescence.

The formula used for V_m has many other potential applications including increasing the electrical characterisation of PBMCs in both **Chapter 3** and **Chapter 4** to potentially identify another diagnostic biomarker for ME/CFS, and following RBD-stimulation as a CoP for COVID-19.

6.3. Conclusion

The aim of this thesis was to explore changes in the electrophysiological properties of healthy cells as biomarkers for novel applications. This thesis has presented three studies during which the electrophysiological properties of cells were analysed using the DEPTech 3DEP and Malvern Zetasizer. The first study demonstrated the development of a diagnostic biomarker to ME/CFS, which found the electrical response of PBMCs to hyperosmotic stress was significantly different in ME/CFS compared with healthy controls and MS disease controls. Changes in the σ_{cyto} and ζ -potential of PBMCs following incubation in hyperosmotic NaCl media and hyperosmotic mannitol media led to the development of an optimum diagnostic biomarker which had a sensitivity and specificity of 81.8% and 85.7% respectively. This result is a promising contribution towards the development of a low-cost, high throughput, easily adoptable method of quantitatively diagnosing ME/CFS which aims to improve the quality of life of ME/CFS patients by accelerating diagnosis, preventing misdiagnosis and incorrect treatment, and counters the stigma and trivialisation of ME/CFS. The second study investigated whether the electrical properties of PBMCs could act as a CoP to SARS-CoV-2. Significant decreases in σ_{cyto} were identified in unchallenged PBMCs of donors following three doses of SARS-CoV-2 vaccination compared with COVID-19 naïve donors. Despite differential responses in G_{eff} to RBD-stimulation between COVID-19 naïve and third dose donors, the sensitivity and specificity of electrical changes were too low to be considered a CoP. The final study investigated how the electrical properties of chondrocytes change over time in cell culture. Values of ζ -potential, C_{eff} , σ_{cyto} , and V_m significantly changed over time. TEA treatment demonstrated these electrical changes were not attributable to changes in K^+ efflux. This work acts to facilitate future experiments to correlate dedifferentiation of chondrocytes to a fibroblastic phenotype with an electrical biomarker, to screen the electrical phenotype of chondrocytes before ACI and increase the success of cell-based cartilage regenerative cell therapy.

7. REFERENCES

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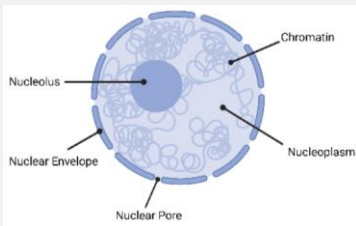
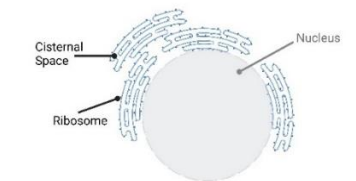
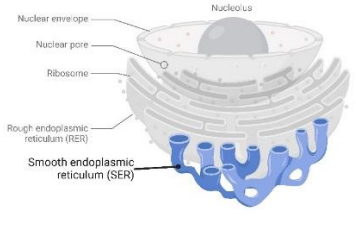
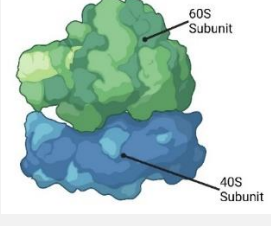
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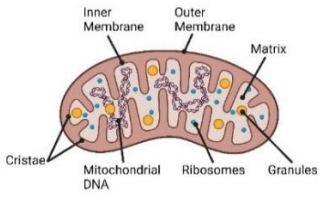
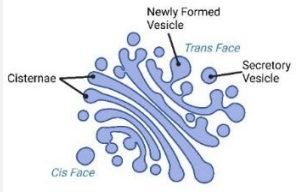
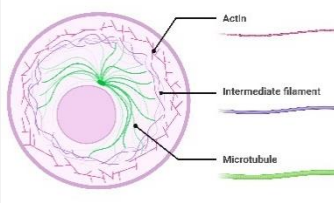
8. APPENDIX A – Supplementary Data

Appendix A.1: Supplementary Information

The Function of Organelles in Eukaryotic Cells

Table 20 | The function of organelles found in eukaryotic cells. Diagrams created and adapted from BioRender.com. (Bustin, 2015).

Organelle	Function	Diagram
Nucleus	Contains the cell's genetic information packaged in the form of chromatin, and is the principal location of DNA replication, transcription, and RNA synthesis. It is surrounded by the nuclear envelope – a double membrane punctuated with large proteinaceous channels called nuclear pore complexes (NPCs). NPCs facilitate the transport of ions, molecules and RNA between the nucleoplasm (a gel-like substance where the chromatin is stored) and the cytoplasm. The nuclear envelope ensures gene expression is regulated (Guo and Fang, 2014, Tripathi and Prasanth, 2011, Zidovska, 2020).	 The nucleus
Endoplasmic Reticulum (ER)	Comprising more than half of the eukaryotic cell membrane and 35% of the cytoplasmic volume, the ER is the largest organelle in the cell (Öztürk et al., 2020). It is a continuous membrane system physically connected to the nuclear envelope, consisting of rough ER and smooth ER domains. The rough ER is an organized network of membrane sheets enveloping a common lumen, studded with millions of ribosomes bound to the cytosolic facing surface, serving as the site of protein synthesis. The smooth ER is a network of interconnected cylindrical tubules which synthesizes and metabolize lipids, phospholipids, and steroids. It is smooth and not studded with ribosomes. Additionally, the ER is a site of calcium storage, where it releases Ca^{2+} or acts as a store to control cellular processes dependent on Ca^{2+} concentrations (Schwarz and Blower, 2016, Öztürk et al., 2020).	 Rough endoplasmic reticulum  Smooth endoplasmic reticulum
Ribosomes	Non-membrane bound organelle. Composed of two subunits, each containing ribosomal RNAs (rRNAs) and ribosomal proteins (r-proteins). Ribosomes are the site of translation (protein synthesized from mRNA templates). In eukaryotic 80S ribosomes the small 40S subunit decodes mRNA (reads nucleotide triplets which correspond to an amino acid), and the large 60S subunit forms peptide bonds between amino acids (Lafontaine and Tollervey, 2001).	 Ribosome

Mitochondria	Otherwise known as the ‘powerhouse’ of the cell, the mitochondria possess two membranes and are the primary site of metabolic respiration. Although the primary role of mitochondria is oxidative phosphorylation to produce cellular ATP, other key functions they perform include ion homeostasis, apoptosis and the production and consumption of reactive oxygen species (Brand et al., 2013). The electron transport chain is located on the inner mitochondrial membrane, and is a collection of proteins which undergo a series a redox-reactions to pass electrons down to liberate energy. The electrical inner membrane potential and ΔpH generates a proton-motive force used in chemiosmosis by ATP-synthase to generate ATP (Lemeshko, 2021, Ahmad, 2022).	 <i>Mitochondria</i>
Golgi Apparatus	Perinuclear organelle that has an integral role in the modification, sorting and packing of macromolecules that have been transported from the endoplasmic reticulum. Furthermore, it is involved in the vesicular transport (small, lipid bilayer membrane-bound spheres which transporting materials) of secreted carbohydrates, proteins and lipids, and the formation of lysosomes (Yang et al., 2015). It is also involved in the removal of excess Ca^{2+} from the cytoplasm (De Loof and Schoofs, 2020).	 <i>Golgi Apparatus</i>
Cytoplasm	A gel-like fluid in which the cell’s organelles reside. It is the main site of protein synthesis and degradation (Alberts et al., 2015).	
Lysosomes	Acidic membrane-organelles with a pH of 4.6 which contain digestive enzymes and act as the major compartment for detoxification of intracellular and extracellular content. Some of their roles include degradation, recycling, cell defence and immunity. To perform these roles, ion homeostasis and lysosomal lumen pH is maintained by an array of ion channels and transporters including the chloride channels and transporters CLC-6 and CLC-7, voltage-gated calcium channels and transient receptor potential channels (Festa et al., 2022).	
Peroxisomes	Regulate synthesis and turnover of complex lipids and reactive oxygen species (ROS) (Schrader et al., 2020).	
Cytoskeleton	A complex, dynamic network of interlinking intermediate filaments, microfilaments and microtubules which extend throughout the cytoplasm and are important for cell shape, cell migration and cell division. For example, microtubules of the cytoskeleton hold sections of cisternae in the endoplasmic reticulum together (Schwarz and Blower, 2016).	 <i>Cytoskeleton</i>

Appendix A.2: ME/CFS Supplementary Data

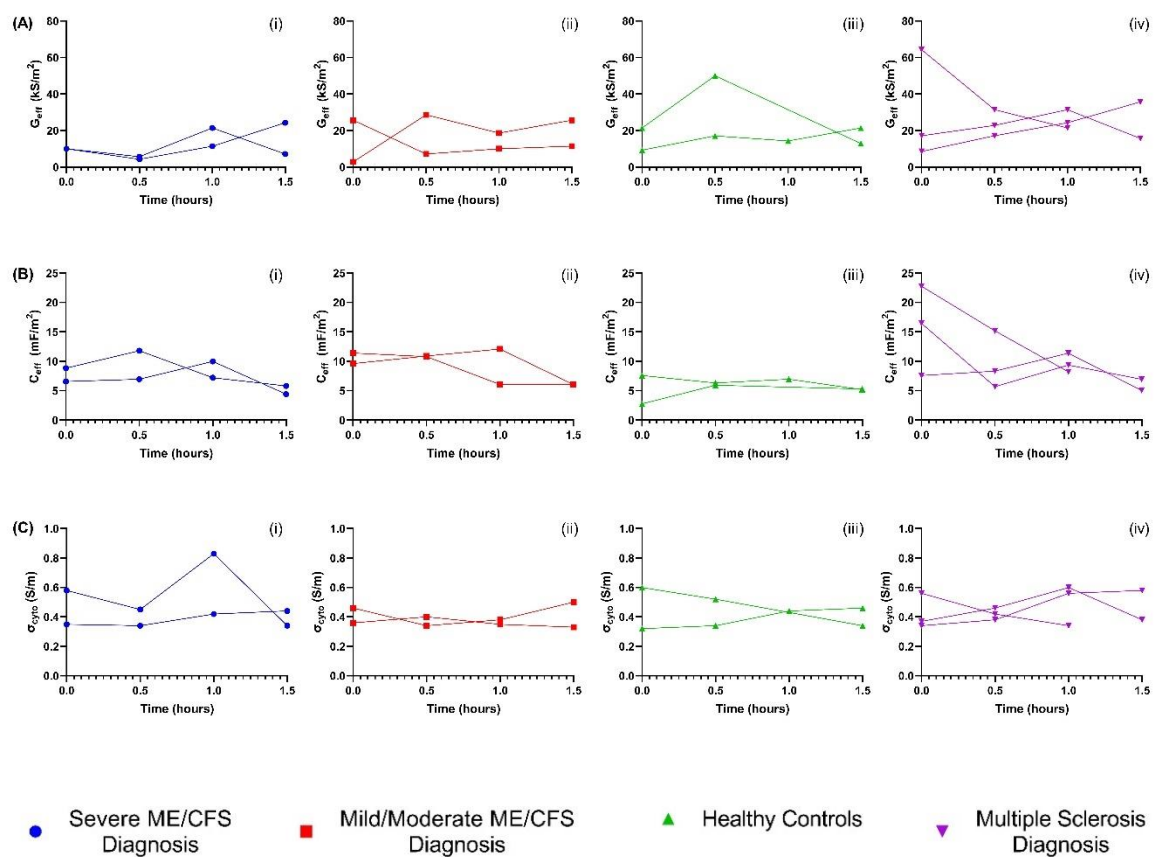


Figure 62 | Values of **[A]** G_{eff} **[B]** C_{eff} **[C]** σ_{cyto} over time during incubation in hyperosmotic NaCl media in (i) severe ME/CFS diagnosis donors (blue circles: $n = 2$), (ii) mild/moderate ME/CFS diagnosis donors (red squares: $n = 2$), (iii) healthy controls (green triangles: $n = 2$) and (iv) MS diagnosis donors (purple inverted triangles: $n = 3$). Values from MATLAB-models with R^2 greater than 0.7.

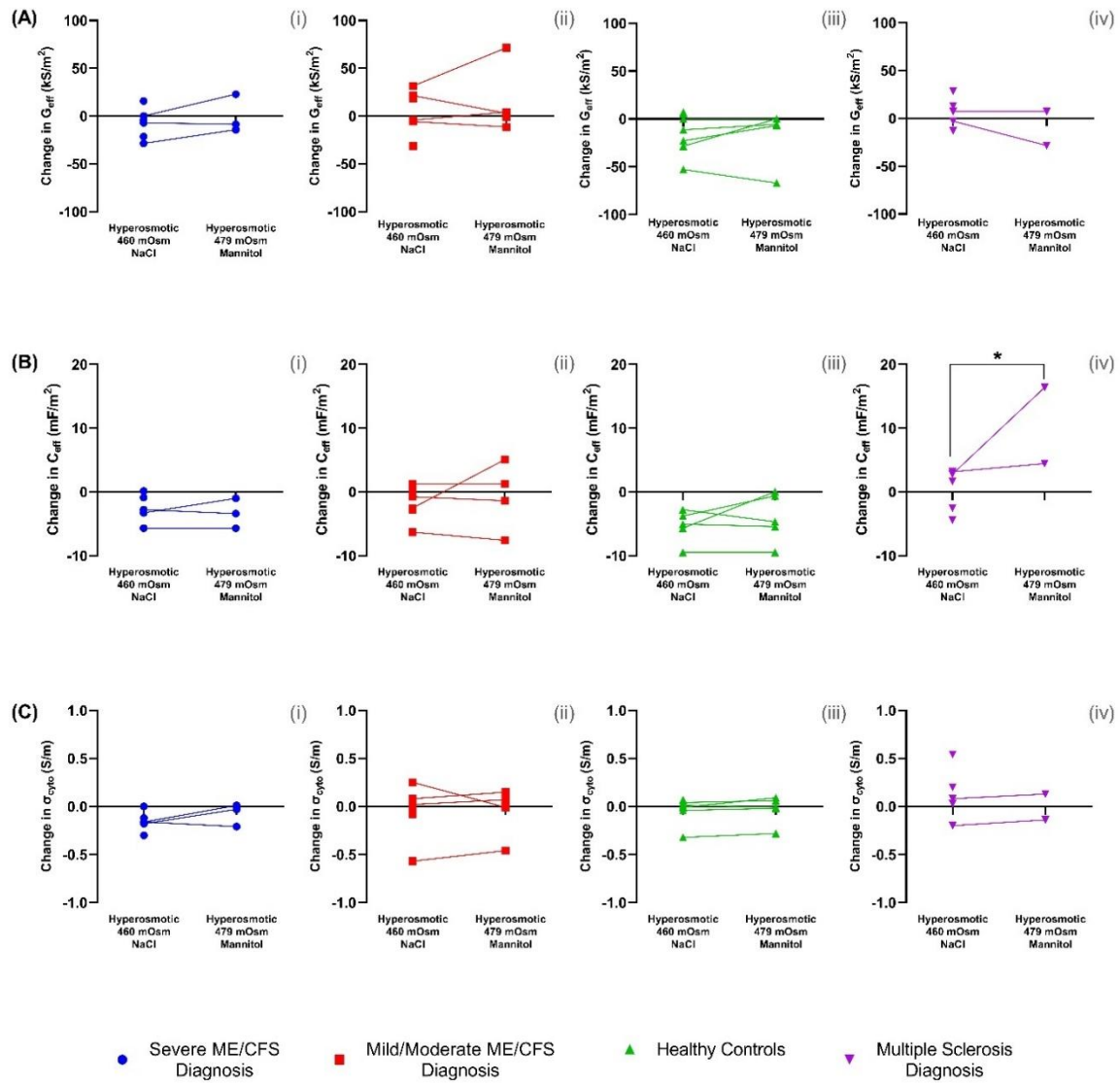


Figure 63 | Matched values of **[A]** change in G_{eff} **[B]** change in C_{eff} **[C]** change in σ_{cyto} following 1.5-hour incubation in hyperosmotic NaCl media and hyperosmotic mannitol media in (i) severe ME/CFS diagnosis donors (blue circles: $n = 3$), (ii) mild/moderate ME/CFS diagnosis donors (red squares: $n = 4$), (iii) healthy controls (green triangles: $n = 5$) and (iv) MS diagnosis donors (purple inverted triangles: $n = 2$). Each line connects matched donor. Values from MATLAB-models irrespective of R^2 value. Statistical significance (* $p < 0.05$) from matched mixed model effects followed by Tukey's multiple comparison, comparing difference between hyperosmotic treatments and donor cohorts.

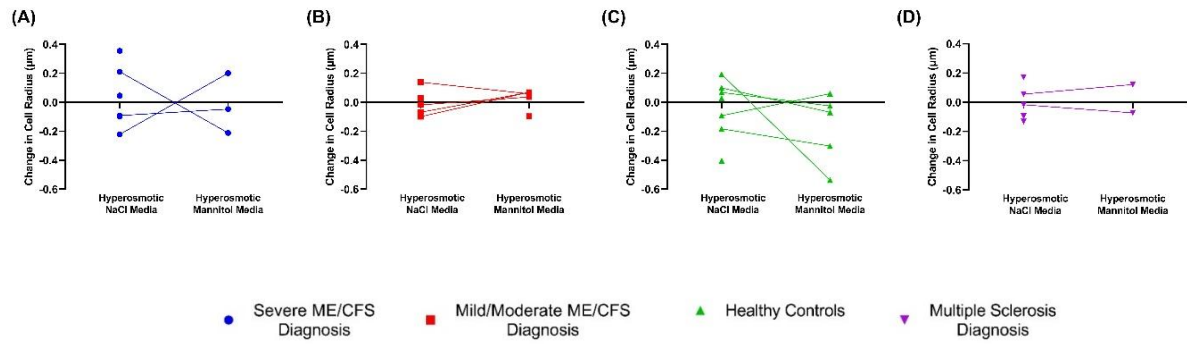


Figure 64 | Matched values of normalised cell radius following 1.5-hour incubation in hyperosmotic NaCl and hyperosmotic mannitol in **[A]** severe ME/CFS diagnosis donors (blue circles: $n = 3$), **[B]** mild/moderate ME/CFS diagnosis donors (red squares: $n = 3$) **[C]** healthy controls (green triangles: $n = 5$) **[D]** MS donors (purple inverted triangles: $n = 2$). Statistical significance ($* p < 0.05$) from matched mixed model effects followed by Tukey's multiple comparison, comparing difference between hyperosmotic treatments and donor cohorts. Statistical significance calculated using matched values mixed effects model followed by Tukey's multiple comparisons ($* p < 0.05$). Each datapoint is a different donor.

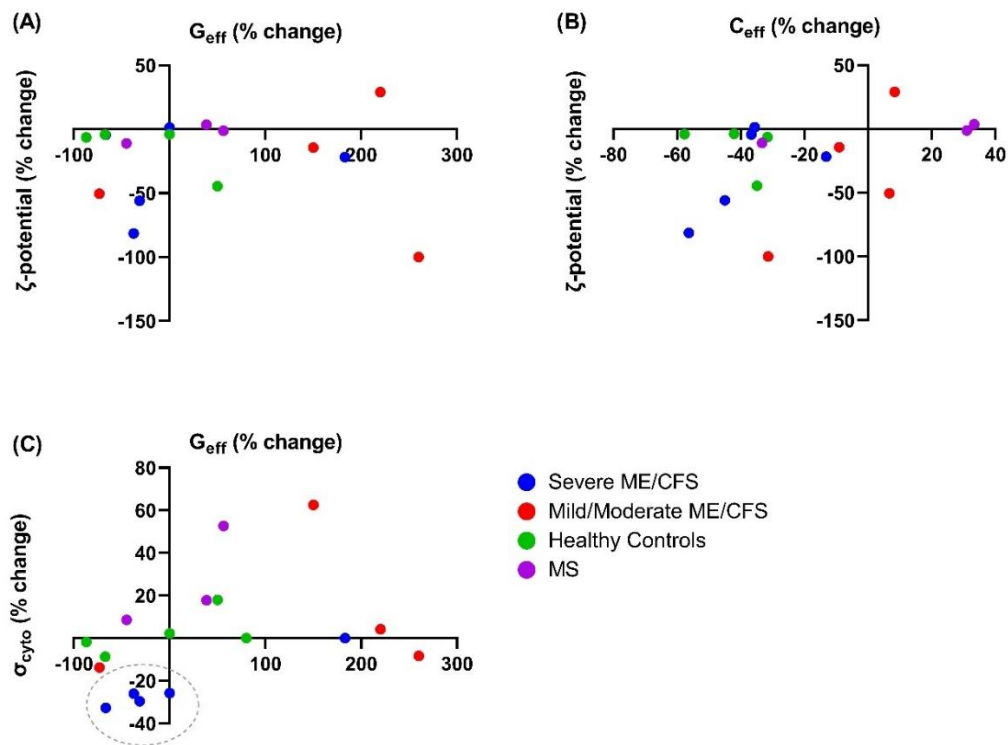


Figure 65 | Plots of **[A]** % change in G_{eff} against % change in ζ -potential **[B]** % change in C_{eff} against % change in ζ -potential **[C]** % change in G_{eff} against % change in σ_{cyto} in severe ME/CFS diagnosis donors (blue circles: $n = 5$), mild/moderate ME/CFS diagnosis donors (red circles: $n = 4$) healthy controls (green circles: $n = 5$) and multiple sclerosis donors ($n = 3$). Grey dashed line shows clustering of severe ME/CFS donors.

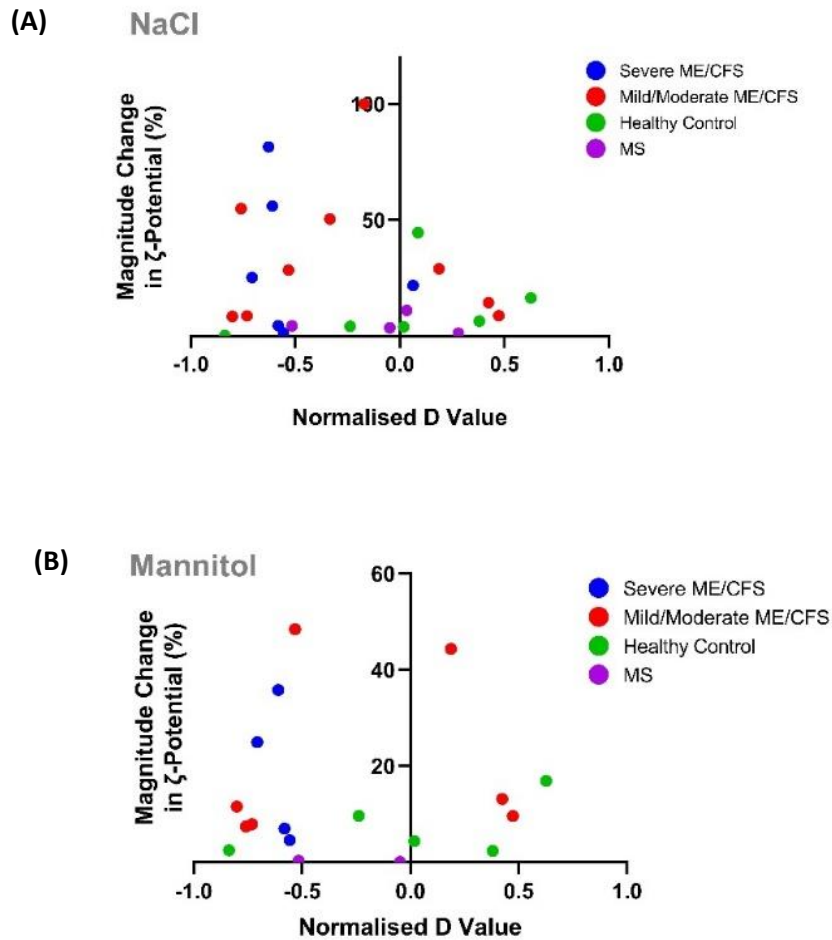


Figure 66 | Plots of **|A|** normalised D value against magnitude of % change in ζ -potential **|B|** normalised D value against magnitude of % change in ζ -potential in severe ME/CFS diagnosis donors (blue circles: $n = 5$), mild/moderate ME/CFS diagnosis donors (red circles: $n = 4$) healthy controls (green circles: $n = 5$) and multiple sclerosis donors (purple circles: $n = 3$). Grey dashed line shows clustering of severe ME/CFS donors.

Appendix A.3: COVID-19 Supplementary Data

15-minute Stimulation of PBMCs with SARS-CoV-2 Spike Protein

Table 21 | Baseline characteristics of COVID-19 naïve and recovered COVID-19 donor cohorts whose PBMCs were isolated and stimulated for 15 minutes with the RBD of the COVID-19 spike protein. Continuous variables are expressed as (MEAN $\pm$ SD) and categorical variables as fraction of sample size.

	COVID-19 naïve, n = 1	Recovered COVID-19, n = 2
Age (years)	32 ($\pm$ 0)	28.5 ($\pm$ 1.5)
Gender	M (1/1)	M (1/2)
Ethnicity	White (1/1)	White (2/2)
Time Since Symptom Onset (days)	-	57 ($\pm$ 0)

Table 22 | Electrophysiological parameters of PBMCs from COVID-19 naïve (n = 1) and recovered COVID-19 (n = 2) donors when unstimulated vs. stimulated with RBD SARS-CoV-2 spike protein for 15 minutes as measured by the 3DEP. (MEAN $\pm$ SEM).

	COVID-19 naïve (n = 1)		Recovered COVID-19 (n = 2)	
	Unstim.	Stim.	Unstim.	Stim.
σ_{cyto} (S/m)	0.37	0.37	0.39 $\pm$ 0.01	0.36 $\pm$ 0.02
C_{eff} (mF/m ²)	11.8	11.4	11.4 $\pm$ 0.63	11.4 $\pm$ 0.00
G_{eff} (kS/m ²)	4.29	8.86	1.36 $\pm$ 0.79	6.429 $\pm$ 0.7
Cell radius (μm)	3.38	3.46	3.29 $\pm$ 0.067	3.38 $\pm$ 0.005

24 Hour Stimulation with SARS-CoV-2 Spike Protein

Table 23 | Baseline characteristics of COVID-19 naïve (n = 1), recovered COVID-19 (n = 2) and first dose (n = 1) donor cohorts whose PBMCs were isolated and stimulated for 24 hours with the RBD of the COVID-19 spike protein. Continuous variables are expressed as (MEAN $\pm$ SD) and fraction of sample size.

	COVID-19 Naïve n = 1	Recovered COVID-19 n = 2	First Dose n = 1
Age (y)	27	26 ($\pm$ 1)	24
Gender	F (1/1)	M (2/2)	F (1/1)
Ethnicity	White (100 %)	White (1/2) Asian (1/2)	White (1/1)
Time Since Symptom Onset (days)	-	41 ($\pm$ 2)	-
Time Since Vaccination (days)	-	-	12 ($\pm$ 0)

Table 24 | Electrophysiological parameters of PBMCs from COVID-19 naïve (n = 1), recovered COVID-19 (n = 2) and first dose (n = 1) donors when unstimulated vs. stimulated with RBD SARS-CoV-2 spike protein for 24-hours as measured by the 3DEP. MEAN of biological repeats $\pm$ SEM.

	COVID-naïve (n = 1)		Recovered COVID-19 (n = 2)		First Dose (n = 1)	
	Unstim.	Stim.	Unstim.	Stim.	Unstim.	Stim.
σ_{cyto} (S/m)	0.41	0.38	0.34 ± 0	0.42 ± 0.02	0.34	0.36
C_{eff} (mF/m ²)	12.6	10.1	10.12 ± 0.63	11.38 ± 1.90	11.4	11.4
G_{eff} (kS/m ²)	8.57	5.71	1.79 ± 0.36	3.93 ± 2.5	5.71	5.71
Cell radius (μm)	3.21	3.43	3.29 ± 0.11	3.17 ± 0.088	3.34	3.26

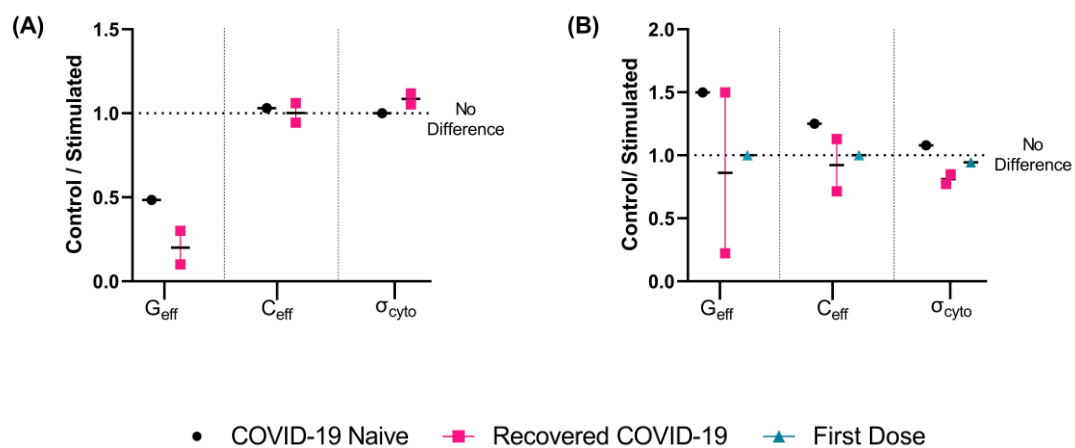


Figure 67 | Control/stimulated values of G_{eff} , C_{eff} , and σ_{cyto} following stimulation with the RBD of the SARS-CoV-2 spike glycoprotein for **[A]** 15-minutes **[B]** 24-hours in COVID-19 naïve (black circle), recovered COVID-19 (pink squares), and first dose donors (teal triangle). Black lines plot mean $\pm$ SEM.

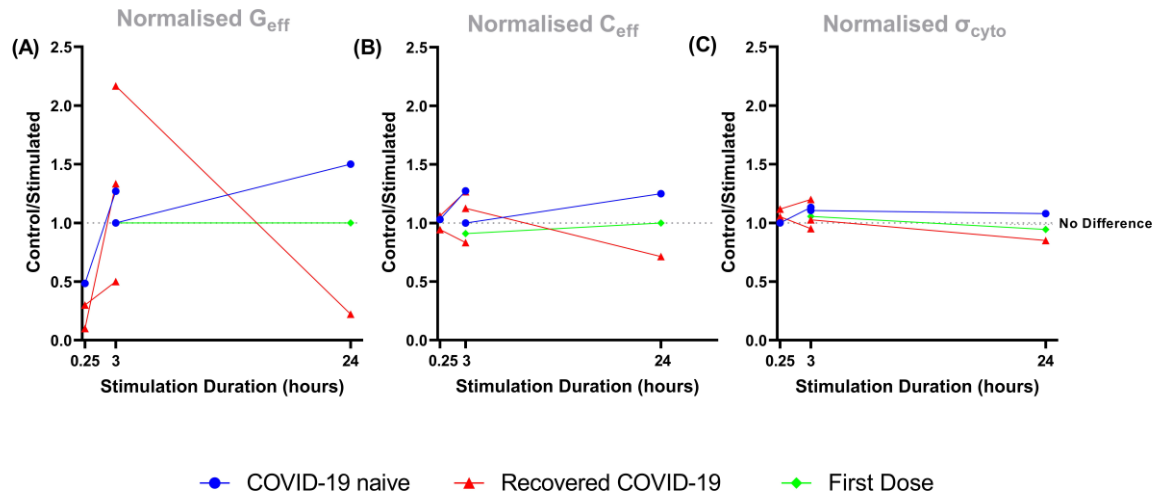


Figure 68 | Scatter plots of normalised values of **[A]** C_{eff} **[B]** G_{eff} **[C]** σ_{cyto} against incubation time with the RBD of the SARS-CoV-2 spike protein for each donor cohort. COVID-19 naïve ($n = 2$; blue circle), Recovered COVID-19 ($n = 3$; red triangle), First Dose ($n=1$; green diamond). Each point is a single donor, with lines connecting paired changes in electrophysiological properties of PBMCs from the same donor after further stimulation with the SARS-CoV-2 spike protein.

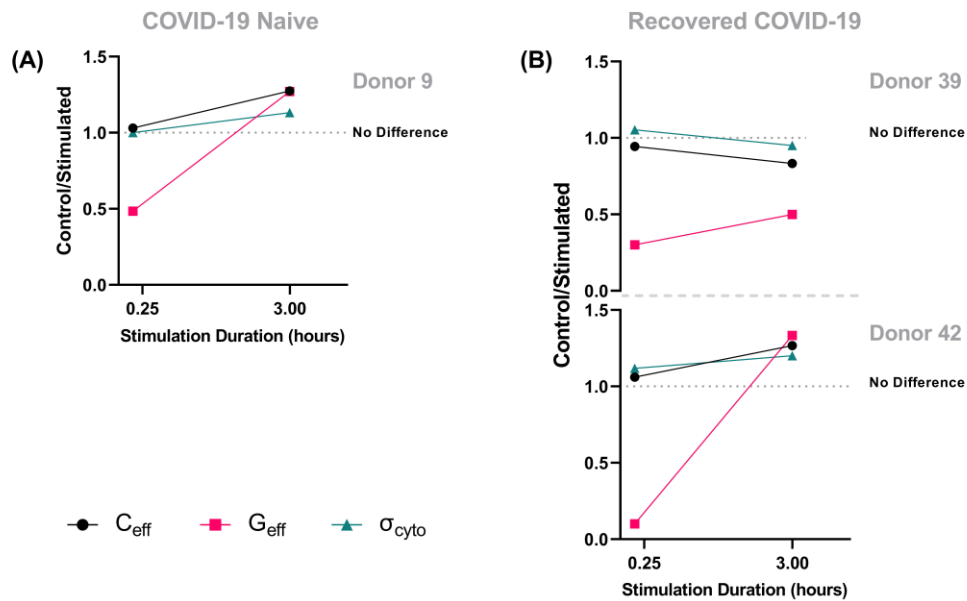


Figure 69 | Changes in the electrophysiological properties of PBMCs from individual donors between 15 min and 3-hour SARS-CoV-2 spike protein stimulation, including C_{eff} (black circle), G_{eff} (pink square), and σ_{cyto} (green triangle). **[A]** COVID-19 naïve donor 9. **[B]** Recovered COVID-19: top: donor 39, bottom: donor 42.

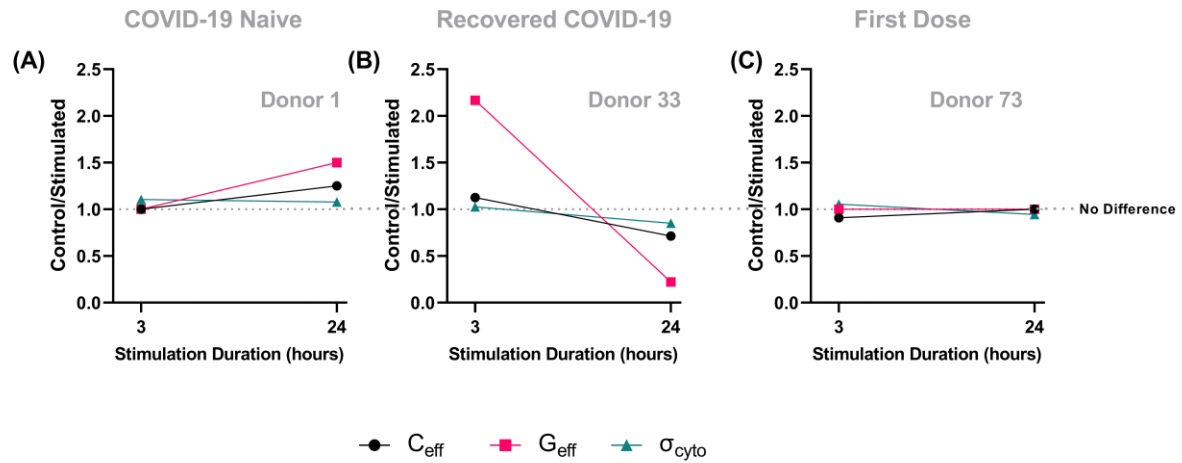


Figure 70 | Changes in the electrophysiological properties of PBMCs from individual donors between 3-hour and 24-hour SARS-CoV-2 spike protein stimulation, including C_{eff} (black circle), G_{eff} (pink square) and σ_{cyto} (green triangle). **[A]** COVID-19 naïve: donor 1 **[B]** Recovered COVID-19: donor 33 **[C]** First Dose: donor 73.

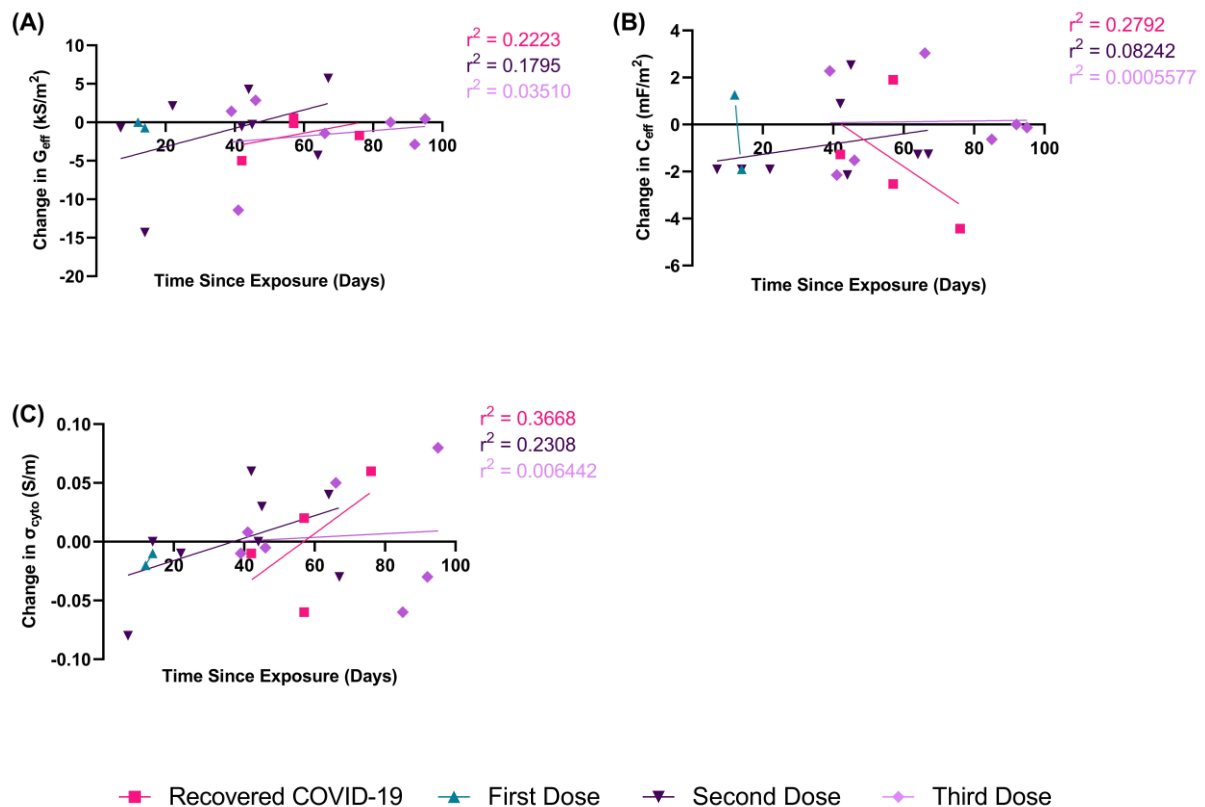


Figure 71 | Normalised three-hour RBD-stimulated-unchallenged in **[A]** G_{eff} **[B]** C_{eff} **[C]** σ_{cyto} against days since COVID-Exposed in recovered COVID-19 ($n = 4$; pink square), first dose ($n = 2$; green diamond), second dose ($n = 8$; purple square) and COVID-19 naïve ($n = 9$, blue circle) donors. r^2 value indicates the goodness-of-fit of the simple linear regression model by determining the variance attributable to the number of days between the date of the experiment and the date of latest COVID-exposure.

Table 25| Simple linear regression models were fitted to indicate the trend in the change in electrical parameters of PBMCs in response to RBD-stimulation between date of experiment and date of exposure, either by natural infection or most recent vaccination, in unmatched donors. Coefficient denotes the gradient of the slope, and the 95 % CI. P-value determines whether the slope is significantly greater than zero (*<0.05, **<0.01). r^2 value indicates the goodness-of-fit of the regression model by determining the variance attributable to the number of days between the date of the experiment and the date of COVID-exposure.

	Donor Cohort	Coefficient	95 % CI	P-value	r^2 Value
G_{eff}	Recovered COVID-19	0.0838	-0.393 to 0.561	0.529	0.2223
	Second Dose	0.130	-0.136 to 0.375	0.296	0.1795
	Third Dose	0.0359	-0.181 to 0.252	0.688	0.03510
C_{eff}	Recovered COVID-19	-0.101	-0.594 to 0.392	0.471	0.2792
	Second Dose	0.0219	-0.0510 to 0.0947	0.491	0.08242
	Third Dose	0.00183	-0.0870 to 0.0906	0.960	0.0005577
σ_{cyto}	Recovered COVID-19	0.00220	-0.00659 to 0.0110	0.394	0.3668
	Second Dose	0.000955	-0.000787 to 0.00270	0.228	0.2308
	Third Dose	0.000154	-0.00205 to 0.00236	0.864	0.006442

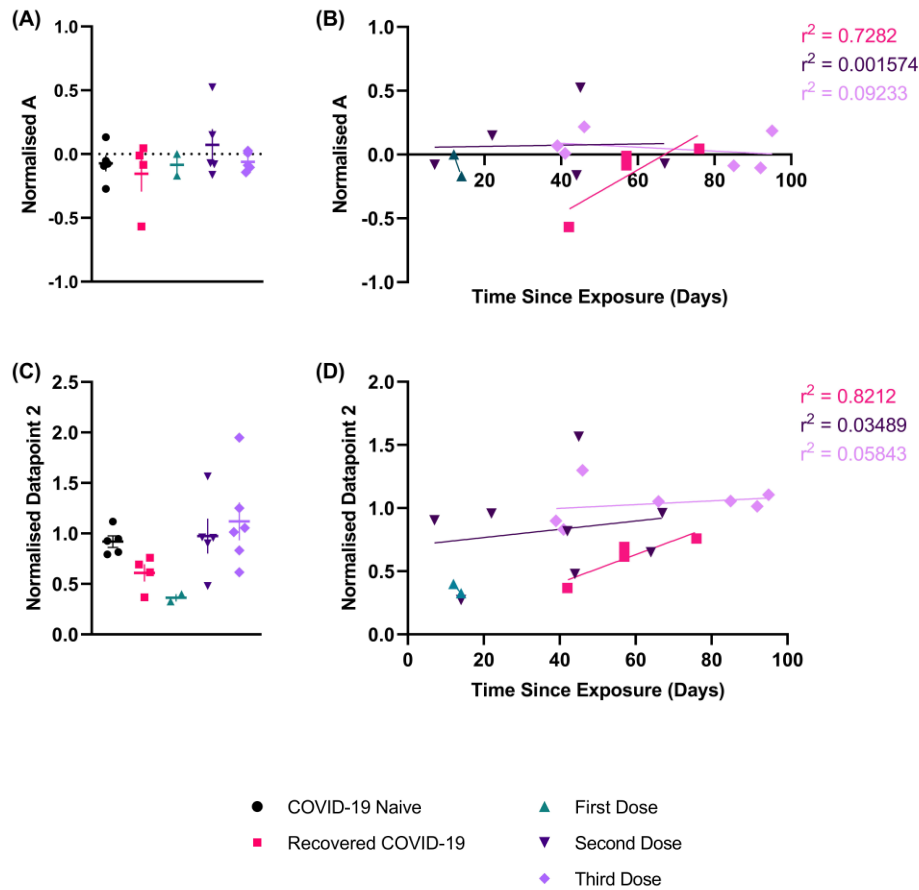


Figure 72 Scatter plots displaying **[A]** Normalised A values **[C]** Normalised datapoint 2 values in COVID-19 naïve (black circles: $n = 5$), recovered COVID-19 (pink squares: $n = 4$), first dose (teal triangles: $n = 2$), second dose (dark purple inverted triangles: $n = 5$), and third dose (lilac diamonds: $n = 5$). Mean lines plotted $\pm$ SEM. Statistical significance assessed using a matched one-way ANOVA followed by Tukey's multiple comparison ($*p < 0.05$). **[B]** Normalised A values **[D]** Normalised datapoint 2 values against days since COVID-Exposed in recovered COVID-19 ($n = 4$; pink square), first dose ($n = 2$; green triangle), second dose ($n = 8$; inverted purple triangle) and third dose ($n = 7$; lilac diamond) donors. r^2 value indicates the goodness-of-fit of the simple linear regression model by determining the variance attributable to the number of days between the date of the experiment and the date of latest COVID-exposure.

Table 26 Simple linear regression models were fitted to indicate the trend in the change in Normalised A and Normalised Datapoint 2 values of PBMCs in response to RBD-stimulation between date of experiment and date of exposure, either by natural infection or most recent vaccination, in unmatched donors. Coefficient denotes the gradient of the slope, and the 95 % CI. P-value determines whether the slope is significantly greater than zero ($*p < 0.05$, $**p < 0.01$). r^2 value indicates the goodness-of-fit of the regression model by determining the variance attributable to the number of days between the date of the experiment and the date of COVID-exposure.

	Donor Cohort	Coefficient	95 % CI	P-value	r^2 Value
Normalised A	Recovered COVID-19	0.01718	-0.01475 to 0.04911	0.1466	0.7282
	Second Dose	0.0004768	-0.02158 to 0.02254	0.9495	0.001574
	Third Dose	-0.001524	-0.008159 to 0.005110	0.5582	0.09233
Normalised Datapoint 2	Recovered COVID-19	0.01114	-0.004672 to 0.02695	0.0938	0.8212
	Second Dose	0.003272	-0.01392 to 0.02046	0.6578	0.03489
	Third Dose	0.001483	-0.006766 to 0.009733	0.6438	0.05866

Appendix A.4: Chondrocyte Data

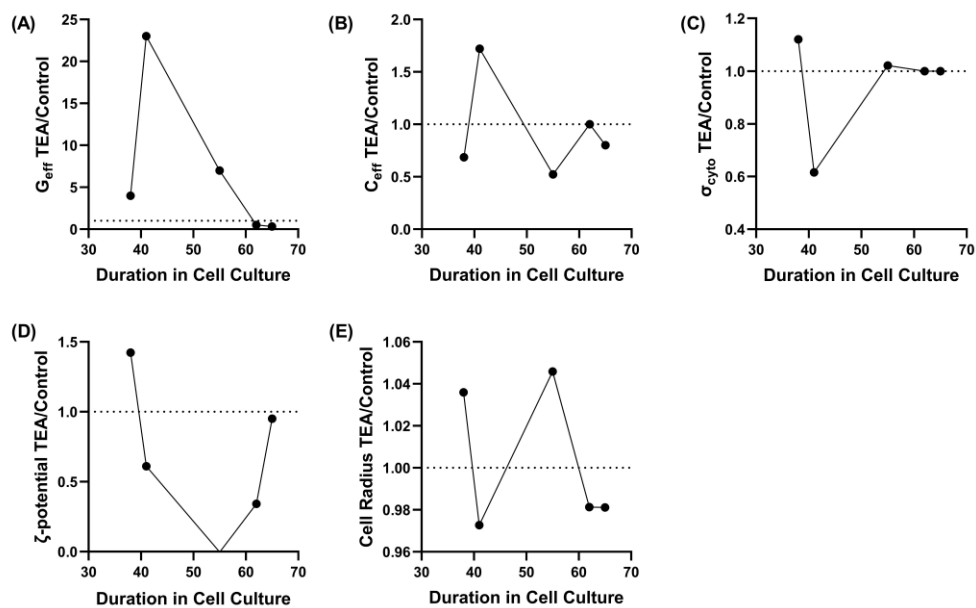


Figure 73 | Normalised values of TEA treated – unchallenged chondrocytes of **[A]** G_{eff} **[B]** C_{eff} **[C]** σ_{cyto} **[D]** ζ -potential **[E]** cell radius over time (n = 5; each datapoint is a separate biological repeat).

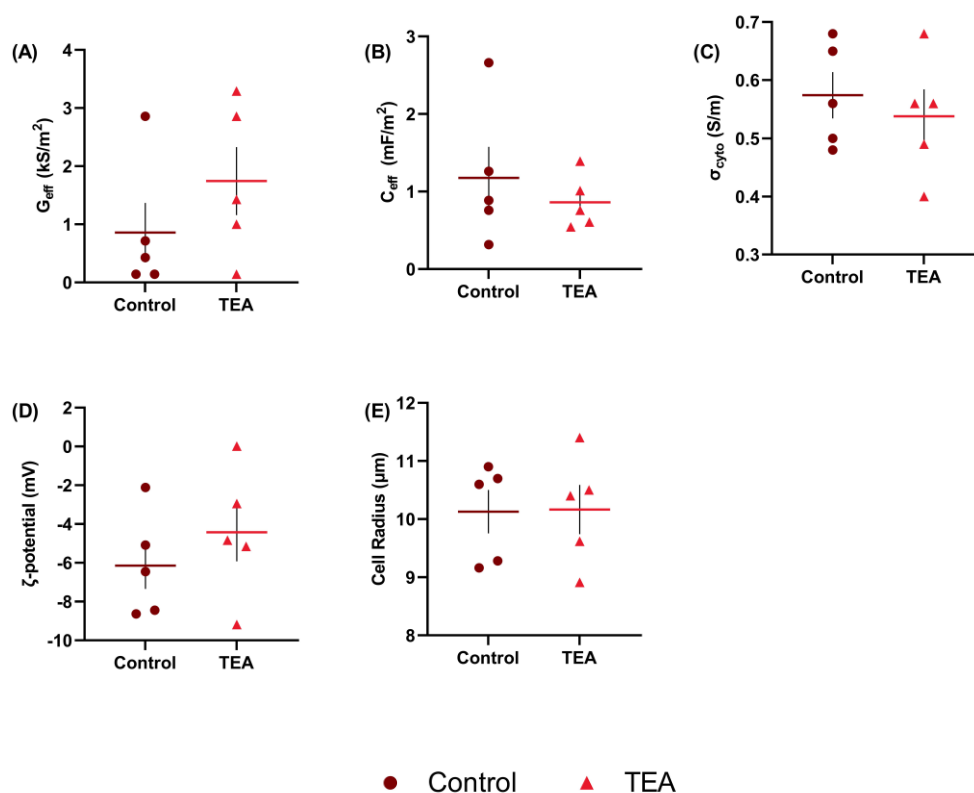


Figure 74 | Values of **[A]** G_{eff} **[B]** C_{eff} **[C]** σ_{cyto} **[D]** ζ -potential **[E]** Cell radius in control (brown circles: n = 5) and TEA treated (red triangles: n = 5) chondrocytes. Mean line plotted $\pm$ SEM. Statistical significance assessed using a paired two-tailed t-test (*p<0.05).

9. APPENDIX B – MATLAB Code

DEP Modelling MATLAB Code

Developed by Professor Michael Hughes and Komal Kaur.

% DATA

data=[];

% FIXED VARIABLES

```
e0=8.854e-12;
j=sqrt(-1);
f1=[1 1.2 1.44 1.2^3 1.2^4 1.2^5 1.2^6 1.2^7 1.2^8 1.2^9 1.2^10 1.2^11 1.2^12];
w=2*pi*[1e3*f1 1e4*f1 1e5*f1 1e6*f1 1e7*f1];
f=w./(2*pi);
e3=78*e0; % Medium Permittivity
```

% KNOWNNS

```
r1=; % Inner shell radius
r2=r1+7e-9; % 7e-9 equals membrane thickness
q3=; % Medium conductivity
```

% CELL VARIABLES

```
e1=; % Cytoplasmic permittivity
e2=; % Membrane permittivity
q1=; % Cytoplasmic conductivity
q2=; % Membrane conductivity
factor1 =; % Scale factor
```

% Low Dispersion 'tick'

```
Ks= ; % Surface conductance
scale=; % Changes amplitude of the tick
```

% TICK MODEL

```
a=(r2/r1)^3;
tau=e0*14*r2/(Ks);
dispersion=scale*(1./(1+j.*tau.*w));
```

% CLAUSIUS MOSSOTTI MODEL


```

ec1=e1-j*q1 ./w;
ec2=e2-j*q2 ./w;
ec3=e3-j*q3 ./w;
ec12=(ec1-ec2) ./ (ec1+2*ec2);
ef2=ec2 .*(a+2*ec12) ./ (a-ec12);
CMF=dispersion+(ef2-ec3) ./ (ef2+2*ec3);
realCMF=real(CMF);

% RESULTS

figure(1)
semilogx(f,realCMF,'b-',data(:,1),factor1*data(:,2),'bo'); % Plots graph
Geff=q2/7e-9 % Displays Geff
Ceff=e2/7e-9 % Displays Ceff

% Recalculation of the model with same frequencies as original data - 65 FREQ TO 20
FREQ (3DEP)

f2 = data(:,1)'; % transpose of the columns and rows
w2 = 2*pi*f2;
dispersion2=scale*(1./(1+j.*tau.*w2));
ec1_2=e1-j*q1 ./w2;
ec2_2=e2-j*q2 ./w2;
ec3_2=e3-j*q3 ./w2;
ec12_2=(ec1_2-ec2_2) ./ (ec1_2+2*ec2_2);
ef2_2=ec2_2 .*(a+2*ec12_2) ./ (a-ec12_2);
cmf2=dispersion2+(ef2_2-ec3_2) ./ (ef2_2+2*ec3_2);
re2=real(cmf2); % modelled curve with the 20 freq

% Plot recalculation

figure(2)
semilogx(f2,re2,'b-',data(:,1),factor1*data(:,2),'bo');
grid on;

fData = factor1*data(:,2)'; % 3DEP results

%ANALYSIS

%Mean Absolute Error

absErrors = abs(fData - re2);
MAE = sum(absErrors,'all')/20;

%Root Mean Squared Deviation

absErrors_1 = abs(fData - re2);
mean_absErrors_1 = mean((absErrors_1).^2);
RMSE = sqrt(mean((absErrors_1).^2));

%R squared formula
Rsq = 1 - sum((re2 - fData).^2)/sum((re2 - mean(re2)).^2);

```

```
%LINEAR PLOTTING (measured vs predicted)
%frequency based model vs measured (linear plot)

figure(3)
plot (re2,fData,'o')

%R coefficient of the linear plot

corr = corrcoef(re2,fData);

%Linear regression model (gives r^2 value of linear graph)

mdl = fitlm(re2,fData);
```

10. APPENDIX C: Research Outputs

Appendix C.1: Conferences and Presentations

Doctoral College Conference – Making the Invisible Visible

15/Jun/2023

Oral presentation – “Significant Electrophysiological Changes in White Blood Cells due to Hyperosmotic Stress in ME/CFS.”

Clock Club Conference

17/April/2023

Poster presentation – “Each Drop of Blood Measures its Time” Electrophysiological Rhythms in Blood Cells.

Physics of Life Conference

27-30/Mar/2023

Two poster presentations –

Identifying Immune Status to COVID-19 Using Dielectrophoresis

Significant Electrophysiological Changes in White Blood Cells due to Hyperosmotic Stress in ME/CFS

University of Surrey Open Research and Research Culture 2023

03/Feb/2023

Presentation to trustees of the ME Association and ME Research UK

19/Jan/2023

Oral presentation and discussion – “Electrophysiological Properties of PBMCs After Incubation in Hyperosmotic Medium in ME/CFS.”

Department of Mechanical Sciences Research Huddle

19/Jan/2023

Oral presentation – Characterising the Electrophysiological Properties of Cells in Health and Disease.

Doctoral College Conference – Exploring New Possibilities

23/June/2022

Oral presentation – “A Diagnostic Tool for Identifying Immune Status to COVID-19.”

Dielectrophoresis Conference 2020.1

26-28/June/2021

Appendix C.2: Journal Publications

Hughes M.P, Clarke K.S.P, Hoque R, Griffiths O.V, Kruchek E.J, Bertagna F, et al. (2024) On the low-frequency dispersion observed in dielectrophoresis spectra. *Electrophoresis*. 1–8. <https://doi.org/10.1002/elps.202300211>

Hughes M.P., Clarke K.S.P., Hoque R, Griffiths O.V., Kruchek E.J., Lewis R., and Labeed F.H. Label-free, non-contact, non-destructive determination of resting membrane potential. *Scientific Reports*. (Submitted).

Clarke K.S.P.*, Griffiths O.V.*, Hoettges K., Lewis R., Hughes M.P., and Labeed F.H. Quantifying the Electrophysiological Properties of Cells Using Dielectrophoresis. *Methods in Molecular Biology*. (Submitted).

Clarke K.S.P., Kingdon C.C., Lacerda E.M., Griffiths O.V., Kruchek E.J., Hoque R., Lewis R., Labeed F.H. and Hughes M.P. Significant Electrophysiological Changes in White Blood Cells due to Hyperosmotic Stress in ME/CFS. *PNAS*. (In preparation).

Clarke K.S.P., Kingdon C.C., Lewis R., Lacerda E.M., Kruchek E.J., Hughes M.P., and Labeed F.H. The search for a biomarker for Myalgic Encephalomyelitis/ Chronic Fatigue Syndrome (ME/CFS): from biochemistry to electrophysiology. (In preparation).

Clarke K.S.P., Stewart A.T., Sinclair E.L., Lewis R., Labeed., Dunn-Walters D.K., and Hughes M.P. Changes in White Blood Cell Electrophysiology in Response to Covid Exposure and Vaccination Observed by Dielectrophoresis. *Scientific Reports*. (In preparation).

Clarke K.S.P., Lewis R., Hughes M.P and Labeed F.H. Chondrocyte-to-fibroblast transformation: observation of cytoplasmic conductivity and membrane potential changes. (In preparation).

Kruchek E.J., Lewis R., Sheppard J.J., Clarke K.S.P., Johnson M.P., Mihut A., Daniels M., Kishore A., Griffiths O.V., Hoque R., Abdallat R., Jabr R.I., Beale A.D., Skene D.J., O'Neill J.S., Gibbins J.M, and Labeed F.H. Post-transcriptional circadian rhythms in isolated human platelets. *Nature Communications*. (Submitted).

Appendix C.3: Other Research Contributions

Circadian Rhythms in the Electrophysiological Properties of Isolated Human Platelets

Assisted in a series of 24-hour and 48-hour experiments with Dr EJ Kruchek and Dr F Labeed's research group to explore the regulation of circadian rhythms in platelets with respect to σ_{cyto} , ζ -potential and the platelet activation marker CD62P.

Electrophysiological Characterisation of Breast Cancer Cells – ANGLE PLC

Worked at ANGLE PLC within the Surrey Research Park, characterising the electrical properties of HS578T (mesenchymal cells), MCF-7 (epithelial cells) and SKBR3 (epithelial cells) to investigate how dielectrophoresis can be applied for cell separation.

Electrophysiological Characterisation of HeLa Cells

Collaborated in experiments to investigate the electrophysiological properties of cervical cells in health vs. disease.

Supervision of Undergraduate Dissertation Projects

- *Comparing the electrical properties of red blood cells obtained from donors of different blood types. (2023)*
- *Electrophysiological characterisation of bladder cancer. (2022)*
- *Analysing changes in the morphology of platelets during blood storage. (2022)*
- *Analysing changes in the morphology of red blood cells during blood storage. (2022)*
- *Effect of temperature and entrainment on red blood cell viability during storage. (2021)*

Appendix C.4: Certificates

Research Involving Human Tissue

09/Jan/2023

NHS Health Research Authority

The Use of Human Sample Research

09/Jan/2023

The Human Tissue Research Operations Group of the University of Surrey

Emergency First Aid at Work (RQF)

12/Sep/2022

QA Level 3 Award

The VIRT²UE Blended Learning Program on Research Integrity

01/Aug/2021

Online Modules on:

- *Introduction to Research Integrity*
- *Introduction of virtue ethics to research integrity*
- *Virtue ethics applied under current research conditions*

Sessions on reflecting ethics in research practice.

Open Research Training Module

University of Surrey



07/Sept/2021

Introduction to Informed Consent

The Global Health Network

06/Jan/2020

Appendix C.5: Research Training/ Workshops

Laboratory Training

Liquid Nitrogen Training	University of Surrey	24/June/2022
Properties, Handling and Hazards of Cryogenic Gases	University of Surrey	22/June/2022
Nikon Tri-Eclipse with A1M Confocal	University of Surrey	24/Nov/2021
Spectramax iD3 Training	University of Surrey	23/Aug/2021
BD FACS Celesta Training	University of Surrey	28/Nov/2029

Scientific Writing

How to Write a Scientific Abstract	Southern Counties Materials, Minerals and Mining Society	02/Feb/2023
The Conversation Media Training	The Conversation	09/May/2022
Writing A Conference Abstract	University of Surrey	21/Mar/2022
Introduction To Endnote	University of Surrey	22/Mar/2021
Getting Started with Thesis Writing	University of Surrey	08/Feb/2021
Thesis Writing: Literature Review	University of Surrey	28/Jan/2021
Writing Concisely	University of Surrey	02/Mar/2021
Writing Coherently	University of Surrey	23/Feb/2021

Writing Critically	University of Surrey	11/Feb/2021
Engaging With Your Literature: Critical Reading And Thinking	University of Surrey	15/Jan/2021

Research Presentations

Better Grant Figures Webinar	BioRender	19/Jan/2023
Poster Presentations	University of Surrey	25/Jan/2021
Presentation Skills Essentials	University of Surrey	22/Jan/2021

Research Practice

Preparing Impactful Research Proposals and Grant Applications	University of East Anglia	03/Mar/2022
Ethics & Governance for PGRs	University of Surrey	02/Dec/2021
How To Develop Research Integrity: A Virtue Workshop	University of Surrey	15/Jul/2021
Research Funding: The Basics	University of Surrey	06/Jul/2021
Building Your Professional Network: An Introduction	University of Surrey	19/Mar/2021
Research Data Management And Open Data	University of Surrey	24/Nov/2020

Professional Development

Postgraduate Funding: Considering the Alternatives	University of Surrey	24/May/2022
Careers Beyond Academia - FHMS	University of Surrey	30/Mar/2022
On the Job: Securing a First Academic Post	University of East Anglia	02/Mar/2022
Marketing Yourself: CV Workshop	University of Surrey	05/Mar/2021
Career Planning for PGRs: Getting Started	University of Surrey	23/Feb/2021
After The Doctorate What Next?	University of Surrey	21/Jan/2021
Time And Project Management for PGRs	University of Surrey	12/May/2020
Preparing For A Postdoc	University of Surrey	06/May/2020

Appendix C.6: Wider Outreach

Labeed F, Tudorache AM and Clarke K (2021) 'Following in Daphne's footsteps: Meet our female mechanical engineers', *University of Surrey News*, 28 June. Available at: <https://www.surrey.ac.uk/news/following-daphnes-footsteps-meet-our-female-mechanical-engineers>

Member of the Institute of Directors 2021-2022

11. APPENDIX D: COVID-19 Impact Statement

This lab-based PhD began on the 1st October 2019. Five months later on the 16th March 2020 special emergency legislation measures were announced by the UK Prime Minister in response to the COVID-19 global pandemic. This involved national UK lockdowns, immediate closure of universities, and a mandate to work from home – prohibiting access to the laboratory and data collection for prolonged lengths of time; significantly impacting the research project. Below is a breakdown of the impact of these emergency legislations and social measures on the ability of the researcher to conduct their research project, along with the contingencies employed:

Impact Delays to starting the ME/CFS experiments – planned to start in April 2020 but were unable to start before August 2022.

Major revisions were made to the original ME/CFS experimental protocol planned pre-pandemic. The original plan was to perform 3DEP experiments at the Royal Free Hospital in London using fresh PBMC samples donated from ME/CFS donors that morning. Due to COVID-19 mandated lockdowns, prohibited travel between areas and restrictions on blood donations due to social distancing measures, it was unforeseeable when these experiments could take place. Funding was thus obtained to purchase frozen PBMCs from the Royal Free BioBank with the intention to perform all 3DEP experiments at the University of Surrey once laboratory work at the University could re-start, with the advantage of increasing donor number and having equipment available to also perform parallel zeta potential experiments.

However, with fewer staff working in the legal departments of the University of Surrey and LSHTM, as well as an unprecedented increase in study applications and amendments to abide COVID-19 regulations, the ethics application, and Material Transfer Agreement (MTA) together took two years to obtain. This significantly reduced the total number of donor experiments were initially planned to perform and meant experiments investigating ion channels were not possible.

Impact Limited number of participants for COVID-19 study (**Chapter 4**).

Restricted laboratory access – lockdowns, work from home orders and the capacity of only one researcher to work in the laboratory at a time to abide social distancing measures until July 2021. The DEP laboratory had a capacity of one researcher at a time to abide social distancing regulations. A combination of these restrictions, problems finding donors and phlebotomists comfortable to participate during social distancing measures and those who fulfil the criteria of each cohort, meant the number of participants were significantly less than optimal.