

The immuno-metabolomics profile of cells presenting differential CCR5 phenotypes

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ABSTRACT

Background

Chemokines are small molecules that form part of the cytokine family and direct the response of cells to chemical stimuli and thus serve as chemotactic factors. Cysteine-cysteine (C-C) chemokine receptor 5 (CCR5), expressed on the surface of various cells, is one of the most studied chemokine receptors. In addition to its more established role in immune function, particularly inflammation, CCR5, and its interaction with its ligand(s), also mediates various metabolic changes. Given the interplay between the immune and metabolic systems, CCR5 immune-directed changes should be measurable at a metabolic level also. However, the CCR5 gene, is prone to various mutations, some of which have an impact on the cell surface expression of the receptor and its associated functions. Although much has been done to characterise CCR5 structurally and from an immune perspective, CCR5-induced metabolic changes are less well described, and have been measured using more traditional analytical techniques, and in an inflamed/diseased model. Immuno-metabolism describes the interplay between the cells' immune and metabolic programs, and represents a novel approach to characterise CCR5 further, in terms of its role during on metabolism. Given the scarcity and difficulties associated with obtaining clinical samples, with varying levels of CCR5, two distinct cell lines with regulatable levels of CCR5 was sourced and their associated immuno-metabolomic profiles characterised. The Affinofile (derived from human embryonic kidney (HEK293) cells, also a control cell line in this study) and Cf2Th (derived from canine thymus cells) cells, were cultured in media containing non-toxic concentrations of antibiotics, to maintain their respective vectors. Treatment of the Affinofile and Cf2Th cells with Ponasterone A (Pon A) and doxycycline, respectively, proved non-toxic and induced differential levels of CCR5, as confirmed via flow cytometry. Analysis of the cell supernatant's cytokine profile by flow cytometry revealed IL-2 as significantly different in cells with differential CCR5 levels as well as between the Affinofile and Cf2Th cell lines. This immunoregulatory cytokine is involved in cell growth, development, and homeostasis. The cells' intracellular metabolic profile as analysed using two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GCxGC-TOFMS), indicated that those cells with higher levels of CCR5 showed metabolite markers indicative of upregulated energy metabolism and receptor stabilisation in line with the growth and metabolic demands of the receptor and its cell which supports the associated detection of IL-2. Investigating CCR5 in a non-activated/disease context informs the design of future experiments that may optimise CCR5-related immune and metabolic functions and its possible therapeutic roles.

Keywords: C-C chemokine receptor 5, CCR5, Affinofile, Cf2Th, metabolomics, cytokine, immuno-metabolism

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ABBREVIATIONS, SYMBOLS AND UNITS

%	Percent	CBA	Cytometric bead array
°C	Degrees Celsius	C-C	Cysteine-cysteine
Δ	Delta	CCL	C-C ligand
μg/ml	Micrograms per millilitre	CCR5	C-C Chemokine receptor 5
μl	Microliter	CCR5Δ32	Mutated CCR5 gene with a 32 base pair deletion
μl/min	Microliter per minute	CCR5-PE	C-C Chemokine receptor 5 conjugated to phytoerythrin
μM	Micromolar	Cf2Th	Canine Thymocyte
3p21	Short arm position 21 on chromosome 3	CHM	Centre for Human Metabolomics
4D	Four-dimensional	CMI	Cell-mediated immunity
4E-BP1	eIF4E binding protein 1	CO ₂	Carbon dioxide
7-AAD	7-Aminoactinomycin	CoA	Coenzyme A
A	Apoptosis	COVID-19	Coronavirus disease 2019
ABS/cell	Antibody binding sites per cell	COX-2	Cyclooxygenase 2
ACC-1	Acetyl-CoA carboxylase I	CRISPR	clustered regularly interspaced short palindromic repeats
AD	Alzheimer's disease	CS&T	Cytometer Setup & Tracking
AFROX	African Oxygen Limited	csv	Comma separated values
AIDS	Acquired immunodeficiency syndrome	CV	Coefficient of variance
AMP	Adenosine monophosphate	DEHP	di(2-ethylhexyl) phthalate
AMPK	AMP-activated protein kinase	DMEM	Dulbecco's Modified Eagle's Medium
ANOVA	Analysis of variance	DMSO	Dimethyl sulfoxide
APC	Allophycocyanin	DNA	Deoxyribonucleic acid
ARDS	acute respiratory distress syndrome	e.g.,	Exempli Gratia
ATP	adenosine triphosphate	ECL	Extracellular loops
BCAA	Branched-chain amino acid	EDTA	Ethylenediaminetetraacetic acid
Bp	Base pairs	EI	Electron impact
BSTFA	n, o-bis(trimethylsilyl) trifluoroacetamide	eIF4E	Eukaryotic translation initiation factor 4E
C1	Antibiotic combination 1	ELISA	enzyme-linked immunosorbent assay
C2	Antibiotic combination 2	eNOS	Endothelial nitric oxide synthase
C3	Antibiotic combination 3	Env	Envelope
C4	Antibiotic combination 4		

ERK	Extracellular signal-regulated kinase	HLA	human leukocyte antigen
ES	Effect size	HMDB	Human Metabolome Database
ETC	Electron transport chain	HREC	Health Research Ethics Committee
eV	Electron volt	i.d.	In diameter
FADH ₂	Flavin adenine nucleotide (reduced)	i.e.,	Id est
FAMES	Standard fatty acid methyl esters	ICL	Intracellular loops
FBS	Fetal bovine serum	IFN	Interferon
fcs	Flow cytometry standard	IL	Interleukin
FITC	Fluorescein isothiocyanate	iNOS	Inflammatory gene-inducible nitric oxide synthase
FL	Fluorescent laser	IPEC-J2	intestinal porcine enterocytes
FL2	Fluorescent laser 2	IS	internal standard
FL4	Fluorescent laser 4	Kbp	Kilobase pairs
FL4-H	Fluorescent laser 4 height	kDa	Kilodalton
FMO	Fluorescence minus one	LCFA	Long chain fatty acid
FSC	Forward scatter	LDL-C	low-density lipoprotein cholesterol
FSC-A	Forward scatter area	LOOCV	Leave-one-out-cross-validation
FSC-H	Forward scatter height	LSD	Least significant difference
FVD	Fixable viability dye	m	Meter
GC	Gas Chromatography	m/z	Mass-to-charge
GCxGC-TOFMS	Two-dimensional gas chromatography time-of-flight mass spectrometry	MAP	Mitogen-activated protein
GM-CSF	granulocyte-macrophage colony-stimulating factor	MAPK	Mitogen-activate protein kinase
GSK-3 β	Glycogen synthase kinase 3 β	MCFA	Medium chain fatty acid
H1N1	influenza A	MCP-2	monocyte chemotactic protein two
HBV	hepatitis B virus	MDSC	myeloid-derived suppressor cells
hCCR5 Δ 24	Heterozygous CCR5 Δ 24	MFI	mean fluorescence intensity
HDL	High density lipoprotein	mg	Milligram
HDL-C	high-density lipoprotein cholesterol	MHC	major histocompatibility complex
HEK	Human embryonic kidney	Min	Minutes
HIV	Human immunodeficiency virus	MIP-1 α	macrophage inflammatory protein one alpha
		MIP-1 β	macrophage inflammatory protein one beta

ml	Millilitre	pg/ml	Picogram per millilitre
mM	Millimolar	PHA-P	Phytohemagglutinin-P
mm	Millimeter	PI3K	phosphoinositide-3-kinase
mRNA	Messenger RNA	PKB/Akt	protein kinase B
MS	Mass spectrometry	PKC	Protein kinase C
MSTUS	Mass Spectrometry Total Useful Signal	PLS-DA	Partial least square discriminant analysis
mTOR	Mammalian target of rapamycin	PMT	photomultiplier tube
M-tropic	Macrophage-tropic	Pon A	Ponasterone A
M-Tropic	macrophage-tropic	PPAR	Peroxisome proliferator-activated receptors
N	Necrosis	ppm	Parts per million
NF-κB	Nuclear factor-kappa B	PR1	Promoter 1
Ng	Nanogram	PR2	Promoter 2
ng/ml	Nanograms per millilitre	Pu	Upstream promoter
NIST	National Institute of Standards and Technology	Q1	Quadrant 1
nm	Nanometer	Q2	Quadrant 2
NMR	nuclear magnetic resonance	Q3	Quadrant 3
NRF	National Research Foundation	Q4	Quadrant 4
NWU	North-West University	QC	Quality Control
ORF	Open reading frame	QC-CV	Quality control coefficient of variance
OXPHOS	Oxidative phosphorylation	q-PCR	Quantitative polymerase chain reaction
P1	Passage 1	RANTES	regulated upon activation, normal T cell expressed and secreted
P2	Passage 2	RNA	Ribonucleic acid
P3	Passage 3	ROS	Reactive oxygen species
P4	Passage 4	S/N	Signal to noise
PBS	Phosphate buffer solution	SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
PC	Principal component	SASBMB	South African Society for Biochemistry and Molecular Biology
PC1	Principal component 1	SSC	Side scatter
PC2	Principal component 2	SSC-A	Side scatter area
PCA	Principal component analysis		
Pd	Downstream promoter		
PE	Phycoerythrin		
PFKFB-2	6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 2		

SSC-H	Side scatter height	TNF	tumour necrosis factor
STAT5	Signal transducer and activator of transcription 5	TOF	Time-of-flight
TCA	Tricarboxylic acid	US	Untreated Stained
TCR	T cell antigen receptors	USA	United States of America
Tet-On	Tetracycline inducible system	UTR	Untranslated region
TG	Triglycerides	UU	Untreated Unstained
Th1	T helper type 1	VC	Viability control
Th17	T helper type 17	VIP	Variable important in projection
Th2	T helper type 2	WNV	West Nile virus
TMCS	Trimethylchlorosilane	α	Alpha
TMD	Transmembrane domains	β	Beta
TMS	Trimethylsilyl	γ	Gamma

CHAPTER 1: INTRODUCTION

1.1 Background and problem statement

Cysteine-cysteine (C-C) chemokine receptor 5 (CCR5) is a protein receptor expressed on the surface of most cells and is involved in innate (inflammation) and adaptive immune responses (increased encountering of antigen-specific cells). CCR5, together with its ligand, chemokine (C-C motif) ligand 5 (CCL5), is released at sites of infection or injury to activate effector cells responsible for chemokine secretion and signal amplification. When CCR5 binds to a ligand, a conformational change occurs in the receptor, affecting effector biological functions. In addition, several mutations within the CCR5 gene exist with the potential to impact the structure and levels of CCR5 present on the cell surface. The structure of CCR5 and its role in the immune system is well known. However, clinical samples with which to characterise CCR5 are scarce (Barmania, 2012). Also, information regarding how CCR5 levels relate to phenotype, and the impact of CCR5 on the metabolism is limited. Where scientists investigated metabolic parameters, the studies focused primarily on an enhanced inflammatory/diseased state (showing contrasting roles for the molecule). Metabolic parameters were also measured using older conventional platforms.

CCR5 has received renewed attention since the 2000s, given reports of the Berlin and London patients being “cured” of human immunodeficiency virus/ acquired immunodeficiency syndrome (HIV/AIDS) following bone marrow transplantations (Jessen *et al.*, 2014). Furthermore, scientists used the clustered regularly interspaced short palindromic repeats (CRISPR) gene-editing tool to genetically modify embryos before transplantation in a woman who subsequently gave birth to babies classified as resistant to HIV infection. This background has extended to CCR5 being revisited and reviewed for its therapeutic roles in cancer, inflammatory conditions such as rheumatoid arthritis, and infectious diseases such as coronavirus disease 2019 (COVID-19).

Most of the attention related to CCR5 has centred around its role as a co-receptor for HIV infection. The envelope glycoprotein of macrophage-tropic (M-tropic) HIV-1 isolates serves as a ligand for CCR5 (Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Doranz *et al.*, 1996; Dragic *et al.*, 1996). However, mutations impact the interaction of the chemokine receptor to its ligands and affect cell signaling. A common mutation in the CCR5 gene is the 32-base pair (bp) deletion denoted as CCR5 Δ 32, resulting in a non-functional receptor that is not expressed on the cell surface. Therefore, regarding HIV infection, homozygous CCR5 Δ 32 genotypes (no CCR5 expression) are classified as protective haplotypes and the homozygous CCR5 wild-type genotypes (higher CCR5 expression) as non-protective haplotypes. In addition, heterozygous CCR5 Δ 32 genotypes (minimal CCR5 expression) are also considered as partially protective haplotypes. Several researchers have attempted to elucidate the role of CCR5 in various other

diseases (Gauthier *et al.*, 2022; Viel, 2022; Zeng *et al.*, 2021). However, these diseases did not show similar distributions of CCR5 Δ 32 genotypes. For example, some studies found CCR5 Δ 32, in either homozygous or heterozygous states, as protective in some cohorts. In contrast, other studies were not able to show the same results. For example, the study conducted by TrehanPati and co-workers (2009) found that CCR5 Δ 32 was associated with protection against Hepatitis B virus infection (TrehanPati *et al.*, 2009). The study performed by Arababadi and co-authors (2010) found that CCR5 Δ 32 was associated with an increased risk for Hepatitis B virus infection (Arababadi *et al.*, 2010a).

Besides viral entry, CCR5 plays a pivotal role in innate immunity (i.e., CCR5 ligands such as CCL5 are released at sites of infection or injury to activate effector cells responsible for chemokine secretion for signal amplification). The adaptive immune response comprising antibody production and cell-mediated immunity (CMI) protects at a later stage of infection. It can efficiently detect any known pathogen since it establishes a memory state. During CMI response, CD4 helper T cells regulate the immune response and activate other immune cells. The cytotoxic CD8 cells eliminate cells that express a foreign antigen. During inflammation, CCR5 expression is upregulated in CD8 cells (Castellino *et al.*, 2006), increasing the chances of CD8 cells encountering antigen-specific cells. Therefore, CCR5 is involved in both innate and adaptive immune responses.

Besides the role of CCR5 in immunity, the binding of a CCR5 ligand to its receptor also mediates metabolic changes (Chan *et al.*, 2012; Gao *et al.*, 2016), which induces a hypermetabolic state. In one study performed by Chan and co-workers (2012), the authors reported that the ligand, CCL5 activates its receptor CCR5 which affects glycolysis and adenosine triphosphate (ATP) production in cancer cells (Chan *et al.*, 2012). Several other studies reported on the role of CCR5 in metabolism (Bonfá *et al.*, 2014; Lin *et al.*, 2017). A clear relationship between immunology and metabolism exists, leading to the term immuno-metabolism, which aims to clarify, amongst other factors, the involvement of inflammation in the pathogenesis of several metabolic disorders. Furthermore, it also describes different metabolic factors that influence the regulation of several different immune cell functions (Raghuraman *et al.*, 2016). Given the role of CCR5 in immune function, subsequent changes should thus be measurable at the metabolic level.

Regarding CCR5, research has tended to focus on its structure and the immune or metabolic profiles, or both, but using diseased models and conventional technologies to measure these parameters. This study will investigate the immuno-metabolic profile of cells with differential CCR5 phenotypes but in a non-inflamed/diseased context using more sensitive technologies capable of measuring multiple cell parameters. Furthermore, the improved characterisation of a clinically relevant molecule involved in immune and metabolic processes makes this study highly relevant in a pandemic era such as the one experienced now. Improved characterisation of a molecule

such as CCR5 can inform the future experimental design and/or identify pathways which can be considered for therapeutic purposes.

1.2 Aim and objectives

1.2.1 Aim

This study aims to characterise the immuno-metabolic profile of cell lines presenting with differential CCR5 phenotypes using flow cytometry and untargeted two-dimensional gas chromatography time-of-flight mass spectrometry (GCxGC-TOFMS). Two cell lines were used to increase the confidence as to whether the immune-metabolic differences observed can be attributed to CCR5 levels.

1.2.2 Objectives

The aim mentioned above requires the following objectives:

- 1) Grow and freeze stocks of the Affinofile/HEK293 and Cf2Th cell lines
- 2) Confirm the antibiotic concentration in which to culture Cf2Th cells (this is known for the Affinofile cells)
- 3) Determine the optimum concentration of CCR5-inducing reagents to achieve low and high CCR5 expression in the respective cell lines
- 4) Culture Affinofile, HEK293, and Cf2Th cells and induce CCR5 levels using the predetermined concentrations of the CCR5-inducing antibiotics as in point three above
- 5) Confirm cell viabilities and respective CCR5 levels for all cell lines using flow cytometry
- 6) Perform immune profiling on the supernatant of all cells
- 7) Analyse the metabolic profile of cells using the standardised method for GCxGC-TOFMS (inclusive of normalisation procedures)
- 8) Stratify samples according to CCR5 levels (per cell line) and employ uni- and multivariate statistical analysis to identify differences in the immune and metabolic profiles of the cells
- 9) Map significantly different immune and metabolic markers to various biological processes and/or pathways

1.3 Structure and outputs

This dissertation is written to comply with the partial fulfilment of the requirements of the North-West University (NWU), Potchefstroom Campus, South Africa, for the completion of the degree *Magister Scientae* (Biochemistry) in dissertation format.

Chapter 1 introduces the critical aspects of this study and the gap it aims to address through the aims and objectives.

Chapter 2 represents the literature review which introduces chemokines, specifically CCR5, and its role in the immune response and metabolism. This section also highlights mutations in the CCR5 gene as these impact CCR5 levels and the biological function of the receptor. Commentary is provided on typical CCR5 levels and/ or percentages recorded in the literature with conclusions regarding the typical immune and metabolic profiles in such instances. Previous researchers used conventional assays to derive most of the immuno-metabolic parameters. Therefore, flow cytometry and metabolomics are presented as investigative tools for the improved characterisation of cells presenting differential CCR5 phenotypes. The chapter is concluded by highlighting the significance that the improved characterisation of CCR5 holds.

Chapter 3 motivates why cell lines serve as model systems to characterise differential CCR5 phenotypes. This chapter provides background on the Affinofile and Cf2th cells with regulatable CCR5 and then describes this study's experimental design, including HEK293 cells without the CCR5-expressing vector as a control. Finally, since the previously engineered Affinofile and Cf2th cells utilise the tetracycline-inducible (Tet-On) system to express distinct levels of CCR5, consideration is given to the influence of the Tet-On system and its associated antibiotics on the immune and metabolic profiles of the cells.

Chapter 4 details the materials and methods used after ethical approval was received. The rationale for using reagents and/or methods employed is also provided.

Chapter 5 presents the results and discussion. The results include the antibiotic concentrations in which to culture the Cf2th cells (this is known for the Affinofile cells), the concentration of CCR5-inducing reagents to add to cells to achieve low and high levels of CCR5, the cell viability and confirmation of various CCR5 levels in the respective cells, the associated extracellular cytokine/immune profile, and the metabolic profile of cells (normalised to 3-phenyl butyric acid) expressing differential CCR5 levels. Discussions supporting or against the arguments in this study accompany the various findings presented.

Chapter 6 provides the conclusion to the study, its strengths and limitations, and considerations for future studies.

Some of the findings of this study was presented at the South African Society for Biochemistry and Molecular Biology's 27th Congress (SASBMB, Annexure E). A review article was published as an output of this study in the International Journal of Molecular Sciences with an Impact factor of 4.556 (Annexure F).

1.4 Study contributions

The primary author of this dissertation is Anandi Rautenbach. The table below summarises the contributions that the co-authors and co-workers made towards this dissertation

Name	Role	Contribution
Anandi Rautenbach	Author	Responsible for the conceptualising, planning, execution, and reporting of this study under the guidance of the supervisor and co-supervisor.
Dr Aurelia Williams	Supervisor	Supervisor She conceptualised, coordinated, and supervised all aspects of the study and performed all the administrative duties linked to the study.
Prof Du Toit Loots	Co-supervisor	Co-supervisor He assisted with the study design, planning, and proofreading of this dissertation and conference outputs.
Emile Jansen van Rensburg	Co-worker	He provided training on all aspects related to metabolomics analysis.
BD Biosciences		For the use of the BD Accuri™ C6 Plus
NWU's Centre for Human Metabolomics (CHM)		For the use of the GCxGC-TOFMS.
Rozanne Adams (BD Biosciences)		For insight regarding the analysis and interpretation of the flow cytometric data.

CHAPTER 2: LITERATURE OVERVIEW

Parts of this chapter was published in an international peer-reviewed journal.

- Rautenbach A, Williams AA. Metabolomics as an Approach to Characterise the Contrasting Roles of CCR5 in the Presence and Absence of Disease. *Int J Mol Sci.* 2020 Feb 21;21(4):1472. doi: 10.3390/ijms21041472.

2.1 Chemokines and chemokine receptors

Chemokines are small protein molecules with molecular weights ranging from 7 to 15 kDa (Groves & Jiang, 1995). Platelet factor four was the first chemokine discovered in 1977 (Walz *et al.*, 1977). Activated platelets release platelet factor four during platelet aggregation to stimulate blood coagulation and are involved in wound repair and inflammation. Chemokines form part of the cytokine protein family. Cells of the immune system secrete cytokines which act as signalling molecules that regulate the interactions between immune cells (Zhang & An, 2007). In addition, chemokines induce chemotaxis – a process where chemical stimuli direct specific cells to sites of injury or infection (Bachmann *et al.*, 2006). Other functions of chemokines include angiogenesis, hematopoiesis, embryonic development, and metastasis (Gerard & Rollins, 2001; Yoshie *et al.*, 2001).

There are four main categories of chemokines based on the structure and number of cysteine residues: C, CC, CXC, and CX3C. The letter 'C' refers to the cysteine molecules - each 'C' in the nomenclature represents two cysteine molecules - and the letter 'X' with the indicated number in the nomenclature denotes the number of amino acids present between the cysteine molecules. For example, CX3C means that three amino acids are present between the cysteine molecules. The letter 'R' is added to the label and refers to the receptor (Murphy *et al.*, 2000). Chemokine receptors on the surface of cells facilitate the chemotactic functions of chemokines (Gosling *et al.*, 1997; Youn *et al.*, 2001). Chemokines interact with a specific receptor to attract immune cells to tissue/organ targets, leading to numerous cellular responses and have therefore been pivotal in many diseases. The role of CCR5 in the immune and subsequent metabolic processes will be the focus of this study.

2.2 CCR5, its location, and structure

Much of the current literature on chemokine receptors focuses on the G-protein coupled chemokine receptor five, CCR5, expressed on numerous immune cells; epithelial and endothelial cells; fibroblasts; vascular smooth muscle cells; and central nervous system cells (Rottman *et al.*, 1997). Raport and co-workers (1996) first located the *CCR5* gene - which encodes for the CCR5

protein - on the short arm position 21 on chromosome 3, denoted as 3p21, using electrophoresis and Southern blotting techniques (Raport *et al.*, 1996). The location of the *CCR5* gene was later confirmed by Liu and co-authors (Liu *et al.*, 1996). In 1997, Mummidi and co-workers (1997) determined that the gene comprises two promoters, two noncoding introns, and three exons, namely exon 1, exon 2a and exon 2b (Mummidi *et al.*, 1997). An illustration of the non-mutated *CCR5* gene, consisting of 6065 bp, is shown in Figure 2-1. The upstream promoter (Pu), also known as Promoter 2 (PR2), is the region of deoxyribonucleic acid (DNA) involved in the initiation of transcription situated close to the 5' end of the sense strand and entails a 1.9 kb region. A coding region consisting of exon 1 (57 bp) and intron 1 (501 bp) follows Pu. The downstream promoter (Pd), also known as Promoter 1 (PR1), includes the regions of both intron one and exon two (Mummidi *et al.*, 2007) - exon two is presented as exon 2a and exon 2b with lengths of 235 bp and 54 bp, respectively. Intron 2 (1.9 kbp) is situated between exon two and exon three - exon three includes the entire open reading frame (ORF), the 3' untranslated region (UTR), and 11 bp of the 5' UTR (Mummidi *et al.*, 1997); these untranslated regions play a pivotal role in the regulation of translation.

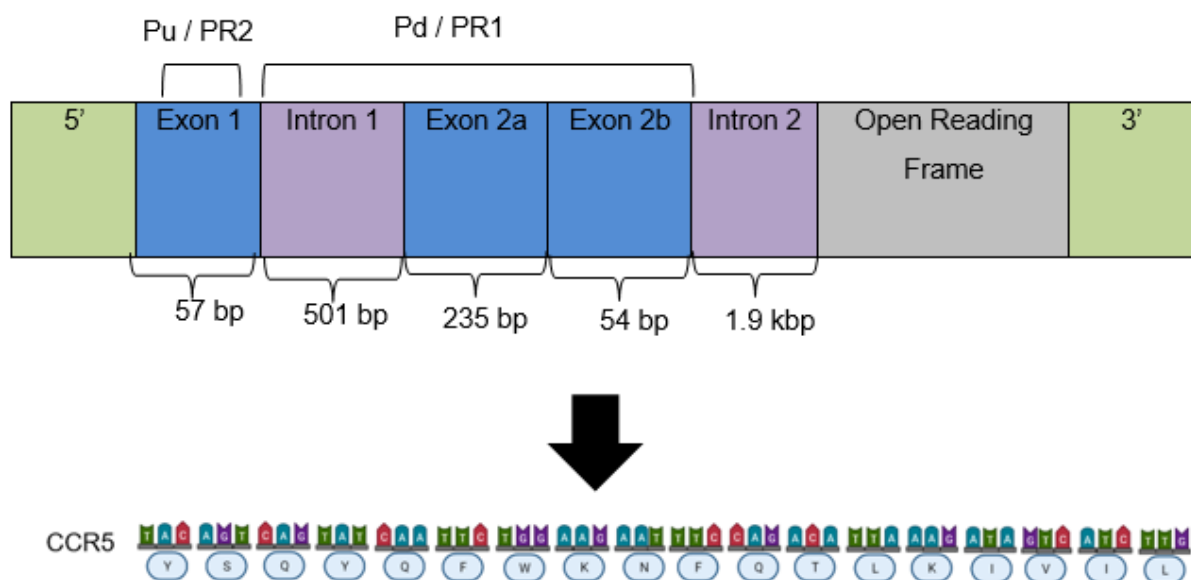


Figure 2-1: Schematic overview of the *CCR5* gene and the resulting amino acid sequence without a mutation (Image created by the author with Biorender.com).

Abbreviations: *CCR5* – C-C Chemokine receptor 5. Nucleotide abbreviations: A – Adenine; C – Cytosine; G –Guanine; T – Thymine in DNA. Amino Acid abbreviations: F – Phenylalanine; I – Isoleucine; K – Lysine; L – Leucine; N – Asparagine; Q – Glutamine; S – Serine; T – Threonine; V – Valine; W – Tryptophan; Y – Tyrosine.

The *CCR5* gene translates to form the *CCR5* protein (graphical representation shown in Figure 2-1 which comprises 352 amino acids (Oppermann, 2004b) and a molecular weight of 40.6 kDa (Samson *et al.*, 1996a). The protein structure starts as an extracellular amino-terminal segment followed by seven hydrophobic transmembrane domains (TMD), which are connected by three extracellular loops (ECL), in addition to another extracellular domain, and three intracellular loops

(ICL). The end of the protein structure is a carboxyl tail, also known as the cytoplasmic tail, at the C-terminal. The protein contains specific conserved residues and motifs, which play a pivotal role in several processes, including ligand-dependent signalling, desensitisation, and receptor trafficking (Oppermann, 2004b) - all of the extracellular domains have cysteine residues. The second intracellular loop contains a conserved amino acid sequence, the DRYLAVHA sequence, associated with G-protein interactions. The third intracellular loop is surprisingly short and contains many positively charged residues. Two disulphide bridges stabilise the CCR5 receptor for ligand binding: the first occurs between two conserved cysteine residues situated on the second and third extracellular loop (Oppermann, 2004b), and the second occurs by a highly conserved cysteine residue on the N-terminal of the protein and a cysteine residue situated in the third extracellular loop (Lee *et al.*, 1999a).

Lee and co-workers (1999) investigated the topology of the CCR5 TMD and reported on some of the structural complexities, and showed CCR5 to exist in multiple conformational states (Lee *et al.*, 1999a). The conformational states were confirmed when Blanpain and co-workers (2002) used antibodies with specific functional properties to inhibit chemokine binding, modulate the activation of intracellular cascades, and influence receptor trafficking. Furthermore, the authors found that the antibodies mapped to several epitopes, including the N-terminal segment and the second extracellular loop (Blanpain *et al.*, 2002). Several researchers established the location of the *CCR5* gene, but further investigations are required to inform on the various confirmations of the CCR5 protein – mainly where a mutation in the *CCR5* gene occurs.

2.3 Ligands and chemokine receptors

A ligand is a molecule that binds to a protein receptor to form a complex. Ligand-receptor binding induces a conformational change in the receptor resulting in a biological response by sending signals to other cells in the body. Ligands for CCR5 include macrophage inflammatory protein one alpha (MIP1- α) (Nibbs *et al.*, 1999), macrophage inflammatory protein one beta (MIP1- β), regulated upon activation of normal T cell expressed and secreted (RANTES) which is also known as CCL5, and monocyte chemotactic protein two (MCP-2) (Combadiere *et al.*, 1996; Gong *et al.*, 1998; Raport *et al.*, 1996; Samson *et al.*, 1996a). Given the role of CCR5 as a co-receptor during HIV infection, as mentioned in Section 1.1, several studies found that the envelope glycoprotein of M-tropic HIV-1 isolates also serve as a ligand for CCR5 (Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Doranz *et al.*, 1996; Dragic *et al.*, 1996).

2.4 CCR5 mutations, association to CCR5 levels and prevalence

The *CCR5* gene is prone to mutations that disrupt the typical structure and function of the receptor, with resulting phenotypes which implicate health and disease. Several publications

reported a large deletion present in the *CCR5* gene structure known as *CCR5*Δ32 (Dean *et al.*, 1996; Liu *et al.*, 1996; Samson *et al.*, 1996b). The ORF has a 32 bp deletion on the extracellular loop (Samson *et al.*, 1996b), from nucleotide position 794 to 825 (Figure 2-2), resulting in a shift in the codon reading frame (Liu *et al.*, 1996) and also the translation mechanism. Consequently, the mutated protein comprises only 215 amino acids instead of the 352 amino acids known to occur in the non-mutated protein.

Figure 2-2: A graphical representation of the *CCR5* gene, originally consisting of 352 amino acids, with the *CCR5*Δ32 mutation, resulting in a premature stop codon due to the frameshift mutation, and an altered amino acid sequence containing 215 amino acids (image created by the author with Biorender.com).

The last three transmembrane domains, along with regions critical for cellular processes such as G-protein interactions and signal transduction, are thus absent in the mutated protein, impacting immune and subsequent metabolic processes. Furthermore, the deletion results in a premature stop codon within the ORF, leading to an unexpressed nonfunctional receptor (Dean *et al.*, 1996; Samson *et al.*, 1996b). CCR5 genotypes have been shown to correlate to the cell surface expression of CCR5 (Benkirane *et al.*, 1997; Liu *et al.*, 1996; Wu *et al.*, 1997), which correlate with infectability/ viral levels (Reynes *et al.*, 2000; Trkola *et al.*, 1996). Wild-type homozygous genotypes relate to high CCR5 levels; heterozygous mutated genotypes relate to low CCR5 levels; while homozygous mutated genotypes relate to basal/absent CCR5 levels (Hütter *et al.*, 2015). Martinson and co-authors (1997) published a report regarding the global distribution of the CCR5 Δ 32 mutations, typically limited to individuals of European ancestry – the mutation is present in about 10% of Caucasians (Martinson *et al.*, 1997).

2.5 Role of CCR5 in the immune system

The immune system consists of several defence mechanisms against any foreign substance - the first is the host's barriers, including physical (such as the skin) and chemical (enzyme secretions) components. When a pathogen passes these barriers and invades the host, the host relies on its innate (inborn) immune responses first, followed by the adaptive (acquired) immune responses.

Memory-lacking cells of the innate immune system circulate through the body; they provide rapid protection and primarily detect the pathogen using receptors. The primary effector mechanism of this immunity is phagocytosis where pathogens are engulfed and become a target for destruction by enzymes or reactive oxygen species (ROS) (Stuart & Ezekowitz, 2005). Innate receptors on cells recognise pathogens that initiate an inflammatory response. The cells secrete cytokines (and chemokines) to increase the phagocytic capacities of the innate immune cells (Luster *et al.*, 2005) and recruit different cells and proteins to the site of infection or injury by increasing vessel permeability (Moser & Leo, 2010). For example, release of the CCR5 ligand, CCL5, is induced by other pro-inflammatory cytokines, prompting the accumulation of several immune cells at sites experiencing inflammation (Korbecki *et al.*, 2020). Immune cells release CCR5 ligands at sites of infection or tissue damage to activate effector cells – these release chemokines to enhance signal amplification (Bachelier *et al.*, 2014; Lederman *et al.*, 2006).

Cells of the adaptive immune system establish some state of memory and protect at a later stage of the infection, efficiently detecting any known pathogen. These qualities are crucial if a specific pathogen displays a high mutation rate since the pathogen may escape recognition by the innate immune system (Bowie & Unterholzner, 2008). T lymphocytes, including CD4 T helper cells and CD8 T cytotoxic cells, are activated when they encounter an antigen and subsequently undergo clonal expansion to increase the number of T cells (Curtis *et al.*, 2005; Pape *et al.*, 1997). Some T cells participate in the immune reaction, while others become memory cells. An antigen-presenting cell takes up the pathogen and digests it through phagocytosis (Gordon, 2002), producing antigen fragments. These fragments bind to the major histocompatibility complex (MHC) proteins of the antigen-presenting cell to form a complex - the MHC on the cell surface of the antigen-presenting cell uses the complex to present the antigen to the receptors of either a CD4 or CD8 cell (Mantegazza *et al.*, 2013). MHC class I proteins form a complex with antigenic fragments of intracellular pathogens and present the fragments to CD8 cells. In contrast, MHC class II proteins form a complex with antigenic fragments of extracellular pathogens and present the fragments to CD4 cells. The different T cells serve distinguishing purposes: CD4 cells regulate immune responses and activate other immune cells; CD8 cells eliminate target cells expressing a foreign antigen. The inflammation process upregulates CCR5 expression on CD8 cells, and cells move towards sites where interactions between antigen-presenting cells (e.g. dendritic cells)

and CD4 T cells (Castellino *et al.*, 2006) occur. This process increases the chance of CD8 cells encountering antigen-specific cells. Therefore, CCR5 expression impacts inflammation (innate immunity) and adaptive immune responses.

Immune cells expressing the CD4 receptor are characterised as T helper type 1 (Th1) or T helper type 2 (Th2) cells depending on the immune response they elicit. Some cells can also elicit a Th17 immune response. Th1 cells produce and secrete cytokines that induce CMI responses and inflammation, including cytokines such as interleukin (IL)-2, interferon-gamma (IFN- γ), and tumour necrosis factor-beta (TNF- β). In contrast, Th2 cells produce and secrete cytokines that induce an antibody response, including IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (Romagnani, 2000). Usually, the Th2 immune response counteracts the effect of the Th1 response (Bonecchi *et al.*, 1998). However, the Th1 and Th2 immune responses should be balanced to eliminate pathogens effectively (Berger, 2000). The expression of CCR5 is mainly limited to Th1 cells (Kutukculer *et al.*, 2016; Loetscher *et al.*, 1998; Yamamoto *et al.*, 2000), potentially altering the balance between Th1 and Th2 immune responses. Th17 cells secrete cytokines such as IL-17A, IL-17F, and IL-21; the secreted cytokines can be related to Th1 or Th2 T cells to induce or alleviate inflammation, respectively (Agaloti *et al.*, 2018). Regarding CCR5, CCR5 Δ 32 has been shown to drive Th2 immune responses in some instances, but various authors failed to mention whether the participants carried a homozygous or heterozygous CCR5 Δ 32 genotype (Muntinghe *et al.*, 2012). Table 2-1 summarises the cytokines and their immune function that will be the focus of this study.

Table 2-1: Summary of the cytokines and their functions that will be the focus of this study.

Cytokine	T helper cell subset	Functions	Reference
IFN-α; IFN-β	Th1	↓ inflammatory genes; ↑ anti-viral properties; ↓ cytokine-mediated lethality and autoimmune disease; ↓ angiogenesis; ↑ MHC class I expression	(Nakhband <i>et al.</i> , 2021; Sosa <i>et al.</i> , 2021)
IFN-γ	Th1 / Th17	Pro-inflammatory; ↑ the Th1 response; ↑ clonal expansion of cytotoxic T cells; ↑ MHC class II molecules; ↑ macrophage activation; associated with autoimmune Th17 responses.	(Adams <i>et al.</i> , 1993; Gajewski & Fitch, 1988; Kamijo <i>et al.</i> , 1994)
TNF-α	Th1	Pro-inflammatory; pro-atherogenic; ↑ inflammatory mediators and innate immune responses.	(Caldwell <i>et al.</i> , 2014; Kalliolias & Ivashkiv, 2016)
IL-17A	Th17	Pro-inflammatory ↑ Th17 autoimmune response	(Douzandeh-Mobarrez & Kariminik, 2019; Iwakura <i>et al.</i> , 2008)
IL-10	Th2, Treg	Anti-inflammatory; ↓ inflammatory genes, ↓ cytokine-mediated lethality and autoimmune disease.	(Borish, 1998; Ouyang <i>et al.</i> , 2011)
IL-6	Th1, Th2	Pro/anti-inflammatory, pro-atherogenic, pro-metastatic, ↑ B cell activation; ↑ neovascularisation.	(Gadient & Otten, 1997)
IL-4	Th2	↑ clonal expansion of cells; ↑ antibody production.	(Nelms <i>et al.</i> , 1999)
IL-2	Regulatory	Control differentiation and homeostasis of T cells	(Knoechel <i>et al.</i> , 2005; Konjević <i>et al.</i> , 2019; Ross & Cantrell, 2018)

Cytokines: IFN: interferon; TNF: tumour necrosis factor; IL: interleukin.

T helper cell subset: Th1: T helper cell type 1; Th2: T helper cell type 2; Th17: T helper cell type 17; Treg: T regulatory cells

Biological role: MHC: major histocompatibility complex; ↑: Increase; ↓: Decrease

Stimulated B cells can divide and differentiate into memory B cells and plasma cells (Burnet, 1957; Hodgkin *et al.*, 2007) and partake in the humoral immune response. In addition, the plasma cells return to the bone marrow, producing antibodies of a particular specificity (Agaloti *et al.*, 2018) - antibody-producing plasma cells can thus facilitate adaptive immunity.

The role of CCR5 in the immune response was studied in several diseased states and/or models. An in-depth discussion of the specific immune responses induced during each of these diseased states is beyond the scope of this dissertation. Instead, representative studies are highlighted to show where immune responses and/or cytokine production are associated/correlated with CCR5

levels. Alzheimer's disease (AD) patients typically present with a neuroinflammatory response (Ardura-Fabregat *et al.*, 2017; Heneka *et al.*, 2015; Zhang & Jiang, 2015). Bajetto and co-workers (2001) reported increased CCR5 and CCR2 expression in AD patients compared to healthy controls, suggesting that these chemokine receptors may be involved in neurodegeneration (Bajetto *et al.*, 2001). Cancer cells produce chemokines responsible for leukocyte infiltration (Balkwill, 2003; Balkwill, 2004). In a study by Lavergne and co-workers (2004), the authors induced CCL5 expression at a mouse tumour site. Mice with increased CCL5 expression presented reduced tumour growth due to increased leukocyte infiltration compared to mice with regular CCL5 expression. The authors reported that the anti-tumour response was absent in CCR5-knockout mice (Lavergne *et al.*, 2004). With regards to infectious diseases, such as hepatitis B virus (HBV) or West Nile virus (WNV) infection, an effective immune response requires the participation of CCR5 (Glass *et al.*, 2005). Rahbar and co-authors (2009) showed that immune cells are dependent on the presence of CCR5 to be recruited at the HBV-infected site in the liver (Rahbar *et al.*, 2009). Christmann *et al.* (2011) showed that when CCR5 is present, the encephalomyocarditis virus influences the expression of inflammatory genes and macrophages and activates various signalling pathways. An example of this is mitogen-activated protein (MAP) kinase (MAPK), nuclear factor-kappa B (NF- κ B), inflammatory gene-inducible nitric oxide synthase (iNOS), and cyclooxygenase 2 (COX-2). In addition, cytokines such as IL-1 β and TNF were synthesised (Christmann *et al.*, 2011). Other signalling pathways affected by CCR5 include phosphoinositide-3 kinase (PI3K), protein kinase C (PKC), and MAPK (Corbisier *et al.*, 2015; Mueller *et al.*, 2006). The signalling cascade initiated is dependent on the particular ligand that binds to CCR5. For example, the CCR5/ MIP-1 α signalling cascade triggers the PI3K/Akt and MAPK/ extracellular signal-regulated kinase (ERK) pathways to enhance cell migration (Kodama *et al.*, 2020), while the CCR5/CCL5 signalling cascade triggers the PI3K/Akt pathway to form the central signalling pathway in cancer cells (Kranjc *et al.*, 2019). Furthermore, CCR5 acts as an insulin signalling regulator in the hypothalamus and blocking CCR5 results in better insulin responsiveness by impairing glucose metabolism (Chou *et al.*, 2016). Regarding melanoma, Weber and co-workers (2020) reported that IL-6 upregulates CCR5 and arginase I, an enzyme involved in the urea cycle, in a STAT3-dependent manner. In myeloid-derived suppressor cells (MDSC), IL-6 upregulated CCR5 expression and increased CD8 T cell activation (Weber *et al.*, 2020). The studies mentioned above highlight various immune molecules and/or pathways in which the degree of activity might be affected by surface CCR5 levels.

More relevant to recent times, a worldwide pandemic of the novel coronavirus took place in November 2019, termed coronavirus disease 2019 (COVID-19). COVID-19 is an infectious disease caused by the SARS-CoV-2 virus and is currently the leading cause of morbidity and mortality since the Spanish Flu in 1918 (Lodise & Rybak, 2020). Acute respiratory distress syndrome (ARDS) attributes somewhat to the high mortality rate, although the leading cause of

ARDS is immune hyperactivation, also known as a cytokine storm (Tisoncik *et al.*, 2012). Autopsies done on deceased COVID-19 patients revealed increased inflammatory cytokines, such as IL-6 and CCL5, leading to multi-system organ failure (Harrison, 2020).

In a study performed by Cuesta-Llavona *et al.* (2021), healthy controls presented with decreased CCR5-transcript expression compared to severe COVID-19 positive patients. Although not statistically significant, the authors reported that non-intensive care patients had lower CCR5-transcript expression than intensive care patients. In addition, the authors found that 1% of the healthy controls presented with the homozygous CCR5 Δ 32 genotype, which was absent in patients (Cuesta-Llavona *et al.*, 2021). Therefore, several researchers investigated CCR5 as a potential target for therapy (Agresti *et al.*, 2021; Cuesta-Llavona *et al.*, 2021; Patterson *et al.*, 2021; Patterson *et al.*, 2020). One therapeutic strategy included the use of Leronlimab, a CCR5 antagonist usually used for the treatment of HIV infection, to block the binding of CCL5 to CCR5 (Clinicalinfo.hiv.gov – Drug Database: Leronlimab) - decreasing the release of downstream pro-inflammatory cytokines (Jacobson *et al.*, 2010; Scurci *et al.*, 2018).

In a study performed by Patterson and co-authors (2020), ten critical COVID-19 positive patients presented with increased plasma IL-6 and CCL5 and decreased CD8 T cell levels. The use of Leronlimab restored the CD4/CD8 T cell ratio, reduced plasma IL-6 levels, and showed overall reduced plasma SARS-CoV-2 viral load (Patterson *et al.*, 2020). Similarly, in a case study by Agresti and co-workers (2021), the researchers showed that the use of Leronlimab (to block CCR5) resulted in better clinical outcomes in four critically ill COVID-19 positive patients that presented with ARDS (Agresti *et al.*, 2021). CCR5 plays a critical role during COVID-19 infection due to its role in the immune system. A recent review by Batabyal and co-workers (2021) discusses therapeutics targeting immune and metabolic pathways in COVID-19 patients with an underlying metabolic condition. These treatment options might be effective due to the hyperinflammatory states measured in COVID-19 patients (Batabyal *et al.*, 2021).

While various studies have investigated the influence of CCR5 on cytokine profiles, several cytokines such as IFN- γ (Hariharan *et al.*, 1999; Zella *et al.*, 1998), TNF- α (Marsden *et al.*, 2015), IL-6 (Weber *et al.*, 2020), IL-2 (Yang *et al.*, 2001) and IL-10 (Sozzani *et al.*, 1998) can also modulate CCR5 expression.

Table 2-2: Table shows representative literature and how CCR5 levels relate to various immune changes.

CCR5 levels	Disease	Immune effect	Reference
High	Yes	↑ inflammation	(Bajetto <i>et al.</i> , 2001)
High	Yes	↑ Leukocyte infiltration ↓ Tumour growth	(Lavergne <i>et al.</i> , 2004)
High	Yes	↑ Expression of inflammatory genes and macrophages ↑ IL-1β ↑ TNF-α	(Christmann <i>et al.</i> , 2011)
High	Yes	↑ IL-6	(Patterson <i>et al.</i> , 2020)

CCR5 levels ↑ - Increased; ↓ - Decreased. Abbreviations: IL- Interleukin; TNF-α – Tumour necrosis factor alpha

Altogether, the literature mainly shows increased CCR5 levels to be associated with a Th1 immune profile. High levels of CCR5 are thus particularly associated with inflammation, increased inflammatory cytokines and their associated pathways, chemotaxis, viral clearance, and tumour growth inhibition. Low CCR5 levels are mainly associated with regulation and a Th2 immune profile which can suppress Th1 responses and the subsequent susceptibility of cells to infection.

2.6 Role of CCR5 in metabolism

CCR5 also affects metabolism, which represents all the chemical processes within a living organism. Metabolism divides into two subsections, namely anabolism and catabolism. Anabolism includes the chemical processes that synthesise molecules needed, such as proteins, whereas catabolism includes the chemical processes that break down molecules for energy production, creating metabolic pathways (Chandel, 2021). The molecules involved in these reactions, also known as metabolites, are usually found in low concentrations and can inform what occurs in the body - the metabolism is thus the closest representation of a system's phenotype (Fiehn, 2002).

The role of CCR5 in metabolism was studied in several diseased states and/or models. An in-depth discussion of the specific metabolic changes induced during each of these diseased states is beyond the scope of this dissertation. Instead, representative studies are highlighted to show metabolic changes associated/correlated with CCR5 levels. Hyde and co-workers (2010) investigated possible associations between the homozygous CCR5 Δ 32 genotype (basal/absent CCR5 levels) and lipid levels in the context of cardiovascular disease. The authors reported low levels of CCR5 to be associated with increased high-density lipoprotein cholesterol (HDL-C) and decreased triglycerides (TG) levels. The function of HDL-C is to assist with the removal of low-density lipoprotein cholesterol (LDL-C) present in the arteries. The authors concluded that CCR5 Δ 32 offers protection against coronary artery disease (Hyde *et al.*, 2010). Bonfá *et al.*'s (2014) study on *Toxoplasma gondii* infection showed deficient CCR5 to be associated with low levels of inflammatory cytokines and elevated triglycerides, reduced cell recruitment and inflammation (Bonfá *et al.*, 2014). In a study performed by Bing and co-workers, a rat model was used to determine the role of CCR5 in diabetes mellitus type 2. In addition, an antibody blocked CCR5 (limited CCR5), and the authors measured metabolic changes. In this study, blocking CCR5 improved the course of diabetes by decreasing low-density lipoprotein (LDL) and TG levels and increasing high density lipoprotein (HDL) levels (Bing *et al.*, 2018).

Kim and co-workers (2018) determined that, in the presence of CCR5, CCL5 decreased TG levels and decreased enzyme expression, including fatty acid synthetase, acetyl-Coenzyme A (CoA) carboxylase, and stearyl-CoA desaturase. The authors reversed this effect by deleting the CCR5 gene and concluded that CCL5-CCR5 binding reduced TG synthesis (Kim *et al.*, 2018). Regarding cholesterol and CCR5, recent evidence showed that the presence of cholesterol in the vicinity of CCR5 affected the binding affinity of maraviroc, a CCR5 antagonist, suggesting that cholesterol might influence the conformational state of the receptor (Calmet *et al.*, 2020). In cancer cells, Chan *et al.* (2012) showed that CCL5 activates CCR5, influencing glycolysis and ATP production (Chan *et al.*, 2012). CCR5 activation results in elevated expression of glucose transporters which finally leads to increased glycolysis. Cancer cells treated with CCL5 presented with elevated anabolic pathways, resulting in high ATP production (Gao *et al.*, 2016).

From a metabolic perspective, high levels of CCR5 appear to enhance metabolic processes which favour the growth of cancer cells and the pathogenesis of infectious and acquired diseases. These processes include increased glycolysis, ATP production, anabolic reactions, and lipid metabolism. At the same time, low levels of CCR5 and/or blocking of CCR5 function improve disease profiles through the following metabolic processes and/or alterations, i.e., increased HDL-C and HDL and decreased glycolysis. Table 2-3 summarises representative studies which show an association between CCR5 levels and various metabolic pathways. Additionally, of the

numerous studies which report on the role of CCR5 during metabolism, conventional indicators such as spectrometric HDL and LDL were mainly used (Lohmann *et al.*, 2002, Bing *et al.*, 2018).

Table 2-3: Table shows representative literature and how CCR5 levels relate to various metabolic changes.

CCR5 levels	Disease (Y/N)	Metabolic effect	Reference
Low	Yes	↑ HDL-C	(Hyde <i>et al.</i> , 2010)
		↓ TG	
Low	Yes	↑ TG	(Bonfá <i>et al.</i> , 2014)
Low	Yes	↓ LDL	(Bing <i>et al.</i> , 2018)
		↑ HDL	
		↓ TG	
High	No	↓ TG	(Kim <i>et al.</i> , 2018)
		↓ fatty acid synthetase expression	
		↓ acetyl CoA carboxylase expression	
		↓ stearoyl-CoA desaturase expression	
High	Yes	↑ glycolysis	(Chan <i>et al.</i> , 2012)
		↑ ATP production	
High	Yes	↑ anabolic pathways	(Gao <i>et al.</i> , 2016)
		↑ ATP	

Metabolic effect: ↑ - Increased; ↓ - Decreased. Abbreviations: HDL-C – High-density lipoprotein-cholesterol; TG – Triglycerol; LDL – Low-density lipoprotein; ATP – Adenosine triphosphate.

2.7 Role of CCR5 in immuno-metabolism

The relationship between immunology and metabolism has led to the term immuno-metabolism – this explains the intracellular metabolic pathways present in immune cells (Murray *et al.*, 2015), the involvement of inflammation in the pathogenesis of several metabolic disorders, and different metabolic factors that influence the regulation of several other immune cell functions (Raghuraman *et al.*, 2016). In addition, a review of the main metabolic pathways involved in immune cells is available (O'Neill *et al.*, 2016).

Cells in the quiescent state, a reversible state where the cell does not actively divide but can re-enter the cell proliferation cycle, mainly utilise catabolic reactions favoured by mitochondria, e.g., oxidative phosphorylation (OXPHOS). Activated cells, such as those expressing high CCR5 levels, exit the quiescent state driven by the breakdown of various nutrients to generate sufficient energy needed for cellular growth; the main metabolic differences induced by activation include elevated glycolysis and glutaminolysis, and upregulation of mitochondrial functions and biogenesis (Chapman *et al.*, 2020). While activated, effector cells switch to aerobic glycolysis for energy, inactive naive T cells depend on glucose and fatty acid metabolism for energy (Newton *et al.*, 2016). The expansion of T cells depends on the non-essential amino acid serine (Ma *et al.*, 2017). Memory T cells require fatty acids to produce an immune response (Pan *et al.*, 2017). In a recent review, Saravia *et al.* (2020) discussed cellular signalling pathways involved in the immuno-metabolic regulation of T cells. They showed that several signalling pathways could alter the metabolism in T cells (Saravia *et al.*, 2020).

Chan *et al.* (2012) showed that CCL5-CCR5 interactions modulate messenger RNA (mRNA) translation by activating the mammalian target of rapamycin (mTOR) / Eukaryotic translation initiation factor 4E (eIF4E) -binding protein 1 (4E-BP1) pathway - mTOR is a central nutrient sensing and glycolysis regulator. Activating the mTOR pathway contributes to chemotaxis by synthesising chemotaxis-related proteins. CCL5 mediates glucose uptake in an mTOR-dependent manner and ATP production, and CCL5 treatment of CD3⁺ T cells *ex vivo* induced activation of AMP-activated protein kinase (AMPK) and downstream substrates Acetyl-CoA carboxylase I (ACC-1), 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 2 (PFKFB-2) enzyme, and Glycogen synthase kinase-3 β (GSK-3 β). By inhibiting glucose uptake and AMPK, the data revealed that CCL5-mediated T cell chemotaxis is dependent on glucose (Chan *et al.*, 2012).

Bonfa and co-workers (2014) used CCR5 wild-type and CCR5-deficient mice and inoculated the mice with a lethal dose of the *Toxoplasma gondii* parasite, causing toxoplasmosis and reported immuno-metabolic changes. They showed that the CCR5-deficient mice presented with decreased levels of several inflammation-related cytokines and transcriptional factors during disease, elevated serum triglycerides, and finally, liver metabolic dysfunction (Bonfá *et al.*, 2014). In the study conducted by Bing and co-authors (2018), a rat model was used to investigate the immune and metabolic profiles of mice suffering from diabetes type 2. The authors reported on the affected metabolic (HDL which was initially significant lower in diabetic rats compared to healthy controls, and LDL and TG which was significantly increased in diabetic rats compared to healthy controls) and immune (IL-6 and TNF- α which was higher in diabetic rats compared to controls, and IL-10 which was lower in diabetic rats compared to healthy controls) profiles before treatment with the CCR5 antibody, which was reversed upon blocking the CCR5 protein with an

antibody. In addition, the authors investigated the expression of proteins related to the PI3K/Akt/mTOR pathway. This rat model reported that blocking CCR5 ultimately improved the course of diabetes type 2 influenced by the PI3K/Akt/mTOR pathway, since the pathway was not activated upon treatment with the antibody (Bing *et al.*, 2018). To investigate the protective role of CCR5 with context to HIV infection, Jaumdally and colleagues (2017) investigated inflammation and T cell activation in the plasma of HIV-negative participants that were unexposed and exposed to HIV, respectively. The authors found that uninfected exposed individuals had lower frequencies of CCR5-positive immune cells (protective against infection) than their uninfected unexposed counterparts. The uninfected exposed individuals thus over-expressed protective CCR5 haplotypes and presented with reduced IFN- γ , IL-2, and granulocyte-macrophage colony-stimulating factor (GM-CSF) levels (reduced Th1 response as documented for models with low CCR5 levels). While inflammatory cytokine concentrations did not differ among their groups, the authors showed that lower CCR5 expression and plasma cytokine concentrations were the dominant signatures of HIV infection resistance (Jaumdally *et al.*, 2017). This data provides crucial biological information regarding samples presenting differential CCR5 expression even before actual infection. The abovementioned study is one of few papers which investigated the role of CCR5 in a non-diseased context.

2.8 CCR5 mutations and/or CCR5 levels and their association with diseases

An extensive review regarding the contrasting roles of CCR5 in the presence and absence of disease was published (Rautenbach & Williams, 2020). Overall, increased CCR5 (wildtype CCR5) was associated with protective functions in the context HIV infection (Balotta *et al.*, 1997; Biti *et al.*, 1997; Heiken *et al.*, 1999; Michael *et al.*, 1998; Rana *et al.*, 1997; Sheppard *et al.*, 2002; Theodorou & Meyer, 1997), West Nile virus infection, (Glass *et al.*, 2005; Glass *et al.*, 2006; Lim *et al.*, 2008), influenza A (H1N1) (Falcon *et al.*, 2015; Keynan *et al.*, 2010; Rodriguez *et al.*, 2013), Hepatitis B infection (Ahmadabadi *et al.*, 2013; Arababadi *et al.*, 2010a; Arababadi *et al.*, 2010b; TrehanPati *et al.*, 2009), liver inflammation and liver failure due to Hepatitis B infection (Ajuebor *et al.*, 2006; Moreno *et al.*, 2005; Stevens *et al.*, 2019), HIV/HBV co-infection (Ellwanger *et al.*, 2020), cancer (due to improved DNA repair pathways (Jiao *et al.*, 2021)), cardiovascular disease and resulting conditions (Ghilardi *et al.*, 2008; Karaali *et al.*, 2010; Mettimano *et al.*, 2003; Pai *et al.*, 2006; Sharda *et al.*, 2008; Zhang *et al.*, 2015) and multiple sclerosis (Kantor *et al.*, 2003; van Veen *et al.*, 2007). Increased CCR5 levels showed non-protective roles in gastric cancer prognosis (Sugasawa *et al.*, 2008) and metastasis (Cao *et al.*, 2011).

Low levels of CCR5 was shown to have protective roles in context to HIV infection (Dean *et al.*, 1996; Jaumdally *et al.*, 2017; Marmor *et al.*, 2006; Rana *et al.*, 1997; Samson *et al.*, 1996b) (Liu

et al., 1996; Marmor *et al.*, 2006), Hepatitis B (Abdolmohammadi *et al.*, 2016) and recovery of Hepatitis B (Thio *et al.*, 2008), clinical outcome of patients with Hepatitis C (Hellier *et al.*, 2003), cardiovascular disease and resulting conditions diseases (Afzal *et al.*, 2008; Balistreri *et al.*, 2008; Gonzalez *et al.*, 2001; Hyde *et al.*, 2010; Lassner *et al.*, 2014; Szalai *et al.*, 2001), asthma (Hall *et al.*, 1999; Srivastava *et al.*, 2003), diabetes mellitus type 1 (Song *et al.*, 2014); diabetes mellitus type 2 (Bing *et al.*, 2018; Muntinghe *et al.*, 2009); diabetes mellitus type 1 and type 2 clinical course (Bing *et al.*, 2018; Kalev *et al.*, 2003); rheumatoid arthritis clinical course (Garred *et al.*, 1998; Gómez-Reino *et al.*, 1999); rheumatoid arthritis susceptibility (Pokorny *et al.*, 2005; Rossol *et al.*, 2009) and systemic lupus erythematosus susceptibility (Carvalho *et al.*, 2014; Schauren *et al.*, 2013). Low CCR5 levels showed non-protective roles in asthma (Abousaidi *et al.*, 2011; Gomulska *et al.*, 2014; Mitchell *et al.*, 2000); systemic lupus erythematosus susceptibility (Baltus *et al.*, 2016); and development of lupus nephritis in systemic lupus erythematosus patients (Schauren *et al.*, 2013), multiple sclerosis survival rate, especially in female patients (Gade-Andavolu *et al.*, 2004), and multiple sclerosis disease clinical course (Pulkkinen *et al.*, 2004).

There were also instances where CCR5 levels were not associated with disease, i.e. Chagas disease (Fernandez-Mestre *et al.*, 2004), influenza A (Maestri *et al.*, 2015; Matos *et al.*, 2019; Sironi *et al.*, 2014), Hepatitis B (Ellwanger *et al.*, 2020), Hepatitis C susceptibility or clinical course (Goulding *et al.*, 2005; Promrat *et al.*, 2003; Ruiz-Ferrer *et al.*, 2004), cardiovascular disease, and susceptibility to resulting conditions (Apostolakis *et al.*, 2007; Simeoni *et al.*, 2004; Wang *et al.*, 2013) (Zhang *et al.*, 2006), asthma (Song *et al.*, 2014); diabetes type 1 (Gambelunghe *et al.*, 2003; Szalai *et al.*, 1999; Yang *et al.*, 2004); rheumatoid arthritis (Kohem *et al.*, 2007; Lindner *et al.*, 2007); systemic lupus erythematosus (Gómez-Reino *et al.*, 1999); or development of lupus nephritis in systemic lupus erythematosus patients (Zhou *et al.*, 2014), Alzheimer's dementia (Balistreri *et al.*, 2006; Combarros *et al.*, 2004) or Parkinson's disease (Huerta *et al.*, 2004), multiple sclerosis (Bennetts *et al.*, 1997; Kaimen-Maciel *et al.*, 2007; Silversides *et al.*, 2004), COVID-19 prevalence or mortality or severity (Bernas *et al.*, 2021; Cantalupo *et al.*, 2021; Čizmarević *et al.*, 2020).

Several factors account for these discrepancies, and some examples include the cohort's ethnicity, the type of model used for each study (e.g., blood, urine, cell lines, etc.), and the difference in assays performed. Given the role of CCR5 in immune function, subsequent changes should be measurable at the metabolic level. Although literature highlighting the role of CCR5 in immune system function and metabolism is available, the latter is very limited and was mainly recorded using older conventional systems. Furthermore, there is minimal evidence about CCR5's role in the absence of an enhanced inflammatory state, such as when an infection persists - an aspect addressed in this study (see also Table 2-2). The aim is to characterise the immuno-metabolic profile of uninfected cells presenting with distinct levels of CCR5 by employing

flow cytometry and mass spectrometry (MS). The literature shows that CCR5 mutations have a low prevalence, leading to a scarcity of clinical samples, making it challenging to study these mutations and their role on CCR5 levels and associated biological processes in depth. More research regarding the role of CCR5 is needed, best studied at this point using cell lines as models and employing advanced multiparameter analytical instrumentation, such as flow cytometry and MS.

2.9 Flow Cytometry as a tool to profile cells presenting differential CCR5 phenotypes

Researchers use various methods to investigate the immune system and its associated processes. Examples include the enzyme-linked immunosorbent assay (ELISA) (Wild, 2013), mass cytometry (Ornatsky *et al.*, 2008), and flow cytometry (Bendall *et al.*, 2012).

The flow cytometer was used to determine cell viability, quantify CCR5 receptor levels, and investigate the Affinofile/HEK293 and Cf2Th cells' immune profiles due to the sensitivity and time-efficiency of the machine while collecting quantifiable data on multiple aspects. Before an investigation, researchers often consider the advantages and disadvantages of the methods at their disposal. Flow cytometry is a commonly employed technique for immune-based analysis. This technique characterises particles, often cells, on a chemical and physical level by non-utilisation or utilising a staining approach using fluorochromes. Fluorochromes are photoreactive molecules that absorb energy in the form of light to manifest as a molecule with a higher energy state. They can also emit light of a specific wavelength when transitioning to a less-excited state – commonly known as fluorescence (Shapiro, 2005). The fluorescent signals are directed towards their respective detectors by various mirrors and beam splitters in the system (Adan *et al.*, 2017). Fluorochromes can bind to a molecule of interest (such as a cell surface receptor) to provide information on additional parameters other than cell size and complexity. The quantification of fluorescence is directly related to the number of fluorochromes moving to a less energetic state, i.e., undergoing fluorescence. The use of more than one fluorochrome with different wavelength specifics allows the researcher to investigate more than one cell characteristic, contributing to the time-efficient aspect of flow cytometry. When considering cells specifically, fluorochromes can be conjugated to an antibody of interest on the cell surface for a variety of reasons (e.g. to identify specific cell types and quantify proteins on the surface of cells), allowing the researcher to identify several subpopulations within a sample (Berny-Lang *et al.*, 2013). Flow cytometry can also sort cells based on preselected characteristics and inform on cell viability.

A typical flow cytometer consists of three components, namely, the fluidics, optics, and electronics systems. The basic functioning of a flow cytometer is shown in Figure 2-3. Briefly, the fluidics

system is responsible for focusing a sample into a stream of single cells. Sheath fluid surrounds the cells to transport the cells in a capillary (hydrodynamic focusing). Changing the sample core size adjusts the sample flow rate, affecting the number of cells entering the stream simultaneously. Since an increase in the sample flow rate results in more cells in the sample core, the cells are less in line when the laser intercepts the cells, resulting in less resolved data. A reduced flow rate limits this occurrence, yielding data with improved resolution. The optics system is responsible for the light aspects of the flow cytometer, such as the laser light source and detection of light emissions of the fluorochromes. Optical filters are required to direct the fluorescence signals to photomultiplier tubes (PMTs). Because the PMTs detect weak fluorescence signals, bandpass filters are placed before the PMTs to ensure that a small range of wavelengths can reach the detectors (Sanders & Mourant, 2013). Based on the physical properties of the cell, a light scattering pattern can be observed and measured by the various optical filters and detectors that form part of the optical system. Although the cell shape and surface structure can influence the overall light scatter pattern, the cell size determines the forward scatter (FSC) pattern. In contrast, cell complexity is informed by the side scatter pattern (SSC) (Figure 2-3). Fluorochromes (if any) absorb and emit fluorescent light. The electronics system is responsible for converting any detected signal to a digital signal and performing data analysis. In addition, the electronic system allows the researcher to set thresholds to limit the number of events collected during flow cytometric analysis. The thresholds are often helpful in filtering out components such as cell debris. Setting the threshold value allows the flow cytometer to collect all events but only process signals with an intensity greater than the threshold value. While acquiring events on the flow cytometer, a gate can define a subset of data by using known characteristics of the analysed population. One such example is cell debris (if not filtered out using thresholds) which are small fragments that result in small FSC patterns that are excluded from other data plots by gating on populations with higher FSC patterns (Mandy *et al.*, 1995). In addition, the researcher can obtain additional data such as the number of acquired events and mean fluorescent intensity (MFI) during gated and ungated data analysis. The data collected is stored as flow cytometry standard (*.fcs) files.

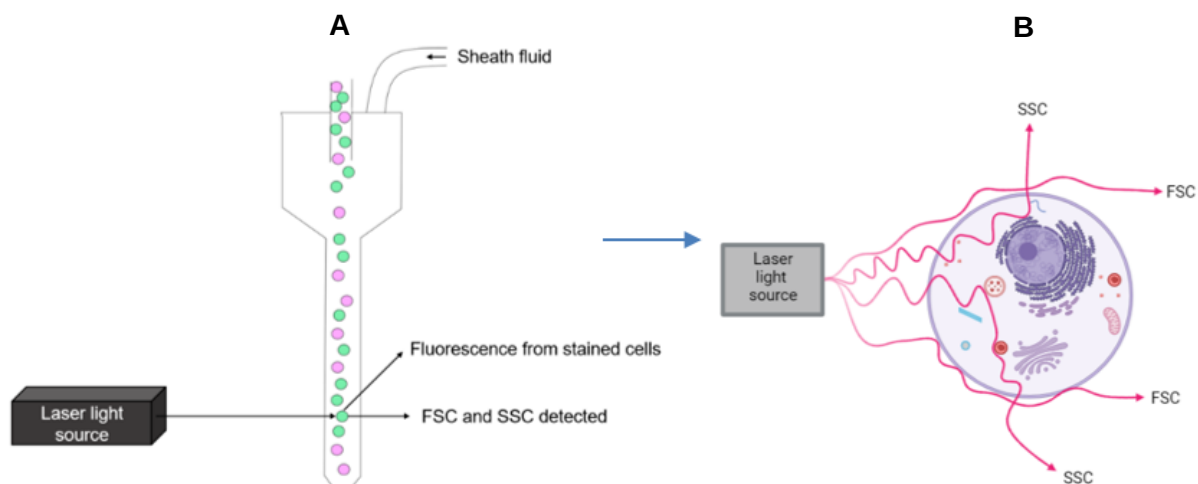


Figure 2-3: Figure illustrating the basic principle of flow cytometry. (A) cells are focused into a single stream to pass the laser light source of the flow cytometer while (B) cell size, measured as forward scatter (FSC), and complexity, measured as side scatter (SSC), can be determined using light scatter patterns produced by the flow cytometer. The FSC and SSC patterns produced by the fluorescent laser light are detected by the detector.

2.10 Metabolomics as a tool to profile cells presenting differential CCR5 phenotypes

Since metabolism represents the final downstream product of the central dogma, it provides a clear indication of what is happening within a living organism—metabolite changes within the living organism link to the system's phenotype. Metabolites are small molecules that participate in metabolic reactions and transform during metabolic processes. Metabolomics entails the study of metabolites (Ramsden, 2015), allowing for the detection, identification and quantification of metabolites in an unbiased manner.

Several metabolomics approaches can be followed depending on the aim of the study. Studying specific metabolites or compounds of interest, often compared to previously established reference ranges, requires a targeted approach. In contrast, a semi-targeted approach allows the study of particular metabolites linked to various metabolic pathways of interest or a specific class of compounds. An untargeted approach enables the analysis of samples without previous knowledge of the metabolites or compounds present in the sample (Esterhuizen *et al.*, 2017; Patti *et al.*, 2012). An untargeted approach allows the researcher to determine the perturbed metabolic profiles of two or more groups (Dudzik *et al.*, 2018). The advantages and disadvantages of untargeted metabolomics have been reviewed (Gertsman & Barshop, 2018). This study followed an untargeted metabolomics approach to identify metabolic changes associated with differential CCR5 levels.

Any of the previously mentioned metabolomics approaches allows the researcher to discover new insights regarding the affected metabolism in any given circumstance. The process uses numerous sensitive and selective analytical techniques (Dunn *et al.*, 2005). While different analytical platforms exist, no one analytical platform can detect and measure all the metabolites present within a sample due to the diversity of the molecules and the different concentrations in which they are present. An analytical platform is selected based on several factors, such as sample type and the biological/research question. Some of the main techniques employed include MS and nuclear magnetic resonance (NMR) spectroscopy, each with advantages and disadvantages. For this project's scope, the focus was directed to MS (for reasons described further in this section).

MS relies on the ionisation of molecules to produce fragments of which the mass can be analysed by the mass analyser and detected by the detector. The result is a mass spectrum showing each compound's mass-to-charge (m/z) ratio. Every fragment is unique to a specific molecule, and the mass analyser sorts the fragments according to m/z ratios. Ionisation converts the molecules of interest in the sample to gas-phase ions, and the ionisation techniques utilised are dependent on the chemical properties of the molecules of interest (Bouchonnet, 2013). For example, thermally stable molecules with low molecular weight are commonly subjected to electrospray ionisation.

In contrast, electron impact (EI) ionisation utilises electrons to bombard the analytes to break into their respective ionised fragments. The mass analyser is responsible for analysing the ion products from the ionisation chamber. Various mass analysers are available, each offering advantages and disadvantages (Awad *et al.*, 2015). An example of a mass analyser is the time-of-flight (TOF) analyser. Time-of-flight MS (TOFMS) determines the m/z ratio by accelerating the ions through a vacuum (flight) tube using an electric field. Since all the molecules have the same acceleration, the velocity of each molecule is dependent on the m/z ratio; smaller molecules reach a higher speed in a shorter time. These molecules thus reach the detector at a different time based on the size of the molecule, which is recorded (Glish & Vachet, 2003). The detector translates this information to a mass spectrum to give quantitative data, which can help elucidate structural properties. Most of the analytical instruments connect to a computer that records spectra, allowing the researcher to observe the abundance of each ionised mass fragment. A brief illustration of the principles of gas chromatography (GC) coupled to MS is shown in Figure 2-4.

The MS-acquired data may be subject to matrix effects; a matrix effect is when analytes are co-extracted with the sample matrix, which alters the quality of the data (Sánchez-Brunete *et al.*, 2005). Mass spectrometers can also be insensitive to some classes of analytes requiring modification of analytes for compatibility, i.e., derivatisation. Furthermore, some analytical platforms destroy the sample, making further analysis on a different platform impossible. In

contrast, the advantages of MS include the use of low sample volumes since acquiring substantial amounts of clinically relevant samples can be difficult. MS can also be coupled to separation techniques such as chromatography, enhancing the system's sensitivity, selectivity, and resolution (Sparkman *et al.*, 2011). Chromatography can separate a mixture of molecules into their components (Glish & Vachet, 2003). Therefore, a mass spectrometer can be extremely useful for detecting and identifying compounds. In addition, the combination of chromatography with MS makes it possible to analyse very complex samples.

The use of GC is limited to volatile yet thermally stable analytes. Analytes are separated based on volatility, while the MS identifies the molecule based on mass and charge ratios (Hübschmann, 2015). The gas chromatograph has four main components, namely (1) the sample inlet point, (2) a column situated in an oven that can be programmed for different temperatures, (3) the carrier gas supply as well as control, and (4) the detector. After sample injection, a carrier gas transports analytes to the column. The carrier gas is an inert gas, hydrogen, helium, or nitrogen. The sample interacts with the stationary phase in the column, resulting in molecules eluting at different times. In theory, a higher temperature of the GC leads to less interaction with the stationary phase, resulting in faster elution times of the more volatile molecules.

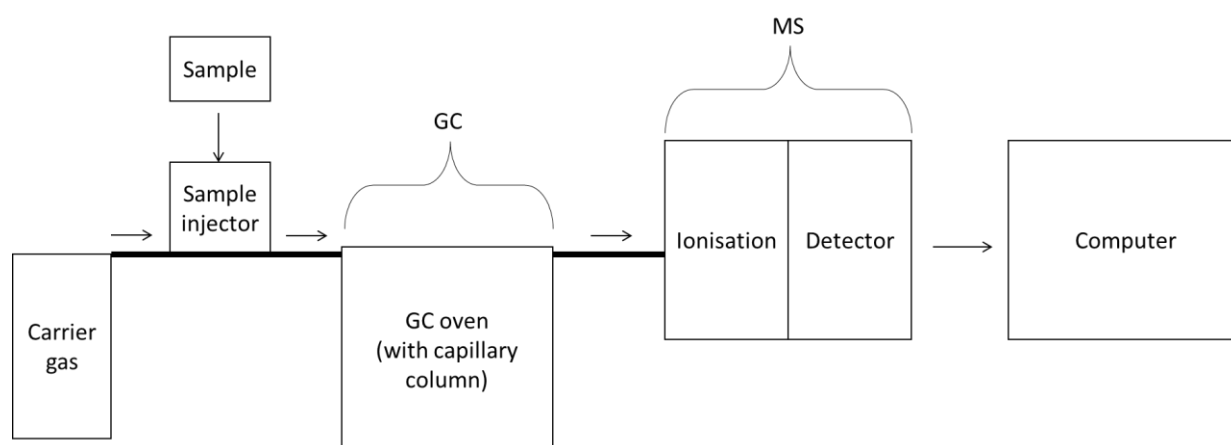


Figure 2-4: The basic principles of GC coupled to MS.

A carrier gas (such as helium) transports the analytes. The sample is injected into the sample injector, where it is transferred to the GC oven in a capillary column. The analytes are separated based on volatility. The analytes elute from the column and enter the MS's ionisation compartment, where the analyte is fragmented. The fragments are then accelerated through the mass spectrometer and reach the detector. A computer connected to the analytical platform translates the data, resulting in mass spectra for each compound.

GC is compatible with a wide variety of other analytical instruments. Technological advancements have made it possible to add another interface to the abovementioned system to extract more information from every sample. An additional GC column before TOFMS analysis makes it

possible to separate co-eluting compounds based on another physical property, such as polarity. Therefore, it is possible to separate and detect more compounds/metabolites using this system (Marriott & Shellie, 2002). For this project, a two-dimensional GCxGC-TOFMS system in an untargeted manner was used to comprehensively characterise cells presenting differential CCR5 phenotypes.

2.10.1 Samples typically used for metabolomics

The biological question and the availability of samples are crucial factors to consider when choosing the type of analytical instrumentation to use. Researchers primarily select samples according to ease of access. Biofluids that can be obtained non-invasively, such as urine, are typically the first choice to use (Chetwynd *et al.*, 2017). Blood is also a popular choice since its collection is minimally invasive and homeostatic (Wishart, 2007). On the other hand, muscle or tissue biopsies are hard to come by, making them less popular for research purposes (Álvarez-Sánchez *et al.*, 2010).

In contrast to clinical samples, which often have cumbersome/lengthy ethical timeframes, researchers can opt to use primary cell cultures or established cell lines for analysis for reasons highlighted in Section 3.1. Before metabolic analysis, sample preparation is needed to make it more compatible with a specific analytical platform. Depending on the analytical platform, sample preparation will vary, although the typical workflow is described in Section 2.10.2. Metabolomics analysis permits the discovery of essential biomarkers and provides valuable biological information. Given that access to clinical samples with CCR5 mutations and varying levels of CCR5 is limited, cell lines were utilised for this project.

2.10.2 Typical MS metabolomics workflow

Upon sample collection from a living organism, metabolic activity continues due to some enzymes present in the sample. Quenching the metabolism of the sample halts the metabolic activity and therefore preserves the sample's current biological state (Winder *et al.*, 2008). Next, the sample is spiked with an internal standard (IS) representing a known amount of a specific compound similar to the analyte of interest. Comparing the analyte signal with that of the IS allows the researcher to quantify the unknown compounds in the sample. An IS offers several advantages, such as improved accuracy and precision, and that one can account for signal suppression or enhancement caused by a complex sample matrix. These can therefore be used to normalise the metabolomics dataset. Normalisation aims to remove variation in the data. In addition to IS, several other approaches exist to normalise metabolic data. Examples include optical density, DNA concentration, and the Mass Spectrometry Total Useful Signal (MSTUS) (Misra, 2020). Spiking with an IS of choice is followed by extraction of the molecules of interest. The extract is

then dried under an inert gas (such as nitrogen) to remove excess water molecules and to limit oxidation in the sample. Derivatisation depends on the analytical platform, or the sample is resuspended in an appropriate solvent/matrix. Derivatisation produces a derivative of a specific compound by using several chemical reactions, making the extract more suitable for a particular analytical platform (Fiehn, 2016). The sample can then be analysed. The sample yields a complex data matrix that requires additional data processing. The computer linked to the analytical platform is equipped with software that processes the data and includes deconvolution, peak detection, alignment, and data standardisation. Various statistical approaches allow the researcher to extract useful biological information from the standardised data matrix. Figure 2-5 below shows a basic metabolomics sample analysis workflow.

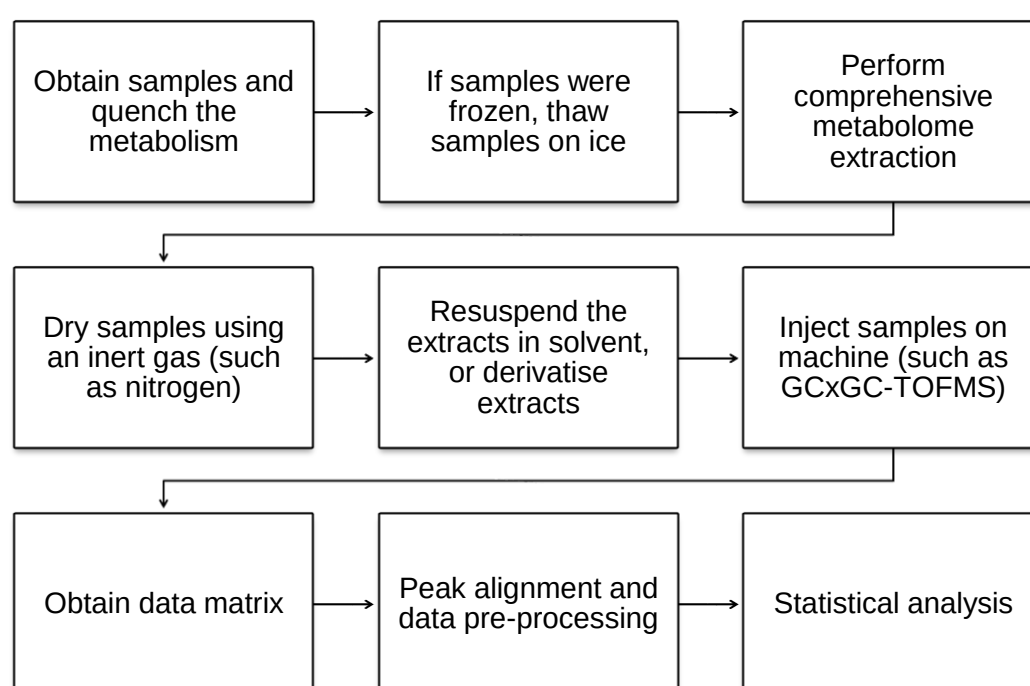


Figure 2-5: Figure showing general workflow for MS-based metabolomics analysis.

2.11 The significance that the improved characterisation of CCR5 holds

This study represents one of the first to report on an uninfected model's CCR5-influenced immune and metabolic changes. With regards to CCR5, previous studies highlighted the distribution frequency of CCR5 Δ 32 in various populations (Carvalho *et al.*, 2004; Martinson *et al.*, 1997; Nkenfou *et al.*, 2013; Ryabov *et al.*, 2004; Vargas *et al.*, 2006), reported on immune data (Jaumdally *et al.*, 2017; Kazemi Arababadi *et al.*, 2009) and/or metabolic data linked to infection or disease (Bing *et al.*, 2018; Hyde *et al.*, 2010). However, the most studied diseases related to

CCR5 were infectious diseases such as HIV/AIDS. Regarding HIV, the virus utilises CCR5 as a co-receptor to enter the cell. Previous studies reported that the homozygous CCR5 Δ 32 genotype protects against HIV infection due to the low/no CCR5 levels present on the cell surface. In contrast, the heterozygous CCR5 Δ 32 genotype offers partial protection against HIV infection due to the low levels of CCR5 and thus results in reduced susceptibility to infection and slower disease progression.

To date, medical personnel successfully cured three patients of HIV infection. The first is known as the “Berlin patient”, the second is the “London patient”; both patients received a transplant of stem cells carrying the homozygous CCR5 Δ 32 genotype (Ding *et al.*, 2021). After the stem cell transplant, both patients presented with virtually absent HIV viral loads (Hübschmann, 2015). The third patient is a woman known as the “New York Patient”. Doctors performed a haplo-cord transplant, i.e. a combination of umbilical cord blood carrying the homozygous CCR5 Δ 32 mutation and partially matched donor stem cells from an adult carrying the wildtype CCR5 genotype (World Health Organization, 2022). To date, medical personnel performed numerous other stem cell transplants with the hope of curing HIV, yet most of these transplants failed. Factors influencing the success of transplants as a cure for HIV infection include the high cost of stem cell transplants and the availability of matched donors. Therefore, although a stem cell transplant is proof that the principle works, it is not a viable option given the number of HIV-infected patients. More recently, a scientist from China used clustered regularly interspaced short palindromic repeats (CRISPR) to silence the *CCR5* gene of two embryos. A female received these embryos and gave birth to HIV-resistant babies (Xu, 2020). In a similar study, Schmidt *et al.* (2020) developed a successful CCR5 gene-editing protocol in *Mauritian cynomolgus* macaque embryos when the authors used CRISPR to edit the *CCR5* gene in these embryos (Schmidt *et al.*, 2020). CCR5 and its ligands have thus shown vast therapeutic potential in the treatment of cancer (Aldinucci & Casagrande, 2018; Hemmatazad & Berger, 2021; Jiao *et al.*, 2019), HIV/AIDS (as explained above), and even COVID-19 (Agresti *et al.*, 2021; Patterson *et al.*, 2021; Patterson *et al.*, 2020) to name but a few. It is thus evident that the CCR5 molecule holds translational value that will be best extracted upon its improved characterisation. Notably, in this study, more information on the fundamental role of CCR5 in a non-activated state will be retrieved to inform the design of future experiments that may optimise CCR5-related immune and metabolic functions and its possible therapeutic roles.

CHAPTER 3: EXPERIMENTAL DESIGN AND CONSIDERATIONS

3.1 Introduction

Cell culture refers to growing cells under specific conditions outside their natural environment (McKeehan *et al.*, 1990). When scientists perform the technique outside the living organism, commonly referred to as *in vitro*, cells can be isolated from various tissues or biofluids. Primary cells, also known as primary culture, are cultured directly from the subject from which it was isolated. When the primary culture becomes confluent, cells must be sub-cultured (or passaged) by transferring the cells to a new, giant flask and fresh growth medium for continuous growth. The limited lifespan of primary cells remains the most significant disadvantage of using these cells for research. Therefore, scientists established immortal cell lines using either tumour cells (cells that continue to divide rapidly) or artificial cell manipulation for indefinite proliferation in a process known as transformation. Transformation can occur naturally or be induced (Chandra *et al.*, 2022). A cell line is a population of cells resulting from a division from a progenitor cell, also known as the sub-clone. The cell line derived from the primary cells is finite and can only divide several times before they reach senescence.

Cell lines are established from primary cells and should thus present functional features similar to the primary cell. The serial passage of cells may result in some genotypic and phenotypic variation over an extended period - genetic drift can influence the cells to become more heterogeneous. Instrumentation such as microscopy and flow cytometry can detect cellular changes. Cell lines can be genetically manipulated according to the aim of the study, changing the phenotype, native functions, and the response of cells to stimuli. Scientists study cells outside of their natural environment, where their interactions with other cell types are absent, which could influence the interpretation of the data, depending on the hypothesis (Kaur & Dufour, 2012). However, continuous cell lines offer several advantages: the population of cells obtained results in consistent homogeneous samples and reproducible results (which are especially important for metabolomics studies). Furthermore, it is cost-effective to use cell lines since researchers can obtain an unlimited amount of easy to use and time-efficient samples since cells can be grown as needed and bypass cumbersome ethical processes (Kaur & Dufour, 2012). Finally, since cells can be grown as needed, large sample volumes can be collected, and various assays can be performed using aliquots of the same sample and thus, resulting in the possibility of more usable information in a study.

The homo- and heterozygous CCR5 Δ 32 genotypes, which yield no to little CCR5, have a low prevalence in Caucasians and are virtually absent in Black Africans (Martinson *et al.*, 1997).

Obtaining clinical samples was thus nearly impossible. Therefore, cell lines imported from two laboratories in the United States of America (USA), was used. The cell lines used for this project have several advantages, the main one being that scientists engineered them to have regulatable CCR5 expression.

3.2 Description of cells with differential CCR5 levels as used in this study

3.2.1 Affinofile cell system

Johnston *et al.* (2009) first described the Affinofile cell line that originated from human embryonic kidney (HEK) 293 cells (Johnston *et al.*, 2009). Briefly, the authors transfected HEK293 cells with a Tet-On system, i.e., pcDNA5/TO-CD4 and pIND-R5 vectors, for regulatable CD4 and CCR5 expression. Regarding Affinofile cells, the target genes are CD4 and CCR5 since the Affinofile cell line expresses both the transactivators and the inducible promoters that drive CD4 and CCR5 expression. As a result, tetracycline (or a derivative such as doxycycline) can regulate the expression of CD4. Furthermore, Ponasterone A (Pon A) – a compound similar to the insect steroid hormone Ecdysone – controls the expression of CCR5 in Affinofile cells. For more detail on the molecular methods used to create the Affinofile system, the reader is referred to the work published by Johnston and co-workers (2009) (Johnston *et al.*, 2009).

The Affinofile cells with regulatable CD4 and CCR5 levels were gifted to us by Dr Benhur Lee's laboratory through Shreyas Kowdle at Icahn School of Medicine at Mount Sinai, New York, USA. The parent cell line (HEK293) expresses low levels of CCR5 (Lehner *et al.*, 2001; Qi *et al.*, 2016) and therefore served as a control cell line against the Affinofile cells (Howard *et al.*, 1999).

3.2.2 Cf2Th cell system

Scientists used Canine Thymocyte (Cf2Th) cells with stable expression of CD4 to generate Cf2Th cells with regulatable CCR5. Briefly, these scientists transfected the Cf2Th cells with the Tet-On system, which uses tetracycline (or a derivative such as doxycycline) to regulate the expression of a target gene (CCR5). For more detail on the molecular methods used to create the Cf2Th cells with regulatable CCR5 expression the reader is referred to the work published by Espy and co-authors (2017) (Espy *et al.*, 2017).

Dr Nicole Espy generated the Cf2Th cells with regulatable CCR5 levels. The cells were the generous gift of Professor Sodroski's laboratory at Dana-Faber Cancer Institute and Harvard Medical School, Boston, MA, USA.

3.3 Experimental Design

The experimental design to investigate the immune-metabolic profile of cells expressing differential CCR5 levels is summarised in Figure 3-1. For this study, the cell lines and the project were registered with the NWU's Health Research Ethics Committee (HREC) with ethics number NWU-00975-19-A1. Given that the imported frozen cells were possibly fragile, the cells were thawed and expanded without antibiotics at first (**Objective 1**). Basal levels of CCR5 expressed on untreated Affinofile and Cf2th cells relative to that of the HEK293 cells (which are without the CCR5-inducing vector), respectively, suggested that CCR5 levels had to be induced to achieve cells with differential (higher) levels of the receptor. Subsequent experiments required the respective cell lines be cultured in selection antibiotics to maintain the vectors mentioned in Section 4.1. The concentration of blasticidin (50 ug/ml) in which to culture the Affinofile cells is well-documented (Joseph *et al.*, 2014; Lassen *et al.*, 2009), while the concentration of antibiotics in which to culture the Cf2Th cells varies across the literature and is unclear (Espy *et al.*, 2017; Mirzabekov *et al.*, 1999). An antibiotic concentration study was done on the Cf2th cells using the Annexin V- fluorescein isothiocyanate (FITC) - 7-aminoactinomycin D (7-AAD) kit, and flow cytometry (**Objective 2**). After confirming the concentration of antibiotic needed in which to culture the Cf2th cells, the optimum concentration of Pon A and doxycycline needed to induce low and high CCR5 levels in Affinofile and Cf2th cells, respectively, was determined using BD's Quantibrite™ kit and flow cytometry (**Objective 3**). Affinofile, HEK293, and Cf2th cells were cultured and treated with the respective CCR5-inducing antibiotics to achieve A) basal, B) low, and C) high levels of CCR5 (**Objective 4**). Next, the cell viabilities were determined and CCR5 levels were confirmed (flow cytometry) using aliquots of cells from the respective culture dishes (basal, low and high CCR5 levels) of the individual cell lines (**Objective 5**). The supernatant was collected to determine the extracellular immune profile of cells presenting differential CCR5 phenotypes (**Objective 6**). Aliquots of cells (3×10^6 cells) were collected for GCxGC-TOFMS analysis to measure the individual metabolic profiles of each cell line (**Objective 7**). Samples were stratified according to CCR5 levels (per cell line) and uni- and multivariate statistical analysis applied to identify differences in the immune and metabolic profiles of the cells (**Objective 8**). The significantly different immune and metabolic markers were mapped to various biological processes and/or pathways (**Objective 9**). The cells were cultured in large culture dishes to provide enough cells for parallel assays.

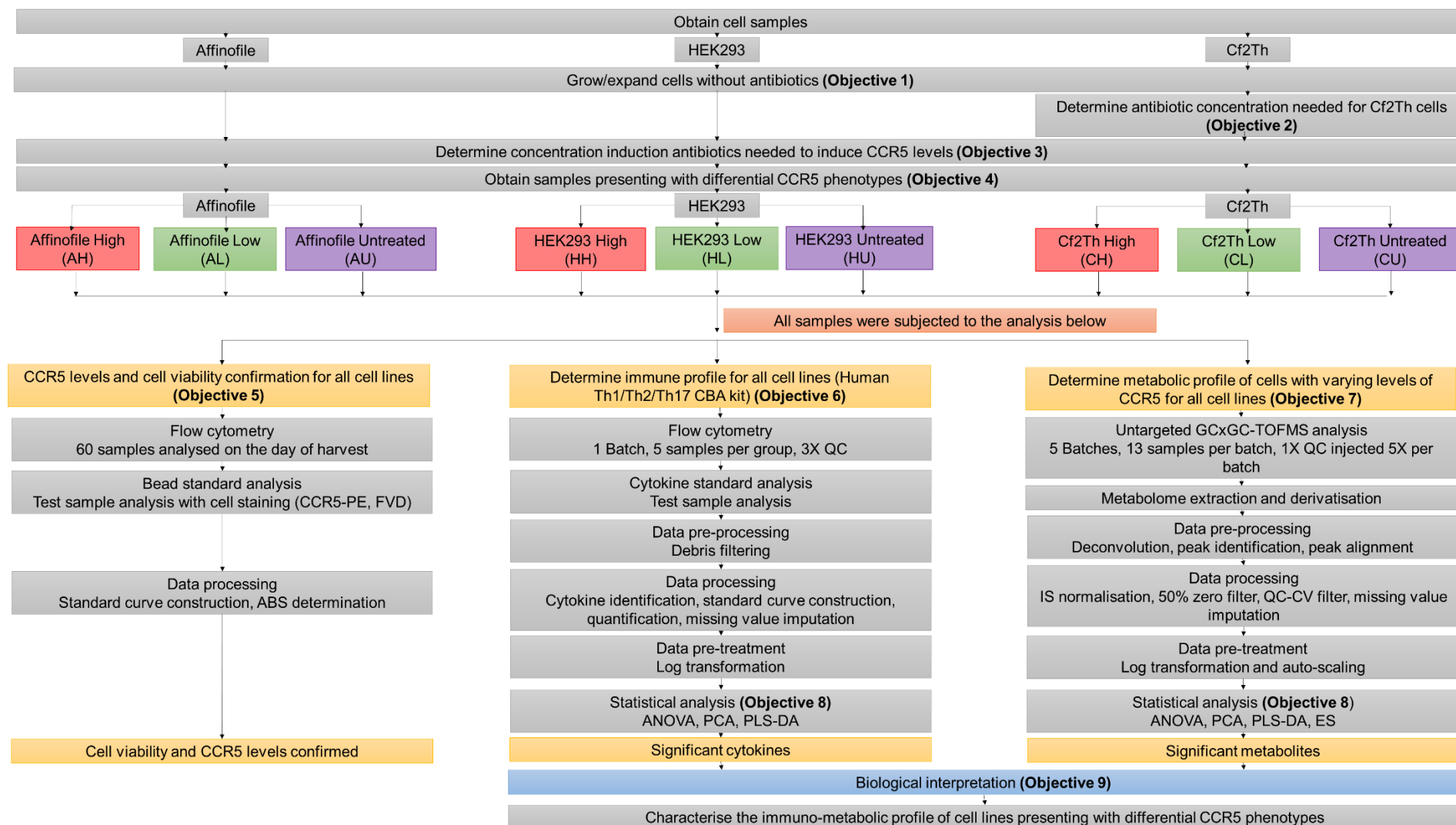


Figure 3-1: General experimental design followed to investigate the immuno-metabolomics profile of cells (Affinofile, HEK293 and Cf2Th) expressing basal, low and high CCR5 levels.

3.4 Considerations regarding the influence of the Tet-On system and associated antibiotic usage

The Pon A used to induce different levels of CCR5 in the Affinofile cells (Chikere *et al.*, 2013; Joseph *et al.*, 2014; Pugach *et al.*, 2009) may influence the immune and metabolic profiles of the cells used in this study. While no literature is available on the metabolic influences of Pon A, literature on its effects on the immune system exists. Constantino *et al.* (2001) investigated the impact of Pon A on cytokine signalling in mammalian cells (pro-B cell line). They reported that Pon A affects cell growth and survival by increasing the effect of IL-3-dependent activation of the PI3K – protein kinase B (PKB) / Akt (PI3K-PKB/Akt) pathway (Constantino *et al.*, 2001). In cancer, activating the PI3K-Akt-mTOR signalling pathway affects the absorption and usage of various metabolites (such as glucose), supporting cancer cell growth (Lien *et al.*, 2016). In addition, the Akt/PKB pathway can influence insulin's metabolic effects (Whiteman *et al.*, 2002). The link between the immune and metabolic systems is known, and changes noted at the immune level will thus reflect in the metabolism. The use of an ecdysteroid-regulated transgenic system (such as the Tet-On system used in this study) holds potential implications when investigating the immune profile of the transactivator, i.e. Pon A can be immunogenic, thus resulting in immune response (Lafont & Dinan, 2003) and, therefore, potential metabolic changes. Table 3-1 shows the various concentrations of Pon A used in other studies, and the respective CCR5 levels obtained. This MSc study used 0.26 μ M and 13 μ M Pon A to obtain CCR5-low and CCR5-high Affinofile cells, respectively. These concentrations are thus in an acceptable range, which should not impact cell viability and associated biological processes.

Doxycycline was used to induce varying levels of CCR5 in the Cf2th cells. Like Pon A, its use may influence the immune and metabolic profiles of the cells used in this study. Doxycycline is an anti-inflammatory agent. Therefore, researchers investigated the effects of doxycycline on cells' immune and metabolic profiles. For example, Bostanci *et al.* (2011) investigated the effects of doxycycline on cytokine secretion in cells stimulated with a periodontal pathogen. The authors reported significant inhibition of pro-inflammatory cytokines by doxycycline (Bostanci *et al.*, 2011). In addition, Han and co-authors (2014) investigated the effect of doxycycline in inflammation-induced lymphangiogenesis - a process in which lymphatic vessels are made from other pre-existing lymphatic vessels - in the cornea of a mouse. The authors reported that doxycycline modulated the PI3K/Akt/eNOS pathway, suppressed inflammatory IL-1 β and TNF- α secretion, and modulated the PI3K/Akt/NF-KB pathway in cells. Ultimately, doxycycline inhibited inflammation-induced lymphangiogenesis (Han *et al.*, 2014).

In a study conducted by Ahler *et al.* (2013), doxycycline elevated glycolytic metabolism, lactic acid secretion, and decreased oxygen consumption in various human cell lines. The authors

suggested that cell lines transfected with the Tet-On system should be accompanied by the appropriate controls (which are cell line-specific) to account for the metabolic shift (Ahler *et al.*, 2013). Moullan and co-workers (2015) reported that doxycycline in $\mu\text{g/ml}$ ranges induced mitonuclear protein imbalance in four distinct cell lines. In addition, doxycycline impacted mitochondrial function and downregulated several pathways. An example of this is ATP synthesis and the electron transport chain (ETC), resulting in reduced cellular respiration. Removing the doxycycline from the media reversed this effect (Moullan *et al.*, 2015). A review also highlighted these findings (Chatzispyrou *et al.*, 2015). Luger and co-workers (2018) analysed the effect of doxycycline on the metabolism of three glioma cell lines in a dose-dependent manner (with concentration ranges of 0.1-10 $\mu\text{g/ml}$). The authors reported that doxycycline inhibited mitochondrial biosynthesis, decreasing oxygen consumption and increasing glucose intake in LNT-229 cells (10 $\mu\text{g/ml}$ doxycycline), G55 cells (0.1 $\mu\text{g/ml}$ doxycycline), and U343 cells (1 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ doxycycline). Increased doxycycline concentrations (e.g. 10 $\mu\text{g/ml}$) decreased cell growth and proliferation in all glioma cell lines (Luger *et al.*, 2018). More recently, Hut *et al.* (2021) investigated the cytotoxic effect of tetracycline on cell viability in a dose-dependent manner at various time intervals and found that cell viability decreased as the concentration of tetracycline increased in the HT-29 cell line. Increased time and tetracycline concentrations lead to morphological changes, reduced cell migration, and increased nuclear fragmentation, a feature of apoptosis (Hut *et al.*, 2021). In this study, 26 ng/ml and 1000 ng/ml (or 1 $\mu\text{g/ml}$) doxycycline was used to obtain CCR5-low and CCR5-high Cf2Th cells, respectively. These concentrations are thus in an acceptable range which should not impact cell viability and associated biological processes.

Affinofile cells must be grown for at least > 3 months without selection antibiotics to obtain cells without the CCR5-expressing vector (Chikere *et al.*, 2013). This was attempted for both cell lines but several technical and logistical issues were experienced which prevented us from achieving this (contamination of a batch of cells, the hard lockdown which halted all experiments, and access to the lab was interrupted in two instances). As a result, there was not enough time to achieve the high passage number. As previously mentioned, the Affinofile cells are HEK293-derived. So instead, HEK293 cells were cultured alongside the Affinofile cells to serve as a control (representing cells without the CCR5-inducing vector).

The studies mentioned previously highlight the possible effects of Pon A and/or doxycycline on the cells' immune and/or metabolic profiles. Researchers should therefore interpret the immune and metabolic data deriving from cells transfected with the Tet-On system requiring induction of the CCR5 with caution. While the reviewed effects on the mitochondria and their associated processes (oxygen consumption and ATP production), glucose metabolism, cell growth, and cytokine production may reflect in this study, the effect should be much less. Low concentrations

of CCR5-inducing reagents were used, especially after accounting for background signals deriving from cells without the CCR5-inducing vector, i.e., HEK293 cells. Table 3-1 summarises the previous studies that reported on the CCR5 levels obtained using various concentrations of Pon A/doxycycline. The table shows that low CCR5 levels are typically 1000 – 20000 antibody binding sites (ABS)/cell while high CCR5 levels are 20000-230000 ABS/cell. The signals measured varied based on the cell culture methods, the flow cytometer's sensitivity to quantify receptor levels, and the amount of induction agent used.

Table 3-1: Summary of CCR5 levels obtained in previous studies after induction with Pon A/doxycycline.

Cell line	Inducing reagent	Concentration of CCR5 inducing reagent used	High CCR5 levels (ABS/cell)	Low CCR5 levels (ABS/cell)	Reference
Affinofile	Pon A	15.6 – 2 000 nM	170 000	10 000	(Chikere <i>et al.</i> , 2013)
		0 – 4 µM	18 719 – 23 235	199 - 1274	(Johnston <i>et al.</i> , 2009)
		0.04 – 1 µM	230 000	22 000	(Agrawal-Gamse <i>et al.</i> , 2009)
				16 000	
					(Pugach <i>et al.</i> , 2009)
		0.0156 – 2 µM	22 000	1 200	(Agrawal-Gamse <i>et al.</i> , 2009)
		0 – 2 µM	50 000	1000	(Loftin <i>et al.</i> , 2010)
		0.0156 – 2 µM	88 000	5 500	(Tilton <i>et al.</i> , 2010)
		15.6 – 2 000 nM	167 000	8 000	(Roche <i>et al.</i> , 2011a)

		0.0156 – 2 μ M	140 000	5 000	(Roche <i>et al.</i> , 2011b)
		0 – 5 μ M	30 472 – 39 162	2 691 – 4 084	(Joseph <i>et al.</i> , 2014)
Cf2Th	Doxycycline	0 – 1 000 ng/ml	13 000	500	(Espy <i>et al.</i> , 2017)

Inducing reagent: Pon A: Ponasterone A

Concentration of CCR5 inducing reagent used: μ M: micromolar; nM: nanomolar; ng/ml: nanogram per millilitre

CHAPTER 4: MATERIALS AND METHODS

4.1 Cell culturing methods

The expansion of cell lines provides the researcher with a reserve of cells for future use. All reagents and consumables were sterilised and handled under aseptic conditions. Various types of media, defined here, were made up afresh as needed. The **thawing media** comprised Dulbecco's Modified Eagle's Medium (DMEM) with 30% filtered tetracycline-free fetal bovine serum (FBS) and without antibiotics. Since the cells were frozen for quite some time, they were deemed fragile, and therefore received an excess of tetracycline-free FBS. The **complete media** comprised of DMEM supplemented with 10% filtered tetracycline-free FBS, also without antibiotics. In contrast, the **culture media** consisted of complete media (described above) supplemented with the appropriate selection antibiotic(s) to maintain the vector (50 µg/ml Blasticidin for Affinofile cells and 0,6 µg/ml puromycin, 400 µg/ml G418, 0.025 µg/ml zeocin and 200 µg/ml hygromycin for Cf2th cells). The **induction media** was prepared by supplementing complete media with Pon A and doxycycline to induce CCR5 in Affinofile and Cf2th cells. The **freeze media** comprised 90% complete media and 10% dimethyl sulfoxide (DMSO). The tetracycline-free FBS used to prepare all media was filtered with 0.2 µm filters.

4.1.1 Reagents for the expansion and culturing of Affinofile, HEK293, and Cf2Th cells

Annexure A (Table A1) shows the specific catalogue numbers for each of the reagents used in the respective experiments.

4.1.2 Thaw of Affinofile, HEK293, and Cf2Th cells

A vial of cells was retrieved and thawed, by submerging it in a 37 °C water bath until only a tiny ice crystal remained, at which point the vial was transferred to the biosafety cabinet. The cell suspension was then transferred to a 15 ml centrifuge tube and the pre-warmed thawing media (4 ml) was gradually introduced to the cells. The cells were centrifuged at 130 x g for 5 minutes at room temperature, the supernatant was removed, and the cells were resuspended in 1 ml of the thawing media. Cells were then mixed to form a single cell suspension and subsequently transferred to a vented T25 flask containing 4 ml of the thawing media. The cells were thus grown in a final volume of 5 ml of thawing media, representing passage one (P1). Cells were transferred to the Nuve EC160 incubator to grow at 37 °C and 5% CO₂.

4.1.3 Transfer of Affinofile, HEK293 and Cf2Th cells

The T25 flask containing the thawed cells was removed from the incubator after three days, inspected under the microscope, and transferred to the biosafety cabinet. The spent media was removed, and the cells were washed with PBS (5 ml). Next, trypsin- Ethylenediaminetetraacetic acid (EDTA) (1 ml) was added to the flasks which were incubated at 37 °C for 3 minutes to detach the cells. Microscopic evaluation confirmed that the cells had detached from the flask. The trypsin-EDTA was neutralised by adding 2 ml of the thawing media to the cells. Cells were mixed to form a single cell suspension and subsequently transferred to a T75 flask and made up to a final volume of 10 ml using thawing media (thawing media was used instead of complete media in this instance due to the fragility of the cells). Lastly, cells (still P1) were transferred to the Nuve EC160 incubator to grow at 37 °C and 5% CO₂.

4.1.4 Expansion of Affinofile, HEK293, and Cf2Th cells

The T75 flask containing cells (P1) was removed from the incubator after three days, inspected under the microscope, and transferred to the biosafety cabinet to passage the cells. The spent media was removed from the cells, washed with PBS and harvested through trypsinisation. Trypsin-EDTA was neutralised by adding 5 ml of complete media. Cells were mixed into a single-cell suspension and split in a 1:3 ratio into T75 flasks by transferring 2 ml of the cell suspension to a T75 flask containing 8 ml of complete media (x 3), representing passage two (P2). Cells were transferred to the Nuve EC160 incubator to grow at 37 °C and 5% CO₂.

After three days, the three T75 flasks were removed from the incubator, inspected under a microscope, and transferred to the biosafety cabinet. The spent media was removed, the cells washed with PBS, harvested through trypsinisation, and neutralised with complete media as described in the preceding paragraph. Cells were then mixed to form a single-cell suspension. The cells from one of the T75 flasks were frozen down as P2. Briefly, the cells were counted using the trypan blue stain, centrifuged, and the pellet resuspended in an appropriate volume of freezing media to a cell concentration of 1×10^6 cells/ml. Cells were frozen down in a Mr Frosty cooler at -80 °C. Cells from the remaining two T75 flasks were split in a 1:3 ratio in T75 flasks and made up to contain 10 ml of complete media, representing passage three (P3), which yielded six T75 flasks that were transferred to the Nuve EC160 incubator to grow at 37 °C and 5% CO₂.

After three days, the cells from P3 were removed from the incubator. The cells were inspected under a microscope and transferred to the biosafety cabinet. As described in the preceding paragraph, the cells from the three T75 flasks were diluted to a 1×10^6 cells/ml concentration and frozen. Cells from the other three T75 flasks were transferred to three T150 flasks, which were transferred to the Nuve EC160 incubator, and grown at 37 °C and 5% CO₂ (still P3). After three

days, the three T150 flasks were removed from the incubator, inspected under a microscope, and transferred to the biosafety cabinet. They were split at a ratio of 1:5 and transferred to an incubator and grown further at 37 °C and 5% CO₂ (now P4). Upon reaching confluency, they were frozen as described above. Early and later passages of the cells were thus frozen at -80 °C, -140 °C, and liquid nitrogen.

4.1.5 Culture of Affinofile, HEK293, and Cf2th cells for respective assays

For the individual assays which followed, vials of cells from P3 were thawed as described previously (Section 4.1.2). These cells were passaged as discussed in Sections 4.1.3 and 4.1.4, but only up to passage number 3. HEK293 cells were treated in the same manner as the Affinofile cells.

4.2 Flow cytometric characterisation of Cell lines

4.2.1 Reagents for flow cytometry

Annexure A (Table A1) shows the specific catalogue numbers for each of the reagents used in the respective experiments.

4.2.2 Antibiotic concentration in which to culture the Cf2Th cell line

Cells genetically engineered often require antibiotics to keep the vector stable. These antibiotics can however be cytotoxic. Since the literature was not clear on the antibiotic concentrations in which to culture the Cf2Th cells, an antibiotic concentration study was performed using the antibiotics recommended by Espy *et al.* (2017) to obtain an antibiotic mixture in which the canine-derived cells remained viable. The concentrations of the various antibiotics tested can be observed in Table 4-1. This was not tested for the Affinofile cells, since the antibiotic concentration for culturing Affinofile cells was previously specified and is consistent in the literature (Johnston *et al.*, 2009).

After culturing the Cf2Th cells in complete media up to P3, cells were harvested through trypsinisation, resuspended in 1 ml media, and counted using trypan blue staining and an automated cell counter (Luna II, Celtic Molecular Diagnostics). Cells were seeded at 5×10^4 cells/well in a 24-well cell culture plate. Each well with cells contained 1 ml of complete media. Cells were left overnight to attach to the plates. Three 24-well plates for each experiment, representing cells harvested on day zero, day one, and day three (Figure 4-1) were prepared. The cells from the plate labelled “day 0” served as a baseline to determine the condition of the cells before antibiotic treatment (harvested on the day that the other plates received antibiotics). The cells from the plates labelled “day 1” and “day 3” were analysed at one and three days, respectively. Samples were in duplicate (two replicates) for each plate and the experiment repeated on three different occasions (three repeats).

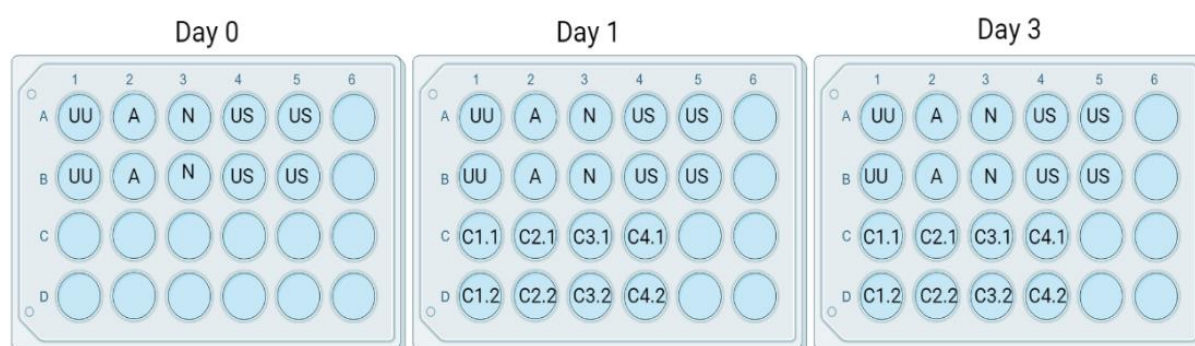


Figure 4-1: A schematic representation of Cf2Th cells seeded in plates for harvest on day 0, day 1 and day 3 (Image created by the author with Biorender.com). The wells of row A and B were combined to ensure enough sample for analysis. The empty wells were filled with 1 ml PBS to minimise evaporation. Abbreviations: UU – Untreated unstained; A – Apoptosis; N - Necrosis; US – Untreated stained; C1.1 – Antibiotic Combination 1 replicate 1; C1.2 – Antibiotic Combination 1 replicate 2; C2.1 – Antibiotic Combination 2 replicate 1; C2.2 – Antibiotic Combination 2 replicate 2; C3.1 - Antibiotic Combination 3 replicate 1; C3.2 - Antibiotic Combination 3 replicate 2; C4.1 - Antibiotic Combination 4 replicate 1; C4.2 - Antibiotic Combination 4 replicate 2.

Once attached and the cells were 75% confluent, the spent media was removed from the wells of all three plates. The plates representing day 1 and day 3 received media containing the various combination of antibiotics as specified in Table 4-1. The plates were incubated at 37 °C with 5% CO₂ and harvested on the respective days.

Table 4-1: Table showing the different antibiotic concentration combinations tested for culture of Cf2Th cells.

	[Puromycin]	[G418]	[Zeocin]	[Hygromycin]
	(µg/ml)	(µg/ml)	(µg/ml)	(µg/ml)
Antibiotic combination 1 (C1)	0,6	400	0,025	200
Antibiotic combination 2 (C2)	0,6	400	0,025	100
Antibiotic combination 3 (C3)	0,6	200	0,05	200
Antibiotic combination 4 (C4)	0,6	400	0,05	100

Abbreviations: µg/ml – micrograms per millilitre

Cell harvesting and the preparation of the cells for flow cytometry analysis was done according to Figure 4-2. Briefly, cells were harvested from all the wells by removing the spent media and washing the cells with PBS (130 x g for 5 minutes at 4 °C). The cells were trypsinised by adding 200 µl trypsin-EDTA to each well, and the plate was incubated at 37 °C for 3 minutes. The detached cells were then observed under the microscope to ensure detachment from the plate. The plate was transferred to the biosafety cabinet, and 600 µl of complete media neutralised the trypsin. Cells were transferred to microcentrifuge tubes and centrifuged at 130 x g for 5 minutes at 4 °C. The supernatant was subsequently discarded, and cells were washed with PBS (130 x g for 5 minutes at 4 °C), and the remaining cell pellets assayed using the Annexin V-FITC - 7-AAD Assay.

4.2.3 Annexin V-FITC - 7-AAD Assay to measure cell viability and mode of cell death (if any) – Antibiotic-concentration study

To determine the viability of the Cf2Th cells grown in various combinations of antibiotics and the mode of cell death (if any), FITC conjugated to Annexin V and 7-AAD was used, hereafter referred to as the Annexin V-FITC-7-AAD kit. Apoptotic cells present with morphological changes, such as shrinkage of the cells and the surfacing of phosphatidylserine (a phospholipid found on the plasma membrane) on the cell surface (van Engeland *et al.*, 1998). The Annexin V-FITC binds to the phosphatidylserine and, when analysed on the flow cytometer, informs on the degree of apoptosis. In contrast, necrotic cells present with changes in the cell membrane, which allows a DNA-intercalating dye, such as 7-AAD, to enter the cell and bind to the DNA. When analysed on the flow cytometer 7-AAD informs on the degree of necrosis.

Before analysing test samples, BD's cytometer setup and tracking (CS&T) beads were analysed according to the manufacturer's instructions. For all test samples, Untreated Unstained (UU), Apoptosis (A), Necrosis (N) controls as well as an Untreated Stained (US) sample was included (Figure 4-2). Control cells were seeded in duplicate and combined, increasing the number of events acquired when doing flow cytometry analysis. The UU control was prepared by resuspending the cell pellet in 500 µl 1X binding buffer and keeping this on ice. The necrosis control (N) was prepared by resuspending the cell pellet in 1 ml ice-cold methanol, followed by centrifugation of the cells at 130 x g for 5 minutes at 4 °C. The methanol supernatant was discarded, and cells were washed with PBS at 130 x g for 5 minutes at 4 °C. The PBS supernatant was discarded, and the cell pellet was resuspended in 98 µl 1X binding buffer. The apoptosis control (A) was prepared by resuspending the cell pellet in 1 ml complete media and adding 2 µg/ml phytohemagglutinin P (PHA-P), a mitogen known to induce apoptosis (Kochubei *et al.*, 2015). These cells were incubated in the Nuve EC160 incubator at 37 °C and 5% CO₂ for 60 minutes. After 60 minutes, 250 µl of 37% formaldehyde was slowly added to the cells to fix them. The cell fixing process preserves the cell conditions to keep the phosphatidylserine on the surface of the cells (Schmiedeberg *et al.*, 2009). The cells were incubated in the Nuve EC160 incubator at 37 °C and 5% CO₂ for an additional 30 minutes. The cells were centrifuged at 130 x g for 5 minutes at 4 °C, the supernatant was discarded, and cells were washed with PBS at 130 x g for 5 minutes at 4 °C. Next, the supernatant was discarded. The cell pellet was resuspended in 98 µl 1X binding buffer and kept on ice. Two µl of Annexin V-FITC was added to the apoptosis control, while 2 µl 7-AAD was added to the necrosis control. The US, A, and N controls served as fluorescence minus one (FMO) control samples. FMOs rely on using all the fluorochromes used for an experiment except one (Maecker & Trotter, 2006). In this experiment, the apoptosis control was stained with Annexin V-FITC only. In contrast, only 7-AAD was used to stain the positive necrosis sample. The FMOs were used to distinguish between unstained, apoptotic, and/or necrosis populations and define gates during flow cytometry analysis.

Finally, test samples were prepared by resuspending cell pellets in 100 µl staining solution, which consisted of 96 µl 1X binding buffer, 2 µl Annexin V-FITC, and 2 µl 7-AAD per sample. The controls and test samples were incubated in the dark, on ice, for 15 minutes. Before sample analysis, 400 µl 1X binding buffer was added to each sample. Before acquiring data on the BD Accuri™™ C6 Plus, BD CS&T calibration beads were run. Then, samples were analysed on the BD Accuri™™ C6 Plus. A total of 10 000 events in the single-cell gate were acquired for each sample. Upon completion, the data was exported as *.fcs files and imported into FlowJo (v10.8). After import, compensation was done to correct for any spectral overlap, and gates were applied using the FMO controls that were prepared alongside the samples.

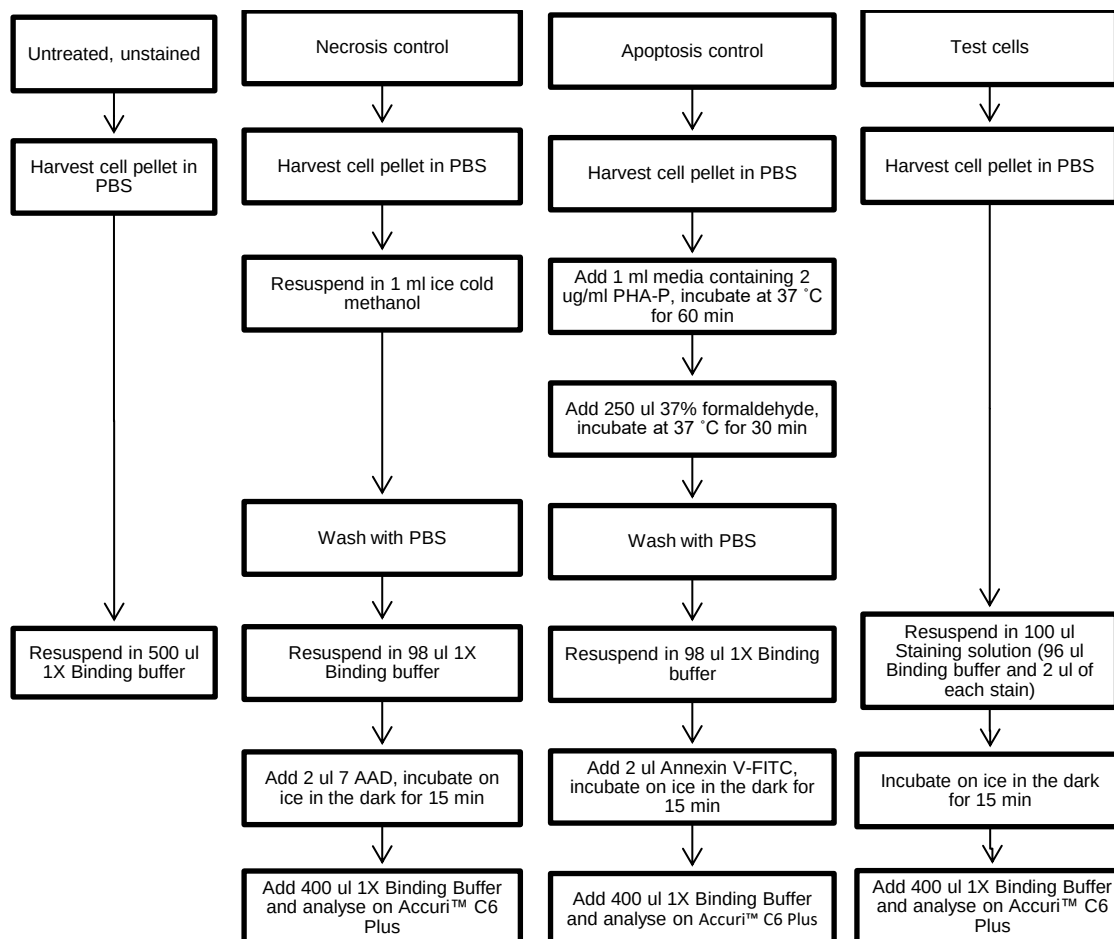


Figure 4-2: Workflow followed for preparing cells for flow cytometric analysis using the Annexin V-FITC-7-AAD apoptosis kit.

Cells were grown, and the different combinations of antibiotics were added to respective wells. All cells were harvested using trypsin-EDTA and transferred to microcentrifuge tubes on the day of analysis. All samples were handled on ice. Cell pellets were collected by centrifuging the cells at $130 \times g$ for 5 minutes at 4°C . The media supernatant was discarded, and cells were washed with PBS. The untreated, unstained control samples were resuspended in $500 \mu\text{l}$ 1X binding buffer and stored on ice until analysis. The necrosis control was resuspended in 1 ml ice-cold methanol, centrifuged, and washed with PBS. The Apoptosis control was resuspended in media containing $2 \mu\text{g/ml}$ PHA-P and incubated at 37°C for 60 minutes. After 60 minutes, 37% formaldehyde solution ($250 \mu\text{l}$) was added, and cells were incubated at 37°C for 30 minutes. After 30 minutes, the cells were centrifuged and washed with PBS. The test samples, apoptotic and necrotic control samples were all resuspended in $100 \mu\text{l}$ staining solution, which consisted of 1X binding buffer and the dyes of interest. The samples were incubated in the dark on ice for 15 minutes. After 15 minutes, $400 \mu\text{l}$ 1X binding buffer was added to each sample, and samples were immediately analysed on the BD Accuri™ C6+ flow cytometer.

Figure 4-3 shows the gating strategy followed. First, the cell population was identified through a side scatter (cell complexity) and forward scatter plot (cell size). Next, singlets (non-clumped cells) were identified through an FSC area and FSC height plot. Finally, a quadrant specifying Annexin V-FITC against 7-AAD was used to distinguish between viable, apoptotic, necrotic, or double-positive cells. From these plots, statistics determining the percentage of cells in each quadrant were exported into Microsoft Excel (2016) which was subsequently used to draw a summary graph to show the effect of the different antibiotic concentrations on cell viabilities over the respective days (Figure 5-1).

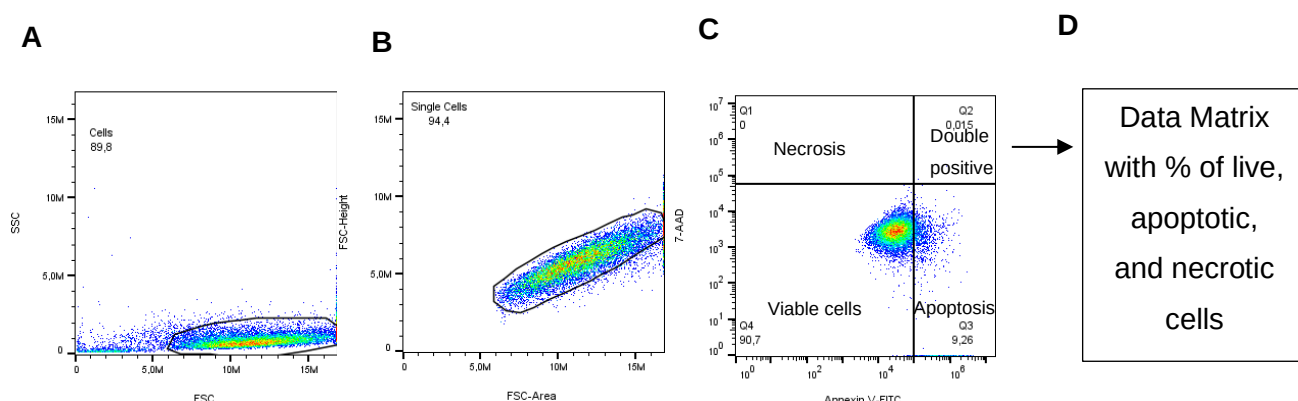


Figure 4-3: Representative gating strategy followed while processing Annexin V-FITC - 7-AAD flow cytometry data in FlowJo (v10.8).

The population of interest was identified (A) and the single cells gated on (B). The quadrants were placed based on the data obtained from FMO samples and applied to all samples to inform cell viability (C). Q1 (quadrant 1) shows the percentage of cells stained positively for 7 AAD and labelled as necrotic. Q2 (quadrant 2) shows the percentage of cells stained with both dyes and labelled as double positive. Q3 (quadrant 3) shows the percentage of cells stained with Annexin V-FITC and labelled as apoptotic. Q4 (quadrant 4) shows the percentage of cells that did not take up any stain and represents the viable cells. Finally, D) represents the final step of the workflow in which the data matrix containing the percentages of the live, apoptotic, and necrotic cells was populated.

As previously mentioned, the antibiotic concentration to use for culturing Affinofile cells is known. Therefore, an antibiotic concentration and time study to determine the best antibiotic combination for culturing Cf2Th cells was done. Next, the baseline CCR5 levels was determined using BD's Quantibrite PE beads (similarly as described in Section 4.2.5) in the respective cell lines. This analysis confirmed that CCR5 levels had to be induced in the individual cell lines.

4.2.4 Determining the optimal concentration of CCR5-inducing reagents (Pon A and doxycycline) to achieve CCR5-low and CCR5-high cells.

The optimal concentration of the CCR5-inducing reagent needed to induce CCR5-low, and CCR5-high cells were determined using previously published protocols for Affinofile cells (Webb & Lee, 2016) and Cf2Th cells (Espy *et al.*, 2017). Before analysing test samples, BD's CS&T beads were analysed according to the manufacturer's instructions. Briefly, Affinofile/HEK293 and Cf2th cells were thawed and passaged as described in Section 4.1, but in T25 flasks (as less material was required for this experiment) and up to P3. First, the cells were allowed to reach confluency. These were then harvested through trypsinisation, washed, resuspended in complete media, and counted using trypan blue. Next, 5×10^5 cells/well were seeded into 24-well cell culture plates. Cells were incubated until they reached approximately 75% confluency. Next, various concentrations of Pon A and doxycycline (induction antibiotics) were added to the Affinofile and Cf2Th cells, respectively but still within the physiological range (Lee *et al.*, 1999b). Finally, empty

wells in the plate were filled with cell culture-grade water to minimise evaporation. The Pon A and doxycycline concentration range used (μM and ng/ml , respectively) are indicated by the values on the respective wells in Figure 4-4. Extra wells with cells were prepared to serve as controls, namely untreated unstained (UU) cells and a viability control (VC) (see Figure 4-4). The experiment was repeated once with two replicates of each sample as it mirrored published data.

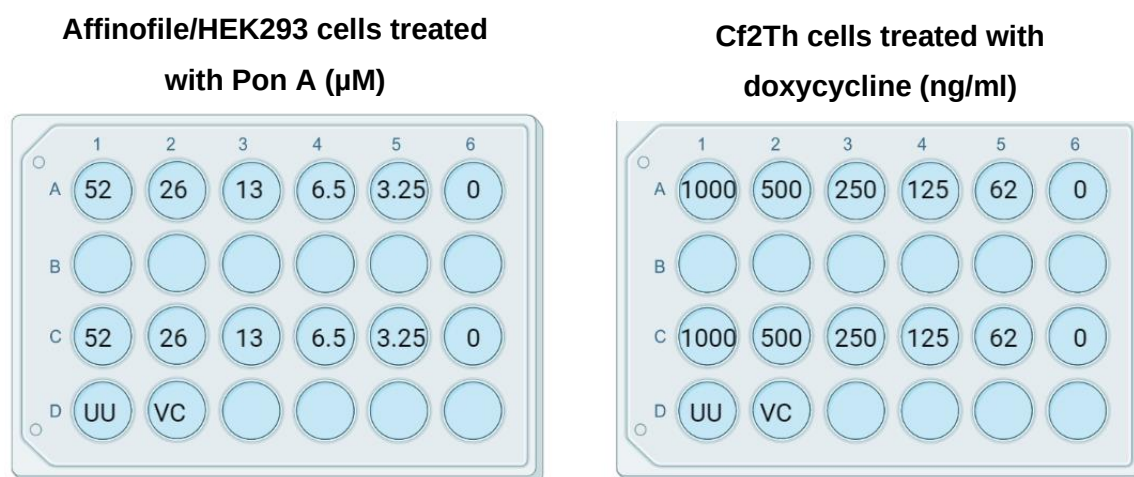


Figure 4-4: Plate layout to determine the optimal concentration of CCR5-inducing reagent needed to induce CCR5-low and CCR5-high cells in Affinofile/HEK293 and Cf2th cells, respectively.

The plate for the Affinofile cells (left) contained various concentrations of Pon A (μM), with two replicates of each concentration to be tested. The plate for the Cf2Th cells (right) had multiple concentrations of doxycycline (ng/ml) with two replicates of each concentration tried. Empty wells were filled with cell culture-grade water to minimise evaporation in the incubator. Cells were treated with respective antibiotics for 18 hours, followed by receptor quantification using flow cytometry.

After 18 hours of treatment (Webb & Lee, 2016), the Affinofile and Cf2th cells treated with Pon A and doxycycline were analysed using flow cytometry to determine the CCR5 levels associated with each treatment. As per published literature, cells were prepared for flow cytometry (Webb & Lee, 2016). Briefly, plates were removed from the incubator and transferred to the biosafety cabinet. The induction media was removed, and cells were washed with PBS twice ($300 \times g$ for 5 minutes at 4°C). The use of trypsin-EDTA can remove the receptors on the cell surface and should be avoided (Nowak-Terpiłowska *et al.*, 2021). Scraping cells in the 24-well plate was not feasible due to the size of the wells. All cells were thus harvested using cold PBS supplemented with 5 millimolar (mM) EDTA instead. Detachment of the cells was confirmed using a microscope. Cell suspensions were transferred to microcentrifuge tubes and centrifuged at $300 \times g$ for 5 minutes at 4°C , and the supernatant was discarded.

The untreated, unstained control sample was prepared by resuspending the cells in 200 µl PBS supplemented with 1% tetracycline-free FBS. The positive control for dead cells was prepared by resuspending the cells in 1 ml ice-cold methanol. Cells were centrifuged at 300 x g for 5 minutes at 4 °C. The supernatant was discarded, and the cells were washed in PBS (300 x g for 5 minutes at 4 °C). The “viability control” was resuspended in 49.8 µl PBS supplemented with 1% tetracycline-free FBS and stained with 0.2 µl eBioscience™ Fixable Viability Dye eFluor™ 660, hereafter referred to as fixable viability dye (FVD). The FVD can permanently stain dead cells before fixation without compromising the intensity of the dye (Coder, 2001). The positive control for CCR5 was the sample with the highest expression of CCR5 post-induction. Untreated, stained samples included those that received 0 µM Pon A (Affinofile cells) and 0 ng/ml doxycycline (Cf2Th).

The test samples were resuspended in a staining solution comprising 47.8 µl PBS supplemented with 1% tetracycline free FBS, 2 µl CCR5 conjugated to phytoerythrin (PE) (CCR5-PE) and 0,2 µl FVD per sample. Regarding CCR5-PE, the 2D7 clone was used for consistency and comparison with other studies since the use of different clones results in different CCR5 expression estimates (Lee *et al.*, 1999a). The 2D7 epitope refers to the part of the antigen to which the antibody binds. All test and control samples were incubated at 4 °C for 60 minutes in the dark. After 60 minutes, cells were washed twice with 1 ml PBS (300 x g for 5 minutes at 4 °C). The cell suspensions were resuspended in 200 µl of 2% paraformaldehyde solution to fix the samples (control and test samples). Cells were transferred to flow cytometry tubes and stored in the dark at 4 °C for 30 minutes and then immediately analysed on the Accuri™ C6 Plus using BD's Quantibrite™ PE beads (Becton Dickson, San Jose, USA).

4.2.5 CCR5 quantification using Quantibrite™ PE beads

The Quantibrite™ kit is supplied with ten tubes. Each tube contains standard beads conjugated to four different levels of PE representing low, medium-low, medium-high, and high CCR5 ABS (the manufacturers provide the exact number of PE molecules per bead). The kit thus allows for determining the amount of CCR5 ABS/cell. Each tube in the kit contains a lyophilised pellet, which was resuspended in 500 µl PBS supplemented with 0.5% tetracycline-free FBS and mixed using a vortex. Before analysing test samples, BD's CS&T beads were analysed according to the manufacturer's instructions. The Quantibrite beads were subjected to flow cytometry analysis, where 20 000 events in the gate were collected. By gating on the population of interest and plotting a histogram (Figure 4-5A), the MFI of the beads conjugated to four different levels of PE could be observed (Figure 4-5B).

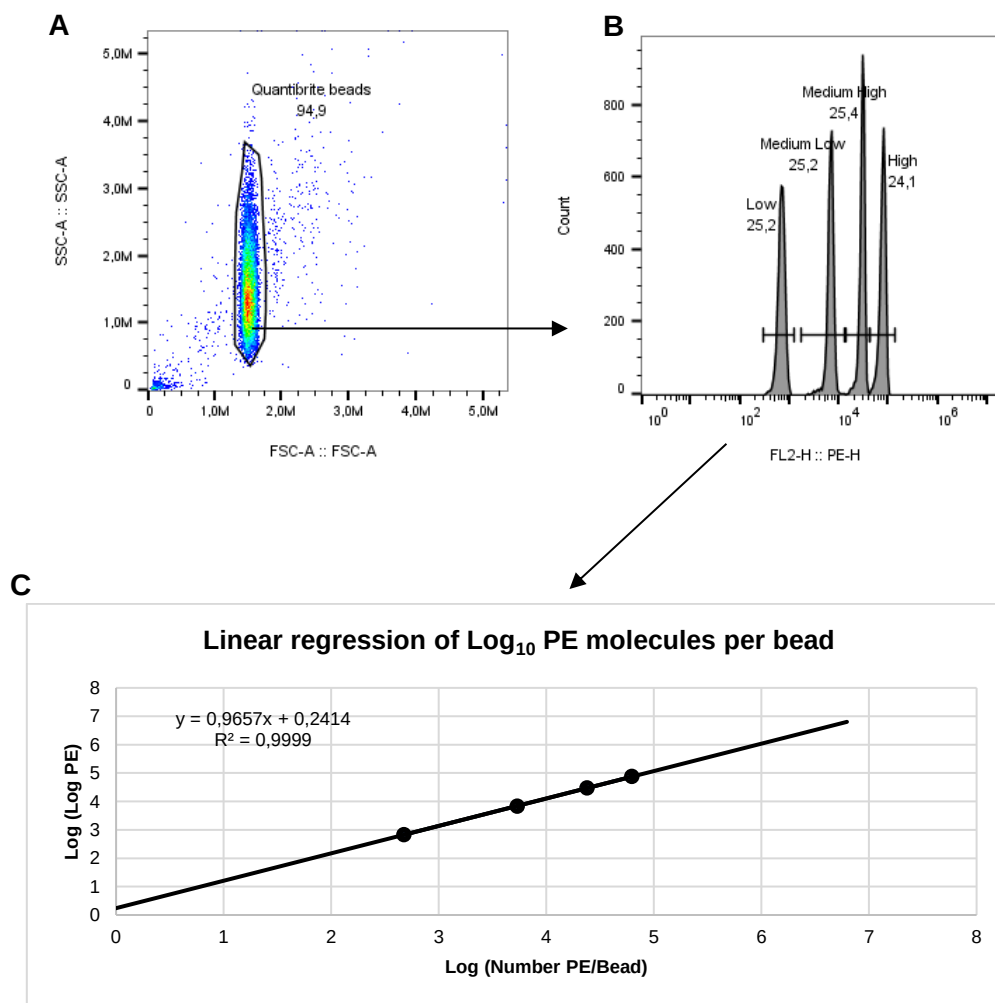


Figure 4-5 shows the gating strategy applied for the BD Quantibrite™ PE standards.

A) Gating on the bead population allows the generation of a B) histogram that shows the MFI of the bead standards, which is used to create a standard curve. C) The ABS/cell can then be estimated using the standard curve. PE – Phytoerythrin; MFI – mean fluorescence intensity.

The test samples were prepared as described in Section 4.2.4 and then subjected to flow cytometric analysis using the same settings used to analyse the Quantibrite™ beads. The test samples were randomised before analysis. Twenty thousand events were collected in the single-cell gate for all samples. Following analysis, the data was exported as *.fcs files and imported into FlowJo (v10.8) for data analysis.

In FlowJo (v10.8), gating was employed to eventually extract geometric means for CCR5 PE in test samples (Figure 4-5C). Gating was achieved by plotting the FSC-A against the SSC-A. Once the gate was applied, a plot of FSC-H against FSC-A was used to gate on the single cells. A plot of SSC-A vs fluorescent laser 4 (FL4) was used to gate on the live cells since the FVD emitted light in that FL4 region. Since a FVD that stains dead cells was added, the cells that did not stain with FVD were considered to be the viable population. After gating on the live cells, a histogram

was generated, which showed the shift in CCR5-PE (bell-shaped). The CCR5-PE geometric mean was obtained for each sample. The data was then exported as an Excel file. The information provided for standard BD Quantibrite™ PE beads was used to plot a linear regression graph of Log₁₀ PE molecules per bead against Log₁₀ fluorescence for FL2, seeing as that PE emits light in the FL2 region. The geometric mean of CCR5-PE (Figure 4-5B) for each sample was used to quantify ABS/cell, i.e. the straight-line equation given by the graph in Figure 4-5C was used to calculate the ABS/cell for the unknown test samples. In the equation $y = mx + c$, the Log₁₀ FL2 geomean value (y) for each was substituted followed by solving for x. The antilog of the answer resulted in the ABS/cell for each sample. The data obtained from this experiment was used to choose the optimal concentrations of Pon A and doxycycline needed to induce low and high CCR5 levels in the respective cell lines. This complete workflow from sample preparation and flow cytometric analysis is shown in Figure 4-6.

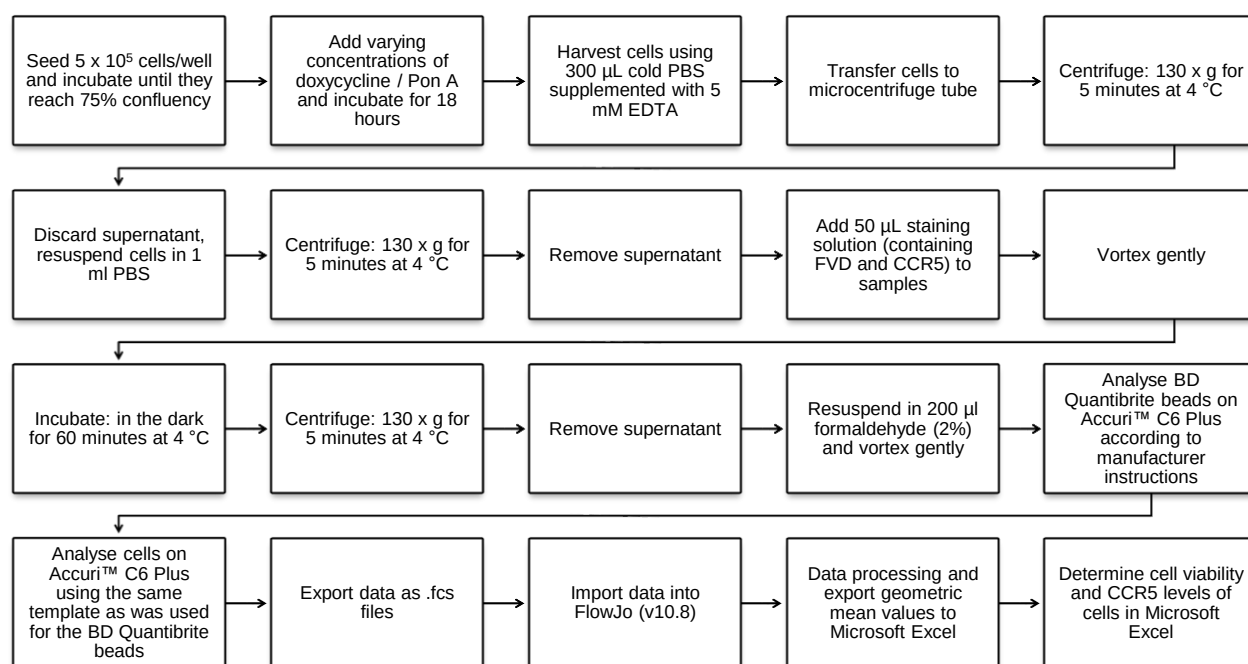


Figure 4-6: Workflow used to determine cell viability and CCR5 expression.

Briefly, cells were seeded and allowed to reach 75% confluency. Then, the respective induction antibiotics were added, and cells were incubated for 18 hours. After 18 hours, cells are harvested using PBS-EDTA and transferred to microcentrifuge tubes. The cells were centrifuged and washed with PBS. Next, cells are resuspended in staining solutions incubated at room temperature in the dark for 60 minutes. After 60 minutes, cells are washed and fixed with formaldehyde. The cells were immediately analysed on the flow cytometer, and the data were processed using FlowJo (v10.8) and Microsoft Excel.

4.2.6 Determination of optimal amount of CCR5-PE for 1×10^6 cells

Saturating amounts of CCR5-PE were needed to determine the amount of CCR5 present on the surface of the test samples. A titration experiment was conducted to determine the required CCR5-PE per sample. A titration experiment requires scientists to prepare aliquots of the same number of cells for flow cytometry using different concentrations/volumes of the antibody of interest. The optimal concentration of CCR5-PE needed to stain CCR5 in 1×10^6 cells was determined using previously published protocols for the Affinofile cells (Webb & Lee, 2016) and the Cf2Th cells (Espy *et al.*, 2017). Before analysing test samples, BD's CS&T beads were analysed according to the manufacturer's instructions. Briefly, Affinofile/HEK293 and Cf2th cells were thawed and passaged as described in Section 4.1 in T25 flasks. The cells were allowed to reach confluency and harvested through trypsinisation, washed with PBS, and resuspended in complete media. Cells were transferred to a T75 flask and allowed to grow in 10 ml complete media until confluent. Cells were then harvested through trypsinisation, washed with PBS, and resuspended in complete media. Cells were split in a 1:3 ratio in T75 flasks. Cells were allowed to reach approximately 75% confluency before adding predetermined concentrations of the induction antibiotics. The Affinofile/HEK293 cells representing low and high CCR5 received 0.26 μM and 13 μM Pon A, respectively (determined in Sections 4.2.4 and 5.1.2). The Cf2Th cells with low and high CCR5 were treated with 26 ng/ml and 1000 ng/ml doxycycline, respectively (Sections 4.2.4 and 5.1.2). For each cell line, one flask remained untreated. The cells were treated with the induction antibiotics for 18h. After 18h, the media was removed, and cells were washed twice with 5 ml PBS. Complete media (10 ml) was added to each flask, and cells were allowed to recover and reach confluency (3 days). These were then harvested by scraping (since trypsin removes the receptors from the cell surface), washed, resuspended in complete media, split in a 1:4 ratio in 60 mm cell culture dishes, and grown in 10 ml complete media. When cells reached confluency, the cells were harvested by scraping, washed with PBS, and counted using trypan blue. Replicate aliquots containing 1×10^6 cells were collected ($n=4$ per sample). Extra cells were collected and prepared to serve as controls, namely untreated unstained and viability controls. The samples were centrifuged at $130 \times g$ for 5 minutes at 4°C . The PBS supernatant was discarded, and cells were resuspended in the respective staining solutions containing CCR5-PE as indicated in Table 4-2 (volumes of CCR5-PE are listed as the product sheet does not supply an exact concentration). Cells were incubated in the dark at room temperature for 60 minutes. Cells received 300 μl fixing solution (2% formaldehyde solution). Cells were centrifuged at $130 \times g$ for 5 minutes at 4°C . The supernatant was discarded, and the cells resuspended 300 μl fixing solution again before storing the samples in the dark at 4°C for 30 minutes after which the samples were immediately analysed on the flow cytometer.

Table 4-2: Staining solutions containing different volumes of CCR5-PE in PBS to determine the optimal amount of CCR5 needed for 1×10^6 cells.

	Staining solution 1	Staining solution 2	Staining solution 3	Staining solution 4
Volume CCR5-PE	2 μ l	4 μ l	10 μ l	20 μ l
Volume PBS	48 μ l	46 μ l	40 μ l	30 μ l
Total volume staining solution (supplemented with 1% FBS)	50 μ l	50 μ l	50 μ l	50 μ l

Abbreviations: CCR5-PE – C-C Chemokine receptor 5 antibodies conjugated to Phycoerythrin; PBS – Phosphate buffer saline; FBS – Fetal bovine serum; μ l – microliter

4.2.7 CCR5 induction in Affinofile/HEK293 and Cf2Th cells

Having determined the concentration of CCR5-inducing reagent to add to Affinofile and Cf2th cells, respectively, (Section 4.2.4), the volume of CCR5 PE needed to stain the induced cells could be determined (Section 4.2.6). For the induction of CCR5 in Affinofile/HEK293 cells, the cells in T75 flasks at P3 were cultured to reach confluency and plated out. In the case of the Affinofile/HEK293 cells, a few dishes with cells were left untreated (topped with complete media only). In contrast, other dishes received induction media, i.e., 0.26 μ M Pon A and 13 μ M Pon A in 10ml complete media, respectively, to achieve low and high CCR5 levels. Similarly, for the Cf2th cells, some dishes with cells were left untreated (topped with complete media only). In contrast, other dishes received induction media, i.e., 26 ng/ml doxycycline and 1000 ng/ml doxycycline in 10 ml complete media, respectively, to achieve low and high CCR5 levels in these cells. The cells were incubated at 37 °C and 5% CO₂ for 18 hours. After 18 hours, cells were removed from the incubator and transferred to the biosafety cabinet. The induction media was discarded, and the cells were washed twice with PBS (130 x g for 5 minutes at 4 °C). Since the antibiotics (whether used for induction or maintenance of the vector) make the cells fragile, 10 ml of complete media were added to the cells allowing for recovery/rest at 37 °C for three days, with 5% CO₂. For Affinofile and Cf2Th cells, there were eight CCR5 high, eight CCR5 low, and four basal CCR5 dishes. For the HEK293 cells, there were five CCR5 high, five CCR5 low, and five basal CCR5 dishes.

4.2.8 Harvest of Affinofile/HEK293 and Cf2Th cells

Culture dishes were removed from the incubator and transferred to the biosafety cabinet to harvest CCR5-low and CCR5-high cells of the respective cell lines. Supernatant was collected into 15 ml falcon tubes for subsequent immune assays. This was further aliquoted for use with the cytometric bead array (CBA) assay (Section 4.2.10). Next, the adherent cells were washed with PBS twice (130 x g, 5 minutes, 4 °C). Trypsinisation is known to remove receptors from the cell surface, and the cells were harvested by scraping in 10 ml PBS at this point (Nowak-Terpiłowska *et al.*, 2021). The cell suspension was transferred to a 50 ml centrifuge tube and counted using the trypan blue method and an automated cell counter (Luna II, Celtic Molecular Diagnostics). Trypan blue confirmed the cell viabilities to be greater than 80%. Flow cytometric analysis verified the cell viabilities and allowed for the determination of CCR5 levels for all samples.

4.2.9 Confirmation of cell viability and CCR5 levels

After harvesting the cells, cell viabilities and CCR5 levels for all samples were confirmed by transferring aliquots of induced cells (1×10^6 cells, Sections 4.2.7 and 4.2.8) to 1.5 ml low bind Eppendorf tubes. Excess cells (1×10^6 cells/aliquot) were used to make up the “untreated unstained” and “viability control” samples for flow cytometry. The cells in the respective tubes were centrifuged at 130 x g for 5 minutes at 4 °C, and the PBS supernatant was discarded. The untreated, unstained control was resuspended in 300 µl PBS and incubated on ice until analysis. The “viability control” was treated with 1 ml ice-cold methanol and centrifuged at 130 x g for 5 minutes at 4 °C. The methanol supernatant was discarded, and cells were washed with PBS by resuspending in 1 ml PBS and centrifuged at 130 x g for 5 minutes at 4 °C. The PBS supernatant was discarded, and cells were resuspended in 49.8 µl PBS supplemented with 1% tetracycline-free FBS and 0.2 µl FVD. All samples (including the untreated, stained sample) were resuspended in a staining solution comprising 29.8 µl PBS supplemented with 1 % tetracycline-free FBS, 20 µl CCR5-PE, and 0.2 µl FVS per sample. Cells were incubated in the dark at room temperature for 60 minutes. After 60 minutes, the samples were fixed by adding 300 µl of 2% formaldehyde solution to each sample. Samples were centrifuged at 130 x g for 5 minutes at 4 °C to wash away unbound antibodies. Cells were resuspended in 300 µl 2% formaldehyde solution and stored in the dark at 4 °C for 30 minutes after which the samples were immediately analysed on the flow cytometer.

Before analysing test samples, BD's CS&T beads were analysed according to the manufacturer's instructions. Once the flow cytometer passed the quality control analysis, BD Quantibrite™ PE beads were analysed according to the manufacturer's instructions (Section 4.2.5). Test samples were analysed in a randomised order. A pre-set medium flow rate of 35 µl/min and 16 µm core

was used to collect the events. The gating strategy followed to determine CCR5 levels within the respective cell lines is shown in Figure 4-7. The first plot (time vs FL4) ensured the fluidics stability of the machine during analysis. The second plot reflects single cells (FSC-A vs FSC-H). Based on the events collected, a threshold of 150 000 was applied to both FSC and SSC to filter out debris. The third plot shows the population of interest (FSC-A vs SSC-A). A gate on the population of single cells was applied, and 20 000 events within this gate were collected. Next, the live cells were gated by excluding the population that took up the FVD. The live population was observed by plotting SSC-A against FL4 (FVD), and gates were applied based on the regions specified by the FMOs to distinguish between CCR5-positive and –negative cells (FL2 axis). A histogram plot of FL2 (since the Quantibrite beads and CCR5-PE reads in the FL2 region) evaluated the MFI of CCR5 during the run and was used to determine the geometric mean in FlowJo (v10.8). Test samples were analysed using the same settings in a randomised order. The data obtained were exported from the Accuri™ C6+, imported into FlowJo (v10.8), and processed as per Figure 4-5. The data were exported from FlowJo (v10.8) into Microsoft Excel, where the MFI was converted into ABS/cell, as explained as part of Figure 4-5 and Figure 4-6. For metabolomics analysis, aliquots containing 3×10^6 cells were transferred to 1.5 ml low bind Eppendorf tubes (see section 4.4.3).

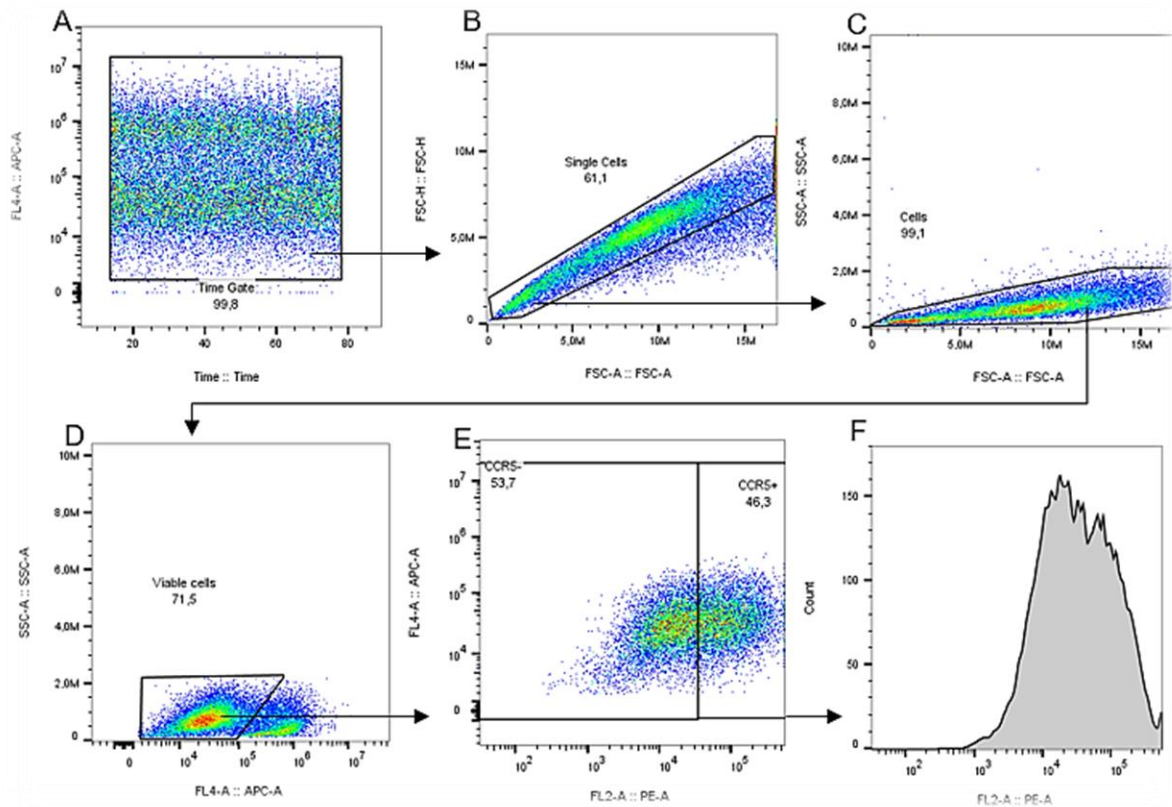


Figure 4-7: Gating strategy used for determining CCR5 levels in the respective cell lines.

Here an Affinofile sample with high CCR5 levels is used as a representative sample. A) A time gate was used to select events that were acquired while the machine showed optimal performance. B) A gate on the single cell population was applied followed by a C) a gate on the entire cell population. D) Viable cells did not take up any FVD and a gate could be applied on those cells. E) The CCR5 levels on viable cells is shown here and by transforming this plot to a F) Histogram allowed for the determination of the geometric MFI which was used to determine the ABS/cell. FVD – Fixable viability dye; MFI – mean fluorescence intensity; ABS/cell – Antibody binding sites per cell.

4.2.10 Immune profile of cells presenting differential CCR5 phenotypes

The role of CCR5 in the immune system was described in Section 2.5. Therefore, it will be helpful to characterise the cytokine profile of cells with low and high CCR5. As previously mentioned, the role of CCR5 in an immune context has not been extensively investigated in the absence of infection. Scientists developed and introduced various methods to measure immunological parameters. One example is to perform an ELISA, which utilises a plate coated with a capture antibody that can bind to an antigen or protein of interest (Crowther, 1995). Unbound products are washed away, and a fluorescent-labelled detection antibody is added. The fluorescence signal produced can be translated into concentrations. More recently, flow cytometry to investigate immune parameters has received increased attention due to the machine's sensitivity,

the lower sample volume required, and cytometric bead array kits which can detect multiple cytokines simultaneously in an assay that is more time-efficient than an ELISA (Shapiro, 2005).

The human Th1/Th2/Th17 CBA kit obtained from BD Biosciences (Lot number: 1032083) was used, and the samples were prepared according to the manufacturer's instructions. The CBA kit utilises capture beads of which the size and fluorescence are known and can detect a range of cytokines. The beads capture cytokines, which can then be detected using a flow cytometer. The cytokines measured using this kit included IL-2, IL-4, IL-6, IL-10, IL-17A, TNF- α , and IFN- γ . Recombinant standards are included in the kit and when mixed with capture beads and PE and analysed on the flow cytometer, it can produce a standard curve of the concentration (x-axis) against MFI (y-axis). The fluorescence intensity detected by the flow cytometer is directly proportional to the amount of that specific cytokine present in the sample. When mixing the capture beads with the unknown samples, the capture beads bind to the cytokines of interest produced by the sample. The addition of PE followed by sample analysis on the flow cytometer produces a signal, and the intensity can be related to concentration using the non-linear logistic graphs (standard curves) generated by FCAP Array. The non-linear logistic graphs are regression models that are constructed for each cytokine bead by plotting the concentration (x-axis) against the MFI (y-axis), giving rise to a sigmoidal-shaped curve since the assay is only linear in a particular concentration range. Further data processing is done using Microsoft Excel.

Cell culture supernatant of the respective cell lines was previously collected as 50 μ l aliquots in 5 ml Eppendorf DNA low bind tubes. A pooled quality control (QC) sample was prepared by thawing the supernatant of untreated cells and cells expressing low and high CCR5 of the respective cell lines. The supernatant was vortexed, and 50 μ l of each sample was transferred to a new 15 ml falcon tube. The pooled sample was vortexed, and 50 μ l aliquots were collected in 5 ml Eppendorf DNA low bind tubes. The assay was performed according to the manufacturer's instructions.

Briefly, the Allophycocyanin (APC)-stained cytokine standards (7 spheres) were transferred to a 15 ml falcon tube. The standards were resuspended in 2 ml of Assay Diluent and incubated at room temperature for 15 minutes. After 15 minutes, the solution was mixed gently through pipetting as vortexing damages the proteins. A serial dilution of the cytokine standards was prepared in 12 different 5 ml Eppendorf DNA low bind tubes, and the appropriate amount of Assay Diluent was added to each tube. The tubes mentioned above were labelled as 'Cytokine Standard Solutions', ranging from 20 pg/ml to 5000 pg/ml. An additional tube was prepared to contain only Assay Diluent to represent a negative control (0 pg/ml). Each standard solution was transferred to control tubes (50 μ l) to prepare the standard solutions for analysis. The capture beads for the cytokines are packaged individually in the kit. The capture beads were vortexed and pooled by transferring 590 μ l of each capture bead solution to the same 15 ml falcon tube. The pooled capture bead solution was vortexed, and 50 μ l was transferred to 5ml Eppendorf low bind tubes

containing samples/ QC's/ standards. To each assay tube, 50 µl of Human Th1/Th2/Th17 PE detection reagent (to detect the cytokines) were added, and all samples were allowed to incubate for 3 hours in the dark at room temperature. Samples were vortexed every 30 minutes. After 3 hours, each sample received wash buffer (1 ml), followed by centrifugation at 200 x g for 5 minutes at 4 °C. The supernatant was discarded, and pellets were resuspended in 300 µl wash buffer.

Three pooled QC samples (50 µl) were prepared and analysed on the flow cytometer in a randomised order to inform on analyst repeatability. In addition, one pooled QC sample (200 µl) was prepared by adjusting the initial ratios of reagents used to prepare a 50 µl aliquot and injected several times during analysis to inform on machine repeatability. After analysing BD's CS&T beads, the test samples were analysed on the BD Accuri™ C6+. Events were acquired according to the template obtained from BD Biosciences. Thresholds of 500 000 were applied for both FSC and SSC parameters. A medium flow rate (35 µl/min) was used to acquire 3000 events in the single-cell gate. The cytokine standards were analysed first from the highest to lowest concentration. The analysis of the test samples followed in a randomised order.

The BD bead library was obtained from BD Biosciences and imported into FCAP Array. The acquired data were exported as *.fcs files and imported into FCAP Array (v3.0). The bead library corresponding to the kit used in this study was selected (bead lot number: 1032083). The beads were then assigned to their corresponding peaks, separated into seven clusters based on their FL4-H MFI (the cytokine populations stained with APC are detected in the FL4 region). Next, the debris were filtered by gating on the populations of interest. After removing the noise from the samples, seven populations in each sample were identified. A built-in calculator was used to assign a concentration to each standard sample to obtain the standard curves for the cytokine standards. The standard curves were automatically generated, and a four-parameter logistic graph was used to fit the data on the standard curve (standard curve graphs are shown in

Figure B1). A regression model was constructed for each cytokine bead by plotting the concentration (x-axis) against the MFI (y-axis), giving rise to a sigmoidal-shaped curve since the assay is only linear in a specific concentration range. These standard curves were used to automatically quantify the cytokine measurements in the test samples using FCAP Array (v3.0). The quantified data from FCAP Array was exported as an Excel file. The data was prepared for statistical analysis by renaming the samples, removing the information related to the standards as these have already been used for quantification, and only keeping the test samples (in rows) with the corresponding cytokine concentrations (in columns). Group labels were assigned to each sample, and the data matrix was saved as a comma separated values (*.csv) file for statistical analysis, e.g., analysis of variance (ANOVA), using MetaboAnalyst (Arts *et al.*, 2016; Tarentini *et al.*, 2021).

4.3 Reagent and sample preparation to determine the metabolomics profile of cells presenting differential CCR5 levels

4.3.1 General reagents needed for GCXGC-TOFMS

Annexure A (Table A1) shows the specific catalogue numbers for each of the reagents used in the respective experiments.

4.3.2 Preparation of specific reagents for GCXGC-TOFMS

The same internal standard stock solution was made once and used throughout the entire study, while the methoxyamine solution was freshly prepared each day, as described below:

4.3.2.1 Internal standard

According to the manufacturer's instructions, the consumables used to prepare the internal standard solution were washed with Liquinox phosphate-free detergent and dried overnight in an oven at 60 °C. The IS used in this experiment included 3-phenyl butyric acid and L-norleucine at a concentration of 50 parts per million (ppm). The internal standards' stock solution was prepared by weighing 100 mg of each compound using separate glass weighing boats. The compounds were washed off into a volumetric flask, and the volumetric flask was filled up to 100 ml with MS-grade water. The respective solutions were sonicated for 30 minutes adequate mixing, and 5 ml of the stock solution was transferred to a clean 100 ml volumetric flask and filled up to 100 ml with MS-grade water.

4.3.2.2 Methoxyamine solution

For derivatisation purposes, a solution of methoxyamine hydrochloride was required. Methoxyamine hydrochloride (400 mg) was weighed using a weighing bottle, followed by the addition of pyridine to a final volume of 20 ml in a volumetric flask, to ensure a final concentration of 20 mg/ml.

4.4 Metabolomics characterisation of cell lines with differential CCR5 levels

4.4.1 Machine and analyst repeatability

Data quality was determined by the machine and analyst repeatability by using a pooled QC sample. The QC sample consists of small amounts of each test sample pooled into one and is thus representative of the sample group. The machine repeatability indicates how repeatable the

analytical instrument functioned and is determined by the coefficient of variance (CV) values of one QC sample, injected multiple times within a batch. The analyst repeatability reports on the repeatability of the analyst being able to perform the extraction, or sample preparation method, and is determined by the CV values of different QC samples injected across multiple batches. These results are presented as quality control-coefficient of variance (QC-CV) plots (Figure 5-18). Analyst and machine repeatability for an untargeted metabolomics approach are deemed acceptable for use when most compounds show a CV of below 50% (Schoeman & Du Preez, 2012).

Five QC samples were prepared and extracted as described previously (Beukes *et al.*, 2019), and were injected in a randomised order on the GCxGC-TOFMS for the purpose of determining the analyst repeatability. A single QC sample was injected consecutively, five times to report on the machine repeatability. Findings are reported in Section 5.3.1.

4.4.2 Optimising sample quenching

Metabolic quenching preserves the metabolic profile of samples, by halting all metabolic activity, ensuring a metabolite snapshot of the sample at the exact same point in time (Pinu *et al.*, 2017). Adherent cells are typically harvested by scraping off the cells, in the presence of an extraction solvent which quenches the metabolism, and in so doing prevents change / loss / leaking of the metabolites. This approach however was not feasible in the context of this experiment since these extraction solvents are known to affect cell viability and some of the cells were subsequently needed for parallel flow cytometric analysis. After harvesting, four alternative quenching strategies were subsequently tested using 3 aliquots of cells per quenching method tested, and the workflow is shown below in Figure 4-8.

Briefly, the metabolic quenching of cells was investigated using 1) ice-cold 0.9% NaCl (0,5 °C) which was added after the cells were harvested, counted and aliquoted (n=3), 2) ice-cold 0.9% NaCl (0,5 °C) added directly after the cells were harvested (prior to cell counting and aliquoting) (n=3), 3) 0.9% NaCl (room temperature) which was added after the cells were harvested, counted and aliquoted (n=3), 4) ice-cold methanol which was added after the cells were harvested (n=3) based on a protocol previously described (Grob *et al.*, 2003). Control samples (n=3) remained unquenched, and these samples were simply kept in 1 ml PBS until whole metabolome extraction could be performed. All samples were stored on ice during this investigation. For this investigation, cells were cultured as described in Section 4.1.4 up to passage 3 in T75 flasks (n = 3), and the supernatant was discarded. The cells were washed with 5 ml PBS, the PBS was discarded, and the cells were harvested by scraping in 10 ml PBS. Next, 7.5 ml of the cell suspension was transferred to an empty 50 ml falcon tube (kept on ice until cell counting), while the remaining 2.5 ml cell suspension was transferred to a 50 ml falcon tube containing 10 ml 0.9% NaCl (0.5 °C,

kept on ice until cell counting), based on a protocol previously described (Sellick *et al.*, 2011). The cells were then counted using trypan blue and the Luna II automated cell counter (Celtic Molecular Diagnostics), and 1.5×10^6 cells were transferred to microcentrifuge tubes. This protocol was followed for both Affinofile/HEK293 and Cf2Th cells and the microcentrifuge tubes contained 3×10^6 cells (1.5×10^6 cells from each cell line). Cells were centrifuged at $130 \times g$ for 5 minutes at 4°C . The supernatant was discarded, and the cells were resuspended in the respective quenching reagents (Figure 4-8). Cells were centrifuged at $130 \times g$ for 5 minutes at 4°C and the cell pellets were stored in the quenching reagents at -80°C until whole metabolome extraction and analysis on the GCxGC-TOFMS the following day.

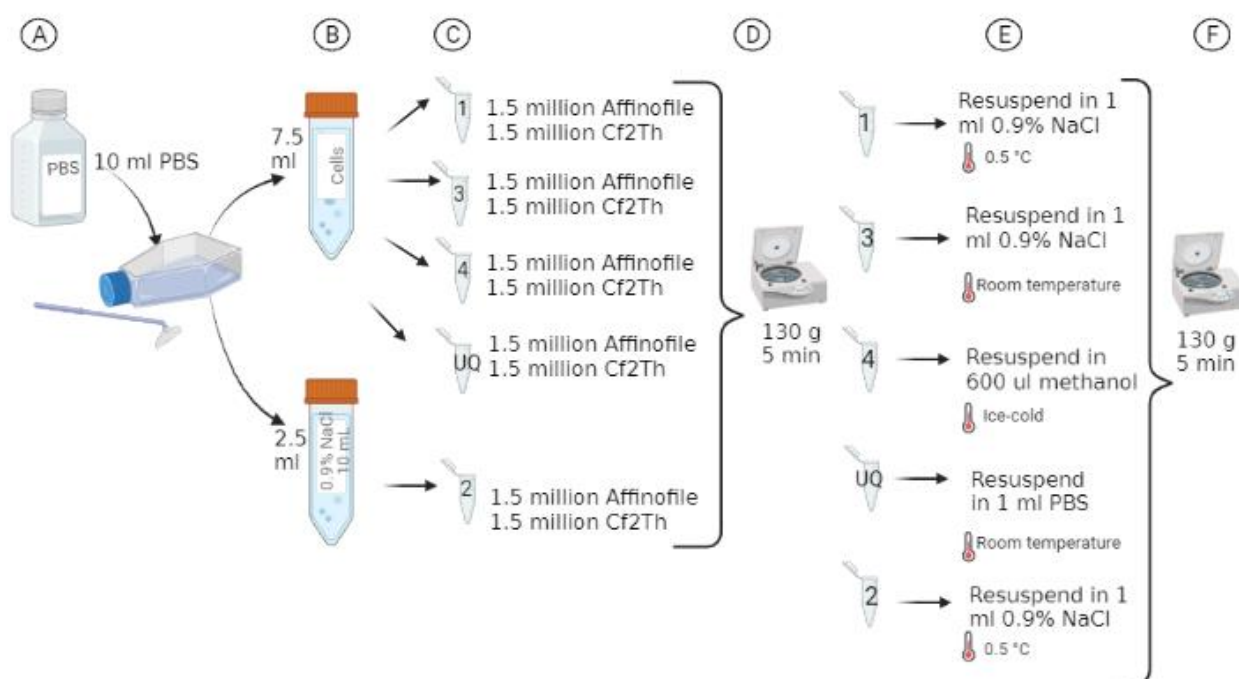


Figure 4-8: Workflow followed to test metabolic quenching using different reagents.

A) The Affinofile cells in a T75 flask were scraped in 10 ml PBS and mixed to form a single cell suspension. B) 7.5 ml of the cell suspension was transferred to one empty 50 ml falcon, while the remaining 2.5 ml of the cell suspension was transferred to a 50 ml falcon tube containing 10 ml 0.9% NaCl (0.5 °C). C) The cells were counted, and 1.5×10^6 cells were transferred to microcentrifuge tubes. The procedure was repeated for the Cf2Th cells, and the microcentrifuge tubes finally contained 3×10^6 cells (1.5×10^6 cells from each cell line). D) The cells were centrifuged at $130 \times g$ for 5 minutes. E) The cells were resuspended in various quenching reagents i.e., tubes 1 and 2 was resuspended in 1 ml 0.9% NaCl (0.5 °C), tube 3 was resuspended in 0.9% NaCl (room temperature), tube 4 was responded in 600 µl ice-cold methanol. The unquenched (UQ) control was resuspended in 1 ml PBS. Each quenching method had three replicate samples. F) The cells were centrifuged at $130 \times g$ for 5 minutes. All tubes were stored overnight at -80 °C, prepared further and analysed on the GCxGC-TOFMS the next day.

The next day, both the quenched and unquenched samples were retrieved from -80 °C freezer and thawed on ice. The samples were centrifuged at $130 \times g$ for 5 minutes at 4 °C. The PBS in the previously centrifuged unquenched samples was decanted, discarded, and washed again with 1 ml cold 0.9% NaCl (since PBS needs to be removed prior to GCxGC-TOFMS analysis, due to its high phosphate content). The three samples quenched with methanol, received 200 µl chloroform and 200 µl water. All other samples were extracted by discarding the 0.9% NaCl and adding 1 ml of the modified Bligh-Dyer extraction solvent consisting of chloroform:methanol:water in a 1:3:1 ratio (Bligh & Dyer, 1959). Fifty µl IS solution (50-ppm) was added to all samples. All samples were further prepared as previously described (Beukes *et al.*, 2019). Briefly, ice-cold acetonitrile (100 µl) was added to each sample, the samples was vortexed on high speed for 15 seconds and incubated on ice for 10 minutes. The samples were then centrifuged at $12\,000 \times g$ for 5 minutes at 4 °C. The extracts were transferred to glass GC vials. The samples were dried under N₂ gas followed by derivatisation using methoxyamine hydrochloride followed by BSTFA.

The derivatives were analysed on the GCxGC-TOFMS according to GC and MS methods as described in Sections 4.4.6 and 4.4.7, respectively. Based on the results obtained in Section 5.3.2, quenching via the addition of ice-cold methanol was used for the remainder of the experiments.

4.4.3 Aliquoting samples and pooled QC-sample preparation

QC preparation: The pooled QC sample contains small amounts of each of the test samples and represents the whole sample group. A pooled QC sample is often added to the injection sequence to assure that the quality of analysis maintained throughout the entire duration of the experiment and can also be used to determine if and correct for, any batch effects in the data.

Test sample preparation: Affinofile, HEK293, and Cf2Th cells were grown in Petri dishes and harvested by scraping in PBS as described in Section 4.1. Cell suspensions were transferred to 15 ml falcon tubes and counted using the trypan blue method. Aliquots of 3×10^6 cells in 1,5 ml Eppendorf low bind tubes were prepared. The QC samples containing 3×10^6 cells were prepared by transferring 1×10^6 cells from each sample to each QC tube, and the final QC sample contained a mix of Affinofile, HEK293 and Cf2Th cells. Cells were centrifuged at $130 \times g$ for 5 minutes at 4°C . The PBS supernatant was discarded, and cells were washed. A pipette was used to remove the PBS supernatant as best possible without disturbing the pellet. The cells set aside for metabolomics were then quenched by resuspending the cells in 600 μl ice-cold methanol and stored at -80°C . Detail on the subsequent handling of the cells for metabolomics investigations and associated normalisation procedures are detailed in Section 4.4.4.

4.4.4 Extraction and derivatisation of samples

In this study, a modification of the Bligh Dyer extraction method (Bligh & Dyer, 1959) was used to remove macromolecules (such as proteins) prior to GCxGC-TOFMS analyses, using chloroform:methanol:water for metabolite extraction from the cells in a 1:3:1 ratio, as described below.

On each day of sample analysis, twelve random quenched test samples (see Section 4.4.2) and one QC sample (all quenched and stored in 600 μl methanol) were retrieved from the -80°C freezer. The samples were allowed to thaw on ice. Once thawed, 50 μl internal standard solution, 200 μl water, and 200 μl chloroform were added to each sample, since all samples were stored in methanol. Samples were extracted by adapting the protocol previously described (Beukes *et al.*, 2019). Briefly, samples were homogenised by adding one 3 mm carbide bead to each sample, then shaking the samples in a vibration mill at 30 Hz for 5 minutes. Acetonitrile (100 μl) was added, and the samples were incubated on ice for an additional 10 minutes, to ensure total protein precipitation. Samples were centrifuged at $12\,000 \times g$ for 10 minutes at 4°C . The supernatants

of all samples were transferred to separate GC vials using a glass Pasteur pipette. The uncapped vials were then transferred to the solvent evaporator heating block set at 35 °C, and samples were dried under a gentle stream of nitrogen gas. Samples were removed from the solvent evaporator, and 50 µl methoxyamine hydrochloride solution was added to each. Samples were vortexed, capped, and incubated at 50 °C. After 60 minutes, samples were allowed to cool down to room temperature and vortexed. Next, 40 µl of BSTFA-1%TMCS was added to each sample using a glass Hamilton syringe, and the samples were again vortexed. Samples were capped and incubated at 60 °C. After 60 minutes, samples were allowed to cool down to room temperature, and the derivatised sample extracts were transferred to GC vials with pulled point inserts using a glass Pasteur pipette. Samples were capped and placed on the chilled autosampler tray (set at 4 °C) of the GCxGC-TOFMS. An extraction blank was also prepared alongside the aforementioned samples, to test for chemicals which may stem from other sources than the biological samples (Fiehn, 2016).

4.4.5 Determination of the split ratios to be used for GCxGC-TOFMS analyses

Method standardisation is vital before test sample analysis on an analytical platform such as GCxGC-TOFMS. Method standardisation requires the analyst to run one sample using different parameters on the machine prior to analyses of the samples (Fiehn, 2016). The samples were prepared as previously described (Beukes *et al.*, 2019). Sample analysis in a split configuration on the GCxGC-TOFMS transfers a certain amount to the column while the rest of the sample is discarded. A split ratio of 1:5 refers to one part of the sample that reaches the column while five parts are discarded as waste. The split ratio used in this project was standardised by injecting the same sample on the GCxGC-TOFMS initially at different split ratios. A split ratio ensures that the column is not overloaded with metabolites from the sample, which would affect the analytical repeatability. Metabolites that do not overload the column reflect as narrow, sharp peaks without peak tailing (Fiehn, 2016). The total ion chromatograms for each injection were evaluated to ensure that the column was not overloaded, and the peaks present were unsaturated. Furthermore, the total ion chromatogram should present as many compounds as possible. In this study, a split ratio of 1:5 showed total ion chromatograms with narrow unsaturated peaks and the greatest number of usable compounds.

4.4.6 GCxGC-TOFMS method parameters

The analysis was done using the Pegasus 4D GCxGC-TOFMS system purchased from Leco (St. Joseph, Michigan, USA), consisting of an Agilent 7890B gas chromatograph, Leco TOFMS and Gerstel autosampler with a cooling sample rack (Gerstel, USA). The GCxGC was equipped with a Rxi-5Sil MS (30m x 0.25 mm i.d., 0.25 µm film thickness) primary capillary column, and Rxi-17 (2m x 0.25 mm i.d., 0.25 µm film thickness) secondary capillary column. The carrier gas used by

this system was helium, set at a flow rate of 1 ml/min. A Restek Topaz 4.0 mm liner with glass wool was used and the injector operated in a split mode. The front inlet septum purge flow (3 ml/min) minimised possible contamination by the septum. The derivatised sample extracts (1 μ l) were injected in a random sequence (as described below) using a split ratio of 1:5, with a front inlet split-flow of 5 ml/min.

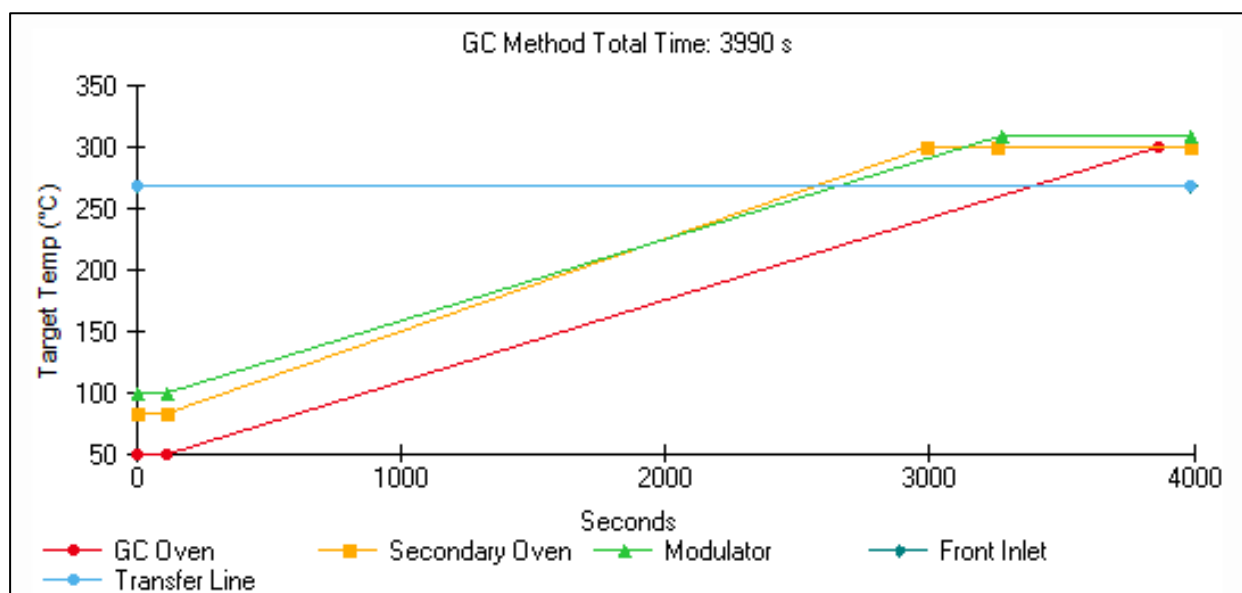


Figure 4-9: Standardised temperatures used during GC analysis of samples.

As shown in Figure 4-9, the front inlet temperature was kept constant at 270 °C for the entire run. The initial oven temperature in the first oven was maintained at 60 °C for 2 minutes. The oven was then heated at a rate of 4 °C per minute until the end of the run. The initial oven temperature in the secondary oven was kept at 85 °C for 2 minutes. The oven was then heated at a rate of 4.5 °C per minute until the end of the run. The modulator was enabled, and the initial temperature of 100 °C was kept constant for 2 minutes. The modulator was then heated at a rate of 4 °C per minute until it reached 310 °C and kept constant for 12 minutes. A modulation period of 300 seconds was used, with the hot pulse time being 0.5 seconds and the cool time between stages being 1 second. A chiller was enabled and set at -70 °C. The transfer line was kept constant at 270 °C throughout the entire run.

The TOFMS acquisition delay was set to 500 seconds. During this time, the filament was turned off to allow solvent elution. Following the solvent delay, 200 spectra per second for masses 50 – 800 m/z were collected. The detector voltage offset was 150V, resulting in the final acquisition voltage of 1490V during analysis. The standard -70 eV was used to achieve fragmentation. The ion source temperature was kept constant at 250 °C. The machine was set to wait for the ion source temperature to reach the target temperature before spectra acquisition.

Figure 4-10 shows the sample injection order followed for each batch of samples. An empty vial, hereafter referred to as the system suitability blank, was placed in position one and was injected during the run to clean the column. A vial containing a 50-ppm FAMES solution was placed in the second position and injected before and after each batch as an additional quality control measure of machine performance. An extraction blank was prepared alongside the samples (prepared the same way as the samples but do not contain cells) and placed in position three to monitor artefacts that stem from the solvents used. Finally, a QC sample was prepared with each batch, loaded in position four, and injected multiple times throughout the batch analysis. Positions five to sixteen were filled with samples, in random order.

Sample	SB	F	EB	Q	Q	S 1-4	SB	Q	S 5-8	SB	Q	S 9-12	Q	Q	EB	SB	F
Position	1	2	3	4	4	5-8	1	4	9-12	1	4	13-16	4	4	3	1	2

Figure 4-10: Injection order of samples on the GCxGC-TOFMS and the position of the respective vials as placed on the chiller.

Abbreviations: SB: System blank; F: FAMES; EB: Extraction blank; Q: Pooled QC; S: Sample.

Following sample analysis of all the samples in a series of batches, the data was processed using Chromatof software (version 4.72, Leco, St Joseph, Michigan, USA).

4.4.7 Data processing method parameters

Chromatof (v4.72) was used to calculate the baseline, detect the peaks above the baseline, identify the peaks by comparison to a library composed from previously injected standards and calculate the areas of the peaks. The baseline offset was set just above the noise, and an automated number of data points were used for smoothing the peaks. A peak width of 50 seconds for the first dimension and 0.2 seconds for the second dimension was selected. The spectral match required to combine modulated peaks was 650 (65%). The minimum required signal-to-noise ratio was set to 50 to retain a subpeak. Peaks were identified by searching the acquired spectra against the National Institute of Standards and Technology (NIST) library with a minimum match of 700 (70%) before assigning a name to the peak.

Peak alignment forms a part of the data processing in Chromatof. The spectral match was set with a mass threshold of 10 (1%) and a minimum similarity match of 600 (60%). The retention time match was set with the maximum number of modulation periods apart as one and the

maximum retention time difference as zero seconds. The signal-to-noise ratio was set to 20 to search for peaks not found by the original peak finding parameters. Matches were kept if they appeared in at least five samples or 50% of samples in a class. The fisher ratio remained undefined.

4.4.8 Data pre-processing

The aligned peak data from Chromatof was exported into Microsoft Excel. Data pre-processing is required to eliminate variation from the data matrix. For each identified analyte, the raw data matrix contained the following information: peak area, retention times for both columns, and the unique mass. Compounds detected inherent to the reagents used (and not the biological samples), such as pyridine for instance, were removed from the data matrix. The peak areas were merged for the same analytes if they had the same retention times and unique masses. Several normalisation strategies were considered initially, but due to the time constraints of DNA and/or protein content determination these strategies were excluded. In addition, normalisation using the total useful signal did not work well with this data set (troubleshooting beyond the scope of this MSc). Therefore, the data were normalised using an internal standard (Section 4.4.9). A 50% zero filter was applied, i.e., the number of zero values for each compound in every group was determined, and if the compound had more than 50% zero values in all sub-groups (basal, low, and high CCR5 levels for all cell lines), the compound was removed from the data set. Due to many of the metabolites' low abundance / concentrations, missing / zero values are often present in MS data matrices (Wei *et al.*, 2018). These are replaced with a value equal to half of the lowest concentration, when processing the data on MetaboAnalyst, since missing values can affect the output (Gromski *et al.*, 2014). The data quality was determined by importing the dataset into MetaboAnalyst and subjecting the data to principal component analysis (PCA). A QC-CV filter was applied, i.e., the coefficient of variation (CV) for all the compounds was determined using all QC samples, and, hypothetically, a cut-off of 50% was applied and the compound was removed from this untargeted dataset if the CV value was greater than 50% (Schoeman *et al.*, 2012).

4.4.9 Data normalisation

Various methods can be applied to normalise metabolomics data. Pre-acquisition normalisation strategies allow the analyst to collect similar samples, e.g., the same number of cells in each sample, adjusting the volume of urine samples so that all samples have a set amount of creatinine, etc. Post-acquisition normalisation approaches allow the analyst to compensate for errors that might have occurred during sample preparation and analysis during data processing by using a known set of values, such as IS concentration and dividing each datapoint in the matrix by the normalisation factor (Sysi-Aho *et al.*, 2007).

This study applied pre-acquisition normalisation by collecting approximately 3×10^6 cells for each sample. During sample preparation for GCxGC-TOFMS, 50 μ l of a 50-ppm solution containing 3-phenylbutyric acid and L-norleucine as internal standards were added to samples to allow for post-acquisition normalisation. Following sample analysis, the background metabolite data of the HEK293 cells was subtracted from the Affinofile data. Next, the entire data matrix was normalised using 3-phenylbutyric acid concentrations. Here, the peak area of the metabolite was divided by the peak area of the internal standard for that specific sample. Using the known concentration of 3-phenylbutyric acid, relative concentrations of the metabolites present in this data matrix was subsequently calculated and expressed as μ g/ml.

4.4.10 Quality assurance and data preprocessing

Transformation: Metabolomics data is often transformed to achieve normality where data skewness occurs. Various data transformation approaches exist (van den Berg *et al.*, 2006). Log transformation calculates the logarithmic value of each data point in the matrix. As a result, the logarithmic values are closer to one another than the original values, making all values in the data matrix more comparable (Karaman, 2017). In this study, log transformation was applied to adjust for the variation in the abundance of different metabolites and thus reduce variation in the data matrix. Log transformation was achieved by applying the $\log_{10}$ equation to all the concentrations in the data set (this was done automatically using MetaboAnalyst).

Data scaling: Much variation when comparing the data between different samples can exist in a metabolomics data matrix e.g. the abundance of metabolites measured (Hendriks *et al.*, 2011). Metabolites with low abundance are often masked by metabolites with a higher abundance in the data. The data can be scaled according to the data dispersion (e.g., standard deviation) or size (e.g., the mean) to account for the variation in the data matrix. Scaling is achieved when each concentration is divided by a factor that differs among variables (Karaman, 2017). An auto-scaling function was used, which centred the data around the mean before dividing by the standard deviation of each variable. Auto-scaling results in a standard deviation of 1 for each metabolite. Scaling aims to give all metabolites equal weights in the dataset, making them more comparable regardless of their abundance. The data were scaled using MetaboAnalyst (van den Berg *et al.*, 2006).

Principal component analysis: Principal component analysis (PCA) is a multivariate statistical technique performed to inspect data and its quality visually. It is an unsupervised method (where group labels are undefined/unknown). It is used to inspect the unbiased natural separation within a group and between groups (Davies & Fearn, 2004). Low intra-group variations will result in the tight clustering of samples within a group (Abdi & Williams, 2010). Each group has a confidence interval observed in the PCA plot. In theory, QC samples should cluster in the middle of the test

samples (as it is a pooled QC and representative of all the samples) with a small confidence interval. PCA allows the researcher to observe batch effects (if any) and is observed when several QCs of the same batch cluster away from the rest of the QCs. The principal component (PC) percentage values portrayed on the x-axis and y-axis refer to the amount of variation explained, i.e., a small percentage relates to a low variation between the groups and thus a low separation between groups. The data is viewed in fewer dimensions, allowing the researcher to observe possible trends. Therefore, PCA is a valuable tool for predicting the outcome of the data (Antonelli *et al.*, 2019).

4.4.11 Statistical methods applied to untransformed data

Univariate statistical analysis aid in examining one variable at a time (Saccenti *et al.*, 2013). The untransformed data was subjected to effect size (ES) statistical analysis, indicating the practical significance, and related to the degree of importance between the two groups. First, each group's average concentration and standard deviations were determined. Then, the difference in average concentrations was divided by the maximum standard deviation for that compound, resulting in a d-value. ES of $d = 0.8$ and higher indicate a large ES (Steyn, 2009). All compounds with a d-value > 1 were considered significant in this study. The strict cut-off values allowed for the identification of biomarkers that differed the most between groups.

4.4.12 Statistical methods applied to transformed data

After data transformation and auto-scaling, samples were stratified according to CCR5 levels (basal, low, and high). The data matrix for each cell line was then subjected to multivariate analysis, including a PCA (to determine if natural separation of the compared groups exists) and partial least squares discriminant analysis (PLS-DA) to identify those variables that best describe the variance between groups. Finally, the data were subjected to one-way analysis of variance to obtain statistically significant variables in the data. Both the PLS-DA and ANOVA will be explained below.

PLS-DA: PLS-DA maximises the covariance of metabolic data points in the data matrix (Antonelli *et al.*, 2019). PLS-DA is a supervised statistical method, since the group/data labels are provided and used by the algorithm to separate the groups. The PLS-DA scores plot allows the researcher to visually inspect the separation caused by the maximum covariance. Additionally, the researcher can obtain a list of variables important in projection (VIP). The VIP metabolites are ranked according to their influence on the PLS-DA scores plot, however, the ratio of samples to the number of metabolites influences this classification. Therefore, it is advised to include exponentially more samples, than the number of metabolites present in the dataset (Westerhuis *et al.*, 2008), which is often not the case for metabolomics analysis. Therefore, the models used

to describe the classification needs to be validated, to determine their accuracy. All compounds with a VIP value > 1 were considered significant in this study if the PLS-DA could be validated.

One such method, is the leave-one-out cross-validation (LOOCV) approach, where a single sample is removed from the data set, and a PLS-DA is obtained for all other samples. Next, the sample is placed back into the PLS-DA model, predicting the classification of the removed sample, and again repeated for the rest of samples individually. The Q^2 value, is the prediction relevance, while the R^2 value is the predictive accuracy of the model. The predictive accuracy can be classified as strong ($R^2 = 0.67$), moderate ($R^2 = 0.33$) or weak ($R^2 = 0.19$). The Q^2 value indicates the discrimination validity (Westerhuis *et al.*, 2008) and determines the reproducibility of the model, hence a Q^2 value close to the R^2 value indicates a reproducible model. The classification model is further validated by permutation analysis to determine the prediction accuracy of the current classification model. The labels are assigned randomly to different samples during the permutation analysis, and a new classification model is determined, and then repeated several times. Permutation analysis results in a statistical p -value related to the model's prediction accuracy.

ANOVA: ANOVA is a univariate statistical approach similar to a t-test (Mishra *et al.*, 2019) although it is used to determine whether the means of three or more groups differ significantly, by determining the origin of the variation within the samples (McHugh, 2011). However, the specific identification of the patterns are analysed with post-hoc tests, in which the means are compared in pairs, e.g. Fisher's least significant difference (LSD) test (Williams & Abdi, 2010). In this study, the adjusted p -value (FDR) cutoff was set to be 0.05 i.e., if $p < 0.05$, the compound was identified as significant.

4.4.13 Confirming compound IDs

Following peak alignment, all compounds were identified by comparison to in-house libraries, containing mass spectra, obtained from previously injected standards. In Chromatof, the spectral matches for each compound can be observed, and possible other identifications based on the spectra also obtained. A forward spectral match refers to the degree to which the spectra obtained for the unknown compound match the spectra of the matched library compound. A reverse spectral match, however, refers to the match of the library spectra to the spectra obtained by the unknown compound. Both spectral matches are represented by a value of 0-999 and read as a percentage. A higher spectral match value relates to a better match between the spectra. The probability is 0 – 10 000 and represents the likelihood that the compound spectra match the library search algorithm's spectra. Each statistically significant compound was evaluated for misidentifications by visually inspecting the spectra and assessing the parameters mentioned above, which is also often useful for finding matches for unidentified compounds. An example of

this is given in Figure 4-11, where the peak (A) and library spectra (C) of glycine are shown. In this example, glycine had a similarity of 937, a reverse similarity of 959, and a probability of 9028, suggesting that the spectra obtained in the test samples are identified as glycine (90.28%) based on the similarities of the spectra, which is a level three identification. To obtain a level five identification, retention time matching is required (Schymanski *et al.*, 2014).

Peak True - sample "C.U.3_1", peak 161, at 1268 , 0.050 sec , sec

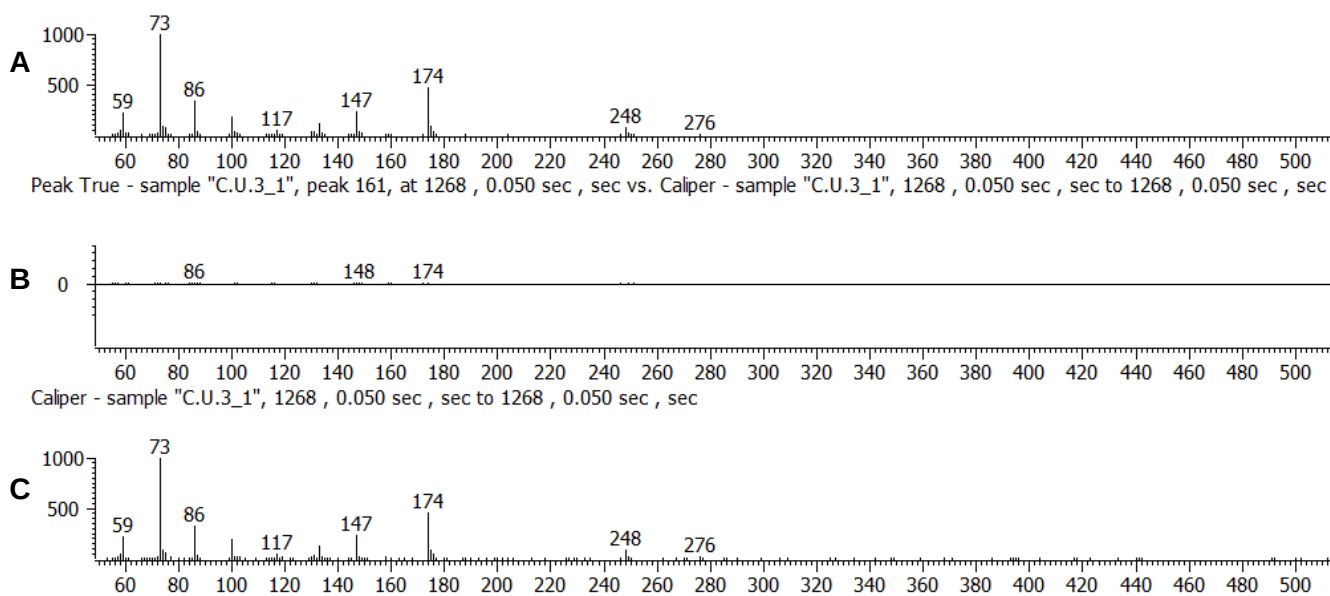


Figure 4-11: Example of peak confirmation as it relates to Glycine 3 TMS. A) Shows the spectra obtained for the unknown sample, B) the difference between the unknown peak spectra and library spectra, and C) shows the library spectra. For this example, the retention times given by the mass spectrum were the same for these compounds.

CHAPTER 5: RESULTS AND DISCUSSION

5.1 Flow cytometric characterisation of cell lines

5.1.1 Antibiotic concentration in which to culture the Cf2Th cells

After expanding the cell lines, a study was conducted to determine the optimal concentrations of antibiotics to use in which to grow the Cf2Th cells. Cells were cultured until confluent before they were passaged; thus, it was important that the cells remained viable in these antibiotics up to this time. Briefly, cells were treated with four different combinations of mixed antibiotics (Section 4.2.2) and cell viabilities were determined after 0, 1, and 3 days, respectively (Figure 5-1), by staining with Annexin V-FITC and 7-AAD (Section 4.2.3). As previously mentioned, the experiment was repeated three times with two replicates of each sample. Repeats of the experiment allowed for the determination of the standard deviation (variation between replicate samples in a day) and the standard error of the mean (variation between repeated experiments based on the average obtained for the replicates) to visualise the deviations that occurred among repeats for each group of the experiment. The untreated samples at day 0 showed cell viabilities of 92% and higher across all repeat experiments (Figure 5-1, green bars). Regarding the treated cells on day 1 (Figure 5-1, blue bars), combinations 1, 2, and 4 presented the highest cell viabilities, while combination 3 presented slightly lower cell viabilities across all repeats. Similar results were obtained on day 3 (Figure 5-1, purple bars), where combinations 1, 2, and 4 presented higher cell viability than combination 3.

Based on these results, combination 3 was excluded as a possibility in which to culture the Cf2Th cells. Combination 1 comprised of 0.6 µg/ml puromycin, 400 µg/ml G418, 0.025 µg/ml zeocin and 200 µg/ml hygromycin and is the same antibiotic concentrations as reported previously (Espy *et al.*, 2017). Combinations 2 and 4 were adapted to contain a lower concentration of hygromycin (100 µg/ml). Chen and co-workers (1995) conducted a previous study that showed that hygromycin induces apoptosis in susceptible cells (Chen *et al.*, 1995). The slightly higher cell viabilities seen for combinations 2 and 4 are thus in agreement with the lower concentrations of hygromycin used. Even though the cells from all combinations presented with cell viabilities greater than 80%, the minimal cell death experienced was caused by apoptosis (data not shown). Combination 3 comprised increased concentrations of zeocin which induced more cell death in the Cf2Th cells. The deviations observed for Day 0 were minor. However, the standard error increased for Day 1 and Day 3. This makes sense since the cells analysed on Day 0 did not receive any treatment and were harvested after less culture time. In comparison, the cells on Day 1 and Day 3 received treatment and were harvested after more culture time, introducing some variance (Čuperlović-Culf *et al.*, 2010). Combination 1, previously reported by Espy and co-

authors (2017) (which is also the source that donated the cells for this study) was thus chosen as the working combination of antibiotics to culture the Cf2Th cells. Taking cell viabilities after 3 days and lower standard error among repeats further supported this choice.

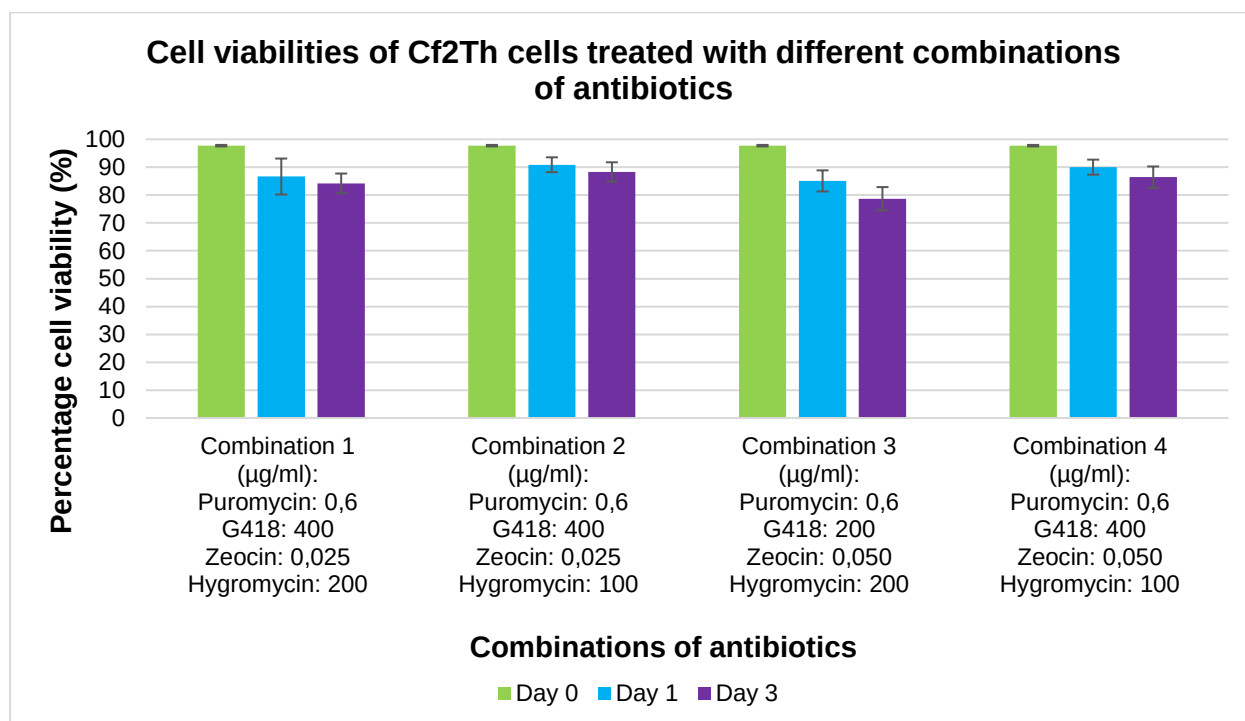


Figure 5-1: Percentage cell viability for Cf2Th cells treated with various concentrations of antibiotics and analysed on the flow cytometer after zero, one, and three days, respectively.

Combination 1 contained the same antibiotic concentrations as reported by Espy *et al.* (Espy *et al.*, 2017). Combinations 2, 3 and 4 were adapted from combination 1. Combination 3 resulted in the most cell death on Day 3, and this combination of antibiotics was excluded as a possibility to culture the Cf2Th cells. Despite combinations 2 and 4 showing higher cell viabilities (especially days 1 and 3) than combination 1, this mixture of antibiotics was chosen for the reasons listed above. Values for each method represent the average of three replicates, and error bars show the average $\pm$ standard error of the mean. Green bars represent day 0, blue bars represent day one, and purple bars represent day 3.

5.1.2 Determination of the optimum concentration of CCR5-inducing reagents (Pon A and doxycycline) to achieve CCR5-low and CCR5-high cells

Cells in different wells received various concentrations of Pon A to induce CCR5 levels in Affinofile cells (as depicted in Figure 4-4). The cells were subjected to flow cytometry after staining the treated cells with the FVD and CCR5-PE. FVD was incorporated to distinguish between live and dead cells. Following analysis on the flow cytometer, the data obtained were processed using FlowJo (v10.8) and Microsoft Excel (2016). The counts collected for the untreated cells (0 µM Pon A) seem slightly higher than those treated with an increasing gradient of Pon A, even though 10 000 events were collected for all samples. The counts between untreated and treated samples (y-axis, Figure 5-2) appear different in Figure 5-2 (Affinofile histogram overlay), which shows

treated samples to be asymmetrical (peak tailing). The asymmetrical peak shape reflects increased CCR5 induction in only a percentage of the cells (not one homogenous shift in CCR5-PE for all cells to the right). CCR5 expression is tightly regulated (Oppermann, 2004a). Indeed, past studies have shown that while maximum induction is achieved, this only happens in some cells (Johnston *et al.*, 2009). The asymmetrical peak shape in the histograms is thus in agreement with previous reports.

It is also evident from Figure 5-2 that Pon A induced a concentration-dependent increase in the levels of CCR5, evident by the increase in MFI of CCR5-PE as the amount of Pon A tested increases except for the highest concentration tested. In this study, maximum induction with 52 μ M Pon A was anticipated. However, this concentration resulted in detachment and a decrease in the number of viable cells (visible through microscopic examination and cell counting). This concentration also yielded less CCR5 than the lower concentrations of Pon A, suggesting that CCR5 expression is more than likely regulated in Affinofile cells. The decreased CCR5 may also be due to the lower cell viability associated with this sample. Based on the MFI shift observed in Figure 5-2A, it is clear that the use of Pon A should remain between 3.25 μ M and 26 μ M to induce maximum CCR5 levels and minimum cell death (Figure 5-2B). To distinguish between live and dead cells, FVD was incorporated into the staining panel. Those cells with an increased shift to the right (dotted line in Figure 5-2B) represent a control for dead cells while the cells with an MFI to the left of the dotted line represent viable cells. As previously discussed in Section 3.4, the use of Pon A influences various signalling pathways and might affect the immune and/or metabolic profiles. Therefore, the aim was to use the lowest possible concentrations of Pon A, which yielded the highest levels of CCR5 without impacting cell viability and integrity. Thus, 13 μ M Pon A was used to induce high levels of CCR5 in the Affinofile cells, given overall increased MFI compared to the other concentrations tested. Since metabolic analysis and comparisons of the metabolism of the cells with low and high CCR5 levels was planned, a small amount (concentration) of Pon A (0.26 μ M) was added to the cells that represented the low-CCR5 group.

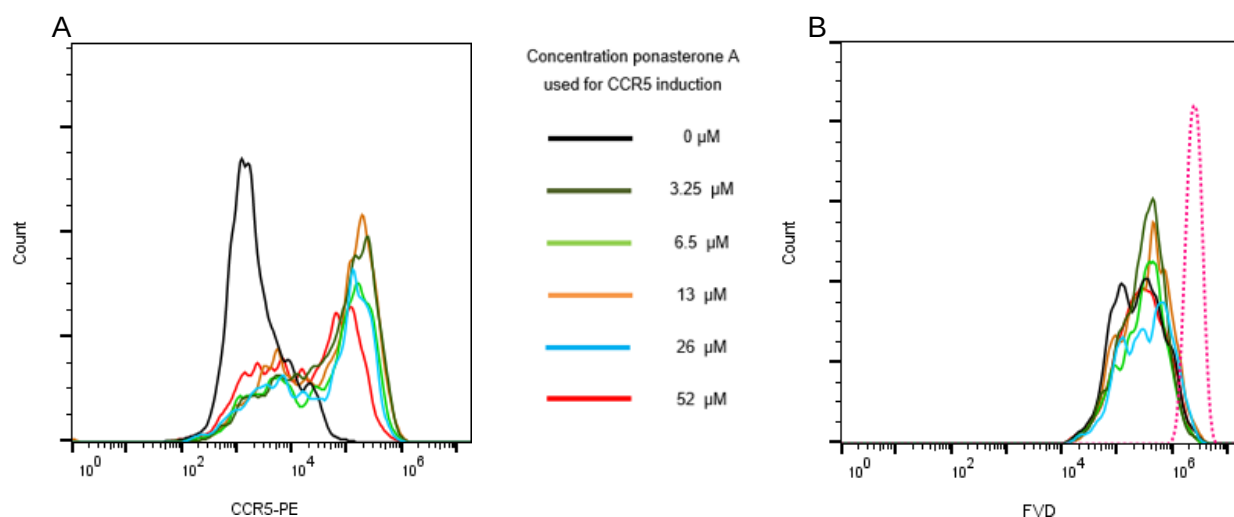


Figure 5-2: Histogram overlays showing the shift in MFI of CCR5-PE of the A) live Affinofile cells when treated with various concentrations of Pon A, and B) the cell viabilities of cells tested with various concentrations of Pon A, with the control for dead cells included (pink dotted line).

Cells in different wells received various concentrations of doxycycline to induce CCR5 levels in Cf2Th cells (as depicted in Figure 4-4). After staining the treated cells with FVD and CCR5-PE, the cells were subjected to flow cytometry. Following analysis, the data were processed using FlowJo (v10.8) and Microsoft Excel. As seen in Figure 5-3 (Cf2Th histogram overlay), the addition of doxycycline increased the levels of CCR5. The MFI of CCR5-PE increases (shift to the right) as the amount of doxycycline tested increases. Like the observations for the Affinofile cells, the counts collected for the Cf2Th cells treated with 0 ng/ml doxycycline appear higher than cells treated with an increasing gradient of doxycycline even though 10 000 events were collected for all samples. Here, the shape of the peak is asymmetrical, reflective of increased CCR5 induction in only a percentage of the cells (not one homogenous shift in CCR5-PE for all cells to the right), ascribed to CCR5 regulation processes, consistent with data reported by others too (Espy *et al.*, 2017).

It is also evident from Figure 5-3A that doxycycline induced a concentration-dependent increase in CCR5 levels – this is shown by the increase in MFI of CCR5-PE as the amount of doxycycline tested increases. All concentrations tested in this study did not influence cell viability significantly, evident by the shift in the signal of the FVD, which remained left of the control for dead cells (right shift) (Figure 5-3B). As previously mentioned in Section 3.4, the use of doxycycline influences mitochondrial function and might affect the immune and/or metabolic profiles. Therefore, the aim was to use the lowest possible concentrations of doxycycline, which yielded the highest levels of CCR5 without impacting cell viability and integrity. Thus, 1000 ng/ml doxycycline was used to induce high levels of CCR5 in the Cf2th cells. In addition, since metabolic analysis and a comparison of the metabolism of cells with low and high CCR5 levels was planned, a small

concentration of doxycycline (26 ng/ml) was added to the cells that represented the low-CCR5 group. Compared to the shape of the Affinofile cells (Figure 5-2B), the shape of the viable Cf2Th cells in Figure 5-3B is sharp, narrow and symmetrical.

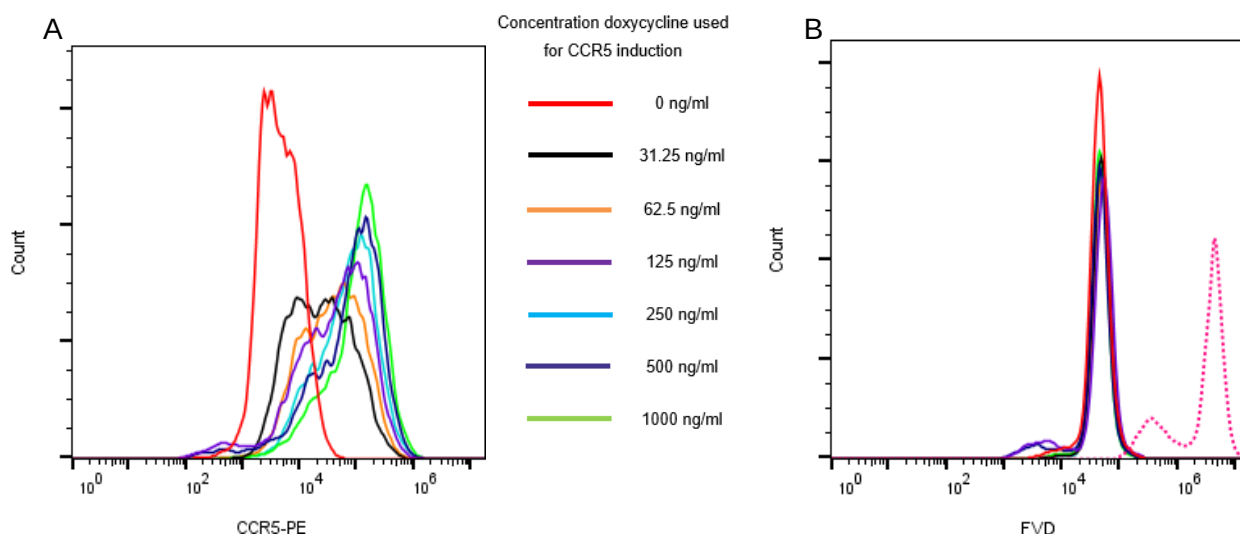


Figure 5-3: Histogram overlays showing the shift in MFI of CCR5-PE of the A) live Cf2Th cells when treated with various concentrations of doxycycline, and B) the cell viabilities of cells tested with various concentrations of doxycycline, with the control for dead cells included (pink dotted line).

5.1.3 CCR5 quantification using BD Quantibrite™ PE beads

After performing the assay to determine the concentration of induction reagents to use (Section 4.2.4), CCR5 levels associated with these induction reagents were quantified using BD's Quantibrite™ PE beads/standards by converting the MFI of CCR5-PE (Figure 5-2A and Figure 5-3B, shift along the x-axis) to a quantitative value, i.e. ABS/cell (Section 4.2.5). As seen in Figure 5-4, the basal ABS/cell for Affinofile cells was approximately 20 000 without treatment and calculated to be approximately 50 000 with low (0.26 μ M) Pon A treatment (calculations explained in Section 4.2.5). The maximum levels of CCR5 induced in the Affinofile cells (using 13 μ M Pon A) were approximately 150 000 ABS/cell. Here, maximum CCR5 levels at 13 μ M Pon A is more clearly visible post quantification of the MFI CCR5-PE shift than simply performing a qualitative visual inspection of Figure 5-2A.

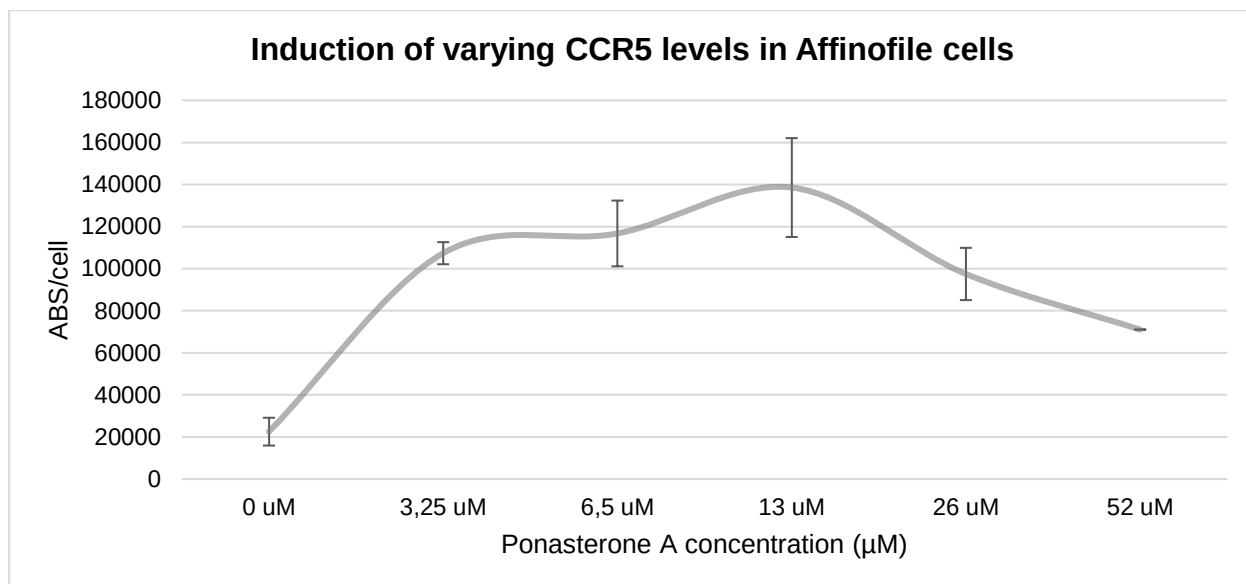


Figure 5-4: Graph showing quantified levels of CCR5 induced by different concentrations of Pon A in Affinofile cells.

Values for each method represent the average of two replicates, and error bars show average $\pm$ standard deviations. ABS – Antibody binding sites; μ M - micromolar.

Similarly, in Figure 5-5, baseline ABS/cell for the Cf2Th cells was approximately 38 000 without treatment and calculated to be approximately 40 000 with low (26 ng/ml) levels of doxycycline treatment. Maximum CCR5 levels were induced by treating the cells with 1000 ng/ml doxycycline, which equated to approximately 105 000 ABS/cell.

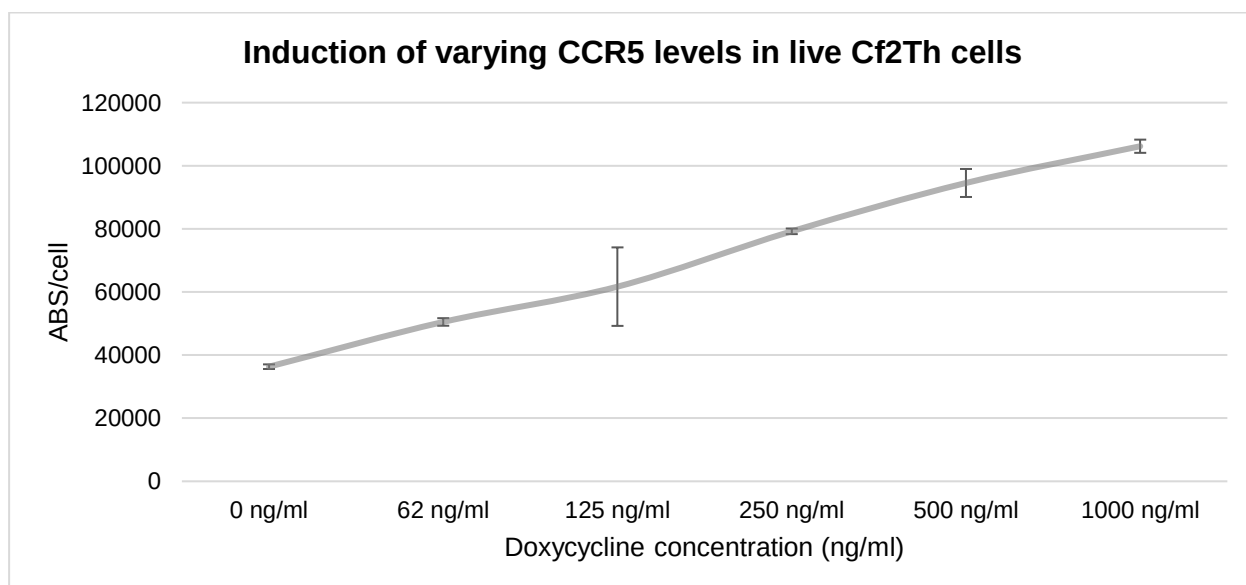


Figure 5-5: Graph showing quantified levels of CCR5 induced by different concentrations of Doxycycline in Cf2Th cells. Values for each method represent the average of two replicates, and error bars show average $\pm$ standard deviations. ABS – Antibody binding sites; ng/ml – nanogram per millilitre.

The peak shapes and associated shifts on the histograms obtained for the test samples (Figure 5-2A and Figure 5-3A) are similar to previously reported studies (Espy *et al.*, 2017). In addition, the basal ABS/cell measured agrees with that obtained in other studies (see Table 3-1).

5.1.4 Determination of optimal amount of CCR5-PE needed for 1×10^6 cells

To determine the optimal concentration of CCR5-inducing reagent needed to induce CCR5-low and CCR5-high cells, the methods of Webb and Lee (Webb & Lee, 2016) and Espy *et al.* (Espy *et al.*, 2017) were followed. These protocols seeded 5×10^5 cells in 24-well plates and stained the cells with 2 μL CCR5-PE. In this study, cells were grown in large Petri dishes for parallel assays to be performed. In addition, an aliquot containing 1×10^6 cells was collected to confirm CCR5 levels in the test samples. Mathematically, for 1×10^6 cells, 4 μL CCR5 would thus be required. However, various volumes of CCR5-PE were used to determine an equivalent amount of CCR5-PE to use for 1×10^6 cells as the manufacturers recommended saturating amounts of CCR5-PE. Therefore, cells were seeded in cell culture dishes described in Section 4.2.6 and treated with Pon A/doxycycline, followed by incubation for 18 hours. The samples were harvested by scraping, and aliquots containing 1×10^6 cells were stained with varying volumes of CCR5-PE (Section 4.2.6, Table 4-2) and subjected to flow cytometry analysis. The acquired data was processed in FlowJo (v10.8) and Microsoft Excel.

For Affinofile cells (Figure 5-6), it is evident that the use of 2 μL CCR5-PE results in the same estimated ABS/cell for untreated and treated cells, which is approximately 1250 ABS/cell. However, the use of higher volumes of CCR5-PE results in an increase in quantifiable CCR5. For example, 20 μL CCR5-PE resulted in the untreated cells presenting 2000 ABS/cell, which represents basal CCR5 levels. In addition, the cells with low levels of CCR5 presented approximately 3000 ABS/cell, and the cells with high levels of CCR5 presented 3500 ABS/cell.

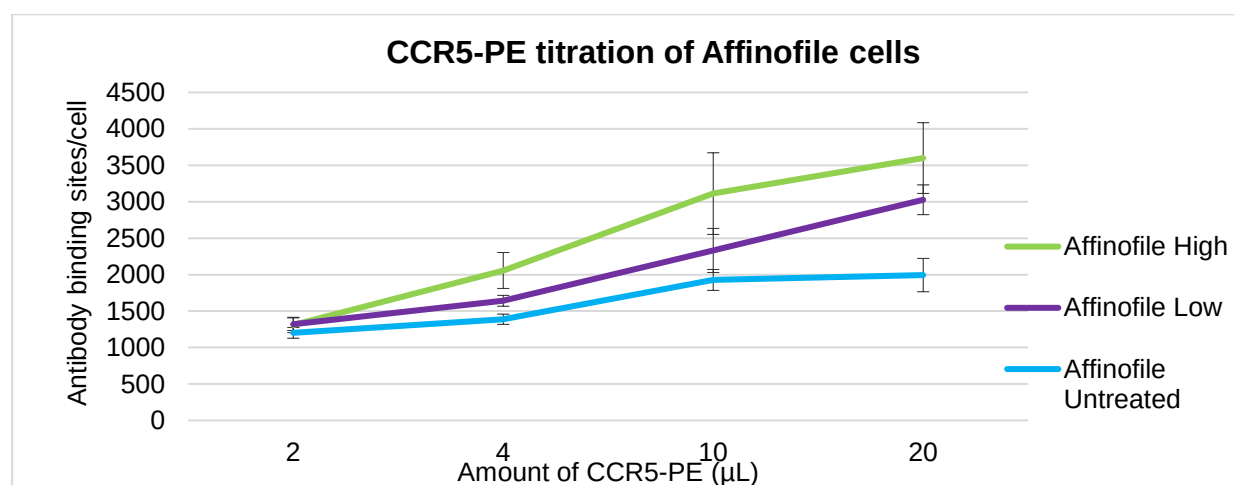


Figure 5-6: Graph depicting CCR5 levels of 1×10^6 Affinofile cells stained with different volumes of CCR5-PE.

For Cf2Th cells (Figure 5-7), the use of 2 μ L CCR5-PE results in a similar estimated ABS/cell for untreated and CCR5-low cells, approximately 2100 ABS/cell. The use of higher volumes of CCR5-PE results in an increase in quantifiable CCR5. Surprisingly, more CCR5-PE up to 20 μ L yielded the same amount of CCR5 for cells with basal and low levels of CCR5, with the maximum estimated ABS/cell for cells with basal and low CCR5 levels being just below 4000 ABS/cell, which suggests that CCR5 may be more tightly regulated in the Cf2Th cells. Twenty μ L CCR5-PE for cells with high levels of CCR5 resulted in approximately 6000 ABS/cell. For the respective cell lines, no more than 20 μ L CCR5-PE volumes were tested, given that this volume differentiated CCR5-low and CCR5-high expressing cells, and the cost associated with the reagent. The use of 20 μ L CCR5-PE was also the manufacturer's recommended volume.

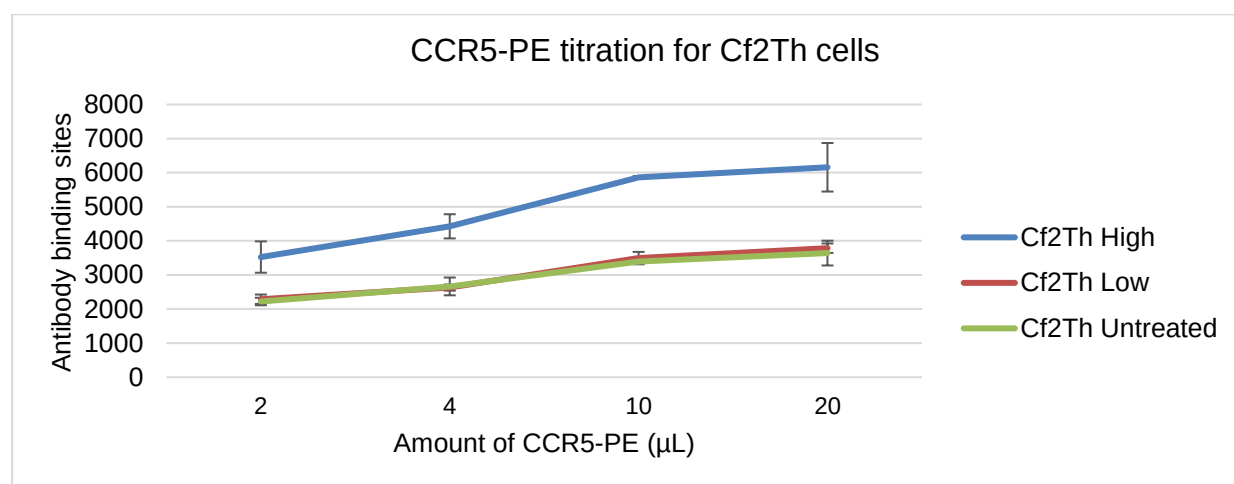


Figure 5-7: Graph depicting CCR5 levels of 1×10^6 Cf2Th cells stained with different volumes of CCR5-PE.

Based on the results shown in this section, CCR5 induction performed in the 24-well plates resulted in much higher CCR5 levels (105 000 ABS/cell, Figure 5-4 and Figure 5-5) CCR5 levels compared to the cells seeded in large dishes (3000 – 6000 ABS/cell, Figure 5-6 and Figure 5-7) despite the higher/equivalent volume of CCR5-PE added. In these experiments where the optimal concentration of CCR5-inducing reagents were tested for, no recovery stage for the cells was incorporated after the 18-hour incubation in the experiments (Sections 4.2.4 and 4.2.5). In the experiments where CCR5 levels were induced and quantified in the test samples (Sections 4.2.7 and 4.2.9), a recovery stage (three days) was incorporated after induction of CCR5 given that antibiotics such as Pon A make cells fragile/prone to cell death (Constantino *et al.*, 2001). The intention with this design is to induce CCR5 and use these cells for future experiments which may require such recovery periods. Similar CCR5 levels to the literature, approximately 10^5 ABS/cell are reported here (Section 5.1.3, Figure 5-4 and Figure 5-5). In the published protocols the recovery stage is omitted. Recovery of the cells results in much lower CCR5 levels, but the induced CCR5 levels remain different between cells with various levels of CCR5 i.e. the cells

presented with basal, low and high CCR5 levels, respectively. The lower CCR5 signal after cell recovery might be due to cell division or other cell processes which dilutes the high CCR5 signal, especially when considering that not all cells were induced to express high CCR5 levels (asymmetric peaks in Figure 5-2A and Figure 5-3A). The asymmetrical peak shapes on the histogram overlays of the Affinofile (Figure 5-2A) and Cf2Th (Figure 5-3A) cells confirm that CCR5 remains uninduced in some cells. In most literature, such a recovery period was excluded, explaining the higher CCR5 signal recorded in these studies and the initial measurements reported here in Figure 5-4 and Figure 5-5.

5.1.5 Cell viability and shift in CCR5 levels

For this study, 1×10^6 cells were harvested from Petri dishes by scraping post CCR5 induction with the respective antibiotics for 18 hours. Samples were stained to check for viability and CCR5 levels (Section 4.2.7, inclusive of the recovery stage) and analysed on the flow cytometer as per Section 4.2.9. The data was exported as *.fcs files and imported into FlowJo (v10.8). One repeat of the assay was done, with each repeat comprising five replicates. The Affinofile and Cf2Th cells showed higher cell viabilities in those samples that remain untreated (dead cells were stained with the FVD and shift to the right, representative samples shown in the histogram overlays in Figure 5-8B and Figure 5-9B). The control sample comprising dead cells prepared for each analysis allowed for the determination of the cut-off MFI values between viable (MFI shift to the left) and dead cells (MFI shifted to the right). In comparison to the Cf2Th and HEK293 cells, the Affinofile cells present lower cell viabilities on average when treated with Pon A. Plotting PE (FL2) against FVD (FL4) revealed that the viabilities of Affinofile and Cf2Th cells ranged from 70% to 95% (data not shown). Based on the literature and considerations discussed in Section 3.4, the small percentage of cell death may be due to specific pathways, e.g., Pon A activates the Akt pathway. Another consideration of the Affinofile cell viabilities is that Pon A makes the cells more fragile (Constantino *et al.*, 2001). As observed in the treatments described in Section 4.2.4, using a higher concentration of Pon A alters the morphology and attachment of the cells to the cell culture vessel.

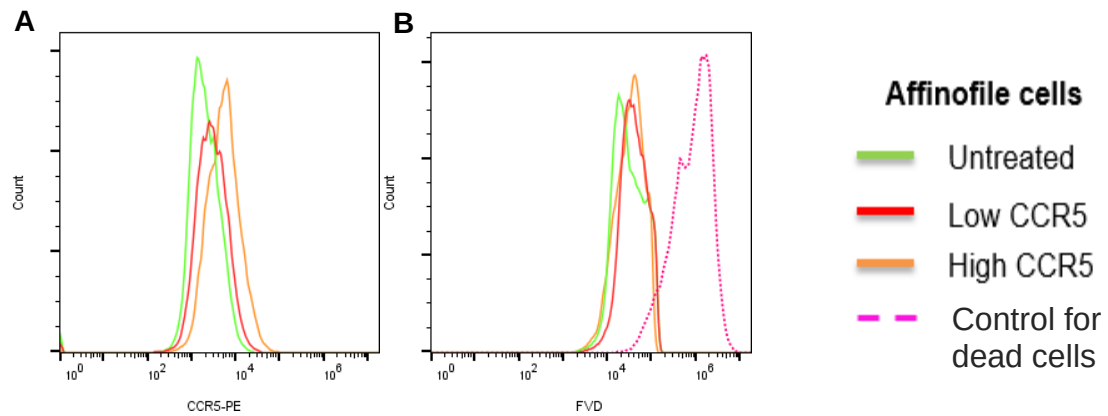


Figure 5-8: Histogram overlays showing the shift in MFI of CCR5-PE of the A) live Affinofile cells harvested in this study, and B) the cell viabilities of the harvested cells, untreated and treated with Pon A, with the control for dead cells included (pink dotted line).

Green: Untreated cells (cells with basal CCR5 levels); Red: Cells with low CCR5 levels; Orange: Cells with high CCR5 levels.

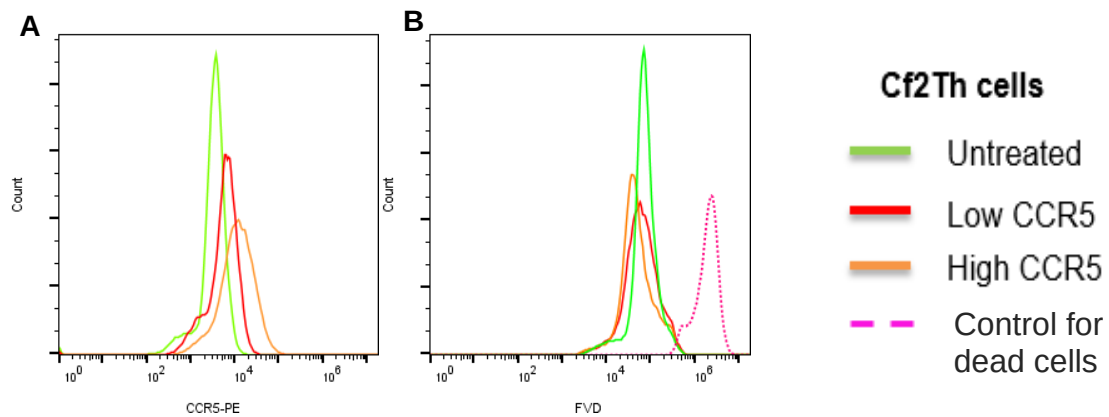


Figure 5-9: Histogram overlays showing the shift in MFI of CCR5-PE of the live A) Cf2Th cells harvested in this study, and B) the cell viabilities of the harvested cells, untreated and treated with doxycycline, with the control for dead cells included (pink dotted line).

Green: Untreated cells (cells with basal CCR5 levels); Red: Cells with low CCR5 levels; Orange: Cells with high CCR5 levels.

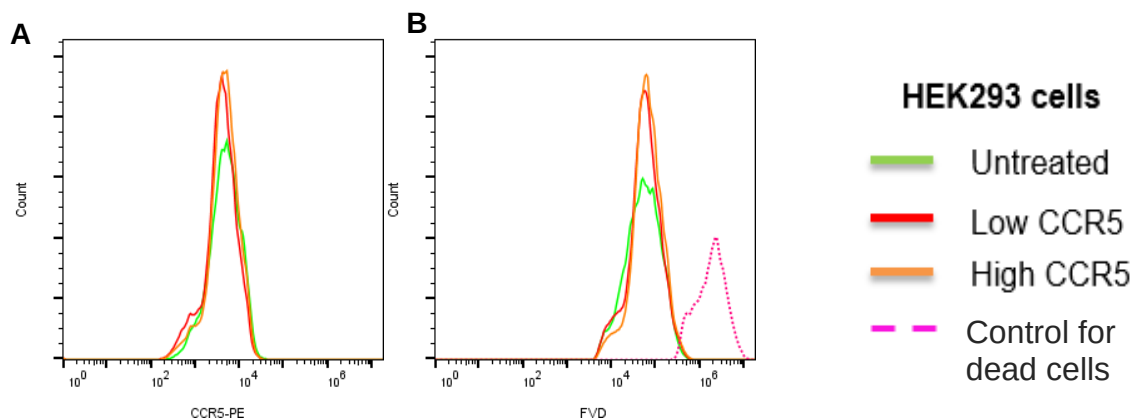


Figure 5-10: Histogram overlays showing the shift in MFI of CCR5-PE of the A) live HEK293 cells harvested in this study, and B) the cell viabilities of the harvested cells, untreated and treated with Pon A) with the control for dead cells included (pink dotted line).

Green: Untreated cells (cells with basal CCR5 levels); Red: Cells with low CCR5 levels; Orange: Cells with high CCR5 levels.

Flow cytometric analysis confirmed those cells experiencing CCR5 induction to be viable (Figure 5-8B, Figure 5-9B, and Figure 5-10B). The metabolites that will potentially be identified during subsequent metabolomics investigations can thus not be ascribed to cell death, since the cells were shown to be more than 70% (Affinofile cells) and 80% (Cf2Th cells) viable upon harvesting the cells. The contribution of cell stress however cannot be ruled out and would have been best estimated by doing for example an assay to determine oxidative stress. In addition, the CCR5 levels were indeed induced in the Affinofile (Figure 5-8A) and Cf2Th (Figure 5-9A) cells, and the cells presented with low and high CCR5.

Figure 5-10A shows that the HEK293 cells did not present with differential CCR5 levels after treatment with various concentrations of Pon A (the same concentrations used for Affinofile cells). Compared to the MFI of CCR5-PE observed for untreated Cf2Th and untreated HEK293 cells, respectively, the untreated Affinofile cells presented slightly increased MFI values indicative of higher basal CCR5 levels. The differential CCR5 levels of Affinofile, HEK293 and Cf2Th, were all within physiological ranges, although lower than those described in the literature for reasons explained under Section 5.1.4

5.2 Immune profile of cells presenting differential CCR5 phenotypes

The supernatant of cells with confirmed basal, low, and high CCR5 levels were prepared and analysed using a BD Human Th1/Th2/Th17 CBA kit as described in Section 4.2.10. The original data matrix obtained contained a considerable amount of zero values indicative of low cytokine concentrations in the test samples, i.e., concentrations below the limit of detection of the instrument. The low concentrations of cytokines detected in the Affinofile/HEK293 cells were unexpected, considering the effect of Pon A on various signalling pathways as described in Section 3.4. Low cytokine concentrations in the Cf2Th cells were expected since a human CBA kit was trialled on these samples.

Annexure B contains several graphs used to interpret the data in the sections below. The non-linear standard curves (fitted using FCAP Array v3.0, Figure B1) were used to translate the MFI for all samples to the relative concentrations. Table B1 shows the theoretical detection limit for each cytokine determined by BD Biosciences. The graphs are discussed in Section 5.2.2.

The MFI detected for the CBA cytokine standards (Figure B2) and the concentrations of the CBA cytokine standards (Figure B3) confirmed that the assay worked optimally, thus test samples were subsequently analysed. Since the cytokine standards were only analysed once, standard deviations are not shown on the graphs of the cytokine standards. Furthermore, Annexure B

contains all the graphs of cytokines detected in the pooled QC samples (MFI in Figure B4, related concentrations in Figure B5), Affinofile cells without subtracting the HEK293 background (MFI in Figure B12, related concentrations in Figure B13), HEK293 cells (MFI in Figure B14, concentrations in Figure B15), and Cf2Th cells (MFI in Figure B9, concentrations in Figure B10). When the MFI of the HEK293 background signal was subtracted from the signal of Affinofile cells, it yielded data with negative values (Figure B6). The related cytokine concentrations of Affinofile cells after subtracting the HEK293 background signal are shown in Figure B7. All test sample graphs show the average $\pm$ standard deviations obtained based on replicates for the cells with basal CCR5 (n=3), low CCR5 (n=5) and high CCR5 (n=5) levels.

The generation of the standard curves are described in Section 4.2.10 and shown in

Figure B1. The data were best fitted using a 4-parameter logistic curve with R^2 values greater than 99.9. These standard curves were used to automatically quantify the cytokine measurements in the test samples using FCAP Array (v3.0).

5.2.1 Quality assurance

Machine and analyst repeatability was measured as described in Section 4.2.10. The data was processed using FCAP Array (v3.0), quantified, and exported to Microsoft Excel (2016). The data matrix was saved as a *.csv file and uploaded to MetaboAnalyst. The data was log-transformed (Section 4.4.10), and a PCA scores plot was constructed to show the machine and analyst repeatability using the cytokine concentrations detected (Figure 5-11). Due to the low MFI and cytokine concentrations measured in the test samples (as seen in Figure B4 and Figure B5), the data matrix contained several zero values which are problematic for statistical analysis. Therefore, the zero values were replaced using small random values determined by MetaboAnalyst. Ideally, the pooled QC samples should cluster closely since the pooled QC sample represents all the test samples. Clustering of the QC samples within the confidence interval was noted after zero value replacement (Figure 5-11).

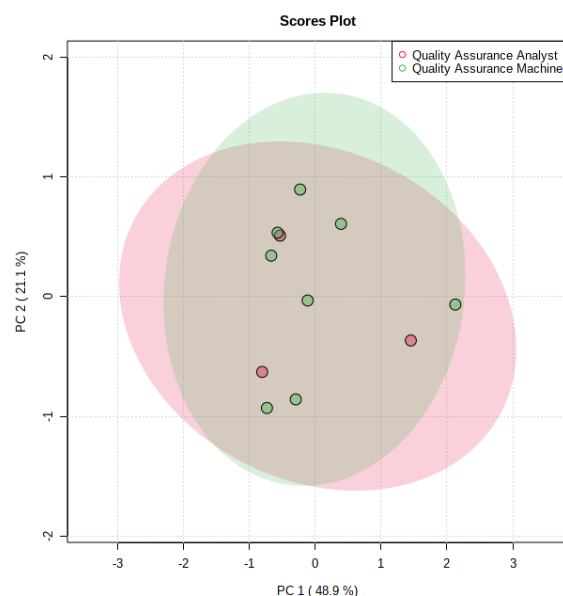


Figure 5-11: A PCA scores plot showing the machine and analyst repeatability for the Human Th1/Th2/Th17 CBA kit after missing value imputation

The zero values were replaced in the entire dataset for further statistical analysis. First, the overall quality of the data was determined by considering all test cytokine and QC samples in a PCA plot (Figure 5-12). All samples were included as test samples irrespective of the cell line or CCR5 levels. QC samples (machine and analyst repeatability QCs) clustered in the middle of the test samples showing that the data is of good quality (Figure 5-12).

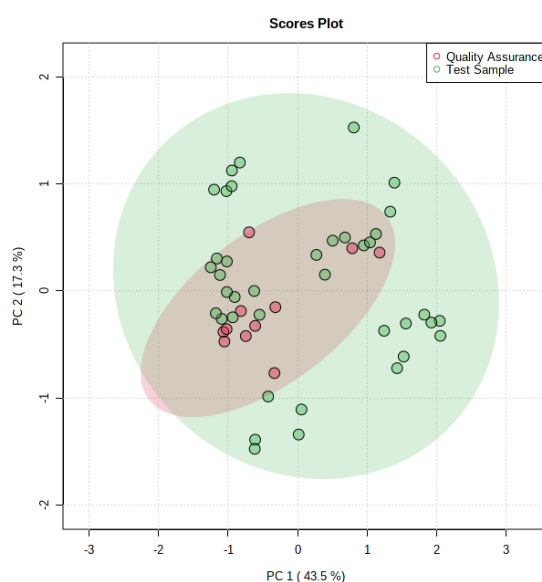


Figure 5-12: PCA scores plot showing the clustering of all test cytokine and QC samples irrespective of the cell line or CCR5 levels. The close clustering of the QC samples among the test samples indicates low variation within QC samples.

The MFI detected in the QC samples (Figure B4) were lower than the MFI detected in the cytokine standards (Figure B2). The lower MFI in the QC samples relates to lower cytokine concentrations (Figure B5) compared to the cytokine concentrations detected in the cytokine standards (Figure B3). Based on the MFI, the analyst repeatability (Figure B4, blue bars) and machine repeatability (Figure B4, green bars) was good based on the small standard deviations present. However, based on the concentrations, the analyst repeatability (Figure B5, blue bars) and machine repeatability (Figure B5, green bars) showed large deviations. The supernatant samples were collected and stored at -80 °C until analysis. The samples did not undergo any freeze/thaw cycles. Cytokines are degradable proteins (Ozbey *et al.*, 2014), which might attribute to the large standard deviations (Figure B5). The largest standard deviation occurred for IL-17A, which has the highest theoretical limit of detection (Table B1). Therefore, the standard deviations shown in Figure B5 might be attributed to concentrations below the limit of detection in the QC samples.

Despite the low concentrations detected in the QC samples, the cytokine standards analysed confirmed that the machine and assay worked, and test samples were subsequently inspected for trends. Cytokine trends found in the respective cell lines with basal, low, and high CCR5 is thus reported.

5.2.2 The immune profile of Affinofile cells presenting differential CCR5 phenotypes

Following CBA analysis, the MFI data obtained for Affinofile cells (after subtracting the HEK293 background signal, Figure B6) were translated (quantified) to concentrations (Figure B7) using the standard non-linear regression curves shown in Figure B1. After subtracting the HEK293 background, large standard deviations were obtained for the replicate samples when considering the MFI graphs, resulting in large standard deviations obtained upon quantification.

During data processing, the negative values were replaced as previously described, and the data were subjected to log transformation (Section 4.4.10). A PCA scores plot showing the natural separation among Affinofile groups (with the HEK293 background signal subtracted, (Figure 5-13) reflected clear separation among Affinofile cells with basal, low, and high CCR5 levels. Principal component 1 (PC1, x-axis) explains 46.9% of the variance, while principal component 2 (PC2, y-axis) explains 19.7% of the variance between the Affinofile groups. Surprisingly, the Affinofile cells with low CCR5 levels did not appear in the middle of the scores plot. This separation profile indicated that the cytokine profiles between untreated cells and cells with low CCR5 levels would differ more when compared to the cytokine profiles of untreated cells (basal CCR5 levels) versus cells with high levels of CCR5. It also suggests increased modulation of the immune system with the slightest increase in CCR5 levels.

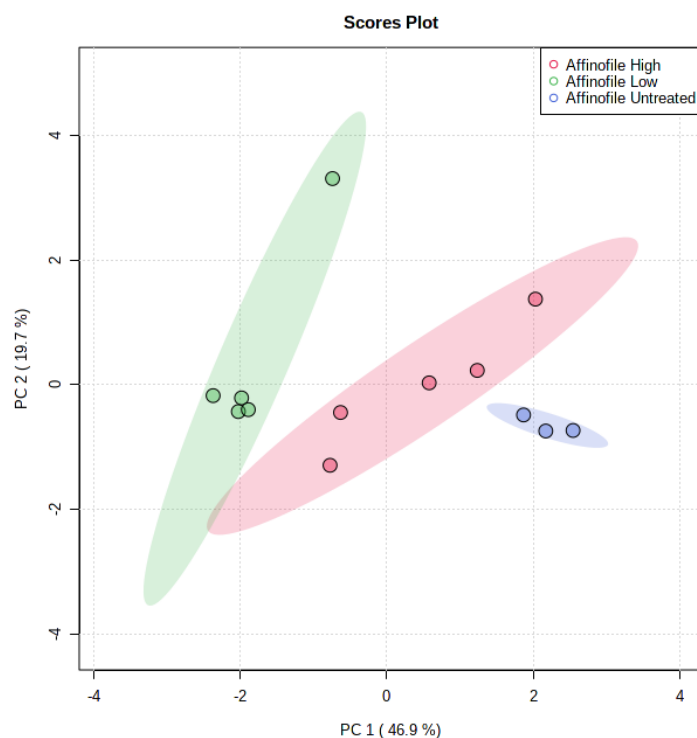


Figure 5-13: PCA scores plot showing group separation based on cytokine profiles measured in the supernatant of Affinofile cells with basal, low and high CCR5 levels (post subtraction of the HEK293 background concentrations).

One-way ANOVA revealed IL-2 ($p = 0.006$) to be statistically significant among the Affinofile groups with varying levels of CCR5, with the highest concentration in the Affinofile cells with basal CCR5 levels, and the lowest concentration in Affinofile cells with high CCR5 levels.

Next, the data were subjected to PLS-DA (Figure B8), validated as described previously (Section 4.4.10). PC1 of the PLS-DA scores plot explained >60% of the variance between Affinofile cells with varying levels of CCR5 (post subtraction of HEK293 cytokine signals, graphs for HEK293 signals are shown in Figure B14 and Figure B15). Variables important in projection (VIP) are values assigned to the compounds responsible for the separation observed in the PLS-DA scores plot. Here, a VIP score of >1 was regarded as significant, which led to the identification of IL-2 (VIP score = 1.5) and IL-6 (VIP score > 2.0) as variables explaining differences between groups (visual difference notable in Figure B9). Finally, cross-validation was used to determine the predictive ability of this model and, in all cases, resulted in an R^2 value < 0.5 and a Q^2 score below 0 (considering 3 components), meaning the PLS-DA model could not be validated. The PLS-DA results (Table B2) were thus excluded to identify possible significant features.

Univariate ES determinations identified several important cytokines ($d > 1$). In Affinofile cells with high levels of CCR5, cytokines that were identified as important included: IFN- γ , IL-10 and IL-6 when compared to Affinofile cells with low CCR5 levels. IL-6 and IL-2 were also important in the

Affinofile cells with increased CCR5 when compared to Affinofile cells with basal CCR5 levels. All 7 cytokines were identified as important when comparing Affinofile cells with low and basal levels of CCR5.

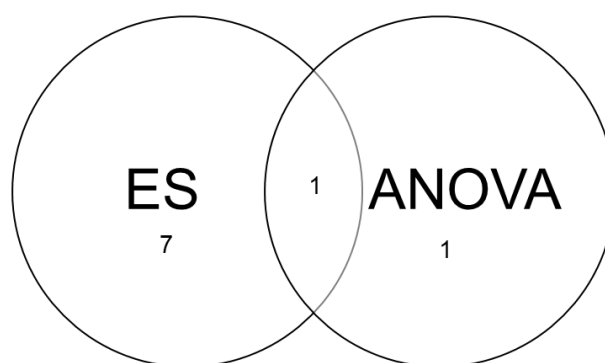


Figure 5-14: Venn diagram showing the important cytokines identified in Affinofile cells with differential CCR5 phenotypes by the various statistical analysis. Only one cytokine (IL-2) was identified as important in both statistical analysis.

Abbreviations: ANOVA – analysis of variance, ES – Effect size.

The identification of IL-2 (highest in Affinofile cells with basal CCR5 levels) as important in different univariate analysis (Figure 5-14) increases one's confidence in the role of this cytokine as a potential feature distinguishing the cells with varying CCR5 levels. IL-2 serves an immunoregulatory role with the ability to enhance and/or suppress immune responses (Abbas, 2020; Knoechel *et al.*, 2005). The decreased IL-2 observed in cells with low CCR5 levels compared to cells expressing high CCR5 levels is in line with the results obtained by Jaumdally and co-workers (2017) where the authors reported that the HIV-exposed cohort expressing reduced CCR5 levels presented with reduced IL-2 (Jaumdally *et al.*, 2017).

As discussed in Section 2.5, low levels of CCR5 are associated with a regulatory and Th2 immune profile, while high CCR5 levels are associated with a Th1 profile when disease/infection is present. For Affinofile cells, as CCR5 levels increased from basal to low levels of CCR5, the cytokine profile tended to shift from a Th1 to a Th2 immune profile, and a further increase in CCR5 levels resulted in a shift towards a Th1 profile.

5.2.3 The immune profile of Cf2Th cells presenting differential CCR5 phenotypes

A human CBA kit to measure cytokines in the supernatant of Cf2Th cells is not well documented. In addition, a non-vector control for the Cf2Th cell line was unavailable. The MFI detected for the cytokines measured for Cf2Th cells (Figure B9) were much lower than the MFI detected in the standards (Figure B2) indicative of the low cytokine concentrations (Figure B10). The standard deviations determined for the MFI were smaller when compared to the standard deviations

obtained for the cytokine concentrations. All cytokine concentrations were below the limit of detection (Table B1). The zero concentration values in the data matrix were replaced using MetaboAnalyst, and the data were log-transformed, followed by PCA to determine the natural separation of the groups (Figure 5-15).

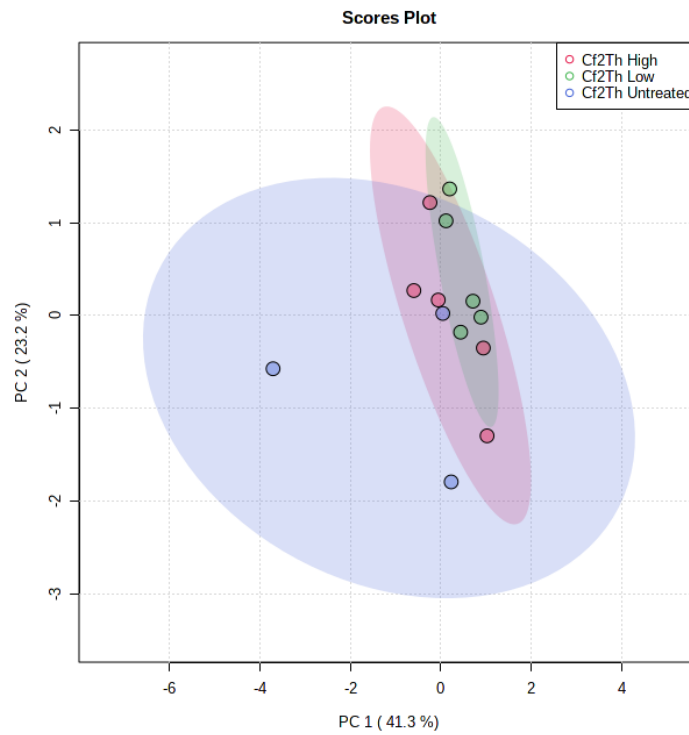


Figure 5-15: PCA scores plot showing the overlap of cytokine profiles measured in the supernatant of Cf2Th cells with basal, low and high CCR5 levels.

As shown in Figure 5-15, the groups mainly overlap, which might be because of the unavailability of a non-vector control. The clustering profile seen on the PCA scores plot suggests that Cf2Th cells with basal CCR5 levels (not treated with doxycycline) present with a more heterogenous immune profile, while cells that received doxycycline treatment (an anti-inflammatory agent) present with more homogenous immune profiles.

One-way ANOVA did not find any important cytokines distinguishing between the Cf2Th cells expressing differential levels of CCR5.

A PLS-DA scores plot (Figure B11) shows the clustering of the Cf2Th cells. A VIP scores plot based on the PLS-DA identified IL-2 (VIP score = 1.6) and IFN- γ (VIP score = 1.3) as the only cytokines with a VIP score above 1.0. The untreated Cf2Th cells (with basal CCR5) presented with the lowest IL-2 concentrations, whereas the highest IL-2 concentration was measured in Cf2Th cells with low CCR5 levels. Furthermore, the lowest IFN- γ concentration was measured in

Cf2Th cells with low CCR5 levels (Figure B10), and the highest concentration was found in Cf2Th cells with basal CCR5 levels (not treated with doxycycline). However, cross-validation for the model confirmed low Q^2 and R^2 values (considering 3 components, Table B3), and the PLS-DA results were thus not considered when identifying possible significant features.

IL-2 was identified as an important cytokine during univariate (ES) analysis (Figure 5-16)., with the highest concentrations in Cf2Th cells with low CCR5 levels The role of IL-2 was discussed in Section 5.2.2.

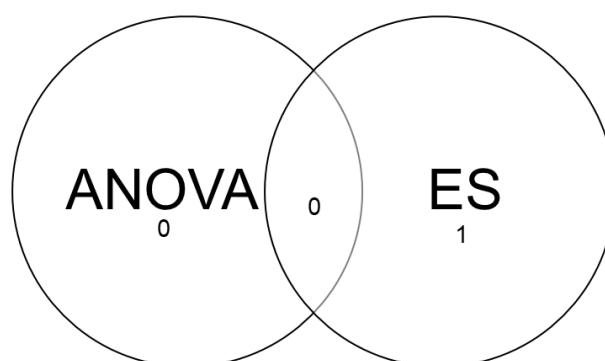


Figure 5-16: Venn diagram showing the important cytokines identified in Cf2Th cells with differential CCR5 phenotypes by the various statistical analysis. No cytokines were identified as important.

Abbreviations: ANOVA – analysis of variance, ES – Effect size.

As with the Affinofile cells, it was attempted to identify shifts in cytokine trends as one moves from basal to high CCR5 levels.

Most noticeable is that the data obtained for Cf2Th cells with differential CCR5 levels differs from the data obtained for Affinofile cells in terms of direction of the trends which indicates the importance of a control cell line when investigating the immune profile of cell samples.

5.2.4 Corresponding immune profile of Affinofile and Cf2Th cell lines

The cytokine profiles of the two cell lines utilised in this project were compared to each other to further characterise the cell lines irrespective of their CCR5 levels. The cell lines used in this study differ in numerous ways, with the most significant being the origin as Affinofile/HEK293 stems from human kidney cells and Cf2Th are canine thymus cells. However, the one thing that is common to the Affinofile and Cf2Th cell lines is the Tet-On vector. Therefore, by comparing the two cell lines with each other, some similar characteristics (corresponding cytokines) between the cell lines that might be attributed to the Tet-On system and CCR5 levels could be reported. Previously, the HEK293 background was subtracted from Affinofile cells. For this evaluation, to compare the cell lines, the HEK293 background was not subtracted from the Affinofile cells due

to the lack of a control cell line for the Cf2Th cells. The samples were stratified according to cell line irrespective of their CCR5 levels. Firstly, the samples were subjected to PCA (Figure 5-17).

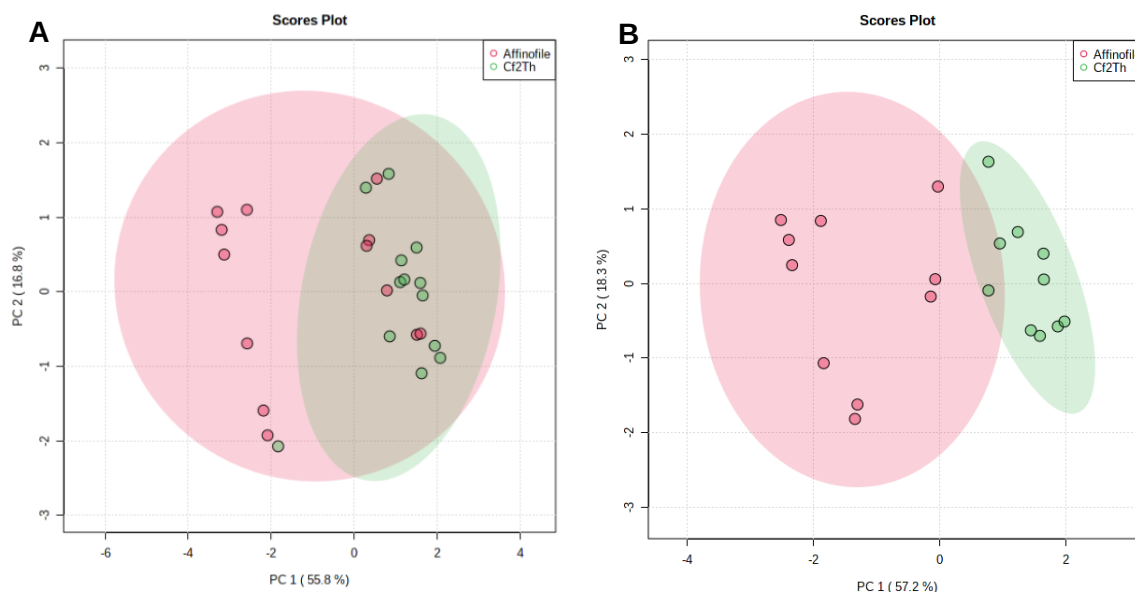


Figure 5-17: PCA scores plots indicate separation of the Affinofile (HEK293 background signal not subtracted) and Cf2Th cells, irrespective of CCR5 levels after imputation of the cytokine data A) All untreated and treated samples were included in the PCA. B) Only treated samples were included in the PCA.

The PCA scores plot shows minimal separation of the Affinofile and Cf2Th cells (Figure 5-17). In Figure 5-17A, the Cf2Th cells display greater homogeneity than the Affinofile cells. By adding sample names, the separation between Affinofile samples was shown to be random (data not shown). In addition, one Cf2Th untreated sample did not fall within the green 95% confidence interval due to the low number of samples ($n=3$ untreated samples per group). Figure 5-17B shows that when the untreated samples for both cell lines were removed from the data matrix, the PCA scores plots presented with improved separation profiles. In addition, the Cf2Th cells still showed more homogenous clustering than the Affinofile cells.

The data was also subjected to PLS-DA (Figure B16 and Figure B17), but the model did not validate (

Table B4). One-way ANOVA did not reveal statistically significant cytokines in the Affinofile cells presenting differential CCR5 levels. Univariate ES identified IL-2 as significant in Affinofile cells with basal CCR5 levels. Next, the Cf2Th data were processed in the same manner. Again, one-way ANOVA did not reveal any statistically significant cytokines in the Cf2Th cells presenting differential CCR5 levels. Univariate ES identified IL-2 as significant in Cf2Th cells with low CCR5

levels. Therefore, the only corresponding cytokine between the two cell lines is IL-2. A control cell line was not used to subtract the background signals, and the secreted cytokine levels might be related to the cell line lineage.

The MFI in the test samples without subtracting the HEK293 background (Figure B12) were much lower (in the hundreds) than the MFI detected in the standards (several thousands, Figure B2), indicative of the low cytokine concentrations in the test samples (Figure B13) and concentrations below the limit of detection (Table B1). The MFI in the HEK293 test samples is shown in Figure B14, while the related cytokine concentrations detected in HEK293 cells are shown in Figure B15.

Weissman *et al.* (2000) reported that IL-2 could upregulate CCR5 expression on the surface of cells (Weissman *et al.*, 2000). Given the regulatory functions of IL-2 (differentiation, proliferation, and homeostasis), the increased levels seen in Affinofile cells with basal CCR5 (untreated with a pro-inflammatory agent, HEK293 background not subtracted), and Cf2Th cells with low CCR5 (treated with a small dosage of anti-inflammatory doxycycline), suggests cell signalling as a mechanism to upregulate CCR5 levels to enhance cell signalling. However, when the HEK293 background was subtracted from the Affinofile cells, similar trends were observed for Affinofile and Cf2Th cells, i.e., IL-2 was increased in untreated cells with basal CCR5 levels and decreased in Affinofile and Cf2Th cells with low CCR5 levels. Therefore, IL-2 is an important feature and can be related to CCR5. Although the trends differ between these cell lines, both cell lines show that IL-2 is associated with CCR5 in the absence of disease/infection. However, further studies are required to investigate the role of IL-2 in light of the limitation noted under Section 6.4 i.e. that CCR5 is involved in signal transduction and the signalling molecules / ligands needed to interact with CCR5, may not necessarily have been present in this system, the media was not supplemented with any such ligands, hence the immune and metabolic differences are not as exaggerated on the PCA scores plots. Future studies can be designed to include ligands. In addition, HEK293 and Cf2Th cells may not express the right toll-like receptors to produce and respond to cytokines. This can be assayed by exposing the cells to the correct toll-like receptor ligands.

5.3 Metabolomics profile of cells presenting differential CCR5 phenotypes

This section investigates the metabolic differences in cell lines with differential CCR5 levels, in the context of the differences in their immune profile as discussed in the previous section. The cells were cultured and harvested as explained in Section 4.2.8. The cell viability and CCR5 levels in all samples were confirmed, as explained in Section 4.2.9. The metabolic differences are not ascribed to cell death as the cells were viable before quenching of the metabolism. A review of

the metabolic markers suggests a minimal role for cell stress in contributing to the observed profile. The samples were prepared for metabolic analysis as per Section 4.4.4, and the data was processed as explained in Sections 4.4.10 - 4.4.12.

5.3.1 Machine and analyst repeatability

The machine and analyst repeatability were determined before applying the GC and MS methods to the experimental samples, using CV values of: 1) the same QC sample, extracted and injected multiple times for machine repeatability, and 2) the separate analysis of a number of different aliquots of the QC sample to determine analyst repeatability.

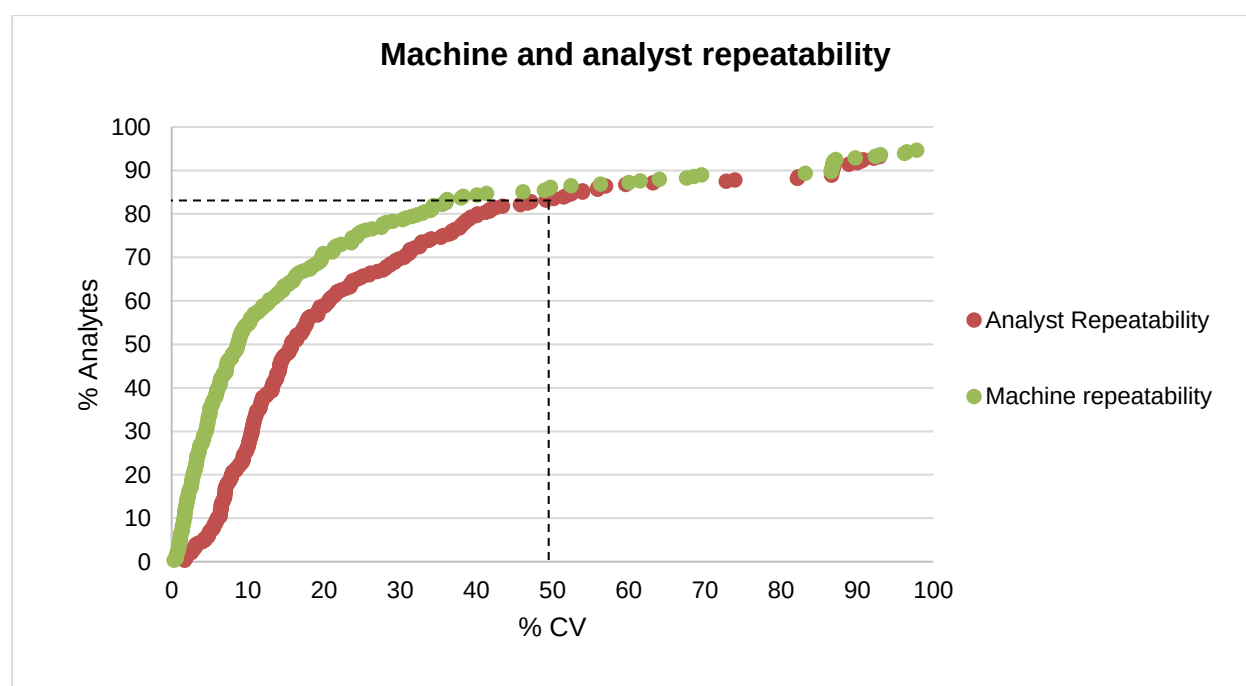


Figure 5-18: Machine and analyst repeatability derived from the QC samples prepared for metabolomics analysis. At least 80% of the compounds detected had CV values smaller than 50%, suggestive that the sample preparation and machine methods are suitable for further analysis these samples.

Both the machine and analyst repeatability graph representative of repeatability (Figure 5-18) shows that at least 80% of the analytes have CV values smaller than 50%, indicating that the sample preparation and machine methodology are suitable for subsequent analysis (Schoeman & Du Preez, 2012).

5.3.2 Optimising sample quenching

As previously described, the standard quenching methods include adding 0.9% NaCl at various temperatures (Sapcariu *et al.*, 2014; Sellick *et al.*, 2011) or ice-cold methanol (Dietmair *et al.*, 2010), added at various time points during sample preparation. While these methods are considered effective, cell damage and metabolite leakage remain a concern (Villas-Bôas *et al.*, 2005; Villas-Boas *et al.*, 2007; Wittmann *et al.*, 2004). Therefore, the previously described

quenching methods were investigated and compared, to determine which of these are most optimal for the metabolomics analyses of the adherent cell lines as used in this study. The efficiency of various quenching methods was evaluated by the determining and comparing the number of usable compounds (all metabolites detected in the sample, i.e., no zero values) and identifiable compounds (all usable compounds excluding the unidentifiable compounds). Figure 5-19 illustrates the percentage of compounds detected in the samples subjected to the different quenching techniques. Compared to the quenched samples, there was a very low percentage of usable compounds detected in the unquenched samples and a comparatively higher number of zero's present in that data matrix (Figure 5-19). This confirms that the quenching methods were successfully employed. The metabolome of the samples treated with ice-cold 0.9% NaCl before cell counting (Figure 5-19, green bars), was comparatively better preserved than in samples treated with ice-cold 0.9% NaCl after cell counting (Figure 5-19, purple bars), not only in terms of number of usable and identifiable compounds, but also in terms of the lower number of zero values in the data matrix. The metabolome of the samples treated with room temperature 0.9% NaCl (after cell counting, Figure 5-19, blue bars) was slightly better preserved than those samples treated with ice-cold 0.9% NaCl (after cell counting) (this quenching method is usually used for suspension cell lines and tested here for the adherent cell lines, since the method allows for rapid quenching of multiple samples). The use of ice-cold MeOH as a quenching reagent is convenient, since the protocol associated with its use is time- and cost-efficient since fewer centrifugation steps are employed. Upon extraction, the remaining extraction solutions can simply be added to the sample, without prior removal of the MeOH. Using MeOH as a quenching solvent preserved the cell's metabolism as well as ice cold 0.9% NaCl added before counting with slightly improved standard deviations.

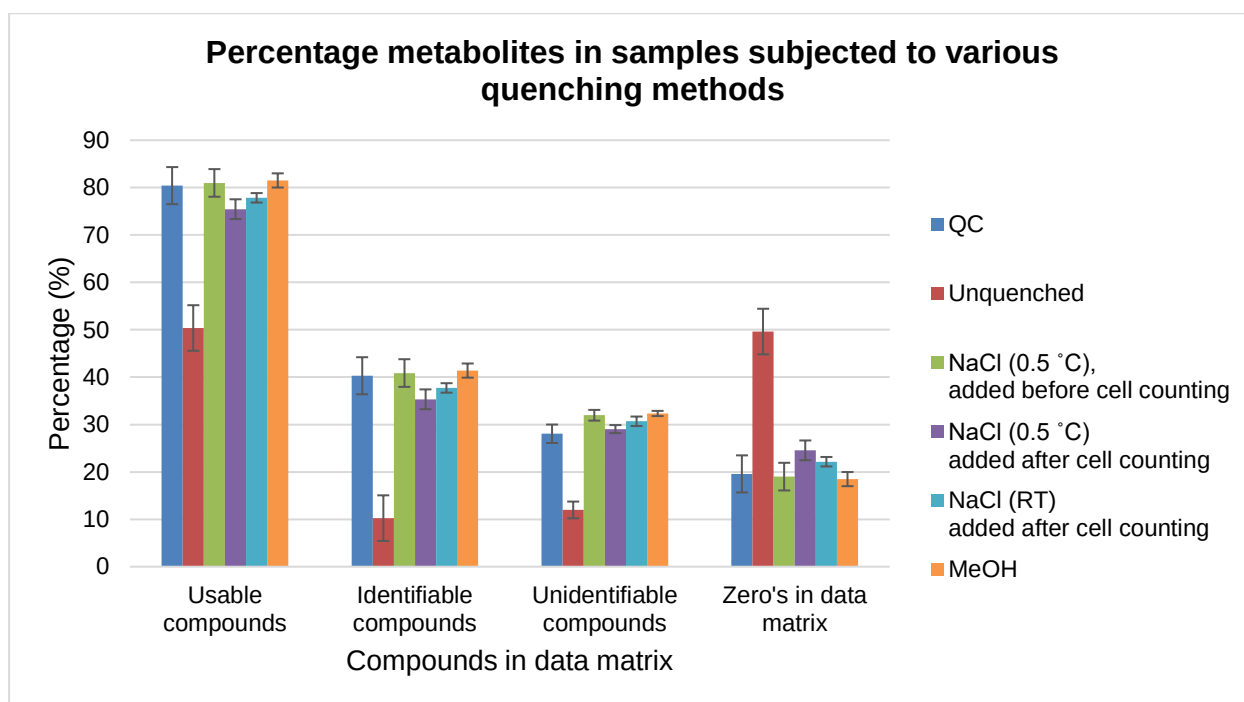


Figure 5-19: Graph illustrating the percentage of usable, identifiable and unidentifiable compounds, and zeros in the GCxGC-TOFMS metabolomics data of samples (n=3) subjected to various quenching methods. Values for each method represent the average of three replicates, and error bars show average $\pm$ standard deviations. Abbreviations: QC- Quality control; NaCl – Sodium chloride; MeOH – Methanol.

Based on these results, it is suggested that the adherent cell samples as used in this study, be quenched using ice-cold methanol, since this solvent reduces the number of experimental steps required, thus reducing the chance of cell death (prior to quenching) and analytical variation during sample preparation, subsequently resulting in a better approach to preserve cell metabolism.

5.3.3 Quality assurance of the metabolomics data

Section 4.4 describes how the GC data were obtained. The quality of the data matrix obtained was determined by visually inspecting the PCA score plots of the data derived from QC samples prepared for metabolic analysis (Figure 5-20). Ideally, the data from the analysed QCs should cluster closely together. Clustering of the QCs showed good machine repeatability between batches. The QCs however clustered a distance from the test samples (usually clustering can be seen at the center of testing samples). This can be explained by the dilution effect (Godzien *et al.*, 2015) in which the metabolites that are abundant in one group of test samples for instance, are reduced or below the detection limit of the pooled QC sample aliquots analysed. Inspection of the raw data indicated several metabolites detected in individual test samples were not detected in the QC's due to their concentrations falling below the detection limit of the GCxGC-TOFMS, due to this dilution effect. Furthermore, the PCA scores plot shows a distinct separation

between the test samples, which when labeled, indicated a distinct separation between the Affinofile and Cf2Th samples (data with labels for the latter not shown in this figure since its purpose is to indicate repeatability of the data).

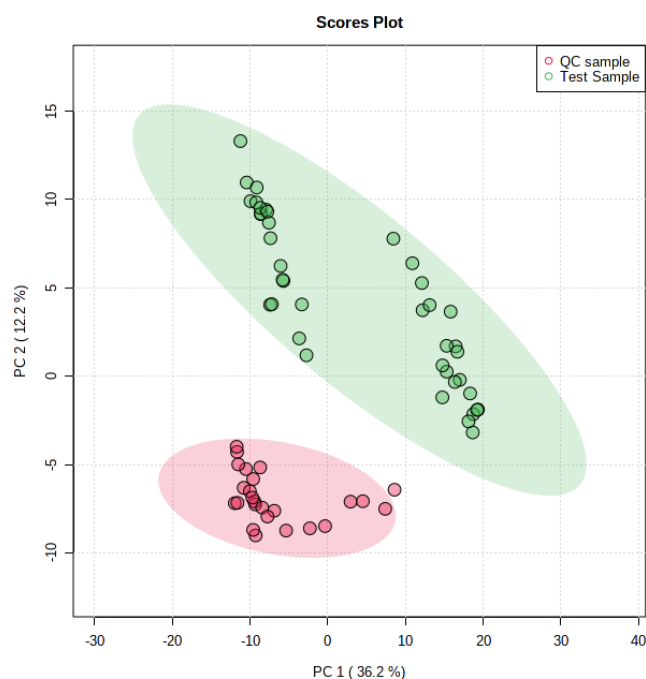


Figure 5-20: PCA scores plot of QCs and all samples used for metabolic analysis shows tight clustering of QC samples and a heterogeneous clustering of test samples.

Next, a PCA scores plot where samples were labelled according to batch numbers was constructed (Figure 5-21). This was done to inspect the data visually for a possible batch effect, which was evident for batch 4 (Figure 5-21A). However, after the QC-CV filter was applied, i.e., the removal of metabolites from the data set if the metabolite was detected in the QC sample with a CV value greater than 50%. After application of the QC-CV filter, no batch effect was evident (Figure 5-21B), and the data was deemed robust for further use.

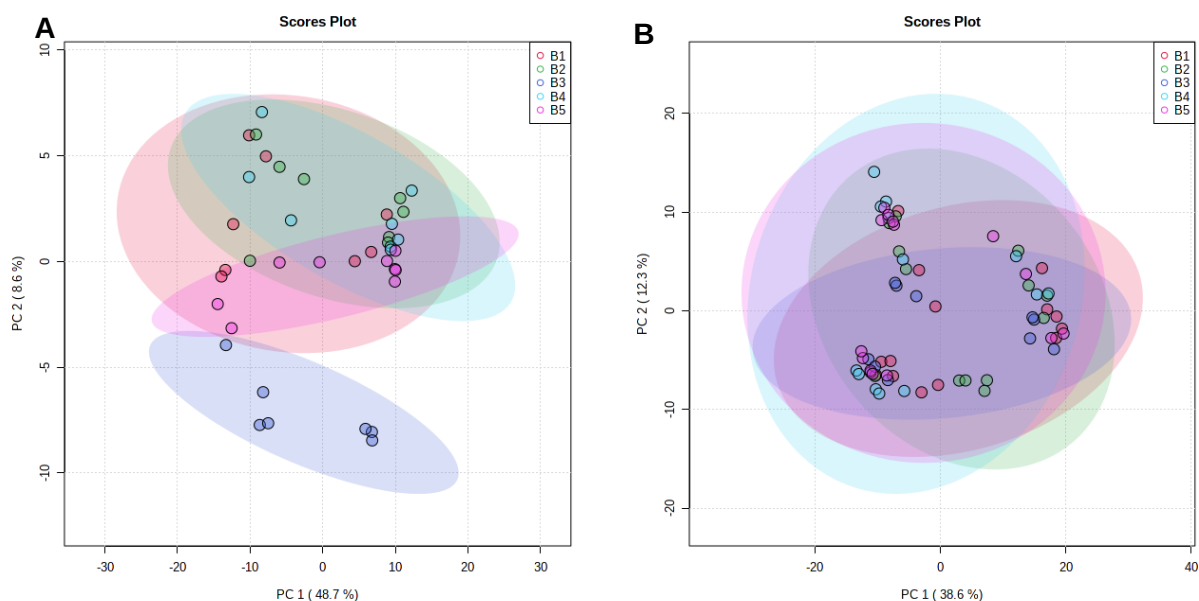


Figure 5-21: PCA plot including all samples used for metabolomics analysis where samples were stratified according to the batch number. A) Before applying a QC-CV filter and B) Post application of a QC-CV filter.

Abbreviations: B - Batch.

Visual inspection of the data in Figure 5-21B, shows a grouping into three, although still within the defined confidence interval. When labels were added, the clustering showed QCs, Affinofile, and Cf2Th groups to be differentiated, suggesting metabolome differences between Affinofile and Cf2Th cells.

5.3.4 Metabolomics profiles of Affinofile cells presenting differential CCR5 phenotypes

The reader is reminded that the cell lines (Affinofile, HEK293 and Cf2Th) were not treated with induction antibiotics and displayed basal CCR5 levels. They were also treated with various concentrations of the respective induction antibiotics to achieve low and high CCR5 levels. All cells were then subjected to GC analysis. After the quality assurance of the data, the GCxGC-TOFMS data collected for the Affinofile cells, were subjected to PCA (Figure 5-22). The PCA plot shows an overlap between Affinofile cells with basal, low, and high CCR5 levels, hence very little metabolic differences are expected to exist between the cells with differential CCR5 levels. This overlap is even more evident between cells with basal and high CCR5 levels. Furthermore, the clustering of the cells with basal CCR5 levels shows little to no variation within this group. The most significant variation within a group, is seen in the Affinofile cells with low CCR5 levels. These results suggest that the small amount of Pon A added resulted in greater metabolic differences at first as this represents a shift when the cells are first triggered. Overall, the variation is still relatively small, as can be observed by the small contributions of the various PCs on the PCA

plot. The separation observed in Figure 5-22 suggests that the metabolic profiles of cells presenting differential CCR5 levels do not differ as much without a disease or infection perturbation.

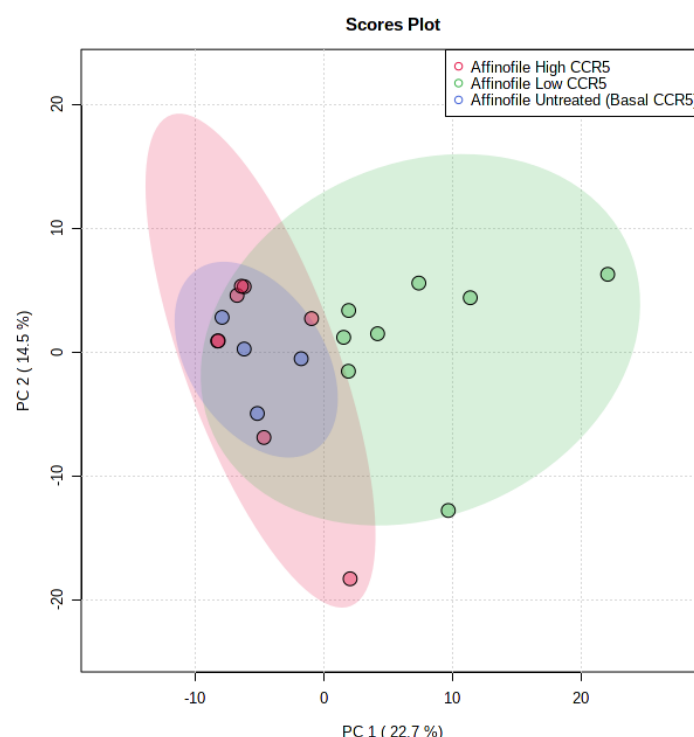


Figure 5-22: PCA scores plot of Affinofile cells with differential CCR5 levels. Shown is the natural separation of cells after imputing metabolic data.

A PLS-DA scores plot (Figure C1) shows the Affinofile cells separated more clearly according to their different CCR5 levels, which is expected since this is a supervised multivariate statistical method. Separation along the first component was however explained by only 12.3%. The best performance of the model was given through three components. Cross-validation of the PLS-DA model showed the data was overfitted (Table C1). The data were subjected to univariate (e.g., ES and one-way ANOVA) statistical analyses to identify potential compounds responsible for separating the cells based on differential CCR5 levels. ANOVA identified 37 while ES identified 176 important metabolites. Among the important compounds, 35 were identified as significant by both the ANOVA ($p < 0.05$) and ES ($d > 1$) statistical analysis. The Venn diagram in Figure 5-23 shows the number of compounds identified by each statistical analysis. The overlap represents the common important metabolites identified by one-way ANOVA and ES.

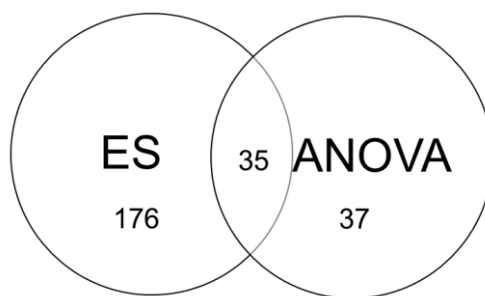


Figure 5-23: Venn diagram showing the number of important compounds identified by each statistical analysis and the metabolites that overlap between ES and one-way ANOVA for Affinofile cells. Abbreviations: ANOVA: analysis of variance; ES: Effect size.

For these 35 common statistically significant compounds, the compound IDs were confirmed, as explained in Section 4.4.13. Briefly, for each compound identified, the spectral matches were evaluated on Chromatof, followed by identifying the underivatised metabolite name using ChemDraw Ultra (v12.0.2.1076). Next, the name of each compound was used to draw the resulting structure. Finally, the groups added by the derivatisation reagents were removed to reveal the name of the metabolite in its underivatised form. Figure 5-24 shows an illustration of this process.

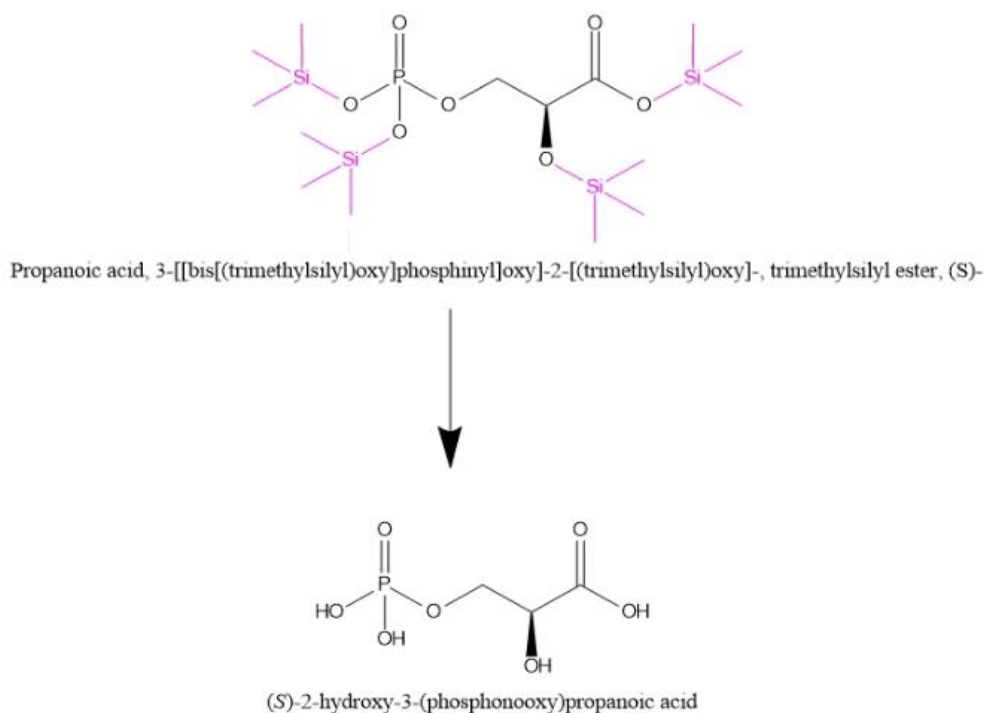


Figure 5-24: Illustration of the process to retrieve the underivatised names of metabolites using ChemDraw Ultra (12.0.2.1076). Original compound IDs obtained from Chromatof were used to draw the original structure. Groups that originated due to derivatisation were removed to reveal each compound's underivatised structure and name.

A summary of the most significant compounds identified, which best describe the metabolic differences of Affinofile cells presenting with differential CCR5 levels, is summarised (in Annexure C, Table C2). Eleven analytes remained unknown since no spectral matches could be made. The discussion below refers either to the common metabolite name or the Human Metabolome Database (HMDB) naming.

Cells with basal CCR5 levels had comparatively elevated phosphate compared to cells with low CCR5 levels. Although cells were collected in PBS, and the majority of PBS was washed off and removed with a pipette before quenching the metabolism with methanol, the phosphate identified more than likely does not stem from the use of PBS, but rather from the intracellular metabolism of the cells. Phosphoric acid plays a vital role in several metabolic pathways, such as OXPHOS, where a free phosphate is released for energy purposes (Gu *et al.*, 2006). Therefore, the phosphate detected might indicate the degree of activity of the energy metabolism-related pathways. Additionally, the elevated oleic acid identified in Affinofile cells with basal CCR5 (compared to Affinofile cells with high CCR5 levels) forms part of the fatty acid oxidation pathway (Lim *et al.*, 2013), suggesting an increased metabolic rate in these cells. Interestingly, phencyclidine was identified as an important marker with the highest occurrence in the untreated Affinofile cells with basal CCR5 levels and the lowest occurrence in the group with high CCR5 levels. During earlier years, phencyclidine was used for anaesthesia and analgesia (pain-relieving medication) (Olmedo, 2002). This metabolite might stem from antibiotics used during cell culturing.

Surprisingly, the Affinofile cells with low CCR5 levels, showed higher concentrations of most metabolites, when compared to the Affinofile cells with basal and high CCR5 levels, respectively. The most significant altered metabolic profile is seen in cells with low CCR5 levels compared to cells with basal and high CCR5 levels, respectively, which was expected based on the PCA scores plot (Figure 5-22). Mammalian cell culture metabolism relies on glutaminolysis, which refers to glucose and glutamine uptake (provided in the cell culture media), lactate and non-essential amino acid secretion (Quek *et al.*, 2010). Therefore, in cell culture, the increased metabolic activity of cultured cells will present elevated metabolites linked to glycolysis, glutamine catabolism, and non-essential amino acid secretion. Two molecules identified as alpha-amino acids: N-acetyl-L-glutamic acid and N-acetyl serine (the N-terminal capped form of L-glutamic acid and serine, respectively), were elevated in the Affinofile cells with low CCR5 levels. The elevated N-acetyl-glutamic acid would activate the enzyme carbamoylphosphate synthetase I, which is involved in the urea cycle and is vital for the effective removal of urea. The cells did not present with elevated urea cycle intermediates in line with its potential removal. Overall, the Affinofile cells with low CCR5 levels showed increased protein catabolism, indicated by the elevated N-acetyl glutamic acid and N-acetyl serine.

Several fatty acids were elevated in the Affinofile cells with low CCR5 levels when compared to cells with high CCR5 levels, i.e., palmitic acid, tridecanoic acid, 4-hydroxybutyric acid, and caprylic acid. Palmitic acid, a saturated long-chain fatty acid (LCFA), is vital to stabilise proteins by side-chain palmitoylation (Towler *et al.*, 1988) and subsequently plays a role in signal transduction (Legrand & Rioux, 2010), suggesting that, as the CCR5 levels increase from basal to low levels, a metabolic shift occurs to stabilise these receptors. The increased concentrations of tridecanoic acid, another saturated LCFA, can be metabolised by β -oxidation, resulting in intermediates that enter the tricarboxylic acid (TCA) cycle. Caprylic acid (also known as octanoic acid) is a saturated medium-chain fatty acid (MCFA) associated with the production of ketone bodies which can be used as an alternative energy source. Oleic acid, a monounsaturated LCFA (previously discussed), acts as a signalling molecule to regulate the rate of fatty acid oxidation (Lim *et al.*, 2013) and regulate palmitic acid production in C6 glioma cells (Gnoni *et al.*, 2010). Overall, the general increased fatty acids detected in Affinofile cells with low CCR5 levels, stabilise CCR5 and may also be metabolised for energy purposes. This statement is further supported by the increased concentrations of 3-phosphoglycerate (a glycolytic intermediate), suggesting increased metabolic activity as the glycolysis and OXPHOS pathways are connected. A previous study reported an increase in the 3-phosphoglycerate dehydrogenase enzyme activity – an enzyme involved in serine synthesis, which can lead to cysteine and glycine production in hepatoma cells, as they enter the proliferation phase (Snell *et al.*, 1987). Affinofile cells with low CCR5 levels, may be utilising less serine, given the increased N-acetyl-serine levels in these cells. Cancer cells are known to undergo metabolic reprogramming, and this includes the pathway affected by the 3-phosphoglycerate dehydrogenase enzyme (Li *et al.*, 2021). The increased 3-phosphoglycerate concentrations found in the Affinofile cells with low CCR5 levels (uninfected model) suggest decreased activity of this enzyme and/or increased glucose uptake compared to Affinofile cells with basal CCR5 levels. Indeed, Affinofile cells with low CCR5 levels, further presented with elevated concentrations of the TCA cycle intermediates (citric acid, α -ketoglutaric acid, succinic acid, malic acid). Glycolysis products (such as acetyl-CoA) enters the TCA cycle and release the CoA molecule. Water is released when citric acid is converted to isocitric acid, which is then converted to α -ketoglutaric acid. Several other metabolic pathways can lead to the formation of α -ketoglutaric acid which can be incorporated into the TCA cycle. The α -ketoglutaric acid in the TCA cycle is eventually converted to succinic acid, which plays an essential role in the electron transport chain (ETC) and continues in the TCA cycle which will result in the conversion of succinic acid to fumaric acid. Fumarase is an enzyme in the TCA cycle that converts fumaric acid to malic acid, which can be converted to oxaloacetic acid. Oxaloacetic acid can then be reverted to citric acid, thus completing the TCA cycle. Therefore, the increased TCA intermediates suggest increased glycolysis and TCA cycle activity related to energy metabolism. The increased aspartic acid, a non-essential amino acid produced by cells with low CCR5 levels, is produced from

fumaric acid in the TCA cycle and serves as a precursor for other amino acids and some nucleotides essential for cell proliferation. Methyl-D-ribofuranoside, a derivative of ribose, is involved in purine/pyrimidine synthesis. The elevated p-hydroxyphenyllactic acid (produced through tyrosine metabolism) suggests increased tyrosine catabolism in these cells. CCR5 undergo posttranslational modifications such as tyrosine sulfation which influences the binding of CCR5 to its ligands (Farzan *et al.*, 1999; Scurci *et al.*, 2021) and increased tyrosine metabolism might be linked to the lower levels of CCR5 expressed on the cell surface, thus reducing the need for tyrosine to allow for posttranslational modifications of CCR5. In addition, p-hydroxyphenyllactic acid and hydroxyphenylacetic acid are antioxidants, produced by the degradation of aromatic amino acids to combat oxidative stress (Beloborodova *et al.*, 2012; Fedotcheva *et al.*, 2008). The elevated 3-hydroxyisobutyric acid detected serves as an intermediate in the amino acid, valine's metabolism and has been found to influence energy metabolism (Viegas *et al.*, 2008). The compound 2,4,7,9-Tetramethyl-5-decyne-4,7-diol, does not occur naturally and is produced by oxidative degradation of decynediol.

Cells with high CCR5 levels presented with elevated myristic acid and pyruvic acid. The increased pyruvic acid in cells with high CCR5 levels is a precursor to producing energy if it enters the TCA cycle. Pyruvic acid can also enter other pathways to produce fatty acids or be converted to glucose if the cell needs it (Randle *et al.*, 1978). The increased pyruvic acid can also stem from possible increased glycolysis since Gao *et al.* (2016) found that high CCR5 levels can increase the glucose transporters on the cell surface of cancer cells (Gao *et al.*, 2016). Myristic acid is a saturated LCFA found in increased levels in cells with high CCR5 levels and is involved in protein fatty acid acylation, known as N-terminal myristoylation (Udenwobele *et al.*, 2017), which is essential for protein stability and subsequently plays a role in enzyme activity (Towler *et al.*, 1988), and also the OXPHOS system (Rioux *et al.*, 2011). Chemokine receptor modifications, via myristoylation can subsequently influence chemotaxis and cell signalling (Neel *et al.*, 2005). Therefore, the Affinofile cells with increased CCR5 levels (after induction) might reflect elevated myristic acid to stabilise the increased number of receptors being expressed.

Based on the metabolic profiles discussed above, cells with basal CCR5 levels showed some degree of energy metabolism based on the elevated free phosphate detected. However, as receptor levels increase from basal to low CCR5 levels, a metabolic shift occurs and cells present with elevated amino acids, fatty acids, and TCA cycle intermediates which can all be related to cell growth and proliferation, the energy demands of the receptor and the cell, and receptor stabilisation. Based on the number of significant metabolites identified in Affinofile cells with low CCR5 levels compared to cells with basal and high CCR5 levels, there is a great shift in the metabolic profile as more receptors are expressed, and when CCR5 levels are increased from

low to high CCR5 levels, this tends to shift back to a profile like the one observed for cells with basal CCR5 levels. Figure 5-25 shows the affected metabolic pathways described above.

5.3.5 Metabolomics profile of Cf2Th cells presenting differential CCR5 phenotypes

After quality assurance, the metabolic data attained for the Cf2Th cells were subjected to PCA (Figure 5-26). The PCA plot shows an overlap between cells with low and high CCR5 levels, respectively. Furthermore, the clustering of the untreated cells (basal CCR5 levels) shows low variation within this group. The most significant variation within a group is seen in the Cf2Th cells with high CCR5 levels, followed by the cells with low CCR5 levels. These results suggest that the doxycycline-induced increase in CCR5 levels might influence the metabolic profile, yet not significant enough to cause a clear separation between the groups. Overall, the variation is still relatively small, as observed by the small PC values (x and y axis) on the PCA plot. The overlap observed in Figure 5-26 suggests that the metabolic profiles of cells presenting differential CCR5 levels will not differ much.

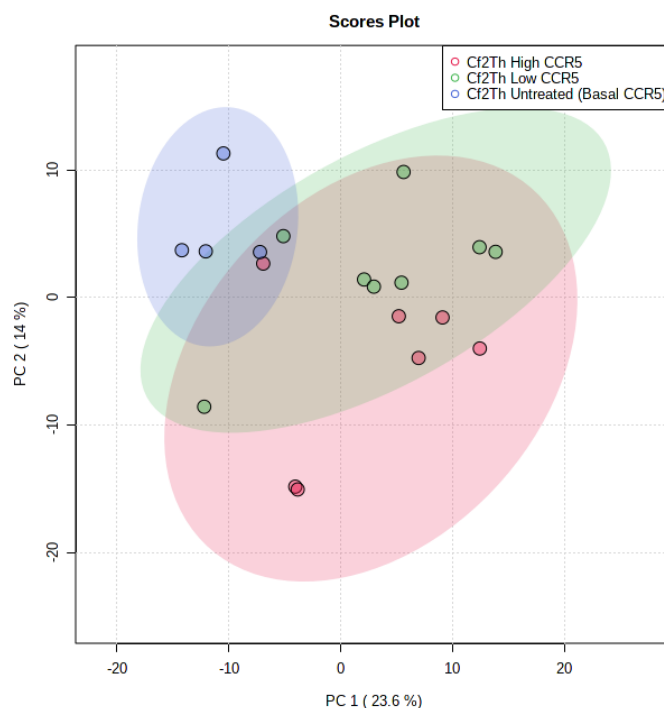


Figure 5-26: PCA scores plot showing the natural separation of Cf2Th cells presenting differential CCR5 levels after imputation of the metabolic data.

Following PCA, the data was subjected to PLS-DA (Figure C2). The PLS-DA separated the Cf2Th cells according to the different levels of CCR5. Separation along the first component was described by only 19.3% (x-axis). The best performance of the model was given through four components. Cross-validation of the model showed the data was overfitted. Univariate one-way ANOVA identified 49 important compounds ($p < 0.05$), while 170 compounds with a large or significant ES ($d > 1$) was identified. The number of unique and overlapping compounds identified by the statistical analysis is presented in the form of a Venn diagram in Figure 5-27.

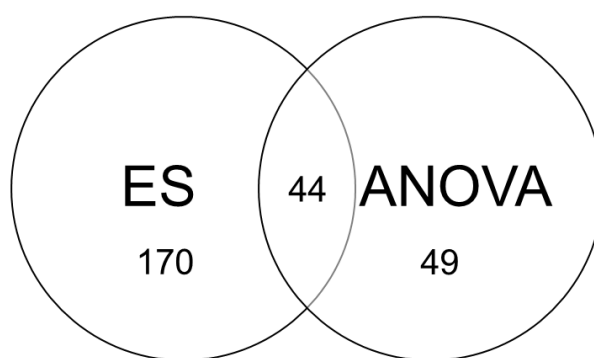


Figure 5-27: Venn diagram showing the number of important compounds identified by each statistical analysis, as well as the overlapping metabolites.

As seen in the Venn diagram, the overlap of all statistical analyses revealed 44 compounds as significant when considering both ES and ANOVA. For these 44 compounds, the compound IDs were confirmed, as explained in Section 4.4.13 and as illustrated in Figure 5-24. A summary of the most significant compounds identified to distinguish the metabolic profiles of Cf2Th cells presenting with differential CCR5 levels is summarised in Table C4.

Of the 44 metabolites, 9 remained unidentified, and as a result, 35 metabolites for which metabolite names could be assigned were interpreted. As previously mentioned, a control cell line for the Cf2Th cells was unavailable, and the resulting metabolic profiles of the Cf2Th cells should be interpreted with caution.

Cf2Th cells with low CCR5 levels presented with elevated succinic acid (TCA intermediate, pathway described in Section 5.3.4) and palmitic acid (saturated long-chain fatty acid, pathway and function described in Section 5.3.4) when compared to cells with basal CCR5 levels. The elevated succinic acid suggests the lower activity of the ETC since no other TCA intermediates were statistically significant in these cells. The increased palmitic acid in Cf2Th cells with low CCR5 levels is considered vital for stabilising proteins by side-chain palmitoylation (Towler *et al.*, 1988). However, the Affinofile cells had a control cell line available (HEK293), while the Cf2Th cells did not, making it impossible to speculate whether increased palmitic acid generally relates to low CCR5 levels.

Most of the metabolites identified as important in the Cf2Th cells with high CCR5 levels, followed similar metabolic trends to the Affinofile cells with low CCR5 levels. To our knowledge, no previous studies have reported on the metabolome of Cf2Th cells, and even more so for those with differential CCR5 levels. Overall, the Cf2Th cells with high CCR5 levels showed elevated sugars, amino acids, and purines/pyrimidines when compared to the Cf2Th cells with basal CCR5 levels. In contrast to the Affinofile cells, where most of the elevated metabolites were found in

cells with low CCR5 levels, Cf2Th cells with high CCR5 levels presented with an increased number of elevated metabolites when compared to the base line cells. This may be due to the lack of a control cell line for Cf2Th cells, as previously mentioned.

Like the Affinofile cells, the Cf2Th cells were cultured in media containing high glucose and glutamine levels. The elevated non-essential amino acids (glutamic acid, proline and glycine) suggest increased metabolic activity (Quek *et al.*, 2010). Purine/pyrimidine metabolism was increased, based on the elevated levels of guanosine (a precursor for the purine guanine), uridine (precursor for the pyrimidine uracil) and uracil. The elevated levels of these purine/pyrimidines are suggestive of the S-phase in the cell cycle (Cohen *et al.*, 1983), since purine/pyrimidines are required for DNA synthesis during mitosis. Elevated putrescine detected in the cells with high CCR5 levels plays a vital role in the cell cycle, since it is a precursor for those components needed to package DNA. The metabolic data suggests increased cell growth and proliferation in the cells with high CCR5 levels. Glycine plays a vital role in the metabolism of cells, by serving as a precursor for numerous metabolic molecules such as creatine, glutathione and nucleic acids (Badenhorst *et al.*, 2013). The elevated glycine detected in Cf2Th cells with high CCR5 levels is a product of purine/pyrimidine synthesis. ROS is a known byproduct of the OXPHOS process (Murphy, 2009). ROS accumulation can be fatal to the cell and the antioxidant effects of glutathione are well known (Franco & Cidlowski, 2009; Marí *et al.*, 2009). Glycine can be conjugated to xenobiotics and/or xenometabolites (foreign chemical molecules such as antibiotics, or in this case also quinic acid) to aid in the metabolism and, ultimately, the excretion of these xenobiotics (Jackson, 1991). One study indicated that serine, formed by excess glycine, affects nucleotide metabolism in cancer cells (Labuschagne *et al.*, 2014). De novo serine and glycine biosynthesis is the primary metabolic routes for glucose-derived carbons (Reina-Campos *et al.*, 2020). Therefore, glycine synthesis was possibly upregulated in cells with high CCR5 levels to meet the metabolic demands of the cells. Cf2Th cells with high CCR5 levels also presented with elevated glutamic acid. Glutamine is a vital intermediate or substrate to several metabolic pathways, including converting glutamine to glutamic acid, for α -ketoglutaric acid synthesis (for the TCA cycle) and also functions as an important signalling molecule (Curi *et al.*, 2016). Since glutamine was specifically given as a growth substrate in the cell growth media, the higher concentrations of glutamine/glutamic acid can be explained by an increased uptake induced by the cells with high CCR5 levels (Curi *et al.*, 2005). Phenylalanine can be converted to tyrosine, which can be converted to acetoacetic acid (a ketone body that enters the ketogenic pathway) and fumaric acid (which can enter the glucogenic pathway via the TCA cycle). Therefore, increased phenylalanine can be indicative of the cells' increased energy demands. Elevated levels of L-aspartic acid, an amino acid that serves as a precursor for several pathways such as protein synthesis, suggests increased protein metabolism in Cf2Th cells with high levels of CCR5. The molecule α -ketoglutaric acid (also known as 2-oxoglutaric acid) plays various roles in energy,

carbon, and nitrogen metabolism, and the elevated α -ketoglutaric acid detected in the Cf2Th cells with high levels of CCR5 suggests that these metabolic pathways are altered. In addition to its role as a metabolic intermediate, α -ketoglutaric acid is a signaling molecule that can influence the activities of enzymes that use α -ketoglutaric acid as substrate (Huergo & Dixon, 2015). For example, Carey and co-workers (2015) reported that the succinic acid/ α -ketoglutaric acid ratio could affect chromatin structure and therefore, cell proliferation and differentiation in stem cells (Carey *et al.*, 2015). α -Ketoglutaric acid is also the precursor for 2-hydroxyglutaric acid (Struys *et al.*, 2007), also elevated in the Cf2Th cells with high CCR5 levels. Fu *et al.* (2015) reported that 2-hydroxyglutaric acid inhibits ATP synthase, resulting in a bio-energetic shift in cells (Fu *et al.*, 2015). Leucine, a ketogenic branched-chain amino acid (BCAA), serves as a nitrogen donor to synthesise glutamic acid (Yudkoff *et al.*, 1994). Leucine is also a regulator for glutamate dehydrogenase enzyme activity, positively stimulating glutamic acid synthesis by regulating the mTOR pathway (Wang *et al.*, 2018b). CoA is classified as a helper molecule or co-enzyme, and thus plays a central role in many metabolic pathways (Leonardi *et al.*, 2005). Furthermore, glutamic acid can also be converted to proline, which was significantly elevated in Cf2Th cells with high levels of CCR5. Additionally, the elevated α -ketoglutaric acid levels detected also suggest the conversion of α -ketoglutaric acid (increased in Cf2Th cells with high CCR5 levels) to succinyl-CoA, a precursor to succinic acid also (increased in Cf2Th cells with low CCR5), which in turn supplies flavin adenine nucleotide (FADH₂) to complex II of the ETC, as previously described. Considering the leucine degradation pathway, a defective 3-hydroxy-3-methylglutaryl-CoA lyase enzyme, results in an accumulation of 3-hydroxymethyl glutaric acid (significantly increased in Cf2Th cells with high levels of CCR5), and subsequent reduction in acetyl-CoA and acetoacetic acid. The 3-hydroxymethyl glutaric acid is subsequently conjugated to carnitine, transferred across the inner mitochondrial membrane and released as the free acid and serve as a substrate for cholesterol synthesis. Amide derivatives of carboxylic acids were also elevated in cells with high levels of CCR5 and included dodecanamide (derivative of the MCFA dodecanoic acid), and acetamide (derived from acetic acid).

Free fatty acids may undergo glycerolysis, converting free fatty acids into various acylglycerols. The Cf2Th cells with high CCR5 levels, presented with significantly increased fatty acids, such as pentadecanoic acid and glycerols. Through the process of lipolysis, triglycerides are broken down into fatty acids and glycerol. The fatty acids can subsequently undergo β -oxidation, and eventually form acetyl-CoA, which in turn is a substrate for the TCA cycle. In this study, the increased fatty acids and glycerol, indicate elevated lipid breakdown and use for energy production in the cells with high levels of CCR5. Phospholipids are also synthesised by ethanolamine. In addition, cell growth and development are dependent on molecules such as bis(2-ethylhexyl) phthalate (Crobeddu *et al.*, 2019) and the sugar derivative arabinonic acid.

D-ribose, a sugar in the pentose phosphate pathway, is used to synthesise DNA, ribonucleic acid (RNA), acetyl-CoA and ATP (Pauly & Pepine, 2000). De novo nucleotide synthesis therefore requires ribose as an essential substrate. Ribose also forms part of a salvage pathway in which cells recycle the end products of ATP. Ribose can be converted to ribitol, a vital riboflavin and flavin mononucleotide component. The role of riboflavin in metabolism has been extensively reviewed (Balasubramaniam & Yapliito-Lee, 2020). The elevated levels of L-sorbose, fructose and glucose suggests decreased glycolysis activity since these compounds enter the glycolysis pathway. However, these metabolites can also have alternative metabolic fates. For example, glucose can be converted to inositol, which can exist in different conformations (such as scyllo-inositol and myo-inositol, both of which were elevated in cells with high levels of CCR5) for use in cell membrane synthesis and purine/pyrimidine synthesis. One study reported that inositol affects insulin signaling, ultimately affecting glucose metabolism (Bevilacqua & Bizzarri, 2018). Increased CCR5 levels can result in insulin resistance since CCR5 regulates the recruitment of macrophages and is involved in adipose tissue inflammation induced by obesity (Kitade *et al.*, 2012). In addition, insulin signaling in dogs can be affected by caprylic acid (not statistically significant in the Cf2Th cells). Sanbar *et al.* (1967) investigated the influence of caprylic acid (a saturated fatty acid) on glucose metabolism in euthanised dogs. They reported that caprylic acid increases insulin secretion (Sanbar *et al.*, 1967). Normally, insulin levels in an organism increase to maintain glucose levels. Therefore, these sugars are considered regulatory molecules in context to the metabolism.

In summary, no significant metabolites were identified in Cf2Th cells with basal levels of CCR5, while Cf2Th cells with low levels of CCR5 has elevated levels of one TCA cycle intermediate and one fatty acid. The Cf2Th cells with high levels of CCR5, however showed the most significantly altered metabolic profile, including sugars, amino acids, fatty acids, and purines/pyrimidines. Like the metabolic changes observed for the Affinofile cells, the altered metabolic profiles can be related to the energy demands of the cell and receptor stabilisation. In addition, given the lineage of the Cf2Th cells, it is expected that the statistically significant metabolites reported in the current study will differ somewhat from that of the human-derived cells (Affinofile/HEK293). Because a control cell line was not available for the Cf2Th cells, whether these metabolites relate to CCR5 levels only or the cell line lineage could not be concluded. Figure 5-28 shows the affected metabolic profiles of the Cf2Th cells expressing differential CCR5 levels as described above. Given the metabolic data pre-subtraction of a control cell line it is evident that, although the metabolites' names will differ, the overall pathways affected may be similar (hence the next evaluation in Section 5.3.6 was done).

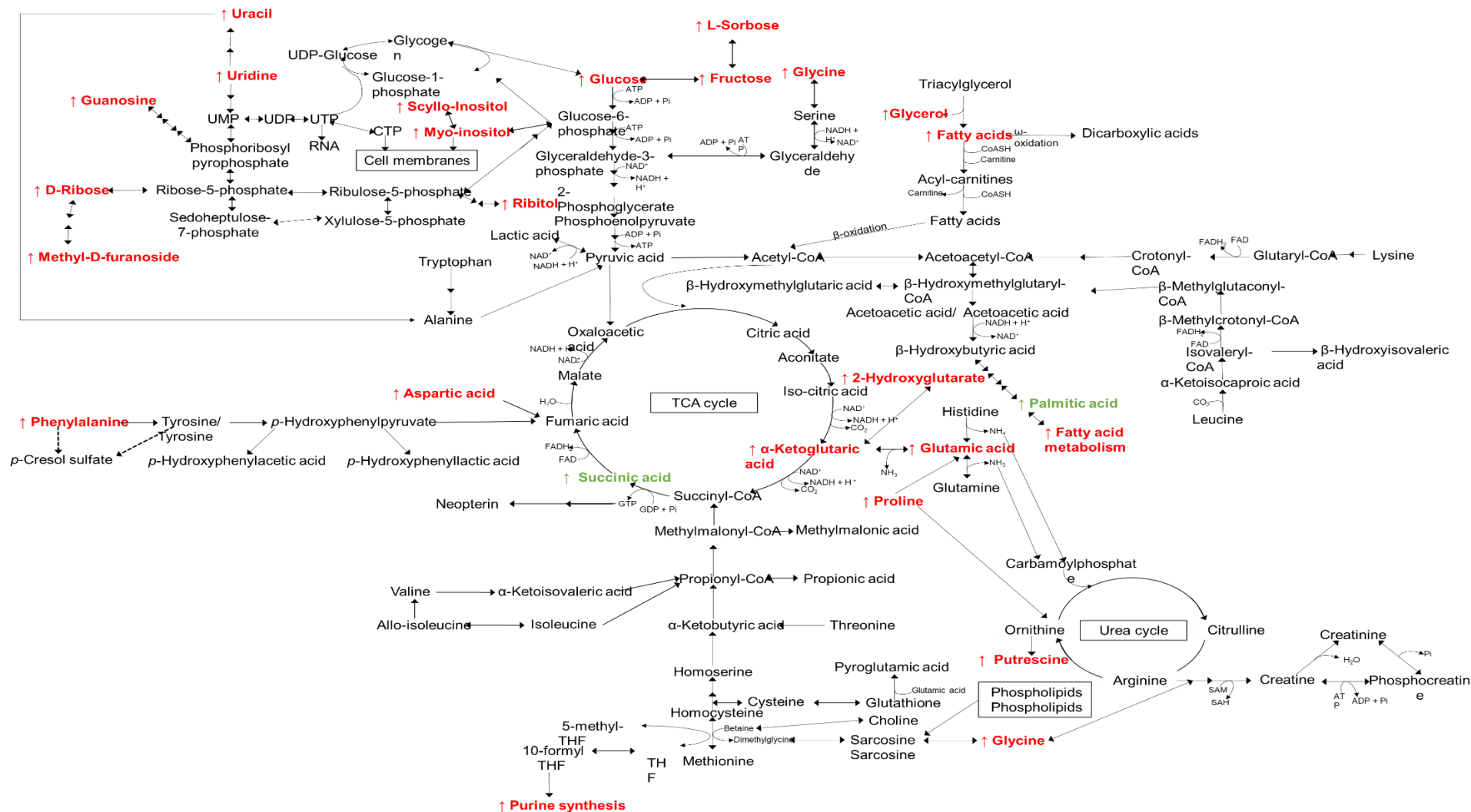


Figure 5-28: Affected metabolic pathways in Cf2Th cells with basal (purple), low (green) and high (red) levels of CCR5. No significant metabolites were identified in cells with basal levels of CCR5. As the receptor levels increase, the metabolic profile of cells shifts to account for the metabolic demands of the receptor and its cell.

5.3.6 Corresponding metabolomics profile of cells presenting differential CCR5 phenotypes

Two different cell lines were used for this project to determine the metabolomics profile of cells presenting with differential CCR5 levels, irrespective of the cell line – in theory, the levels of CCR5 should affect the metabolism similarly in these cell lines, and a general trend should be observed. Since the control cell line for Cf2Th cells was not available for reasons described in section 3, a comparison of the metabolic profiles of the Affinofile and Cf2Th cells (without a control cell line) was also done to identify similar and/or deviating trends.

The metabolic data for each cell line were subjected to PCA, respectively (Figure 5-29). In both cases, minimal separation occurs between the sample groups, suggesting only slight metabolic differences to be evident.

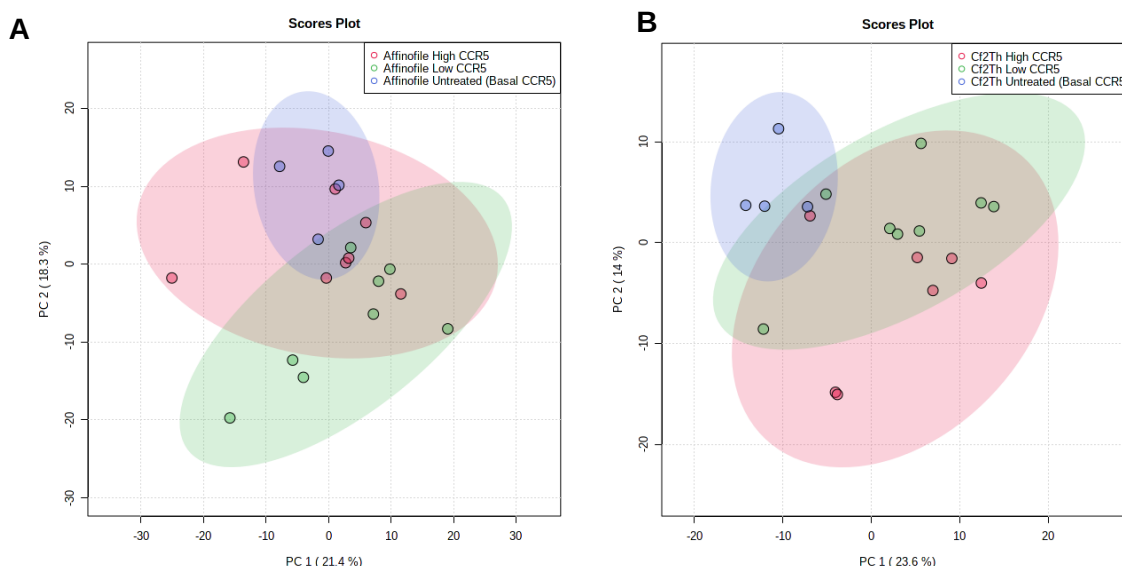


Figure 5-29: PCA scores plot for the metabolic profiles of A) Affinofile cells and B) Cf2Th cells without subtracting a control cell line.

The data for each cell line was further subjected to PLS-DA, respectively. The PLS-DA scores plots are shown in Appendix C (Figure C3). The PLS-DA scores plot failed to validate for the Affinofile (Table C5) and the Cf2Th (Table C3) cells, thus the findings from the PLS-DA were not considered for the identification of important metabolites. Instead, one-way ANOVA was used to identify statistically significant metabolites in Affinofile and Cf2Th cells. The list of important compounds is shown in Annexure C (

Table C6). Eight metabolites showed similar trends in both cell lines and are highlighted in grey.

Both the Affinofile and Cf2Th cells with high levels of CCR5, showed elevated phosphate, suggesting an increased metabolic activity within these cells since phosphate is released during anabolic processes (as previously described in Section 5.3.4). Additionally, the elevated uridine (pyrimidine precursor) and uracil (pyrimidine) metabolism seen in both these cell lines, are vital for nucleotide synthesis needed for cell growth and proliferation (as previously described in Section 5.3.5).

Furthermore, both the Affinofile and Cf2Th cells presenting with low CCR5 levels, showed elevated putrescine, which plays a vital role in the cell cycle, since it is a precursor for components needed to package DNA, further suggesting that cells with increased CCR5 levels (compared to basal CCR5 levels) have increased cell growth and proliferation. The elevated glucose (and L-fucose) and D-glycerate-3-phosphate indicated increased glucose uptake and glycolysis, suggestive of elevated cell proliferation and energy demands related to receptor activation. Increased glutamine uptake (from the cell culture media) could result in elevated L-aspartic acid levels (present in both cell lines presenting with low CCR5 levels). The non-essential amino acid, L-aspartic acid, is a precursor for several other essential amino acids, including methionine, threonine, lysine, and isoleucine. Furthermore, L-aspartic acid can be converted to fumaric acid and oxaloacetic acid, the two TCA cycle intermediates. Both Affinofile and Cf2Th cells, with low CCR5 levels, showed elevated Bis(2-ethylhexyl) phthalate (not shown on the metabolic map), also known as di(2-ethylhexyl) phthalate (DEHP). This molecule forms part of a family of chemical compounds that do not occur naturally. This metabolite was not identified as a significant marker in the Affinofile cells when the HEK293 background was subtracted, the fact that it was identified as a significant metabolite in the Cf2Th cells, suggests that this compound stems from the cell culture procedure and associated Pon A/doxycycline used to induce CCR5 levels.

Taken together, these results suggest that uninfected cells with induced CCR5 levels have increased metabolic activity associated with cell growth, proliferation, and receptor function. Overall, the data suggests that, as CCR5 levels increase, the metabolic profile shifts to meet the metabolic demands of the receptor and the cell. Figure 5-30 shows the affected metabolic profiles of the Affinofile and Cf2Th cells expressing differential CCR5 levels when the background from the control cell line is not subtracted.

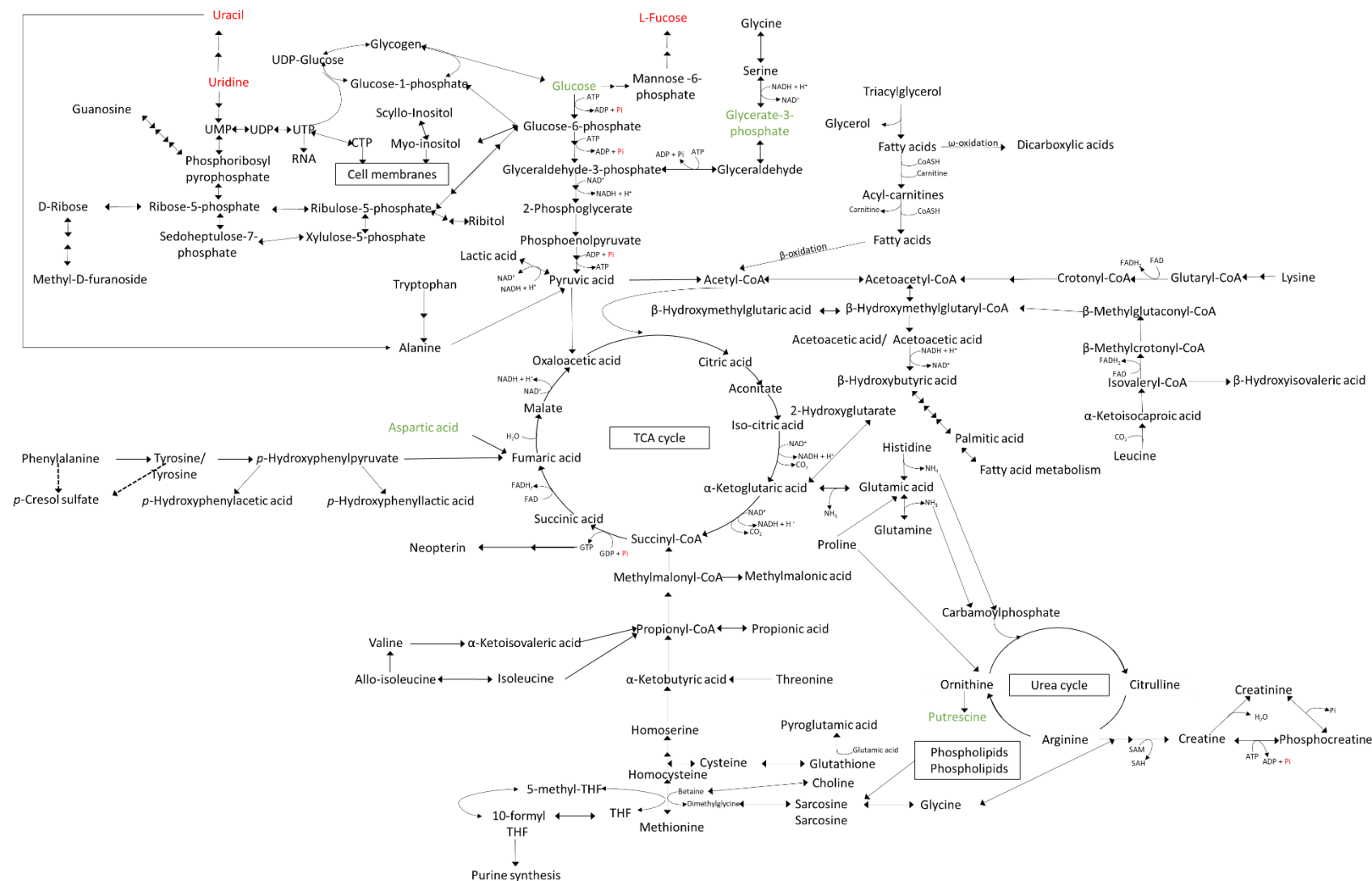


Figure 5-30: Metabolic map showing the overlapping metabolites identified as important in Affinofile and Cf2Th cells when the background signal of a control cell line is not subtracted. Corresponding metabolites found in cells with high levels CCR5 levels are shown in red, while metabolites found in cells with low levels of CCR5 are shown in green. Cells with basal levels of CCR5 did not show any corresponding metabolites in the cell lines.

5.4 Immuno-metabolomics profile of cells presenting differential CCR5 phenotypes

The immune data generated for the Affinofile (Section 5.2.2) and the Cf2Th (Section 5.2.3) cells should be interpreted with caution, due to the generally low concentrations of all cytokines detected in all groups. Additionally, the PLS-DAs failed to validate for all the aforementioned immune and metabolic comparisons (and hence marker selection was done using mainly ANOVA and ES). The immuno-metabolomics profile of the Affinofile cells was subsequently elucidated by association of the respective immune and metabolic profiles through the literature.

5.4.1 Immuno-metabolomics profile of Affinofile cells presenting differential CCR5 phenotypes

The immuno-metabolomics profile of cells presenting differential levels of CCR5 considers if the cytokines and metabolites identified share a relationship, the list of cytokines and metabolites identified as important in Affinofile cells are summarised in Table D1.

Th1, Th2 and Th17 cells are dependent on glycolysis and glutaminolysis to support the function and growth processes of the cells (Chakraborty *et al.*, 2021). Other factors such as the cytokine environment can influence the effector cell phenotype i.e. determine whether the cell differentiate into a Th1 or Th2 effector cell (Santana & Rosenstein, 2003). Upon activation, Th2 cells depend on lipid metabolism for cell growth and division and increase the uptake of glutamine to serve as a nitrogen source, produce TCA cycle intermediates, and ribose. Glutamine favours Th1 responses over Th2 responses (Stark *et al.*, 2019) and promote Th17 responses (Johnson *et al.*, 2018), whereas reduced glutamine levels are associated with reduced IL-2 levels (Cruzat *et al.*, 2018). Resting cells utilise pathways such as TCA to produce ATP via OXPHOS (Norata *et al.*, 2015). In addition, the TCA cycle will produce intermediates required for the biosynthesis of proteins that constitute the biomass of the cell (Pearce & Pearce, 2013).

The Affinofile cells with basal CCR5 levels presented with a general Th1 immune profile, and elevated phosphate and oleic acid. Ross and co-workers (2018) reviewed the signalling pathways and metabolic effects of IL-2 in T cells (Ross & Cantrell, 2018). IL-2 signals can prevent the loss of Glut I transporters on the surface of T cells which regulates glucose uptake (Wofford *et al.*, 2008). Although not in context to CCR5, the immune-metabolic profile in cells have been investigated. In Jurkat cells, oleic acid activates signalling pathways such as PPAR, stimulates TNF- α production (Th1), and modulates the expression of genes related to the cell cycle, proliferation, apoptosis, oxidative stress and cytokine receptors (Cury-Boaventura *et al.*, 2006), thus promoting a Th1 immune profile. In human liver samples, oleic acid upregulated TNF- α and downregulated IL-6, and the authors reported that oleic acid could influence inflammation and

apoptosis in liver cells (Li *et al.*, 2016). Another study has reported that the cytokines IL-6 and TNF- α , inhibits ketogenesis, by influencing the octanoic acid and α -ketoglutaric acid metabolic pathways or modifying the redox state in the mitochondria (Pailla *et al.*, 1998). However, Affinofile cells with basal CCR5 levels presented with the lowest concentrations of TNF- α and the highest concentrations of IL-6 when compared to Affinofile cells with low and high levels of CCR5, respectively. These results suggest that cells with basal CCR5 levels do in fact present with a Th1 immuno-metabolic profile. Upon activation, Th2 cells depend on lipid metabolism for cell growth and division and increase the uptake of glutamine to serve as a nitrogen source, produce TCA cycle intermediates, and ribose (Chakraborty *et al.*, 2021; Johnson *et al.*, 2018). The general immune profile trends for the Affinofile cells showed that cells with low CCR5 levels present with a Th2 / regulatory immune profile, and the metabolic profile showed elevated proteins, fatty acids, TCA intermediates, and methyl-D-ribose. These results suggest that the Affinofile cells with low CCR5 present with an overall Th2 / regulatory immuno-metabolomics profile and regulated by the increased IL-2 levels. Glycolysis enhances the production of IFN- γ by Th1 cells (Stark *et al.*, 2019), and although not statistically significant, the Affinofile cells with low CCR5 levels had the highest levels of IFN- γ .

As CCR5 levels increase from low to high, the trends in Affinofile cells showed an overall Th1 immune profile, and the metabolic profile showed elevated pyruvic acid (a TCA intermediate) and myristic acid (a fatty acid). Myristic acid is a saturated LCFA (n=14) and can serve as an alternative energy source for Th2 cells (Stark *et al.*, 2019) and influence chemotaxis and signal transduction. Based on the immune profile described in section 5.2.2, a Th1 immune profile was expected, but based on the literature, the metabolomics profile suggested a Th2 profile for Affinofile cells with high levels of CCR5. This data suggests that the Th1/Th2 immune profile is regulated by other cytokines in the cell environment (Fishman & Perelson, 1994) which is dependent on the cells' demands.

5.4.2 Immuno-metabolomics profile of Cf2Th cells presenting differential CCR5 phenotypes

The immune profile of Cf2Th cells (no control cell line available to subtract background data) (Section 5.2.3) showed no statistically significant cytokines between untreated cells with basal levels of CCR5, and cells treated with doxycycline to induce low and high levels of CCR5 (summarised in Table D1). Surprisingly, the MFI of TNF- α was detected (Figure B9) but could not be translated to concentrations (Figure B10).

The Cf2Th cells with basal levels of CCR5 presented with an overall Th2 / Th17 immune profile, but the metabolomics profile showed no statistically significant metabolites for the cells with low CCR5 levels (Section 5.3.5) The inflammatory IFN- γ cytokine supports cell differentiation (Farrar

& Schreiber, 1993) and can upregulate glycolysis (Wang *et al.*, 2018a). A previous study found that elevated levels of IL-17A correlated with elevated LDL levels in a mouse model (Cimato *et al.*, 2013), thus showing that IL-17A influences lipid metabolism (Yu *et al.*, 2014). IL-4, is known to be involved in clonal expansion, and influences the mitochondrial TCA cycle in macrophages (Puleston *et al.*, 2019). However, speculations on the association between the immune and metabolic profiles of Cf2Th cells cannot be made with certainty and only trends are reported.

General immune profile trends for the Cf2Th cells with low levels of CCR5 showed a clear regulatory profile since IL-2 levels (significant by ES determination) was the only cytokine that was significantly decreased relative to Cf2Th cells with basal and high CCR5 levels, respectively. Succinic acid and palmitic acid were elevated in the Cf2Th cells with low levels of CCR5. As previously mentioned, palmitic acid exerts pro-inflammatory effects and influences cytokine production (Huang *et al.*, 2012; Takahashi *et al.*, 2012; Zhou *et al.*, 2019). Natural killer cells present with increased glycolysis and OXPHOS activities, in the presence of IL-2 (Keating; Keppel *et al.*, 2015).

As CCR5 levels increased from low to high CCR5 levels, Cf2Th cells presented with an overall Th2 immune profile, together with several elevated metabolites. However, the Cf2Th cells with high CCR5 levels, had the highest number of statistically significant metabolites, associated with several different metabolic pathways, including purine/pyrimidine metabolism, sugar metabolism, amino acid metabolism and fatty acid metabolism. Resting cells utilise pathways such as TCA to produce ATP via OXPHOS (Norata *et al.*, 2015). In addition, the TCA cycle will produce intermediates required for the biosynthesis of proteins that constitute the biomass of the cell (Pearce & Pearce, 2013). As previously mentioned, immune cells require glycolysis and glutaminolysis to support the function and growth processes of the cells (Chakraborty *et al.*, 2021) and Th2 cells depend on fatty acid metabolism. Given the elevated levels, i.e. the accumulation, of metabolites involved in glycolysis and glutaminolysis, it seems that Cf2Th cells with high levels of CCR5 depend on alternative metabolic pathways to sustain the energy demands of these cells. The Cf2Th cells with high levels of CCR5 showed higher IL-10 concentrations compared to Cf2Th cells with basal and low CCR5 levels, respectively. Arginase-2 is a mitochondrial enzyme involved in ammonia detoxification via the urea cycle (Caldwell *et al.*, 2018), and is regulated by the cytokine IL-10, which alters the metabolic profiles in macrophages by influencing the activity of succinate dehydrogenase, thus affecting the TCA cycle (Dowling *et al.*, 2021). Wang and co-authors (2021) treated natural killer cells with IL-10, which resulted in an elevated glycolysis and OXPHOS, via mTOR, resulting in increased IFN- γ secretion and a subsequent cytotoxicity (Wang *et al.*, 2021). Additionally, some metabolites, such as α -ketoglutaric acid and 2-hydroxyglutaric acid (both increased in these cells), can inhibit mTOR (Chin *et al.*, 2014). In addition, IL-4 was slightly increased in Cf2Th cells with high levels of CCR5 compared to cells with low levels of

CCR5. IL-4 regulates glucose and fatty acid metabolism (Tsao *et al.*, 2014) suggesting that, as CCR5 levels increase, metabolic reprogramming occurs.

5.4.3 Corresponding immuno-metabolomics profile of distinct cell lines presenting differential CCR5 phenotypes

By comparing the immune and metabolic profiles of Affinofile and Cf2Th cells (no background data subtracted), corresponding cytokines and metabolites could be identified, e.g., the same cytokines/metabolites increased in cells with the same levels of CCR5 (Table 5-1). IL-2 was the only cytokine identified as important and was identified in Affinofile cells with basal levels of CCR5, and in Cf2Th cells with low levels of CCR5, without subtracting a background cell line from either cell lines. The most significant ES occurred between the cells having basal and low CCR5 levels for both cell lines. As discussed in Section 5.3.6, no corresponding metabolites in cells with basal levels of CCR5 was identified. Cells with low CCR5 levels showed elevated bis(2-ethylhexyl) phthalate, glucose, L-aspartic acid, D-glycerate-3-phosphate and putrescine. Cells with high levels of CCR5 showed increased L-fucose, phosphate, uracil and uridine levels.

Table 5-1: Corresponding cytokines and metabolites in Affinofile and Cf2Th cells with differential CCR5 levels (no subtraction of a control cell line).

	Basal CCR5 levels	Low CCR5 levels	High CCR5 levels
Corresponding significant cytokines	-	-	-
Corresponding significant metabolites	-	bis(2-ethylhexyl) phthalate glucose L-aspartic acid D-glycerate-3-phosphate putrescine	L-fucose phosphate uracil uridine

CHAPTER 6: CONCLUSIONS

6.1 Concluding summary

This study set out to determine the immuno-metabolic profile of two distinct cell lines presenting with various levels of CCR5. The use of two distinct cell lines was to increase the confidence of reporting on CCR5-specific findings. First, the cells were expanded and cultured (Objective 1). The non-toxic antibiotic concentrations optimal for culturing the Cf2Th cells was determined using flow cytometry (Objective 2). Furthermore, the basal levels of CCR5 were determined for Affinofile, HEK293 and Cf2Th cells, which also indicated that CCR5 induction was necessary. After determining the optimal concentration of the induction reagents required to induce the various levels of CCR5 in the cell lines, the MFI shift of CCR5-PE was quantified and represented by ABS/cell (Objective 3), following induction of CCR5 levels (Objective 4), and the viability of cells was determined and shown to be high in all cell lines. Given the recovery stage incorporated in this study, the expected CCR5 signal was diluted, although untreated, low and high levels of CCR5 was still evident and within physiological ranges (Objective 5). Upon confirming both low and high levels of CCR5 using the appropriate volumes of CCR5-PE, the immuno-metabolic profiles of these cells were investigated (Objectives 6 - 9).

Similar trends were observed in the overall immune profiles of the cells i.e., the identification of IL-2 was important in both cell lines. For Affinofile cells, as CCR5 levels increased from basal to low levels of CCR5, the cytokine profile tended to shift from a Th1 to a Th2 immune profile, and a further increase in CCR5 levels resulted in a shift towards a Th1 profile. In contrast, for Cf2Th cells, as CCR5 levels increased from basal to low levels of CCR5, the immune profile switched from a Th2 to a Th1 immune profile, while a further increase in CCR5 levels resulted in a shift to a Th2 immune profile. The contradictory results might be explained by the unavailability of a control cell line for Cf2Th cells.

The second significant finding was the altered metabolic profiles of cells with different CCR5 levels. The TCA cycle and other pathways related to energy metabolism were consistently identified as significant in the cells. For Affinofile cells, the increase in receptors (and receptor activation) reprograms the metabolism of these cells based on the metabolic demands of the receptors and its associated cell(s). As CCR5 receptor increased, metabolites such as myristic acid known for its functionality in receptor stabilisation was also identified. Similar deductions could be made from the metabolic data for the Cf2Th cells. In both cell lines the number of metabolites and/or metabolic pathways impacted change with CCR5 levels and the consequent energy requirements (most evident during the initial shift from basal CCR5 to low CCR5 when the cell is first triggered).

IL-2, a cytokine with known roles in cell processes such as differentiation and homeostasis, was identified as an important molecule in cells with differential levels of CCR5 which agrees with the metabolic differences observed in this study. While correlation analysis could not be performed, general trends and associations between the immune and metabolic profiles were interpreted and associations using the literature as a guide were drawn and aided in the elucidation of the immune-metabolic profiles. Further investigation regarding the role of IL-2 and the shift toward the different immune profiles and its association to CCR5 levels is warranted.

6.2 Strengths of this study

The use of two distinct cell lines for the stated aim offers several advantages. Firstly, the cell lines stem from different lineages, while containing the same Tet-On system, allowing one to compare findings, by investigating and comparing corresponding immune and metabolic molecules (without subtracting control cell lines). Previous studies have used Affinofile cells to investigate HIV-related research questions. Here it was used to better characterise the role of CCR5 in an uninfected context. Due to the availability of a control cell line for the Affinofile cells (HEK293 cells), the immune and metabolic differences of Affinofile cells with differential CCR5 levels could be concluded. Additionally, GCxGC-TOFMS is an extremely sensitive analytical apparatus, which allowed for the detection and comprehensive reporting on the metabolite changes observed. While the instruments and reagents required to perform flow cytometric experiments are costly, and extensive sample preparation is required (Jahan-Tigh *et al.*, 2012), flow cytometry offers a variety of advantages. These advantages include that the flow cytometer has faster run-times comparatively per individual sample, and allows for multiplex assays, with small sample volumes. Furthermore, each fluorochrome can deliver information pertaining to a selected parameter, and multiple fluorochromes can be used in the same experiment. Additionally, the fluorescence intensity of the fluorochromes measured, is directly proportional to the amount of molecule being assayed, making it a suitable method for quantification. Flow cytometry offers excellent sensitivity and specificity due to the specificity of the antibodies used. Hence, in summary, utilising the flow cytometer in this investigation, allowed us not only to investigate multiple parameters of the cells, but also determine the receptor levels accurately, while simultaneously determine cell viability, mode of cell death, and secreted cytokine trends.

6.3 Limitations of this study

The most obvious limitation lies in the fact that genetically engineered cell lines require the appropriate controls. The current investigation was thus limited because an appropriate control for the Cf2Th cell line was not immediately available. Additionally, the use of HEK293 cells, which is the parent cell line for the Affinofile cells, although reported to have low basal CCR5 (Ling *et*

al., 1999), did indeed express slightly higher levels of CCR5 than expected (which was still lower than the Affinofile CCR5 levels). Nevertheless, regarding the metabolomics investigations, the expansion of cell lines in the absence of selection antibiotics, until the cells lose the vector, would serve as a better control, but require time which was already limited for this MSc study as per challenges mentioned in Section 3.4.

While immune-metabolomic associations can be derived using the literature, correlation analysis was lacking, but could provide more meaningful information.

A human Th1/Th2/Th17 kit was used for canine samples and may have contributed to the low concentrations detected (especially TNF- α that was not detected in any Cf2Th cell samples).

Harvesting cells for metabolomics analysis is time and labour-intensive, resulting in small sample groups for each cell line. This study design showed that immediate harvest after induction results in approximately 100 000 ABS/cell, while allowing the cells to recover after induction results in approximately 6000 ABS/cell. Where subsequent experiments will require such recovery, the reduced CCR5 signal warrants an alternative experimental design.

6.4 Future considerations

The current study utilised genetically engineered cells to induce CCR5 using the Tet-On system. It is well known that CCR5 is involved in signal transduction interactions with various molecules, resulting in a biological effect. The critical ligands for CCR5 include: MIP-1 α , MIP-1 β , RANTES and MCP-2 and, and upon binding to CCR5, lead to a conformational change of the receptor and ultimately result in a signalling transduction cascade (Abrol *et al.*, 2014). The various signalling molecules/ligands needed to interact with CCR5, may not necessarily have been present in this system, the media was not supplemented with any such ligands, hence the immune and metabolic differences are not as exaggerated on the PCA scores plots. Future studies can be designed to include ligands. In addition, HEK293 and Cf2Th cells may not express the right toll-like receptors to respond and produce cytokines. Further studies should be designed to determine the presence of toll-like receptors on the surface of these cells by exposing the cells to toll-like receptor ligands.

In this investigation, the addition of the induction antibiotics did not induce CCR5 in 100% of the cells (as can be seen in Figure 5-2 and Figure 5-3). These cells are not presenting maximum CCR5 levels which possibly dilutes some of the immune and metabolic changes observed. Therefore, it would be advisable to perform this investigation using samples in which all the cells in the sample present with either high or low CCR5 levels post CCR5 induction and investigate the immune and metabolic profile in these cells. This can be achieved by cell sorting.

During the cell culture process, cells were incubated with the CCR5-inducing reagents for 18 hours. After the induction period, the supernatant was discarded. It is advisable to collect the supernatant and investigate the extracellular immuno-metabolic profile in this sample. Other abundant metabolites and cytokines excreted into the cell culture media may inform more on intracellular processes.

The use of supernatant samples allowed us to investigate the extracellular immune profiles. The investigation of the immune profile for both cell lines has shown that the absence of infection results in cytokine levels that are often below the detection limit. In addition, for the measurement of cytokines produced by the Cf2Th cell line, and future studies should consider a better suited kit for such experiment. As mentioned, using a human Th1/Th2/Th17 kit might not be optimal for detecting all the cytokines produced by the Cf2Th cells. Therefore, an alternative immune assay, such as the Milliplex canine cytokine/chemokine bead panel (which uses Luminex technology) is suggested to confirm the extracellular immune profile of the Cf2Th cells. Luminex technology has been applied to successfully study cytokines/chemokines in various canine-derived samples (Hussain *et al.*, 2018; Martinez *et al.*, 2018). Measuring the intracellular immune profile would further add to the existing knowledge regarding CCR5 in the absence of infection.

In addition to using antibodies and flow cytometry to measure CCR5 levels on the cell surface, it is possible to use western blots to measure the CCR5 protein or quantitative polymerase chain reaction (q-PCR) to measure the mRNA for CCR5. As evident in the literature and from this study, the use of cell lines transfected with the Tet-On vector, holds some implications, since the addition of induction antibiotics may affect various signalling pathways and, therefore, the immune and metabolic profiles of these cells. Therefore, it may be interesting to assess the effects of induction antibiotics on the signalling pathways as an added parameter to consider, when interpreting the immune and metabolic results.

For the metabolomics analyses, the cell counting during the cell harvesting procedures is labour and time-intensive, which adds to the amount of cell death observed in samples. When performing metabolomics analysis using cell samples, it will be helpful to consider other pre-acquisition normalisation strategies such as weighing wet pellet mass (Misra, 2020) or DNA concentration (Silva *et al.*, 2013), which is often done in plate formats.

Regarding the GCxGC-TOFMS data, it will be helpful to expand the current reference library to address the unknown metabolites identified as statistically significant or use a targeted metabolomics approach to investigate compounds of interest.

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Annexure A: Materials and reagents used in this study

Table A1: Table summarising the main materials/reagents used in this study, arranged alphabetically.

Material/reagent	Supplier	Catalogue #
3-Phenylbutyric acid	Merck	116807-5G
7-AAD	BD Bioscience	BD/559925
Acetonitrile	Anatech	BJ017CS
BD Detergent solution concentrate for BD Accuri™ C6+	BD Bioscience	BD/660585
BD Quantibrite™ beads	BD Bioscience	BD/340495
BD Sheath additive for BD Accuri™ C6+	BD Bioscience	BD/660584
Blasticidin	ThermoFisher Scientific	A1113903
BSTFA	Merck	15238-10x1ML
CCR5-PE (Clone 2D7)	BD Bioscience	BD/555993
Cell culture-grade water	Separations	SH30529.FS
Chloroform	Anatech	BJ049CS
CO ₂ gas	Afrox	201-RC
CS&T Beads	BD Bioscience	BD/656504
Cytometric bead array kit	BD Bioscience	560484
Dimethyl sulfoxide (DMSO)	Sigma Aldrich (St. Louis, Missouri, USA)	D2650
DNA LoBind microcentrifuge tubes	Merck	EP0030108051
Doxycycline Hyclate	Sigma Aldrich (St. Louis, Missouri, USA)	D3072-1ML
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma Aldrich (St. Louis, Missouri, USA)	D6429
Eppendorf Tips: Blue (100 µl – 1000 µl)	Merck	EP0030000919
Eppendorf Tips: Yellow (20 µl – 200 µl)	Merck	EP0030000870
Eppendorf Tips: White (1 µl – 10 µl)	Merck	EP0030000811
Ethanol	SkyLabs	CHEM-E-05100
Fatty Acid Methyl Esters (FAME's)	Merck	1269119-100MG
Fixable viability dye (FVD)	ThermoFisher	65-0864-14
Formaldehyde solution	Sigma Aldrich (St. Louis, Missouri, USA)	F1635-500ML

G418	Sigma Aldrich (St. Louis, Missouri, USA)	4727878001
GC vial caps	Chemetrix	51855820
GC vials	Chemetrix	YYRES21154
Glass Pasteur pipettes	Sigma Aldrich	612-1702
Helium gas	Afrox	524203-SE-C
Hygromycin	ThermoFisher Scientific	10687010
Iso-Octane	Merck	PHR1915-3X1.2ML
Isopropanol	SkyLabs	CHEM-I-09400
Liqui-Nox	Merck	Z742916-3,8L
Methanol	Anatech	HW34966
Methoxyamine hydrochloride	Sigma Aldrich	89803-5G
L-Norleucine	Merck	N6877-1G
Nitrogen (gas)	Afrox	98-SE
Penicillin-Streptomycin	ThermoFisher Scientific	15140122
Phosphate-buffered saline (PBS)	Separations	SH30028.02
Ponasterone A	ThermoFisher Scientific	H10101
Pulled point GC vial inserts	Chemetrix	51832085
Puromycin	ThermoFisher Scientific	A1113803
Pyridine	Merck	270970-4x25ml
Rxi-17 Cap. Column (30m 0.25mm ID 0.25um)	Leco	YY-RES-13523
Rxi-5Sil MS Cap. Column (30m 0.25mm ID 0.25um)	Leco	YY-RES-13623
Tetracycline-free FBS	SepSci	P30-3602
Topaz Liner, Split Precision Liner w/Wool (4mm x 6.3 x 78.5)	Leco	YY-RES-23305
Trypsin-EDTA	ThermoFisher Scientific	15400054
Zeocin	ThermoFisher Scientific	R25001

Annexure B: Immune profile supplementary data

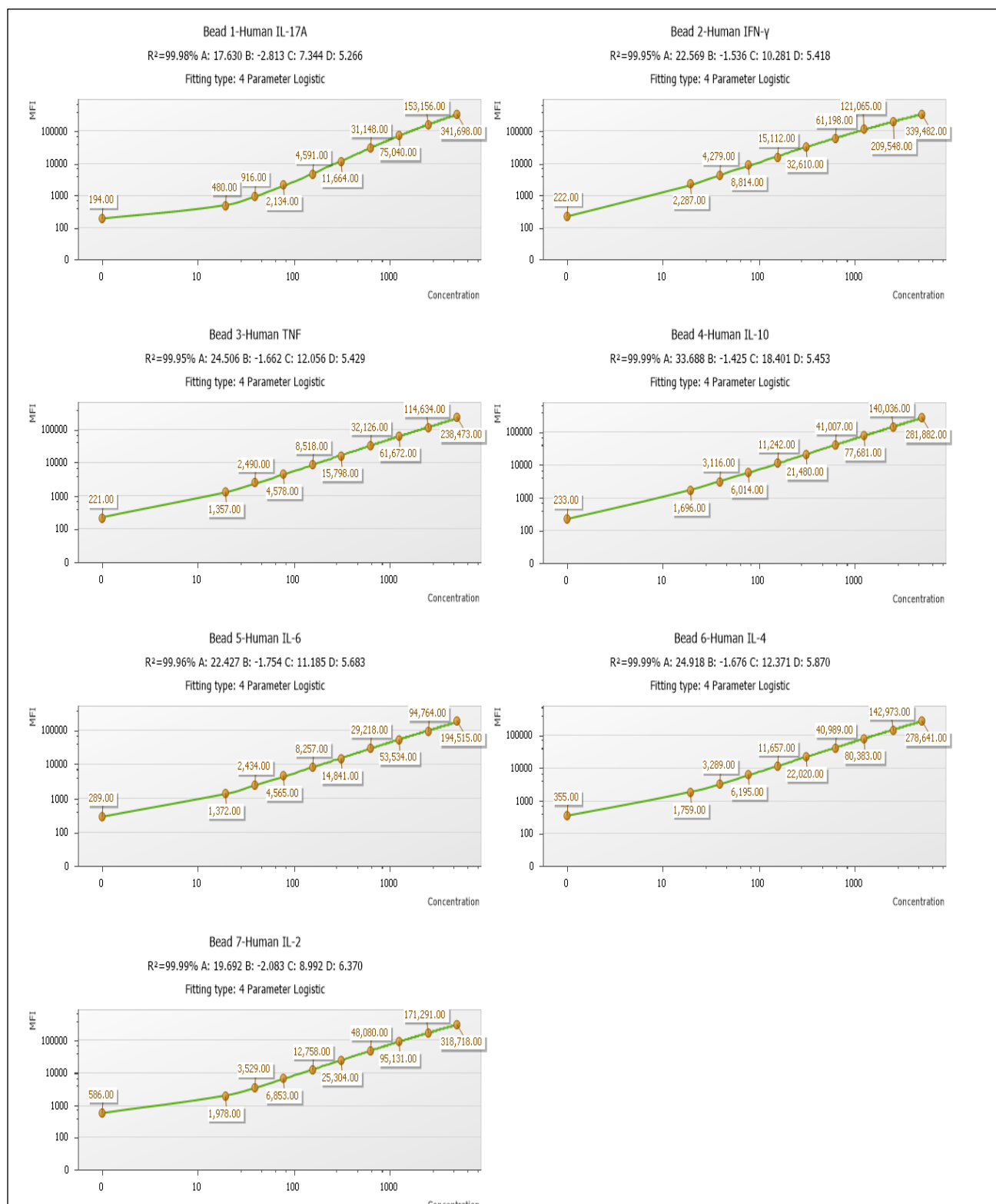


Figure B1: Standard curves for each cytokine standard measured post use of the human Th1/ Th2/ Th17 CBA kit and flow cytometric analysis. The non-linear standard curve fitting was done using FCAP Array.

Abbreviations: IFN- γ - Interferon-gamma; MFI – mean fluorescence intensity; TNF – Tumor necrosis factor; IL – interleukin.

Table B1: Theoretical limit of detection for each cytokine

Cytokine	Limit of detection (pg/ml)
IL-2	2.6
IL-4	4.9
IL-6	2.4
IL-10	4.5
TNF-α	3.8
IFN-γ	3.7
IL-17A	18.9

Abbreviations: pg/ml – picograms per millilitre; IFN- γ - Interferon-gamma; TNF- α – Tumour necrosis factor-alpha; IL – interleukin.

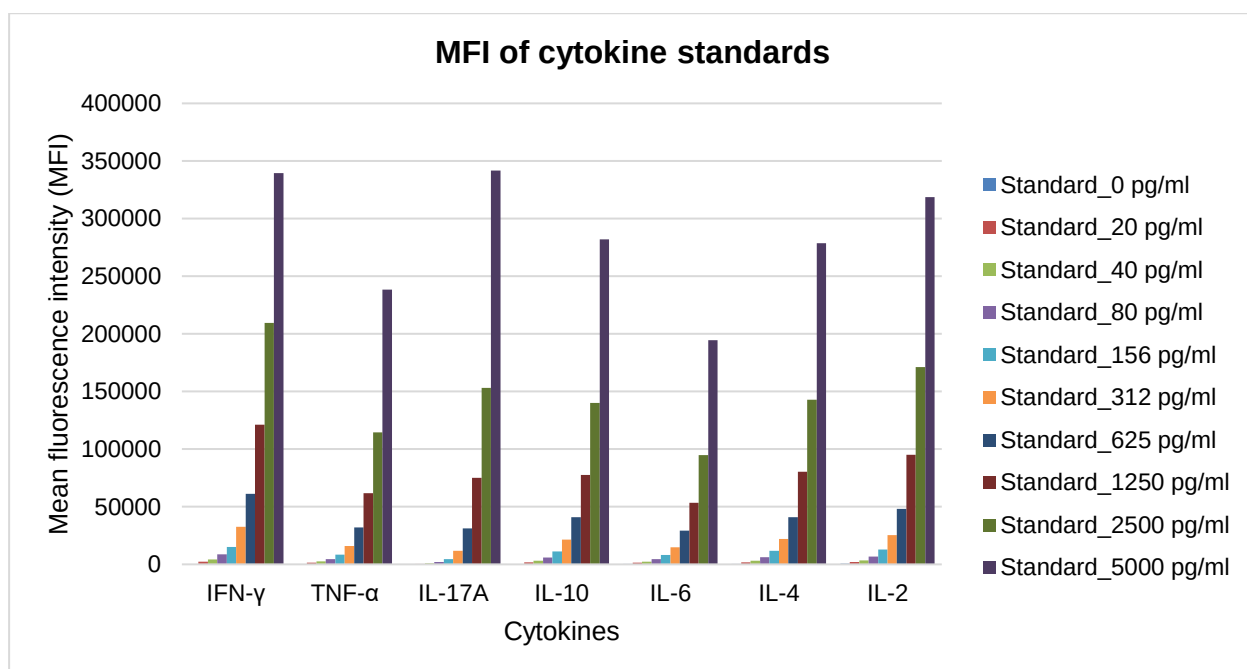


Figure B2: Bar graph showing the mean fluorescence intensity for each cytokine standard.

Abbreviations: IFN-γ - Interferon-gamma; MFI – mean fluorescence intensity; pg/ml – picograms per millilitre; TNF-α – Tumour necrosis factor-alpha; IL – interleukin.

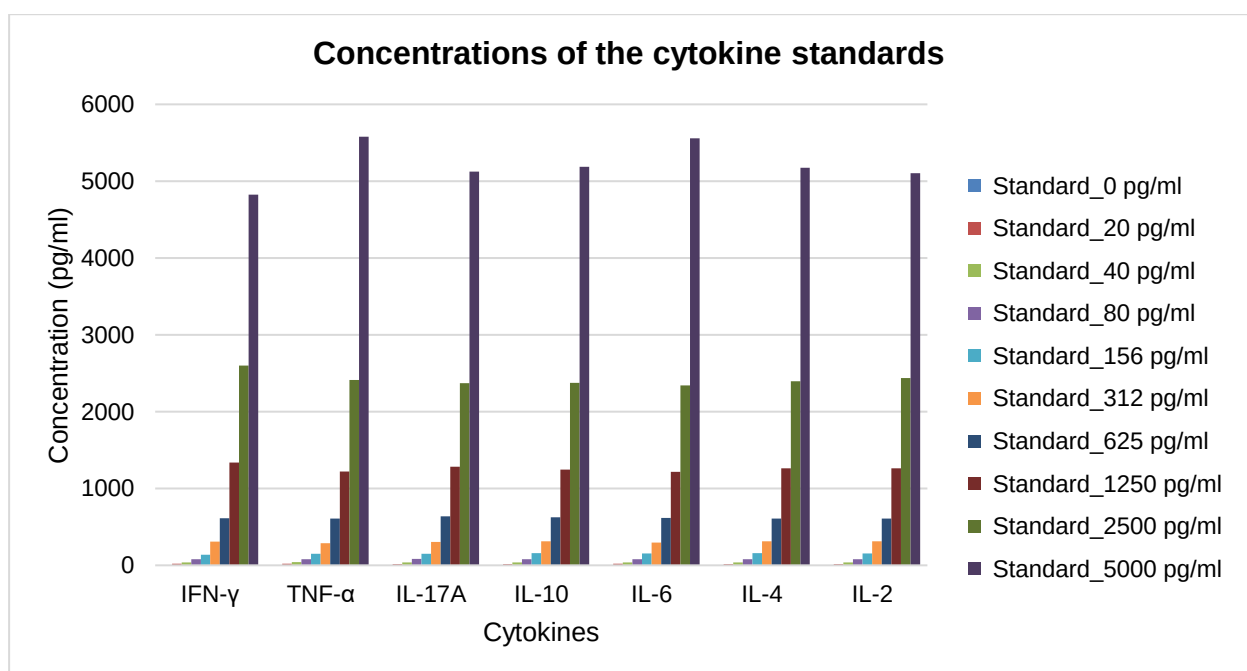


Figure B3: Bar graph showing concentrations for each cytokine standard.

Abbreviations: IFN-γ - Interferon-gamma; MFI – mean fluorescence intensity; pg/ml – picograms per millilitre; TNF-α – Tumour necrosis factor-alpha; IL – interleukin.

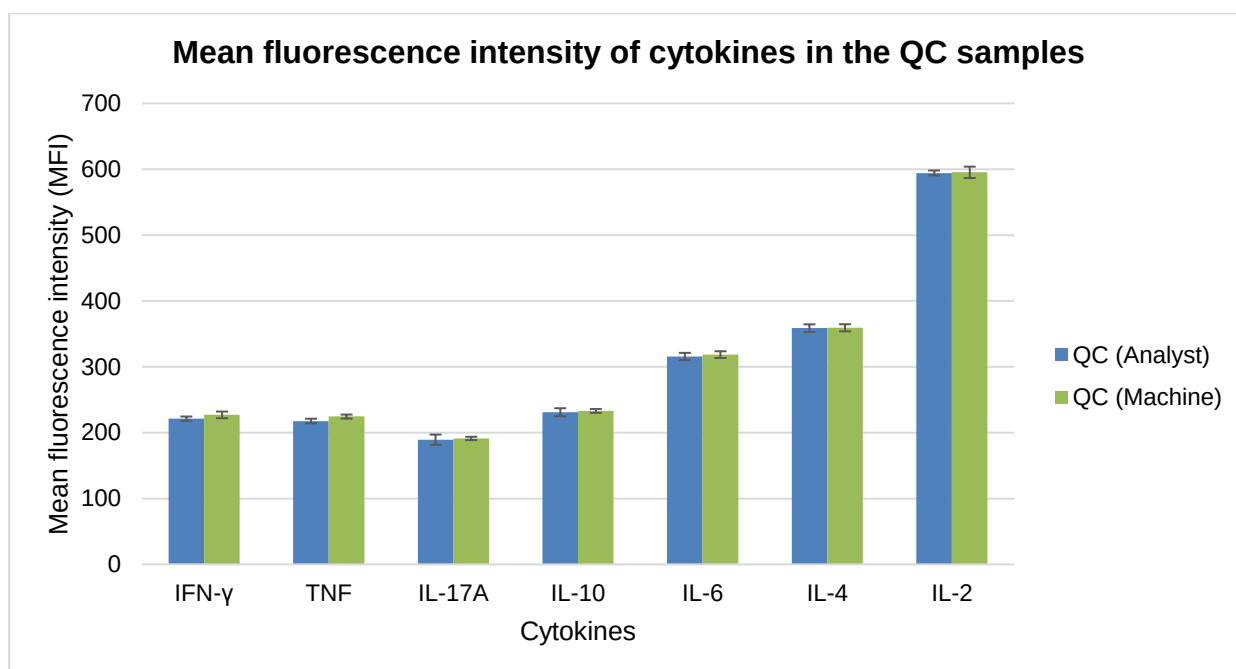


Figure B4: The cytokines' mean fluorescence intensity (MFI) measured in QC samples, The MFI values for each cytokine represent the average of three replicates (analyst repeatability) and eight replicates (machine repeatability), respectively, and error bars show average $\pm$ standard deviations.

Abbreviations: IFN- γ - Interferon-gamma; MFI – mean fluorescence intensity; QC – Quality control; TNF- α – Tumour necrosis factor-alpha; IL – interleukin.

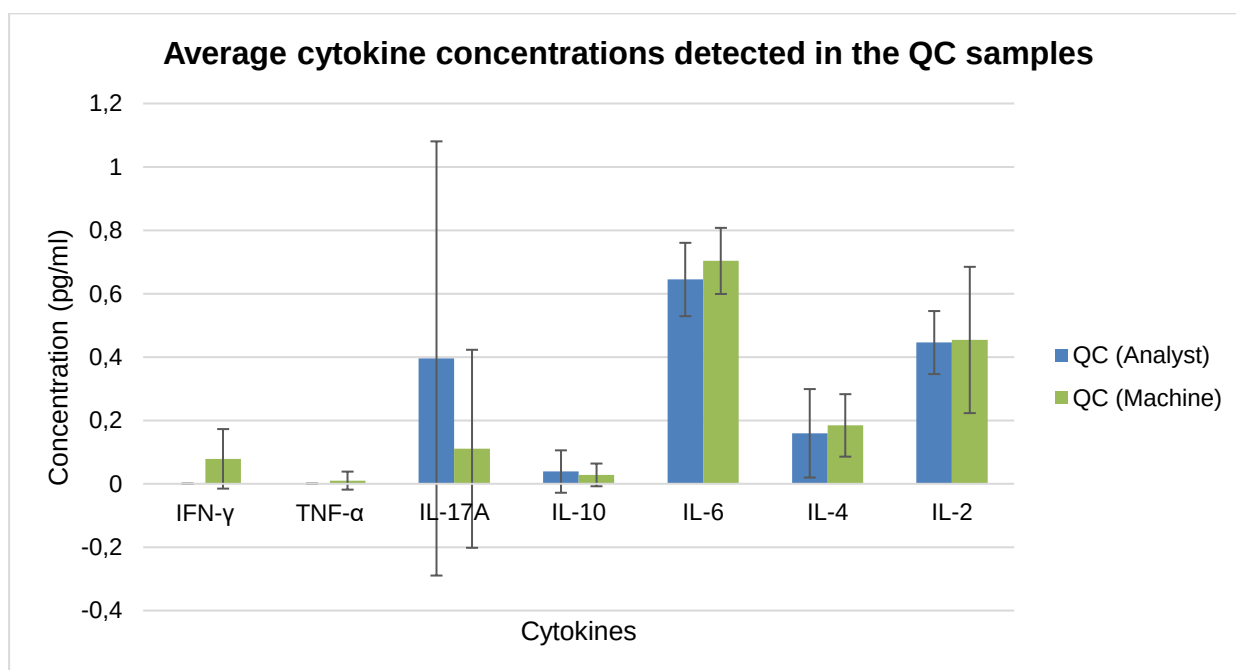


Figure B5: Cytokine concentrations measured in QC samples. The concentrations for each cytokine represent the average of three replicates (analyst repeatability) and eight replicates (machine repeatability). The error bars show average $\pm$ standard deviations.

Abbreviations: pg/ml – picograms per millilitre; IFN- γ - Interferon-gamma; TNF- α – Tumour necrosis factor-alpha; IL – interleukin.

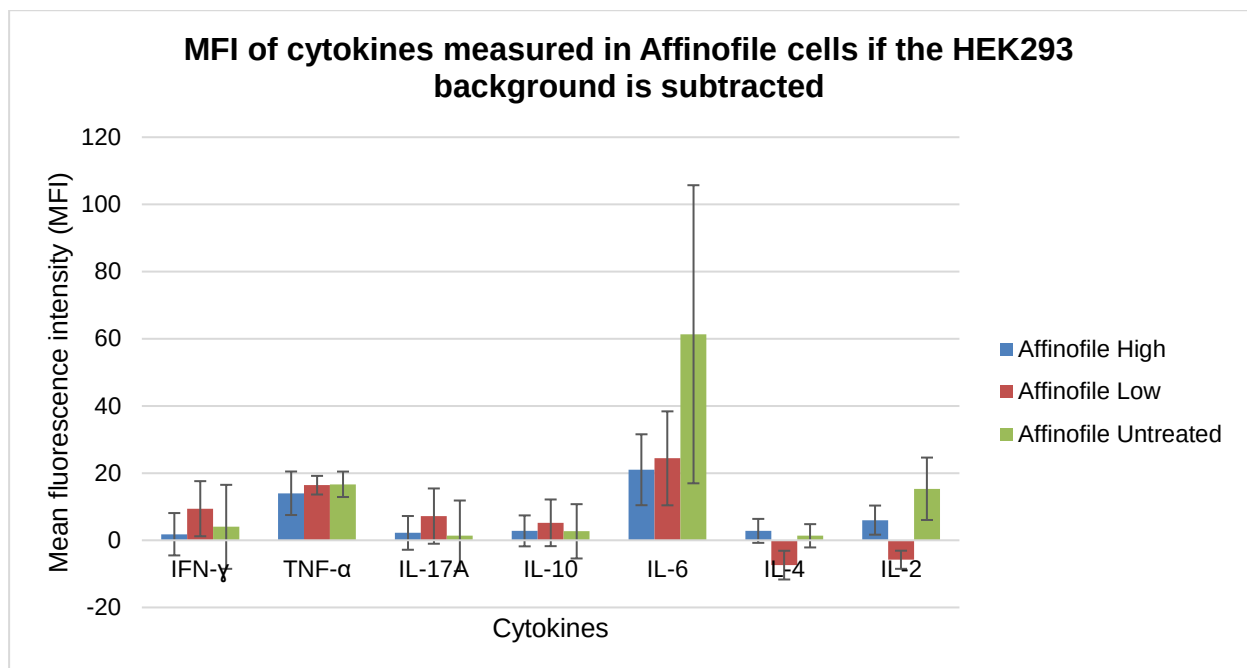


Figure B6: Cytokines' mean fluorescence intensity (MFI) measured in Affinofile cells if the HEK293 background is subtracted. The MFI values for each cytokine represent the average of five replicates (high and low CCR5, respectively) and three replicates (basal CCR5). The error bars show average $\pm$ standard deviations.

Abbreviations: HEK293 – Human embryonic kidney; IFN- γ - Interferon-gamma; MFI – mean fluorescence intensity; TNF- α – Tumour necrosis factor-alpha; IL – interleukin.

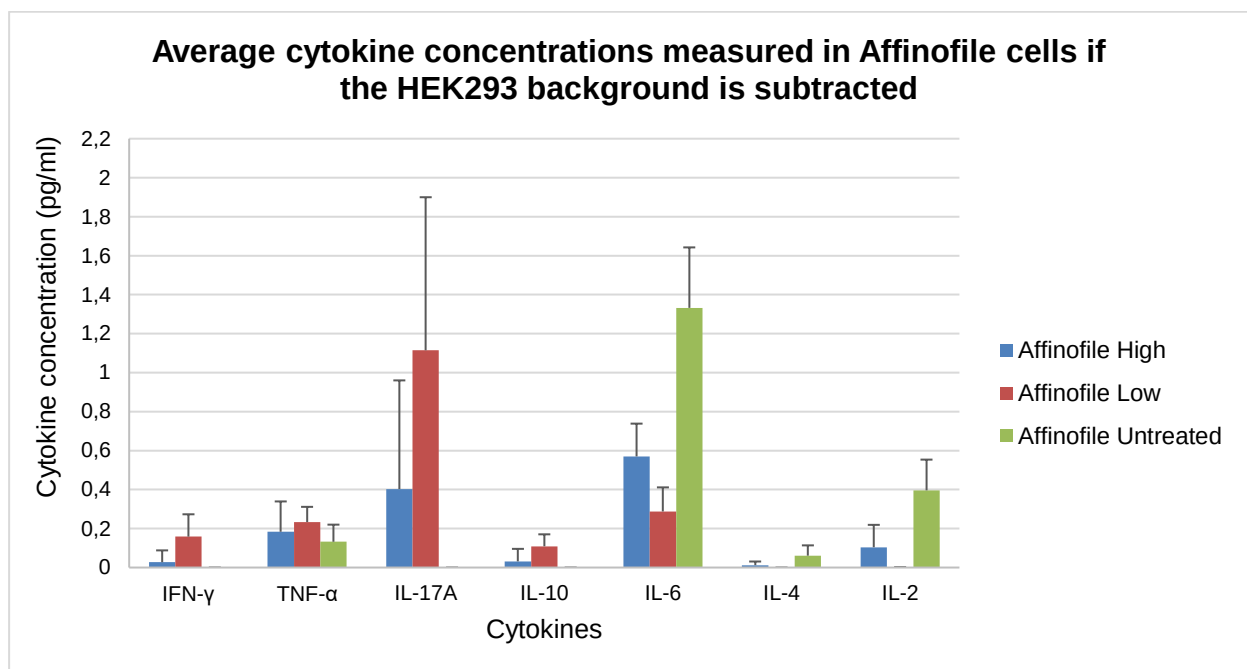


Figure B7: Cytokine concentrations measured in Affinofile cells if the HEK293 background signal is subtracted. The concentrations for each cytokine represent the average of five replicates (high and low CCR5, respectively) and three replicates (basal CCR5). The error bars show average $\pm$ standard deviations.

Abbreviations: Cf2Th – canine thymocytes; HEK293 – Human embryonic kidney; pg/ml – picograms per millilitre; IFN- γ - Interferon-gamma; TNF- α – Tumour necrosis factor-alpha; IL – interleukin.

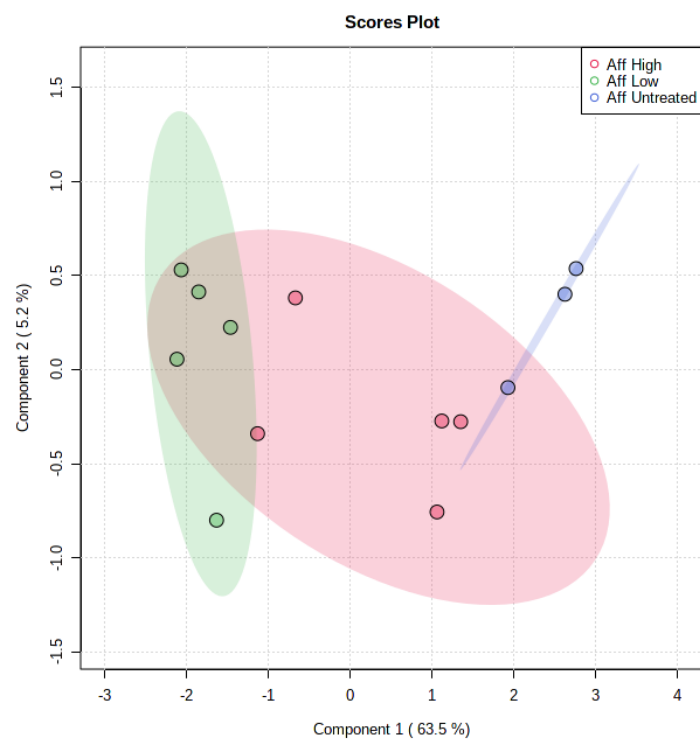


Figure B8: PLS-DA scores plot showing group separation based on cytokine profiles measured in the supernatant of Affinofile cells with basal, low and high CCR5 levels (post subtraction of the HEK293 background concentrations).

Table B2: Cross validation results of the PLS-DA for Affinofile cells

Measure	1 Component	2 Components	3 Components
Accuracy	0.38462	0.30769	0.46154
R ²	0.098045	0.32113	0.46881
Q ²	-0.37308	-2.5518	-2.7105

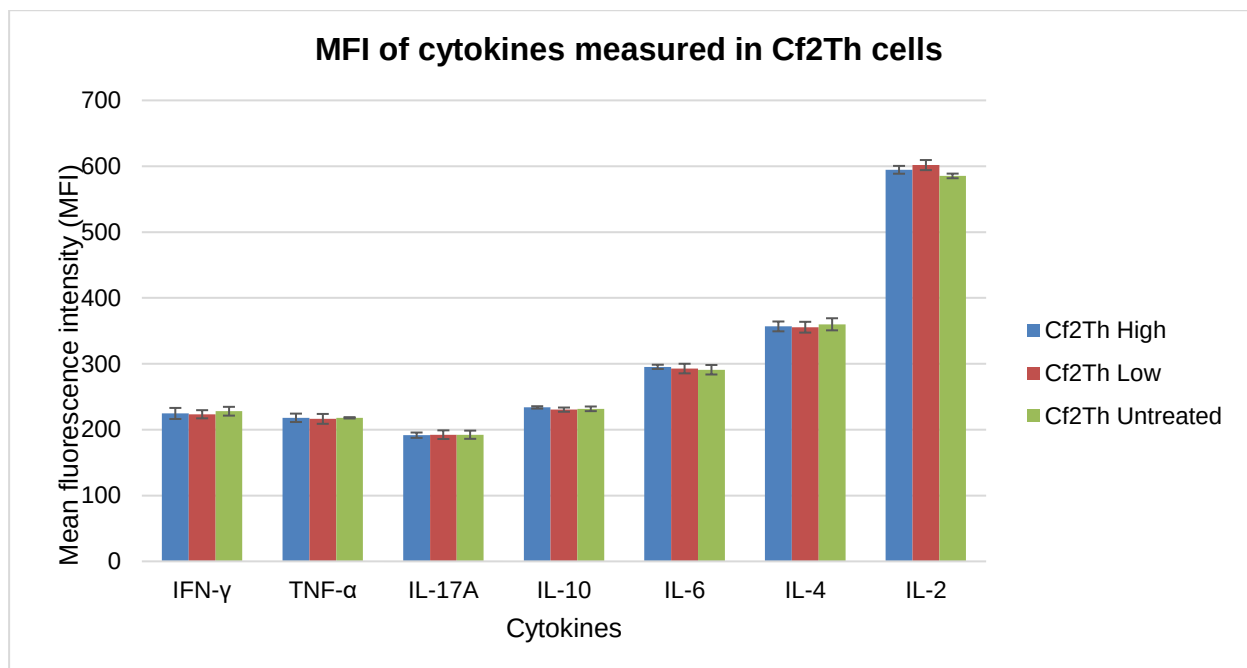


Figure B9: Cytokines' mean fluorescence intensity (MFI) measured in Cf2Th cells. The MFI values for each cytokine represent the average of five replicates (high and low CCR5, respectively) and three replicates (basal CCR5). The error bars show average $\pm$ standard deviations.

Abbreviations: Cf2Th – canine thymocytes; IFN-γ - Interferon-gamma; MFI – mean fluorescence intensity; QC – Quality control; TNF-α – Tumour necrosis factor-alpha; IL – interleukin.

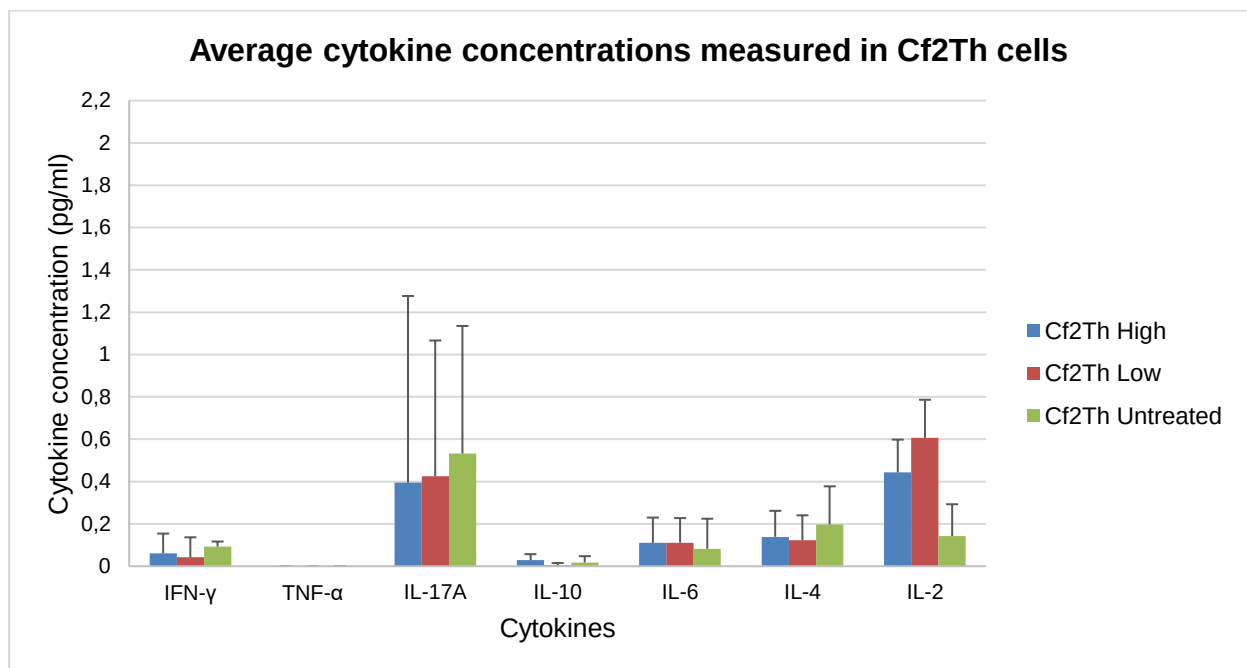


Figure B10: Cytokine concentrations measured in Cf2Th cells. The concentrations for each cytokine represent the average of five replicates (high and low CCR5, respectively) and three replicates (basal CCR5). The error bars show average $\pm$ standard deviations.

Abbreviations: Cf2Th – canine thymocytes; pg/ml – picograms per millilitre; IFN-γ - Interferon-gamma; TNF-α – Tumour necrosis factor-alpha; IL – interleukin.

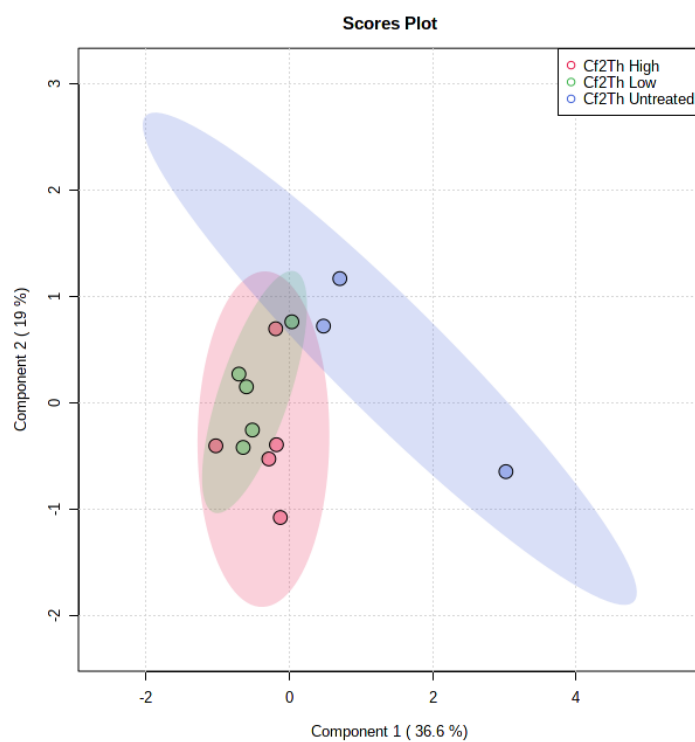


Figure B11: PLS-DA scores plot showing the overlap of cytokine profiles measured in the supernatant of Cf2Th cells with basal, low and high CCR5 levels.

Table B3: Cross validation results of the PLS-DA for Cf2Th cells

Measure	1 Component	2 Components	3 Components
Accuracy	0.076923	0.0	0.23077
R ²	0.37183	0.57985	0.68445
Q ²	-0.34754	-0.54174	-0.27147

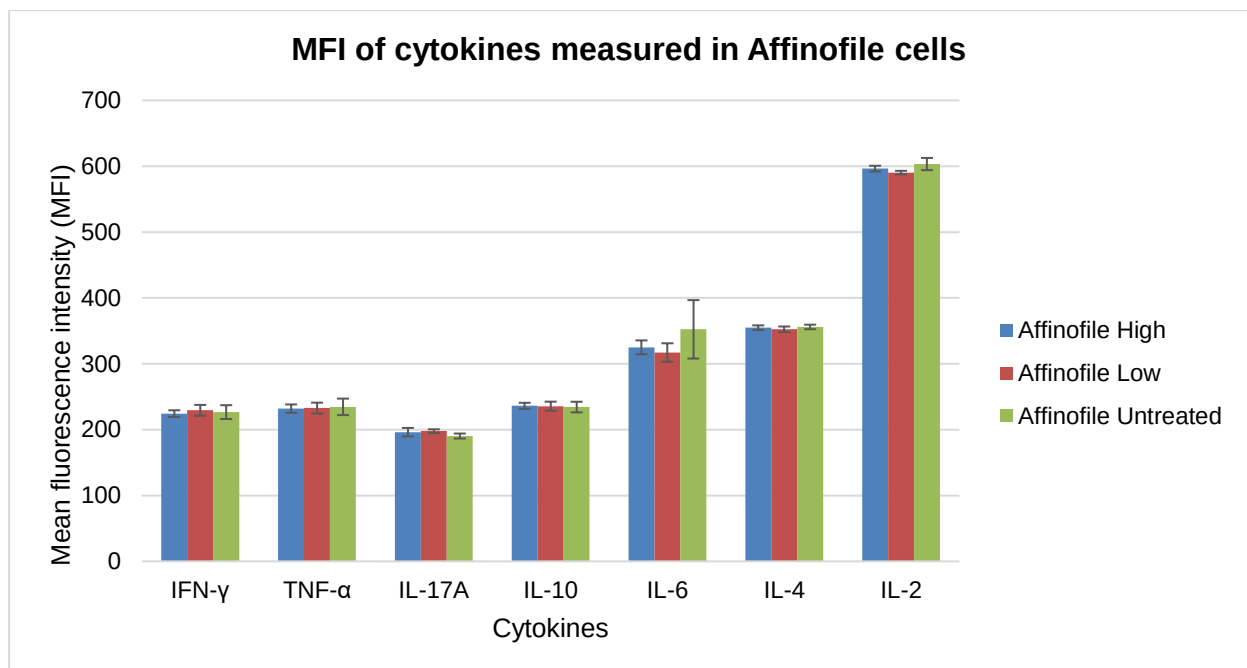


Figure B12: The cytokines' mean fluorescence intensity (MFI) as measured in Affinofile cells, without subtracting the MFI measured for HEK293 cells. The MFI values for each cytokine represent the average of five replicates (high and low CCR5, respectively) and three replicates (basal CCR5). The error bars show average $\pm$ standard deviations.

Abbreviations: Cf2Th – canine thymocytes; HEK293 – Human embryonic kidney; IFN- γ - Interferon-gamma; MFI – mean fluorescence intensity; TNF- α – Tumour necrosis factor-alpha; IL – interleukin.

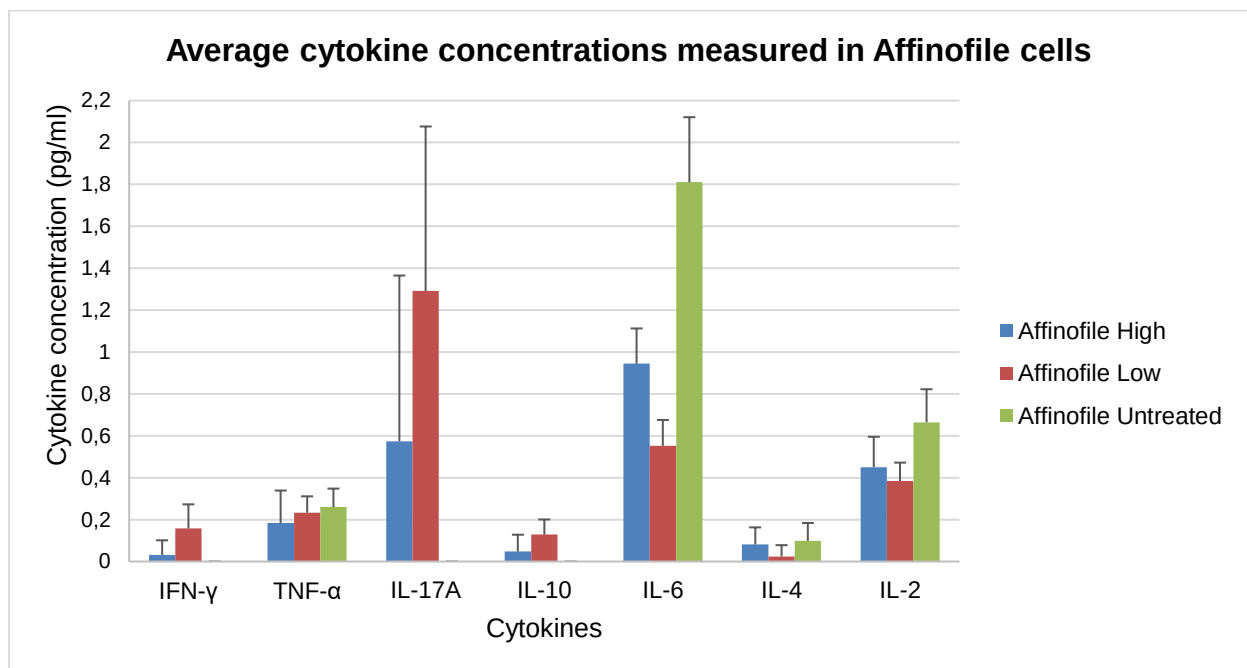


Figure B13: Cytokine concentrations measured in Affinofile cells. The concentrations for each cytokine represent the average of five replicates (high and low CCR5, respectively) and three replicates (basal CCR5). The error bars show average $\pm$ standard deviations.

Abbreviations: pg/ml – picograms per millilitre; IFN- γ - Interferon-gamma; TNF- α – Tumour necrosis factor-alpha; IL – interleukin.

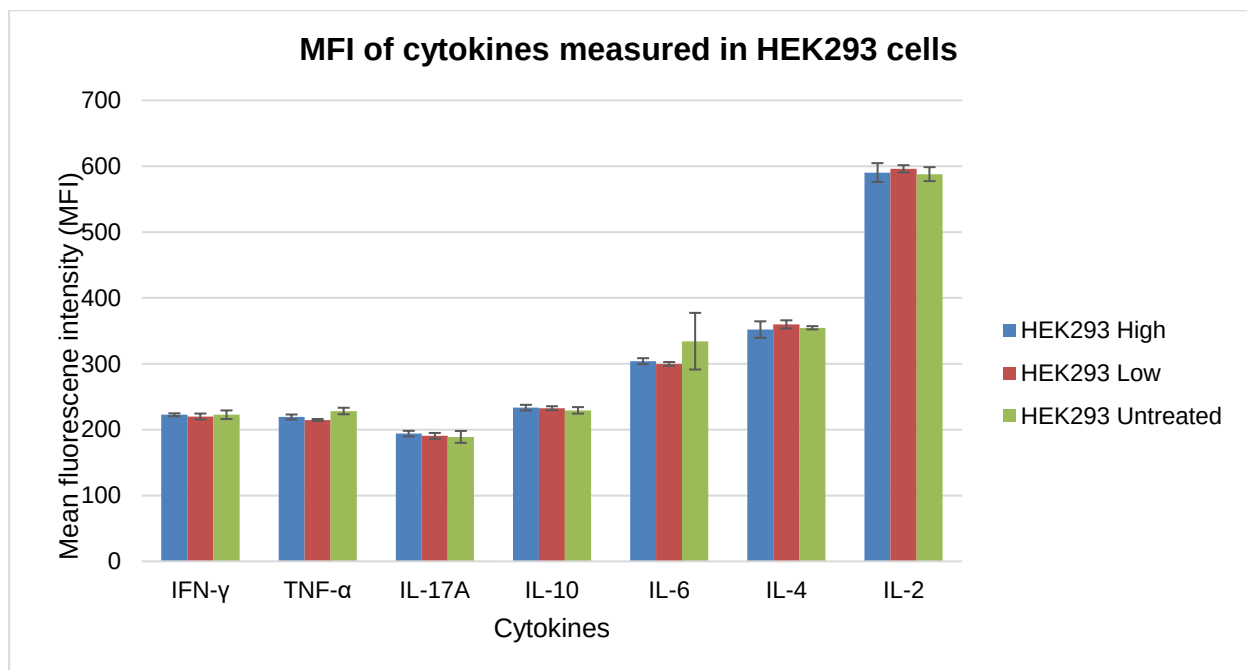


Figure B14: Cytokines' mean fluorescence intensity (MFI) measured in HEK293 cells (the control cell line used for Affinofile cells, since the Affinofile cells are HEK293-derived). The MFI values for each cytokine represent the average of five replicates (high and low CCR5, respectively) and three replicates (basal CCR5). The error bars show average $\pm$ standard deviations.

Abbreviations: Cf2Th – canine thymocytes; HEK293 – Human embryonic kidney; IFN- γ - Interferon-gamma; MFI – mean fluorescence intensity TNF- α – Tumour necrosis factor-alpha; IL – interleukin.

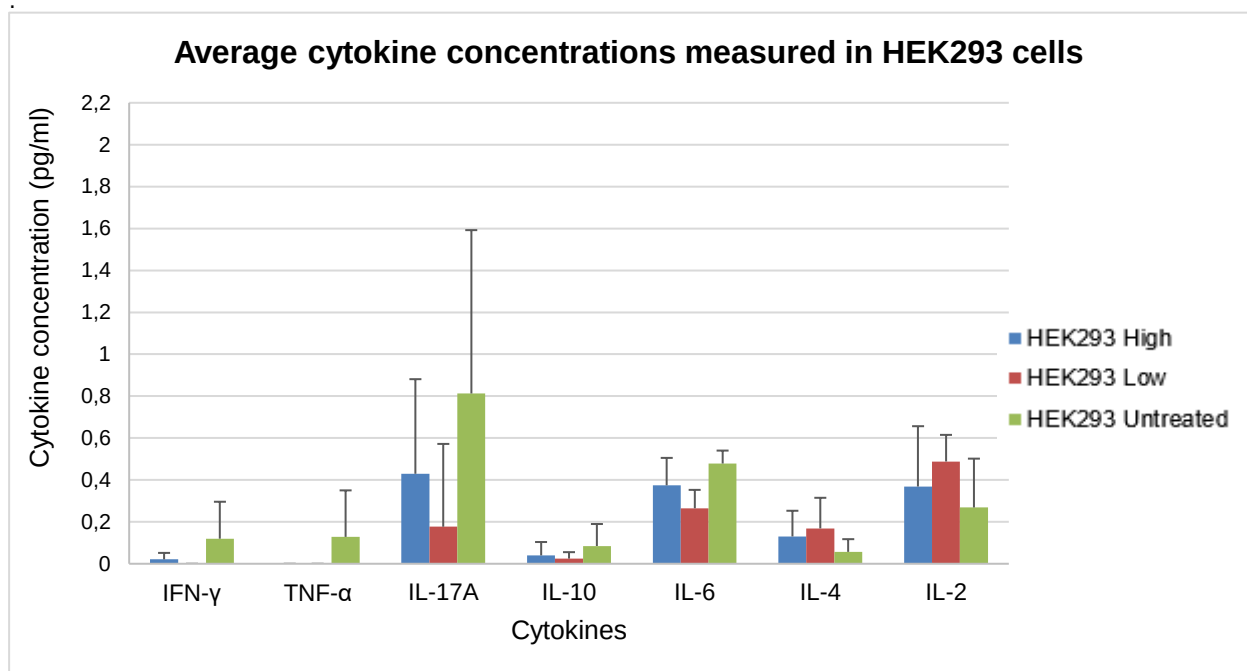


Figure B15: Cytokine concentrations measured in HEK293 cells (the control cell line used for Affinofile cells, since the Affinofile cells are HEK293-derived). The concentrations for each cytokine represent the average of five replicates (high and low CCR5, respectively) and three replicates (basal CCR5). The error bars show average $\pm$ standard deviations.

Abbreviations: Cf2Th – canine thymocytes; HEK293 – Human embryonic kidney; pg/ml – picograms per millilitre; IFN- γ - Interferon-gamma; TNF- α – Tumour necrosis factor-alpha; IL – interleukin.

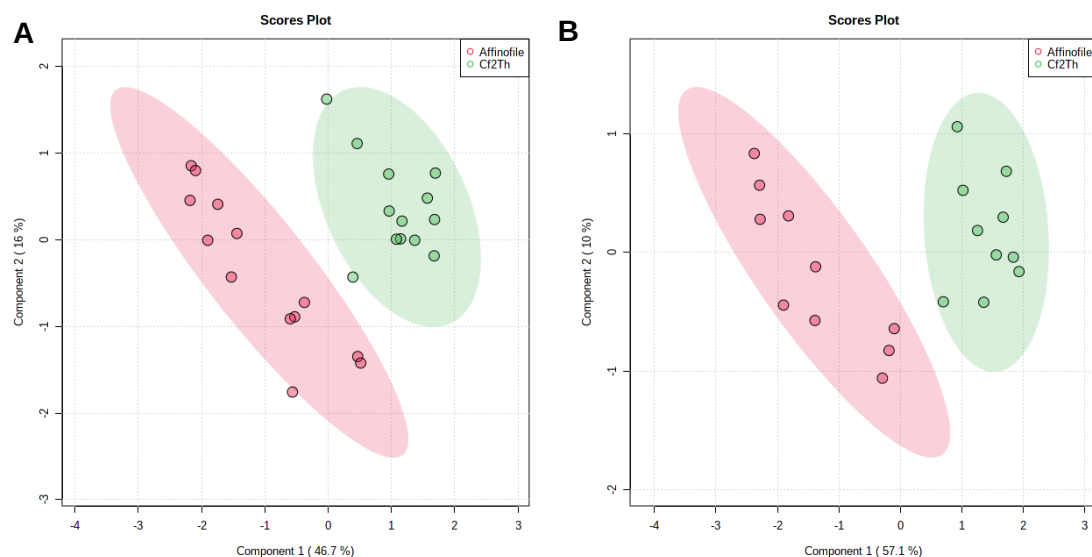


Figure B16: PLS-DA scores plots indicate separation of the Affinofile (HEK293 background signal not subtracted) and Cf2Th cells, irrespective of CCR5 levels after imputation of the cytokine data A) All untreated and treated samples were included in the PLS-DA. B) Only treated samples were included in the PLS-DA.

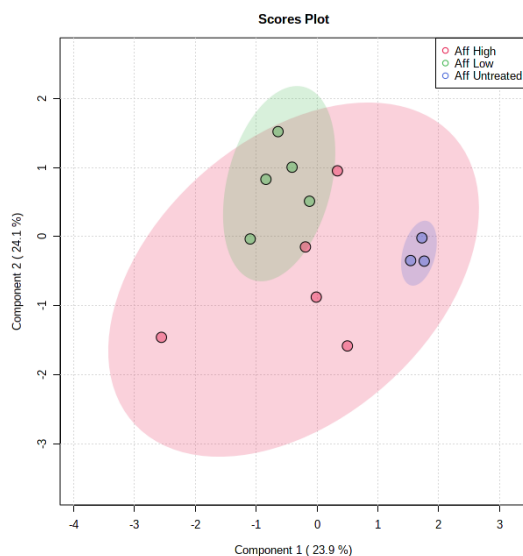


Figure B17: PLS-DA scores plot indicate the separation of the Affinofile (HEK293 background signal not subtracted) after imputation of cytokine data.

Table B4: Cross validation results of the PLS-DA for Affinofile cells if the HEK293 background is not subtracted

Measure	1 Component	2 Components	3 Components
Accuracy	0.38462	0.61538	0.46154
R ²	0.35087	0.41958	0.47304
Q ²	-0.56593	-1.0452	-3.7451

Annexure C: Metabolic profile supplementary data

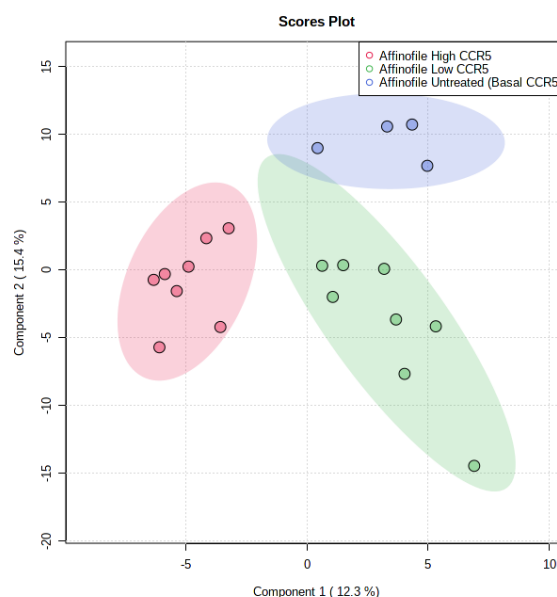


Figure C1: PLS-DA scores plot of Affinofile cells with differential CCR5 levels. Shown is the natural separation of cells after imputing metabolic data.

Table C1: Cross-validation results for PLS-DA for Affinofile cells

Measure	1 Component	2 Components	3 Components	4 Components
Accuracy	0.75	0.8	0.85	0.85
R ²	0.64651	0.89286	0.95396	0.97749
Q ²	-0.10529	0.15177	0.40836	0.36439

Table C2: Summary of significant metabolites identified (after control cell line subtraction) for Affinofile cells with differential CCR5 levels. Metabolites are listed here in order of mention as per Section 5.3.4, i.e. according to processes identified as important as one shifts from basal to low to high CCR5 levels.

Library Hit Compound	Metabolite	HMDB name	HMDB ID	Highest in group	Lowest in group
Phosphoric acid, bis(trimethylsilyl)monomethyl ester	Phosphoric acid	Phosphate	HMDB0001429	Affinofile Untreated	Affinofile Low
Oleic Acid, (Z)-, TMS derivative	Oleic acid	Oleic acid	HMDB0000207	Affinofile Untreated	Affinofile High
Phencyclidine	Phencyclidine	Phencyclidine	HMDB0256399	Affinofile Untreated	Affinofile High
N-Acetyl-L-glutamic acid, 2TMS derivative	N-Acetyl-L-glutamic acid	N-Acetyl-L-glutamic acid	HMDB0001138	Affinofile Low	Affinofile High
N-Acetyl serine 2 TMS	N-Acetyl serine	N-Acetyl serine	HMDB0002931	Affinofile Low	Affinofile High

Palmitic acid 1 TMS	Palmitic acid	Palmitic acid	HMDB0000220	Affinofile Low	Affinofile High
Tridecanoic acid, TMS derivative	Tridecanoic acid	Tridecanoic acid	HMDB0000910	Affinofile Low	Affinofile High
4-Hydroxybutanoic acid, 2TMS derivative	4-Hydroxybutanoic acid	4-Hydroxybutyric acid	HMDB0000710	Affinofile Low	Affinofile High
Octanoic acid, TMS derivative	Octanoic acid	Caprylic acid	HMDB0000482	Affinofile Low	Affinofile High
Propanoic acid, 3-[[bis[(trimethylsilyl)oxy]phosphinyl]oxy]-2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	3-Phosphoglyceric acid	3-Phosphoglyceric acid	HMDB0000807	Affinofile Low	Affinofile High
Citric acid 4 TMS	Citric acid	Citric acid	HMDB0000094	Affinofile Low	Affinofile High
Oxoglutaric acid 2 TMS; 1 MEOX	Oxoglutaric acid	α -ketoglutaric acid	HMDB0000208	Affinofile Low	Affinofile High
Butanedioic acid, 2TMS derivative	Succinic acid	Succinic acid	HMDB0000254	Affinofile Low	Affinofile High
L-Malic acid 3 TMS	L-Malic acid	L-Malic acid	HMDB0000156	Affinofile Low	Affinofile High
L-Aspartic acid, 2TMS derivative	L-Aspartic acid	L-Aspartic acid	HMDB0000191	Affinofile Low	Affinofile High
4-Hydroxyphenyllactic acid, 3TMS derivative	4-Hydroxyphenyllactic acid	p - Hydroxyphenyl lactic acid	HMDB0000755	Affinofile Low	Affinofile High
2,4,7,9-Tetramethyl-5-decyne-4,7-diol, 2TMS derivative	2,4,7,9-Tetramethyl-5-decyne-4,7-diol	2,4,7,9-Tetramethyl-5-decyne-4,7-diol	HMDB0245484	Affinofile Low	Affinofile High
Propanoic acid, 2-methyl-3-[(trimethylsilyl)oxy]-, trimethylsilyl ester	3-Hydroxy-2-methylpropionate	(R)-3-Hydroxyisobutyric acid	HMDB0000336	Affinofile Low	Affinofile High
Myristic acid, TMS derivative	Myristic acid	Myristic acid	HMDB0000806	Affinofile High	Affinofile Low
Propanoic acid, 2-(methoxyimino)-, trimethylsilyl ester	Pyruvic acid	Pyruvic acid	HMDB0000243	Affinofile High	Affinofile Low
Methyl α -D-ribofuranoside, 3TMS derivative	Methyl α -D-ribofuranoside	Methyl-D-Ribose	N/A	Affinofile Low	Affinofile High
5-Ethyl-1,2,4-oxadiazol-3-amine	Unknown	Unknown	Unknown	Affinofile Low	Affinofile High

Analyte 1115	Unknown	Unknown	Unknown	Affinofile Low	Affinofile High
Analyte 1175	Unknown	Unknown	Unknown	Affinofile Low	Affinofile High
Analyte 1275	Unknown	Unknown	Unknown	Affinofile Low	Affinofile High
Analyte 241	Unknown	Unknown	Unknown	Affinofile Low	Affinofile High
Analyte 602	Unknown	Unknown	Unknown	Affinofile Low	Affinofile High
Analyte 486	Unknown	Unknown	Unknown	Affinofile High	Affinofile Low
Analyte 61	Unknown	Unknown	Unknown	Affinofile Low	Affinofile High
Analyte 971	Unknown	Unknown	Unknown	Affinofile Untreated	Affinofile High
Analyte 688	Unknown	Unknown	Unknown	Affinofile Low	Affinofile High
Analyte 703	Unknown	Unknown	Unknown	Affinofile Low	Affinofile High
Analyte 895	Unknown	Unknown	Unknown	Affinofile Low	Affinofile High

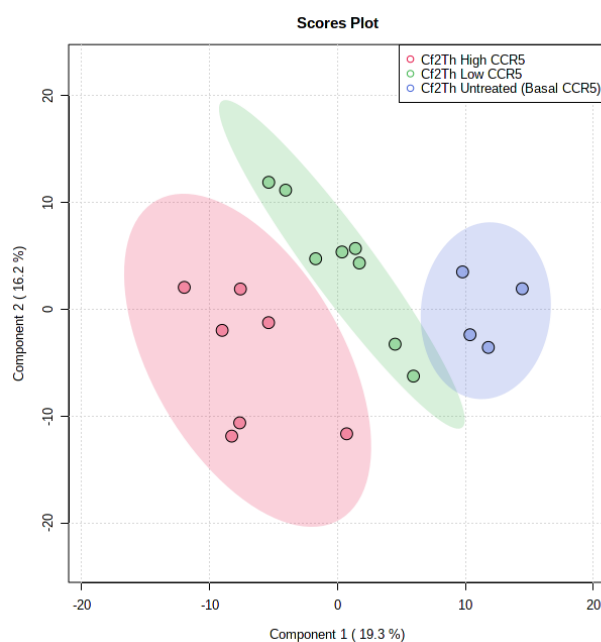


Figure C2: PLS-DA scores plot showing the separation of Cf2Th cells presenting differential CCR5 levels after imputation of the metabolic data.

Table C3: Cross-validation results for PLS-DA for Cf2Th cells

Measure	1 Component	2 Components	3 Components	4 Components
Accuracy	0.10526	0.89474	0.89474	0.89474
R ²	0.77407	0.91512	0.98052	0.99393
Q ²	0.30869	0.38124	0.42169	0.56317

Table C4: Compounds identified as significant in Cf2Th cells presenting with different CCR5 levels in order of mention in section 5.3.5. i.e. according to processes identified as important as one shifts from basal to low to high CCR5 levels.

Library Hit Compound	Metabolite	HMDB name	HMDB ID	Cf2Th Highest in group	Cf2Th Lowest in group
Butanedioic acid, 2TMS derivative	Butanedioic acid	Succinic acid	HMDB0000254	Cf2Th Low	Cf2Th Untreated
Palmitic Acid, TMS derivative	hexadecanoic acid	Palmitic acid	HMDB0000220	Cf2Th Low	Cf2Th Untreated
L-Glutamic acid, bis(trimethylsilyl) ester	L-Glutamic acid	L-Glutamic acid	HMDB0000148	Cf2Th High	Cf2Th Untreated
L-Proline, TMS derivative	L-Proline	L-Proline	HMDB0000162	Cf2Th High	Cf2Th Untreated
Glycine 3 TMS	Glycine	Glycine	HMDB0000123	Cf2Th High	Cf2Th Untreated
Guanosine 4 TMS	Guanosine	Guanosine	HMDB0000133	Cf2Th High	Cf2Th Untreated
Uridine 3 TMS	Uridine	Uridine	HMDB0000296	Cf2Th High	Cf2Th Untreated
Uracil 2 TMS	Uracil	Uracil	HMDB0000300	Cf2Th High	Cf2Th Untreated
Putrescine 4 TMS	Putrescine	Putrescine	HMDB0001414	Cf2Th High	Cf2Th Untreated
Quinic acid (5TMS)	Quinic acid	Quinic acid	HMDB0003072	Cf2Th High	Cf2Th Untreated
Oxoglutaric acid 2 TMS; 1 MEOX	Oxoglutaric acid	α-Ketoglutaric acid	HMDB0000208	Cf2Th High	Cf2Th Low
L-Phenylalanine, TMS derivative	L-Phenylalanine	L-Phenylalanine	HMDB0000159	Cf2Th High	Cf2Th Untreated
L-Aspartic acid, 2TMS derivative	L-Aspartic acid	L-Aspartic acid	HMDB0000191	Cf2Th High	Cf2Th Untreated
Pentanedioic acid, [(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester	2-hydroxypentanedioic acid	2-Hydroxyglutaric acid	HMDB0059655	Cf2Th High	Cf2Th Low

Pentanedioic acid, 3-methyl-3-[(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester	3-hydroxy-3-methylpentanedioic acid	3-Hydroxymethylglutaric acid	HMDB0000355	Cf2Th High	Cf2Th Untreated
Dodecanamide, TMS derivative	Dodecanamide	Dicyanamide	HMDB0251566	Cf2Th High	Cf2Th Untreated
Ethanimidic acid, N-(trimethylsilyl)-, trimethylsilyl ester	Ethanimidic acid	Acetamide	HMDB0031645	Cf2Th High	Cf2Th Untreated
Pentadecanoic acid, TMS derivative	Pentadecanoic acid	Pentadecanoic acid	HMDB0000826	Cf2Th High	Cf2Th Untreated
Ethanolamine 3 TMS	Ethanolamine	Ethanolamine	HMDB0000149	Cf2Th High	Cf2Th Untreated
Bis(2-ethylhexyl) phthalate	Bis(2-ethylhexyl) phthalate	Bis(2-ethylhexyl) phthalate	HMDB0249243	Cf2Th Low	Cf2Th High
Arabinonic acid, 2,3,5-tris-O-(trimethylsilyl)-, • -lactone, d-	Arabinonic acid	Arabinonic acid	HMDB0000539	Cf2Th High	Cf2Th Untreated
Arabinofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)-	5-(hydroxymethyl) tetrahydrofuran-2,3,4-triol	D-Ribose	HMDB0000283	Cf2Th High	Cf2Th Untreated
Ribitol 5 TMS	Ribitol	Ribitol	HMDB0000508	Cf2Th High	Cf2Th Untreated
L-(-)-Sorbose, pentakis(trimethylsilyl) ether, methyloxime (syn)	L-Sorbose	L-Sorbose	HMDB0001266	Cf2Th High	Cf2Th Untreated
D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime	D-Fructose	D-Fructose	HMDB0000660	Cf2Th High	Cf2Th Untreated
D-Glucose 5 TMS; 1 MEOX	D-Glucose	D-Glucose	HMDB0000122	Cf2Th High	Cf2Th Untreated
Scyllo-Inositol, 6TMS derivative	scyllo-Inositol	scyllo-Inositol	HMDB0006088	Cf2Th High	Cf2Th Low
myo-Inositol 6 TMS	myo-Inositol	myo-Inositol	HMDB0000211	Cf2Th High	Cf2Th Low
Methyl ¹⁴C-D-ribofuranoside, 3TMS derivative	Methyl-D-ribofuranoside	Methyl-D-ribofuranoside	Unknown	Cf2Th High	Cf2Th Untreated
d-Erythrotetrofuranose, tris-O-(trimethylsilyl)-	d-Erythrotetrofuranose	N/A	N/A	Cf2Th High	Cf2Th Untreated
1-cis-Monovaccenylglycerol bis-trimethylsilyl ester	N/A	N/A	N/A	Cf2Th High	Cf2Th Untreated
d-Erythrotetrofuranose, tris-O-(trimethylsilyl)-	(3R,4R)-tetrahydrofuran-2,3,4-triol	N/A	N/A	Cf2Th High	Cf2Th Low
Analyte 1175	Unknown	N/A	Unknown	Cf2Th High	Cf2Th Untreated
Nonanamide	Nonanoic acid amide	N/A	N/A	Cf2Th High	Cf2Th Untreated
Analyte 1613	Unknown	Unknown	Unknown	Cf2Th High	Cf2Th Untreated

Analyte 216	Unknown	Unknown	Unknown	Cf2Th Low	Cf2Th High
Analyte 508	Unknown	Unknown	Unknown	Cf2Th High	Cf2Th Untreated
Analyte 597	Unknown	Unknown	Unknown	Cf2Th High	Cf2Th Untreated
Analyte 602	Unknown	Unknown	Unknown	Cf2Th Low	Cf2Th Untreated
Analyte 838	Unknown	Unknown	Unknown	Cf2Th Low	Cf2Th High
Analyte 890	Unknown	Unknown	Unknown	Cf2Th Low	Cf2Th High
Analyte 946	Unknown	Unknown	Unknown	Cf2Th High	Cf2Th Untreated

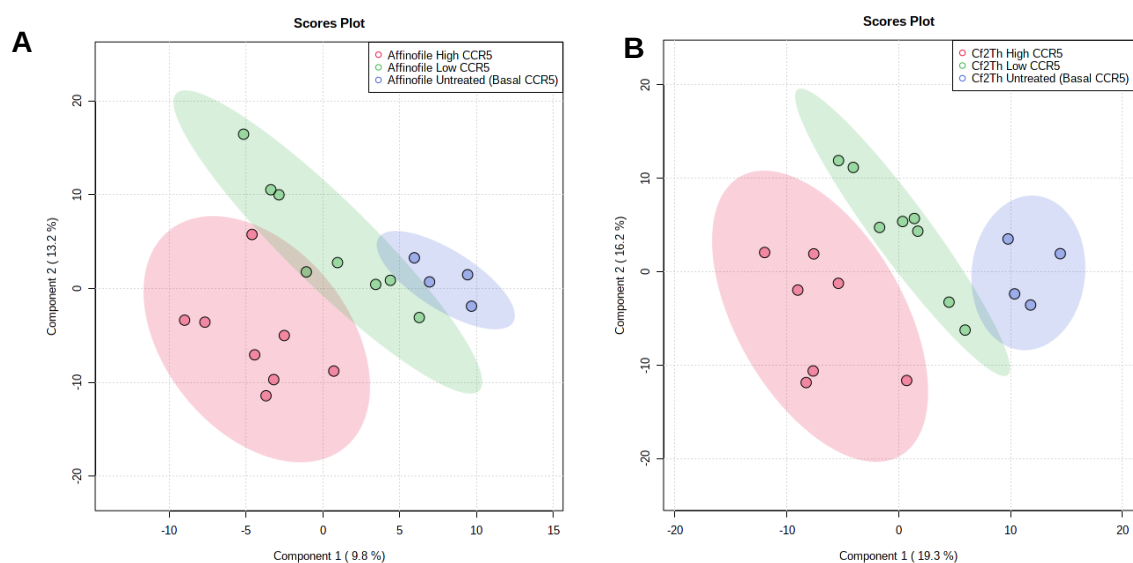


Figure C3: PLS-DA scores plots of the A) Affinofile and B) Cf2Th cells with varying levels of CCR5, without subtracting a control cell line.

Table C5: PLS-DA cross-validation results for Affinofile cells if the background is not subtracted

Measure	1 Component	2 Components	3 Components	4 Components
Accuracy	0.75	0.65	0.75	0.75
R²	0.66093	0.86587	0.95122	0.98639
Q²	-0.59425	-0.22866	-0.15483	-0.17093

Table C6: Significant metabolites identified in Affinofile and Cf2Th cells presenting with differential CCR5 phenotypes (no subtraction of control cell line). Metabolites are listed according to the order of mention in section 5.3.6, i.e. according to CCR5 level and pathways affected.

Library Hit Compound	Highest in Cf2Th group	Highest in Affinofile group	HMDB ID	HMDB name
Phosphoric acid, bis(trimethylsilyl)monomethyl ester	Cf2Th high	Aff High	HMDB0001429	Phosphate
Uracil 2 TMS	Cf2Th high	Aff High	HMDB0000300	Uracil
Uridine 3 TMS	Cf2Th High	Aff High	HMDB0000296	Uridine
Putrescine 4 TMS	Cf2Th low	Aff low	HMDB0001414	Putrescine
D-Glucose 5 TMS; 1 MEOX	Cf2Th Low	Aff Low	HMDB0000122	Glucose
‡-L-Galactopyranoside, methyl 6-deoxy-, (S, S,R,R,S)-, 3TMS derivative	Cf2Th high	Aff high	HMDB0000174	L-Fucose
Propanoic acid, 3-[[bis[(trimethylsilyl)oxy]phosphinyl]oxy]-2-[(trimethylsilyl)oxy]-, trimethylsilyl ester, (S)-	Cf2Th low	Aff low	HMDB0060180	D-Glycerate 3-phosphate
L-Aspartic acid, 2TMS derivative	Cf2Th low	Aff Low	HMDB0000191	L-Aspartic acid
Bis(2-ethylhexyl) phthalate	Cf2Th low	Aff Low	HMDB0249243	Bis(2-ethylhexyl) phthalate
Analyte 597	Cf2Th high	Aff Low		
Analyte 722	Cf2Th high	Aff Low		
Butanedioic acid, 2TMS derivative	Cf2Th High	Aff Low		
D-Ribose, 2,3,4-tris-O-(trimethylsilyl)-, 5-[bis(trimethylsilyl) phosphate]	Cf2Th high	Aff Low		
Ethanimidic acid, N-(trimethylsilyl)-, trimethylsilyl ester	Cf2Th high	Aff Low		
Heptacosane	Cf2Th high	Aff Low		
L-Glutamic acid, bis(trimethylsilyl) ester	Cf2Th High	Aff Low		
L-Proline, TMS derivative	Cf2Th high	Aff Low		
Ribitol, 5TMS derivative	Cf2Th high	Aff low		

Corresponding metabolites are highlighted in grey.

Annexure D: Immuno-metabolic profile supplementary data

Table D1: Summary of the statistically significant cytokines and metabolites in Affinofile and Cf2Th cells with varying levels of CCR5.

		Affinofile cells*	Cf2Th cells**
Basal CCR5 levels	Significant cytokines	IL-2	-
	Significant metabolites	Oleic acid Phencyclidine Phosphate	
Low CCR5 levels	Significant cytokines	-	-
	Significant metabolites	Palmitic acid Succinic acid Methyl-D-ribofuranoside 2,4,7,9- 3-Phosphoglyceric acid 4-Hydroxybutyric acid Caprylic acid Citric acid decyne-4,7-diol (R)-3- Hydroxyisobutyric acid Heptacosane Hexadecane L-Aspartic acid L-Malic acid Methyl-D-Ribose N-Acetyl-L-glutamic acid N-Acetyl serine p – Hydroxyphenyl lactic acid Tetramethyl-5- Tridecanoic acid α -ketoglutaric acid	Palmitic acid Succinic acid Bis(2-ethylhexyl) phthalate
High CCR5 levels	Significant cytokines	-	-
	Significant metabolites	Myristic acid Pyruvic acid	D-Glucose D-Fructose L-Glutamic acid L-Proline Glycine Guanosine α -Ketoglutaric Uracil Uridine 2-Hydroxyglutarate 3-Hydroxymethylglutaric acid D-Ribose L-Sorbose Ribitol scyllo-Inositol myo-Inositol L-Phenylalanine Bis(2-ethylhexyl) phthalate Arabinonic acid Dodecanamide Acetamide Ethanolamine L-Aspartic acid Pentadecanoic acid Putrescine Quinic acid

Table showing the important cytokines and metabolites for each cell line. The metabolites that overlap are indicated in bold.

* HEK293 background signal subtracted

** No background signal subtracted

Annexure E: SASBMB 2022 conference participation

Parts of this study were presented virtually at the South African Society for Biochemistry and Molecular Biology (SASBMB) 2022 conference. The poster (presented as a slideshow) is shown below. The contributions of the NRF are acknowledged.



Metabolic profile of Affinofile cells presenting differential CCR5 levels

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Annexure F: Review Article (output)



Review

Metabolomics as an Approach to Characterise the Contrasting Roles of CCR5 in the Presence and Absence of Disease

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Abstract: Chemokine receptors such as C-C chemokine receptor 5 (CCR5) are activated through interaction with their ligands and are well known for their role in chemotaxis and signal transduction. While serving these roles, cellular responses are effected, hence the immune function of these molecules is established. Given the role of CCR5 in immune function and that the immune and metabolic systems are interlinked, subsequent immune-directed changes should be measurable at a metabolic level. Numerous investigations have reported on metabolic changes associated with CCR5 status in the presence of disease, so as to understand whether specific CCR5 genotypes, frequency and/or levels offer protection to the host or not. However, these metabolic changes were recorded using older conventional techniques. Depending on certain factors such as the disease model, the geography of the samples and/or the ethnic group under study, the role of CCR5 in disease differs. In addition, little is known about CCR5's role in the absence of an enhanced inflammatory state, such as when infection persists. Metabolomics is defined as the study of metabolites and informs on metabolic changes within living organisms as induced by various stimuli, such as the interaction of CCR5 with its ligand. Since metabolomics reflects the underlying biochemical activity and state of cells/tissues, this review proposes it as a tool to clarify the contrasting roles of CCR5.

Keywords: CCR5; immunology; metabolism; metabolomics; immunometabolism; disease

1. Introduction

Chemokines are small proteins with molecular weights ranging from 7 to 15 kilodalton (kDa) [1]. These molecules form part of the cytokine family. Cytokines are small proteins secreted by cells of the immune system and act as signaling molecules which regulate the interactions between cells. Cytokines have the ability to maintain the balance between humoral and cell-mediated immune (CMI) responses. Their activities are often redundant since different cytokines have the ability to stimulate similar functions. With respect to the immune system, chemokines may also exert different/dual functions. Chemokines can be pro-inflammatory stimulating inflammation and the production of more chemokines, or anti-inflammatory inhibiting inflammatory reactions as well as the production and/or release of chemokines. Chemokines thus function as chemotactic factors, directing the response of cells to chemical stimuli [2]. More specifically, chemokines are involved in the trafficking of a variety of leukocytes to a site of infection or tissue damage. In addition to chemotaxis, chemokines play a role in several other biological processes such as angiogenesis and hematopoiesis, as well as aid in embryonic development and metastasis [3,4].

The chemotactic function of chemokines is facilitated through the presence of chemokine receptors which occurs on the surface of cells. These receptors are naturally expressed on most cells but are

especially produced during diseased states [5,6]. Chemokines and their receptors are found to play a pivotal role in many diseases. One of the most studied proteins in this regard is C-C chemokine receptor type 5 (CCR5). The CCR5 gene is located on the short arm position 21 on chromosome 3, denoted as 3p21 [7]. The CCR5 gene undergoes translation, resulting in the CCR5 protein which has a molecular weight of 40.6 kDa [8], comprising 352 amino acids [9]. This G-protein coupled chemokine receptor is expressed on numerous cell types. The cell types include cells of the immune system, namely, dendritic cells, macrophages as well as memory T-cells; epithelial cells, endothelial cells; fibroblasts, vascular smooth muscle and cells of the central nervous system such as microglia, astrocytes and neurons [10]. Several ligands for this chemokine receptor exist including: macrophage inflammatory protein one alpha (MIP1- α) [11], macrophage inflammatory protein one beta (MIP1- β), regulated upon activation, normal T-cell expressed and secreted (RANTES) which is also known as C-C chemokine ligand 5 (CCL5) and monocyte chemotactic protein two (MCP-2) [7,8,12,13].

The immune system consists of several defence mechanisms against any foreign substance. During the innate immune response which involves chemokine production, inflammation forms a key part of the response. CCR5 plays a key role during inflammation. More specifically, CCR5 ligands such as CCL5 are released at sites of infection or tissue damage in order to activate effector cells. These effector cells release chemokines for further signal amplification [14,15]. When receptors interact with a specific ligand to perform its specific function, the protein receptor undergoes a conformational change. The interaction between chemokine and receptor thus attracts immune cells to affected tissue/organ sites and allows the receptor to send biological signals to other parts of the body effecting a cellular response [16]. Other changes include the improved movement of the cell(s) and enhanced secretion of lysosomal enzymes involved in the degradation of waste products.

The adaptive immune response comprising antibody production and CMI responses provides protection at a later stage of an infection and is able to efficiently detect any known pathogen since it establishes a state of memory. During CMI, CD4⁺ T-helper cells regulate the immune response as well as the activation of other immune cells. The cytotoxic CD8⁺ T-cells eliminate target cells that express a foreign antigen. The expression of CCR5 on CD8⁺ T-cells is especially upregulated during inflammation [17]. This process increases the chance of CD8⁺ T-cells to encounter antigen-specific cells. Therefore, in addition to the role of CCR5 in the innate immune response, CCR5 expression also has an influence on the adaptive immune response. Cells are often characterised as either T-helper type 1 (Th1) or T-helper type 2 (Th2) depending on the immune response and the cytokine profile they induce. Th2 immune responses usually counteract the effect of the Th1 response [18]. Ideally, the Th1 and Th2 immune responses should be balanced to effectively eliminate pathogens [19]. The expression of CCR5 is limited to Th1 cells [20]. The amount of CCR5 present on the cell's surface can thus influence T-cell immune responses and ultimately alter the balance of Th1 and Th2 immune responses to impact disease outcome.

In addition to the role of CCR5 in immune function, CCR5 also affects metabolism namely, ligands/chemokines such as CCL5 through interaction with their CCR5 receptors, mediates metabolic change [21,22] inducing a hypermetabolic state in the affected system. Studies that have reported on CCR5-influenced metabolic changes in the presence of disease include, for example, that of Gao [22] as well as Bonfa [23], where metabolic changes during cancer and *Toxoplasma Gondii* infection were investigated, respectively. In a previous study done on cancer cells, Gao et al. [22] showed that CCL5 activated CCR5 and that this activation impacted on glycolysis as well as adenosine triphosphate

(ATP) production. Cancer cells, when treated with CCL5, showed increased expression of glucose transporters which increased glucose uptake and therefore glycolysis. Metabolic analysis revealed that cancer cells treated with CCL5 reflected increased anabolic pathways, which are responsible for synthesizing complex molecules. The increase in some metabolic pathways ultimately resulted in increased ATP production [22]. Bonfa and co-workers [23] used a CCR5-deficient mouse model and reported decreased levels of several inflammation-related cytokines and transcriptional factors during *Toxoplasma Gondii* infection. In addition, they reported that CCR5 deficiency resulted in elevated serum

triglycerides and finally liver metabolic dysfunction [23]. Bing and co-workers [24] used a rat model and reported on the expression and function of CCR5 during the course of type II diabetes mellitus. The authors used an antibody to block CCR5 function and measured metabolic changes. Diabetic mice presented increased levels of low density lipoprotein (LDL) and increased fasting blood triglyceride levels. Based on these conventional indicators (i.e., spectrometric high density lipoprotein (HDL) and LDL), the group was able to show that blocking CCR5 function improved diabetes in their model [24].

It is established that there is a clear relationship between immunology and metabolism. This has led to the term immunometabolism which aims to clarify amongst other factors the involvement of inflammation in the pathogenesis of several metabolic disorders [25]. Furthermore, it also describes different metabolic factors that have an influence on the regulation of several different immune cell functions [26]. For example, Ma et al. [27] showed that optimal T-cell expansion is dependent on serine, a metabolite vital for purine metabolism [27]. Pan et al. [28] showed that some memory T-cells are dependent on fatty acids for eliciting effective immune responses [28]. Although these studies are not CCR5-specific, they clearly highlight the immuno-metabolic concept. Given the role of CCR5 in immune function, subsequent changes linked to the chemokine receptor should thus be measurable at the metabolic level. Although literature highlighting the role of CCR5 in immune system function as well as metabolism is available, the latter is very limited and/or recorded using older conventional systems as in the study by Bing and colleagues [24]. Minimal information is also available on CCR5's role in the absence of an enhanced inflammatory state such as when infection persists.

The functionality of CCR5 is compromised when the gene coding for CCR5 is subject to mutations. One such mutation involves the 32-base pair deletion, CCR5 Δ 32, which is present in about 10% of the Caucasian population [29]. This 32 bp deletion from nucleotide position 794 to 825 results in a frameshift mutation, an altered codon reading frame and a dysfunctional translation mechanism. The entire structure of the gene is altered yielding a mutated protein composed of only 215 instead of 352 amino acids. The last three transmembrane domains of CCR5 along with regions critical for cellular processes, such as G-protein interactions and signal transduction, are thus absent in the mutated protein. This has implications for immune and subsequent metabolic processes. The end result is altered expression and function of the receptor [30,31]. Consequently, several CCR5 genotypes exist in this regard i.e., individuals can present with a homozygous 32 bp deletion genotype presenting no CCR5 thus ligands and/or other chemokines cannot bind. Alternatively, individuals can present with a wild type (WT) homozygous genotype where normal levels of CCR5 is expressed or a heterozygous genotype where minimal CCR5 is expressed. The CCR5 genotype can therefore be directly correlated to the cell surface expression of CCR5, also noted as CCR5 levels. Individuals can thus present with no, low or high CCR5 levels depending on the genotype. In more recent studies, authors categorised cohorts according to their haplotype. Haplotype takes into account the different polymorphisms on the same chromosome and are categorised as either protective or non-protective [32].

In the case of diseases where CCR5 is involved in pathogen entry, individuals presenting with the homozygous 32 bp deletion will not express CCR5, while individuals with the heterozygous 32 bp deletion will express lower amounts of CCR5 and are therefore characterised as having a protective phenotype. In contrast, individuals presenting the WT homozygous genotype, express higher levels of CCR5 and are thus characterised as having a non-protective phenotype. A protective haplotype will therefore take polymorphisms leading to lower CCR5 expression into account, while non-protective haplotypes are associated with increased CCR5. More recently, Jaumdally and colleagues [33] in their 2017 study investigated inflammation and T-cell activation in the plasma of uninfected participants, exposed and unexposed to human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS). Exposed uninfected individuals had lower frequencies of CCR5-positive (CCR5⁺) immune cells (protective against infection) than their unexposed counterparts. These individuals when grouped thus expressed protective CCR5 haplotypes and presented with reduced inflammatory cytokines. The data obtained by Jaumdally and co-workers [33] provides important biological information regarding samples presenting differential CCR5 status even before

actual infection. The terms used to describe CCR5 (i.e., genotype, levels, haplotype, etc.) are thus all interlinked.

Much of the attention and clinical relevance related to CCR5 has centred on its role as co-receptor, facilitating viral entry during HIV-1 and other infections. The role of CCR5 in HIV-1 was established when authors showed that CCR5 on the surface of CD4⁺ T-cells has a direct correlation with infectability/viral levels [34]. As such, the role of the 32 bp deletion is increasingly investigated

in context to HIV-1 disease susceptibility [35] and progression [36,37]. As a result of the failed expression of CCR5, the 32 bp deletion is associated with resistance against certain infections, such as HIV-1 [38], while others show the mutation to facilitate disease progression [35], mainly due to failed chemotactic function. Section 2 of this article details more of the contradictory roles of CCR5 in disease.

The therapeutic role of blocking CCR5 has also become of increased research interest. In the case of diseases where CCR5 is needed for viral entry into the cell, such as with HIV-1 infection, a CCR5 antagonist inhibits viral entry into the cell and therefore limits disease development and progression. Maraviroc is a common CCR5 antagonist used to inhibit HIV-1 entry. In addition, maraviroc is also shown to be successful at attenuating disease severity in several other conditions [39]. While the mini-review by Vangelista and co-workers [40] also reports on the role of CCR5 in a few diseases, the authors focus on the therapeutic benefit of blocking CCR5 function, biochemically or through molecular therapies.

With respect to CCR5, the 32 bp deletion occurring in Caucasians is however not the only mutation known to alter the molecule's expression and/or function. A novel 24-base pair deletion with phenotypic effects similar to that of CCR5Δ32 was recently reported in the coding region of CCR5 in African individuals [41]. Numerous other genetic mutations within the CCR5 gene structure have been studied and linked to disease progression [42–46]. A full discussion of other mutations within CCR5 lies beyond the scope of this article. Further discussions will thus only focus on the 32 bp deletion given its current clinical relevance and media attention received.

In 2009 Hütter and co-workers [47] reported on the first patient to be cured of HIV-1 infection. The patient, commonly known as the “Berlin Patient”, underwent a hematopoietic stem cell transplantation as part of his treatment for leukaemia. The protective homozygous CCR5Δ32 genotype was carried by the stem cell donor [47]. In subsequent years there were reports that yet another patient maintained remission of HIV-1 infection. This patient, known as the “London patient”, underwent two allogeneic hematopoietic stem cell transplants using a donor with a homozygous CCR5Δ32 genotype. Complete remission was observed six months after the stem cell transplant [48]. At the end of 2018, CCR5 made the news once again when a Chinese geneticist, He Jiankui, engineered HIV-1 resistant babies. Clustered regularly interspaced short palindromic repeats (CRISPR) are short DNA sequences present in living organisms. Scientists recently started using Cas9, a protein with the ability to cut DNA strands, in conjunction with these CRISPR DNA sequences to alter an organism's genetic material. It is reported that Jiankui used CRISPR-Cas9 to generate genetically modified embryos carrying the CCR5Δ32 mutation. This results in an individual with cells devoid of functional CCR5 on their surface [49]. Whilst this mutation is generally protective against HIV-1 infection, the long-term effects of such an experiment are not yet known. While some researchers report individuals carrying the 32 bp deletion to live a normal life [40], others report on the negative effects of the mutation (i.e., that it is associated with increased susceptibility to various common diseases and increased mortality). Conclusions regarding the negative effects of CCR5Δ32 were deduced using data from the United Kingdom (UK) Biobank [50]. The article has since been retracted due to genotyping calling bias in the dataset used. Using the same dataset as the preceding article, Maier et al. [51] reported that the homozygous CCR5Δ32 does not influence the lifespan of individuals harbouring the homozygous CCR5Δ32 genotype [51]. This area of research thus remains open for further investigation.

In addition to mutations, the expression of receptors and their ligands is influenced by disease. Cancer, a condition in which cells grow abnormally, presents with the increased expression of CCL5 and CCR5. Furthermore, receptor expression is also influenced by epigenetic modifications such as

DNA methylation [52]. These modifications alter the activity of DNA without changing the actual DNA sequence. In the case of CCR5, DNA methylation is inversely related to the amount of CCR5 present on the surface of T-cells. When T-cells are activated (such as during infection), demethylation occurs resulting in upregulation of CCR5 expression.

Considering the role of chemokines, it is anticipated that the differential expression patterns of chemokine receptors and their respective ligands may play a role in inflammation and impact the immune response and metabolism to ultimately influence disease progression and/or clinical outcome. The metabolic change(s) in particular should then be measurable using metabolomics. The role of CCR5 in this regard, which is also the essence of this review, is summarised in Figure 1. Major diseases in which the role of CCR5 is investigated is reviewed in Section 2 and summarised in Table 1. In the table, a summary of the disease model used to investigate the role of CCR5 is presented, together with the primary assays performed and parameters measured. Where immune and metabolic parameters were measured these are highlighted in the text to further justify the metabolomics approach proposed here. We realise that not all studies are reported in this article and while some of the reported studies overlap with information reported by Klein [53] and Vangelista and co-workers [40], we however highlight a broader range of conditions to show the contrasting roles of CCR5 therein with possible reasons for the discrepancies. Given CCR5's immuno-metabolic effects, metabolomics is proposed as an approach with which to clarify the receptor's role.

2. The Role of CCR5 in Disease

2.1 Cardiovascular Diseases

The cardiovascular system comprises of the heart, surrounding arteries and blood vessels. CCR5 is present on the surface of leukocytes which are transported through the body via circulating blood. Several diseases of the cardiovascular system exist and it is well known that inflammatory processes play a role during such conditions. As a result, chemokines and chemokine receptors are implicated in cardiovascular diseases (CVD). The signalling molecules in the immune system are responsible for maintaining homeostasis and initiating an immune response when necessary. However, continuous inflammatory responses during the diseased state can influence disease progression [54]. Therefore, since inflammatory processes play a role in cardiovascular diseases, CCR5 may be involved in cardiovascular disease progression. Several studies have therefore been conducted to assess the role of CCR5 in diseases related to the cardiovascular system.

Atherosclerosis is caused by a build-up of cholesterol and fatty deposits on the inner walls of the arteries which impedes blood flow, resulting in a variety of conditions [55]. Atherosclerosis can be categorised as an inflammatory disease of the cardiovascular system due to the cellular responses present in atherosclerotic lesions [56]. The inflammatory response is primarily mediated by macrophages as well as specific T lymphocytes involved in the specific stage of atherosclerosis disease [57]. Activation of the lymphocytes results in the release of several inflammatory mediators such as chemokines and cytokines [58]. The chemokines are involved in the migration and accumulation of macrophages within fatty streaks, lesions that develop into fibrous plaque. During the development of atherosclerotic plaques, several immune cells are recruited continuously which is a characteristic of the chronic inflammatory response [59]. An example of a chemokine that is expressed in large quantities in atherosclerotic plaques is monocyte chemoattractant protein-1 (MCP-1) [56]. Readers with an interest in this chemokine are directed to the article of Lin and co-workers [60] which provides a detailed review of MCP-1 [60].

A meta-analysis was performed by Zhang and colleagues [61] regarding the association of CCR5 Δ 32 and susceptibility to atherosclerotic disease. The authors combined 13 case-controlled studies comprising various ethnic cohorts and established that there was no association between CCR5 Δ 32 and atherosclerotic disease susceptibility. Surprisingly, further subgroup analysis revealed an increase in the frequency of CCR5 Δ 32 in Asian patients and a significant association of the mutation

with increased susceptibility to atherosclerotic disease [61]. Atherosclerosis can also result in a variety of sub-conditions, explained in the sections that follow.

Atherosclerosis results in coronary artery disease (CAD) which develops when coronary arteries become narrow or in some cases blocked. Several studies report on the role of CCR5Δ32 in CVD. We highlight a few of these studies which showed CCR5Δ32 to (a) be a risk factor for CVD, (b) offer protection against CVD and (c) have no impact on CVD. Several studies using blood found that, although there was mostly no difference in the frequencies of CCR5Δ32 among patients and controls [62–65], the 32 bp deletion may still be a risk factor for the development of atherosclerosis [62,66]. In the study conducted by Pai and co-workers [62], seven polymorphisms within chemokine receptors CCR5 and CCR2 were studied. The authors found no difference in allele distributions between cases and controls. None of the haplotypes were associated with the risk of coronary heart disease. The authors did not observe the homozygous CCR5Δ32 genotype among patients when cases were stratified according to age and thus found CCR5Δ32 to be associated with early age of onset. In addition, the authors measured immune and metabolic parameters. Inflammatory markers such as C-reactive protein (CRP) and cytokines were measured using an immunoturbidimetric high sensitivity assay and the enzyme-linked immunosorbent assay (ELISA), respectively while cholesterol was measured using standard protocols. Coronary heart disease patients presented with increased inflammatory markers and lipids and decreased HDL-C when compared to controls [62]. In the study conducted by Sharda and co-workers [66], the authors determined CCR5Δ32 genotypes in a North Indian cohort using the polymerase chain reaction (PCR). The authors reported an increased frequency of heterozygous CCR5Δ32 genotype among patients when compared to controls but the increase was statistically insignificant (hence the heterozygous superscript is not shown for [66], Table 1). Furthermore, the homozygous CCR5Δ32 genotype was absent in the cohort. Metabolic analysis of the patients' blood showed significant increases in total cholesterol, triglycerides, LDL and apolipoprotein B compared to controls. The authors did not specify the methods used to determine these parameters. Based on the higher frequency of the mutation within patients, the authors suggested that it might be associated with risk of developing CAD [66].

Some studies found CCR5Δ32 to be associated with protection against CVD-related conditions. Hyde and co-authors [63] used blood samples from two populations enrolled in a cardiovascular prevention trial. The authors investigated possible genetic associations with circulating lipid levels and ultimately the influence of genetic polymorphisms such as CCR5Δ32 on cardiovascular disease. While there was no association between the heterozygous CCR5Δ32 genotype with lipid levels and cardiovascular disease, the authors did show an association between the homozygous CCR5Δ32 genotype, increased high-density lipoprotein cholesterol (HDL-C) and decreased triglycerides (TG) levels, respectively. HDL-C is known to act as a scavenger to aid in the removal of low-density lipoprotein cholesterol (LDL-C) from arteries. TG is linked to the fatty build-up present in the arteries of CAD patients. Therefore, the increased HDL-C and decreased TG levels present in homozygous CCR5Δ32 carriers are associated with protection against CAD [63]. In a study by Afzal and co-workers [67], the role of CCR5Δ32 as a risk factor for CVD in a Bruneck population was investigated. The authors used PCR to determine the CCR5 genotypes of CVD patients. In addition, the authors determined the levels of several inflammatory markers and HDL using immune and enzymatic assays, respectively. Furthermore, an ultrasound was used to determine the thickness of arteries. The authors reported both homozygous and heterozygous CCR5Δ32 to be associated with decreased inflammatory markers, incident of CVD and risk of death. The authors concluded that both homozygous and heterozygous CCR5Δ32 genotypes offer some protection against CVD [67]. Lassner and co-workers [68] determined the CCR5Δ32 frequency in patients compared to controls and investigated the mortality rates of the cohort. Genotypes were determined using PCR. Although there was no difference in frequencies of CCR5Δ32 between the two groups, the study found that the patient cohort carrying either the homozygous or heterozygous CCR5Δ32 mutation presented decreased mortality rates over a six-year period compared to healthy controls, and thus associated

CCR5Δ32 with protection against non-ischemic cardiomyopathy (CM) [68]. Szalai and co-workers [69] investigated various CCR5 related polymorphisms in CAD patients and healthy controls. The authors genotyped all individuals and determined lipoprotein(a) levels using enzymatic methods. While the heterozygous CCR5Δ32 genotype had no effect on CAD susceptibility, the absence of homozygous CCR5Δ32 genotypes in the patient group led the authors to suggest a protective role for homozygous CCR5Δ32 in the context of CAD [69].

In contrast, some studies found that CCR5Δ32 did not have an effect on CAD susceptibility. Apostolakis and co-workers [64] investigated the role of several polymorphisms within the chemokine and chemokine receptor genes and their involvement in susceptibility to CAD. The genotypes of all CAD patients and healthy controls participating in the study was determined by either PCR alone or PCR combined with restriction fragment length polymorphism (RFLP). The DNA purity was determined using spectroscopy. Individuals carrying the homozygous CCR5Δ32 genotype were absent in this cohort. The authors reported no significant differences in allele and genotype frequencies among CAD patients and controls and therefore no association of CCR5Δ32 with CAD [64]. The association of CCR5Δ32 with CAD was investigated by Simeoni and co-authors [65]. Individuals' samples were genotyped using PCR/RFLP methods and individuals were divided into CAD cases according to coronary angiography results. CRP was measured using a nephelometric immunoassay while standard metabolic parameters defining CAD were reported. CRP was increased among patients, but not associated with CCR5Δ32. Homozygous CCR5Δ32, heterozygous CCR5Δ32 and homozygous WT genotypes were present among patients and controls. The allelic and genotypic frequencies did not differ significantly between patients and controls. The authors thus concluded that CCR5Δ32 is not associated with CAD susceptibility [65]. A meta-analysis comprising 10 studies that focused on CCR5Δ32 was performed by Wang and colleagues [70]. The authors found no significant association of the polymorphisms in the CCR5 and CCL5 genes with CAD risk. Four of these studies associated the CCR5Δ32 mutation with protection against CAD, while three found no association between CCR5Δ32 and CAD. Overall, it appears that CCR5Δ32 is not associated with CAD risk. Other studies used in the meta-analysis focused on polymorphisms within the CCL5 gene [70].

Myocardial infarction (MI), more commonly known as a heart attack, occurs when the blood flow to a section of the heart is decreased, resulting in damaged heart muscles. During MI, the chemokine MCP-1 is involved in post infarction remodelling, a process that refers to changes in the structure and function of the heart due to loss of myocardium. Elevated levels of MCP-1 are present in MI patients and are associated with disease prognosis [71]. The CCR5Δ32 mutation is associated with protective effects against MI. In an investigation by Balistreri and co-authors [72], Sicilian patients and healthy controls were genotyped using blood samples. The authors reported a significant increase of allelic and genotypic frequencies of CCR5Δ32 in controls compared to patients. Based on these results, the authors suggested a protective role for CCR5Δ32 against acute MI [72]. In a study performed by Gonzalez et al. [73], CCR5Δ32 was associated with age of onset of MI. Genotypes were determined using PCR. The authors reported absence of the homozygous mutation in patients <55 years and increased CCR5Δ32 allelic frequencies in controls when compared to patients. In addition, CCR5Δ32 was increased in patients >60 years compared to patients <55 years. Therefore, the authors suggested a possible protective effect of CCR5Δ32 against early MI [73]. CCR5Δ32 was also associated with increased risk for MI in a Turkish population. In an investigation performed by Karaali and co-workers [74], genotypes were determined using PCR/RFLP. The authors showed increased allelic frequencies of homozygous and heterozygous CCR5Δ32 in patients compared to controls. A significant increase in cholesterol and LDL-C levels were also reported in patients compared to controls. Consequently, the authors concluded that the CCR5Δ32 genotypes were associated with an increased risk of developing MI [74].

Atherosclerosis can cause carotid stenosis when plaque build-up within the artery carrying blood to the brain, occurs. One study determined the role of various variants within chemokines and chemokine receptors as a risk factor for internal carotid artery stenosis (ICAS). Ghilardi and

co-workers [75] obtained blood samples from an Italian cohort and used PCR to genotype individuals for various polymorphisms. Regarding CCR5Δ32, the authors found that there were no significant differences in genotypic and allelic frequencies of CCR5Δ32 in patient and control groups. Therefore, the authors concluded that CCR5Δ32 does not have an effect on ICAS development [75].

Hypertension refers to the condition in which blood exerts force against the walls of blood vessels causing blood pressure to increase to unhealthy levels. The link between the CCR5Δ32 variant and hypertension formed the central focus of a study by Mettimano and co-workers [76] in which the authors investigated polymorphisms within the CCR5 and CCR2 genes in hypertensive patients, respectively. For this analysis, blood was used and the cohorts included Caucasians due to the increased prevalence of the CCR5Δ32 mutation in this group. The authors reported increased allelic frequency of CCR5Δ32 in the patient group and therefore showed a clear association between CCR5Δ32 and hypertension [76]. Unlike Mettimano, Zhang and co-authors [77] could not confirm this association when they conducted a larger study in 2006 comprising subjects from the United States and Poland. The authors reported that the allelic frequency of CCR5Δ32 was the same in the patient and control groups. In addition, CCR5Δ32 did not have an influence on blood pressure and did not show an association with hypertension in any age group [77].

2.2 Diseases of the Nervous System

The central nervous system is mainly comprised of the brain, spinal cord and peripheral nerves. The overall health of the central nervous system is conserved by a specialised population of cells. These cells include microglial cells which are involved in the removal of damaged neurons, astrocytes which are associated with neuronal synapses and the regulation of transmission of electrical impulses within the brain, as well as specialised cells with the ability to transmit signals to the rest of the body known as neuronal cells [78]. These cells continuously produce chemokines in order to maintain a state of homeostasis within the brain. Both chemokines and their receptors influence the migration and differentiation of these specialised cells. Inflammatory mediators have the ability to enhance the chemokine receptor–ligand interaction. Therefore, chemokines and their receptors are associated with a variety of inflammatory conditions [79], including nervous system diseases such as Alzheimer’s disease (AD), Parkinson’s disease (PD), multiple sclerosis (ms), tumour progression, stroke and AIDS-associated dementia [79], to name a few.

Alzheimer’s disease is a chronic neurodegenerative disease and is the most common form of dementia. It is a progressive condition in which brain cells degenerate [80]. AD patients usually present with beta amyloid plaques, a protein fragment which is eliminated under normal conditions, but forms insoluble plaques within the brain of AD patients. The formation of these plaques result in neuron degeneration. Disease progression is enhanced by pro-inflammatory processes. The chemokines and receptors present in the central nervous system play a pivotal role in neuro-inflammation. The expression of cytokines and other inflammatory mediators is upregulated during the neuro-inflammatory response [81], showing an association with pathological changes seen in AD patients [82]. Similarly, chemokine receptors are also involved in the pathophysiology of AD. It was reported that CCR5 and CCR2 levels/expression increase in AD patients compared to controls. Upregulation of chemokine receptors and their ligands were found in AD brain and that may contribute to plaque-associated inflammation and neurodegeneration [79]. Given the role of CCR5 in the immune system, it is expected that CCR5 deficiency is associated with protection against inflammatory diseases. CCR5 deficiency however can result in activation of the astrocytes as well as amyloid-beta deposits. One of the main consequences of amyloid-beta deposits include memory dysfunction due to enhanced CCR2 expression [83]. Several studies were conducted regarding CCR5Δ32 and its association with AD. Combarros and co-workers [84] conducted a study and reported on the lack of association of CCR5Δ32 with AD. Combarros et al. [84] used PCR to determine the genotypes of their Spanish cohort and did not find a significant difference in the allelic or genotypic frequencies of CCR5Δ32 between AD patients and controls. In addition, the authors reported that CCR5Δ32 did not influence the age of onset of

AD [84]. Balistreri and co-workers [85] investigated the possible protective role of CCR5Δ32 in AD using blood samples from an Italian cohort. The control group consisted of elderly individuals that did not present with dementia. The authors did not find significant differences in the allelic distribution and frequency of CCR5Δ32 between AD patients and controls. In addition, the authors showed neither gender nor disease progression to be associated with CCR5Δ32 [85]. These results confirm findings of a previous study done by Galimberti and co-workers [86] who also used an Italian cohort. The authors obtained blood samples and determined the CCR5Δ32 genetic distributions in patients and healthy controls. The authors found no difference in genotypic frequencies among patients and controls; CCR5Δ32 frequencies did not differ when cases were stratified by gender or age of onset [86]. Similar results were obtained by another study that investigated the role of CCR5 in AD using an Iranian cohort. The CCR5Δ32 frequency was determined post PCR and RFLP genotyping of blood samples. The authors reported a lack of association of CCR5Δ32 with AD due to similar CCR5Δ32 genotypic distributions in patients and controls [87]. Collectively, these studies showed a lack of association of CCR5Δ32 with AD susceptibility, progression and age of onset. However, other mutations linked to inflammation might play a role in AD. Jorda et al. [88] investigated the role of the chemokine system in AD using a specific mouse model generated for this condition. Reverse transcription-PCR (RT-PCR) and Western blots were used to determine the expression of CCR5 and various chemokines. The authors observed that the AD-representing mice showed decreased CCR5 levels compared to the WT mice. In this model the decreased CCR5 suggests a possible increase in astrocytes which is important for transmission of electrical impulses in the brain. The authors concluded that the varying chemokine levels could be used to explain the inflammation seen in the brains of AD patients [88]. An in-depth review article regarding CCR5 and AD was published by Li and Zhu [89].

Parkinson's disease is a progressive nervous system disorder that affects nerve cells within the brain which are responsible for the production of dopamine. Dopamine, a neurotransmitter, is a chemical that transmits signals to other neuronal cells. The role of CCR5Δ32 in PD has not been studied extensively. Huerta and co-workers [90] investigated the influence of various CCR5 polymorphisms in PD susceptibility and progression in a Spanish cohort, using blood as a model. The authors included PD patients as well as late-onset Alzheimer's disease patients (LOAD) and compared these patient groups to healthy controls. No significant difference in genotypic or allelic frequency distribution between both patient groups and controls was found. The authors thus showed that CCR5Δ32 does not contribute to disease susceptibility or clinical outcome for PD and LOAD [90].

Multiple sclerosis is a progressive, immune-mediated disorder that attacks the central nervous system, affecting primarily the brain and spinal cord, causing an impaired flow of information between the brain and the body. The nerves present in the brain and spinal cord are covered with a protective layer, known as the myelin sheath. Demyelination is a process in which the myelin sheath is damaged, resulting in retarded nerve impulses. Lesions, also known as plaques, are an area within the brain which can suffer damage due to inflammation resulting from the immune response in which the myelin sheath is attacked. Ms patients present with demyelination, axonal injury and decreased neurological function [79]. Chemokines and chemokine receptors play a pivotal role in ms by trafficking pro-inflammatory T-cells into the central nervous system. Consequently, mutations within chemokines and receptors may influence disease susceptibility and progression. In a study performed by Gade-Andavolu and co-workers [91], the brain tissue and blood samples of ms patients and healthy controls were obtained. The authors determined the genotypes of the respective samples using PCR. The homozygous and heterozygous CCR5Δ32 genotypes were not associated with ms disease susceptibility but were associated with decreased survival rates. In addition, the authors stratified cases by gender and reported a more significant effect of CCR5Δ32 on mortality in female patients [91]. Pulkkinen and co-workers [92] obtained blood samples from ms patients and healthy controls and used PCR genotyping data to compare CCR5Δ32 distribution among patients and controls. The authors reported increased homozygous CCR5Δ32 genotypes within the patient group compared to controls, suggesting this variant to contribute to ms disease susceptibility. Further analysis showed

that the increased homozygous CCR5 Δ 32 genotypes were more prevalent in primary progressive ms patients compared to other ms subtypes. In addition, the authors used RT-PCR to determine CCR5 messenger ribonucleic acid (mRNA) levels and flow cytometry to determine the amount of CCR5 expressed on the surface of CD4⁺ T-cells. The authors found that CCR5 Δ 32 reduced the amount of CCR5 expressed on the cell surface and that the amount of CCR5 was not associated with ms subtype or disease progression. Therefore, the authors concluded that the absence of CCR5 on the cell surface is not associated with protection against the development of ms, but that CCR5 is rather involved in the clinical course of ms [92].

Other studies reported that CCR5 Δ 32 genotypes offer protection against ms due to the involvement of CCR5 in inflammatory responses. Kantor and co-workers [93] performed PCR on blood samples to genotype all individuals in their study and reported that, although CCR5 Δ 32 does not have an effect on ms disease susceptibility, homozygous and heterozygous CCR5 Δ 32 genotypes were associated with slower disease progression and progression to disability and therefore a more favourable clinical course. Given the role of CCR5 in inflammatory immune responses and upregulation of CCR5 expression during a diseased state, higher levels of CCR5 may contribute to persistent inflammation which may be damaging [93]. In the study conducted by van Veen and co-workers [94], the authors obtained blood samples of healthy controls and ms patients as well as brain tissue from ms brain donors. Similar CCR5 Δ 32 distributions among patients and controls were observed. The authors concluded that CCR5 Δ 32 does not contribute to ms disease development, clinical course, lesion activity, perivascular leukocyte infiltration or axon density. However, the authors found CCR5 Δ 32 to be associated with a reduced black hole ratio (areas with permanent damage of the axons) and increased remyelination. The authors failed to specify whether these effects are attributed to homozygous or heterozygous CCR5 Δ 32 genotypes. Therefore, the authors concluded that CCR5 Δ 32 is associated with a protective phenotype and improved clinical outcome [94].

Some studies found no significant difference in CCR5 Δ 32 allelic frequency between ms patients and control groups. In these instances, CCR5 Δ 32 was thus not associated with ms disease susceptibility [95–97]. In the investigation performed by Bennetts and co-workers [95], PCR was used to screen an Australian cohort for CCR5 Δ 32 genotypes. The authors reported similar allelic frequencies of CCR5 Δ 32 among patients and healthy controls which suggested that CCR5 Δ 32 is not implicated in ms disease [95]. Kaimen-Maciel et al. [96] obtained blood samples from ms patients who were at various stages of the disease as well as from healthy controls. The authors used PCR to determine CCR5 Δ 32 genotypes in this Brazilian cohort and reported that ms patients presented with similar CCR5 Δ 32 frequencies as seen in healthy controls. In addition, CCR5 Δ 32 frequencies did not differ significantly according to disease stage among ms patients. The homozygous CCR5 Δ 32 mutation was absent in this cohort. Patients harbouring the heterozygous CCR5 Δ 32 genotype presented with a later onset of disease. Absence of the mutation in patients was thus protective in this regard. In addition, the

authors showed a lack of association of CCR5 Δ 32 with ms disease susceptibility [96]. Silversides and co-workers [97] obtained blood samples from an Irish population and used PCR to genotype samples and investigate CCR5 Δ 32 frequencies within the cohort. The cohort included healthy controls and ms patients suffering from various types and stages of disease. The authors found similar genotypic and allelic frequencies of CCR5 Δ 32 among patients and controls suggesting that CCR5 Δ 32 is not associated with ms disease susceptibility nor with disease progression. However, when the authors stratified cases according to ms disease types or stages and age of onset, elevated CCR5 Δ 32 genotypes were observed among patients suffering from relapsing-remitting ms disease. This was especially observed in patients with a known family history of ms disease. This led the authors to report that CCR5 Δ 32 is associated with an earlier age of onset in relapsing-remitting patients. These results could not be replicated in a group where the family history of relapsing-relapsing patients was unknown [97].

2.3 Immune System Diseases

The immune system includes organs as well as processes that provide resistance to infection from pathogens. The major organs that form part of the immune system include the thymus, bone marrow and lymph nodes. A dysregulated immune system may cause an overactive or underactive immune response with a pro- or anti-inflammatory fingerprint.

Asthma is considered a chronic inflammatory disease and involves the bronchial tubes within the lungs. Since asthma is a complex condition in which the bronchial tubes are constantly inflamed, chemokines and their receptors are implicated in this disease. The production of chemokines and their receptors is generally upregulated following an allergen challenge. Previous studies reported elevated chemokine levels in patients with asthma [98,99]. Various genetic and environmental factors can also influence the pathogenesis of asthma. The investigation of specific genetic variations and their influence on asthma is thus challenging and contradictory results are often obtained. Hall and co-workers [100] conducted a study regarding CCR5Δ32 and its influence on asthma. The authors obtained a mouthwash sample from Caucasian children and determined CCR5Δ32 genotypes using PCR. Genotypes of randomly selected samples were confirmed by sequencing. The homozygous CCR5Δ32 genotype was absent in children with asthma, whereas the prevalence of asthma was decreased in individuals carrying the heterozygous CCR5Δ32 genotype compared to individuals

carrying the homozygous WT genotype. The authors suggested that, based on the prevalence of CCR5Δ32 within their cohort, CCR5Δ32 offers protection. The protective role may be attributed to the development of allergic inflammation [100]. In a later study conducted by Srivastava and co-workers [101], the protective role of the CCR5Δ32 variant was confirmed in childhood asthma, but not adulthood, suggesting that the protective role of CCR5Δ32 might become lost during the transition from childhood into adulthood. Srivastava and co-workers [101] explained that the protective effect might disappear during adulthood due to external factors such as adverse environmental exposures or host factors such as atopy. Atopy describes the genetic predisposition of an individual to develop various allergic reactions and/or disease [102]. This conclusion was made following the analysis of blood samples from a population in northern Scotland. The study firstly genotyped the blood samples of children. Once again, the homozygous CCR5Δ32 genotype was absent in children with asthma. The authors concluded CCR5Δ32 to be associated with protection against asthma in children. The same cohort was used several years later as young adults, but no protective effect was seen [101]. These results were in contrast to the results obtained by Mitchell and co-workers [103] who investigated the association between CCR5Δ32 and asthma in two respective cohorts. The cohorts consisted of individuals from Western Australia and the United Kingdom, respectively. Genotyping of the blood of the respective cohorts showed no significant linkage to asthma in either of the cohorts [103]. Abousaidi and co-workers [104] investigated the frequency of CCR5Δ32 in asthma patients and healthy controls. Blood samples were obtained from an Iranian cohort. Genotypes were determined using PCR. Since asthma is related to an allergic reaction, serum immunoglobulin E (IgE) was measured using an ELISA. The levels of IgE were higher in asthmatic patients compared to controls. While none of the asthmatic patients presented with CCR5Δ32, the heterozygous CCR5Δ32 genotype was present in 1.5% of the control group. Overall, the CCR5Δ32 genotypic distribution among patients and controls was not significant, implying that CCR5Δ32 does not have an influence on the pathogenesis of asthma [104]. In a study by Cracoviensia and co-workers [105] using a Polish population, no statistical difference in allelic frequencies of CCR5Δ32 in asthma patients compared to healthy controls was found. Consequently, the authors identified a lack of association of CCR5Δ32 with asthma [105]. Within the context of asthma and CCR5, the type of immune response induced may influence disease outcome. During allergic inflammation, Th2 cells are mainly involved due to the anti-inflammatory response [105]. Since a larger Th2 response counteracts the Th1 immune response, it may be possible that the function of CCR5, which is primarily expressed on Th1 cells, is suppressed. For that reason, pro-inflammatory chemokine release may be affected which will ultimately result in an altered immune cell signalling pattern and thus contribute to disease pathogenesis. Despite blood as the main sample type analysed, the role of

CCR5 Δ 32 in asthma remains unclear. More studies regarding immune changes based on the genotypes are necessary to further our understanding regarding CCR5 Δ 32 and its role in asthma disease.

Diabetes mellitus type 2 (T2DM) is also characterised as an inflammatory disease. During this condition, insulin production decreases which leads to insulin resistance as the disease progresses. Muntinghe et al. [106] investigated the effect of CCR5 on cardiovascular mortality in a T2DM patient cohort using blood and urine, respectively. PCR was used to determine the CCR5 Δ 32 genotypes in the samples of all individuals. Haemoglobin A1c (HbA1c), which shows the ability of the body to control blood sugar, was measured using liquid chromatography. Serum lipid levels as well as serum and urine creatinine levels were routinely tested to determine kidney function and measured using various methods. The authors found that the CCR5 Δ 32 allele contributed to elevated blood pressure and HDL levels. Surprisingly, patients carrying the CCR5 Δ 32 allele showed better survival rates. The authors thus concluded that CCR5 Δ 32 is protective in T2DM. The CCR5 Δ 32 allele is alleged to protect against other diabetic complications resulting in better survival rates [106]. Since the genotype contributing to the protective effect is not specified, it is suspected that the homozygous genotype would play a bigger role since CCR5 is involved in the initiation of inflammatory responses. Consequently, loss of CCR5 results in less severe inflammatory responses. Our interpretation is supported by the study of Bing et al. [24] who used a rat model to show low levels of CCR5 improved T2DM [24].

Autoimmune diseases refer to a state in which the immune system of an individual produces antibodies against self. There is some evidence indicating that chemokines are involved in autoimmune diseases [107,108]. Genetic variations and mutations in chemokines and their receptors can therefore result in dysregulation of the immune system due to chronic inflammation. Diabetes mellitus type 1 (T1DM) is considered an autoimmune disease. The immune system attacks pancreatic cells

responsible for the production of insulin. The inflammatory reaction is supported by the infiltration of CCR5⁺ adipose tissue macrophages [109]. Gambelunghe et al. [110] obtained blood samples from an

Italian cohort and performed CCR5 Δ 32 genotyping using PCR and sequencing. The authors reported similar CCR5 Δ 32 frequencies among diabetic patients and healthy controls and confirmed that CCR5 Δ 32 was not associated with susceptibility to T1DM [110]. Szalai and co-workers [111] investigated the distribution of CCR5 Δ 32 among children presenting with T1DM and among nondiabetic individuals. There was no difference in the distribution of CCR5 Δ 32 among the groups; CCR5 Δ 32 was therefore not associated with T1DM [111]. Yang et al. [112] used blood from a British cohort and investigated several polymorphisms in the genes of chemokines with a role in T1DM. CCR5 Δ 32 was not associated with T1DM. However, when combining the effects of various polymorphisms, the authors found that the chemokine system was associated with complications in diabetic patients [112]. Kalev et al. [113] used blood to investigate the frequency of CCR5 Δ 32 in T1DM and T2DM patients as well as healthy controls using PCR. The authors found no difference in allelic frequencies between patient and control groups. The heterozygous CCR5 Δ 32 genotype was however associated with the clinical course of T1DM (i.e., there is a later age of onset of T1DM in heterozygous CCR5 Δ 32 patients compared to homozygous WT patients). With regards to T2DM, heterozygous CCR5 Δ 32 patients presented with decreased frequency of concomitant diseases compared to homozygous WT patients. The authors finally reported CCR5 Δ 32 to be associated with protection regarding clinical course, but not disease susceptibility [113]. In a meta-analysis conducted using a mixed population, Song et al. [114] aimed to investigate the role of CCR5 Δ 32 in diabetes and asthma disease susceptibility. The meta-analysis included fourteen studies of which six were representative of T1DM and three of asthma. In relation to T1DM, CCR5 Δ 32 was considered a protective factor while no association of CCR5 Δ 32 with asthma susceptibility was found [114].

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease which causes swelling and deformities in joints. An auto-antibody called rheumatoid factor (RF) is present in the blood of patients. In addition, several immune cells undergo peripheral activation followed by attraction to the synovial compartment. Kohem and co-workers [115] investigated the role of CCR5 Δ 32 in RA patients and healthy controls as well as the phenotypic expression of CCR5 on T-cells. The authors

used flow cytometry to determine the immunophenotype of individual cells. There was no difference in the frequency of CCR5 Δ 32 among patients and controls. The homozygous CCR5 Δ 32 genotype was absent in both groups, while the heterozygous CCR5 Δ 32 genotype was present in some patients. The heterozygous CCR5 Δ 32 genetic frequencies were similar among patients and controls. The authors found elevated levels of CCR5⁺ cells in the synovial fluid of RA patients. Given the role of CCR5 in the immune system, elevated levels of CCR5 enhance inflammation, thus influencing disease severity. The authors observed that heterozygous CCR5 Δ 32 patients had aggressive RA disease and based on this concluded that CCR5 Δ 32 was not implicated in RA disease [115]. Lindner and co-workers [116] investigated the association of CCR5 Δ 32 with RA and juvenile idiopathic arthritis (JIA). The authors reported no association of CCR5 Δ 32 with both diseases based on similar CCR5 Δ 32 genetic frequencies among patients and controls [116]. Some studies report that CCR5 Δ 32 results in less severe disease and improved clinical outcome in RA patients thus offering a protective role. In a study conducted by Garred and co-workers [117] patients carrying either the homozygous or heterozygous CCR5 Δ 32 genotypes tested negative for RF compared to patients with the homozygous WT genotype. In addition, the prevalence of swollen joints and morning stiffness was decreased in these patients and suggests CCR5 Δ 32 may influence autoimmune disease severity by improving clinical course [117]. Gómez-Reino et al. [118] used blood samples from RA patients, systemic lupus erythematosus (SLE) patients and healthy controls. The authors performed PCR to determine CCR5 Δ 32 genotypes. The authors reported that none of the RA patients presented with the homozygous CCR5 Δ 32 genotype, while the heterozygous CCR5 Δ 32 genotype and homozygous WT genotype caused the same severity in disease. The results obtained suggest a protective role of CCR5 Δ 32 against RA. In addition, the authors investigated the role of CCR5 Δ 32 in SLE; similar CCR5 Δ 32 genotypic and allelic frequencies were observed among patients and controls suggesting a lack of association of CCR5 Δ 32 with SLE [118]. In a study based on a New Zealand cohort, Pokorný and co-workers [119] performed PCR on blood samples to ultimately compare the frequencies of CCR5 Δ 32 between Caucasian RA patients and healthy controls. None of the RA patients presented with the homozygous CCR5 Δ 32 genotype. The frequency of the heterozygous CCR5 Δ 32 genotype was less in RA patients compared to controls. This evidence suggests a protective role of the homozygous CCR5 Δ 32 genotype. Although the allelic frequency of CCR5 Δ 32 was reported to be decreased in patients, the authors reported that frequency of CCR5 Δ 32 did not differ significantly when cases were stratified according to disease severity or outcome [119]. Rossol et al. [120] reported decreased RA disease susceptibility for German individuals carrying CCR5 Δ 32. For this, the authors determined the prevalence of CCR5 Δ 32 in RA patients as well as healthy controls. Blood samples were obtained and genotypes were determined using PCR. The authors found that CCR5 Δ 32 was more prevalent in RA patients with articular, inside joints diseases when compared to RA patients with extra-articular, outside joints manifestations. The authors also used immunofluorescence and immunohistochemistry to determine levels of various antibodies and nephelometry to determine levels of RF in serum. The authors found that CCR5 Δ 32 was associated with lower RF levels. In conclusion, it was shown that CCR5 Δ 32 offers protection against joint erosions and extra-articular manifestations. The authors thus demonstrated that RA disease susceptibility as well as clinical course is influenced by an individual's CCR5 Δ 32 genotype [120].

Systemic lupus erythematosus is a chronic autoimmune disease more commonly known as lupus. Multiple organs are affected in this disease with inflammation mainly attributed to chemokines. Lupus patients present with elevated chemokine levels during active disease [121]. The role of CCR5 Δ 32 in the development of SLE was investigated in a study conducted by Carvalho et al. [122] using a Portuguese cohort. Blood samples were obtained and subjected to PCR analysis to determine sample genotypes. The authors showed that the frequency of heterozygous CCR5 Δ 32 for SLE patients was lower compared to the control group, while the homozygous CCR5 Δ 32 genotype was absent among patients. Since the mutation did not feature much in patients, the authors reported CCR5 Δ 32 to be associated with protection against SLE disease [122]. Schauren et al. [123] investigated the association of CCR5 Δ 32 with SLE in a Brazilian cohort. The CCR5 Δ 32 genotypic frequencies were decreased in European

patients and increased in African patients, respectively compared to healthy controls. The homozygous CCR5 Δ 32 genotype was absent in European patients. The authors therefore showed that CCR5 Δ 32 genotypes are involved in the protection of European patients against SLE. In addition, the authors showed that CCR5 Δ 32 genotypes are associated with development of class IV lupus nephritis [123]. Similar results were obtained by Baltus and co-workers [124] in a female Brazilian cohort. The authors investigated the association of CCR5 Δ 32 genotypes with SLE disease susceptibility and disease activity. Blood samples were obtained and genotypes were determined using PCR. The authors reported increased homozygous and heterozygous CCR5 Δ 32 frequencies within SLE patients suggesting that CCR5 Δ 32 is associated with the development of SLE disease. Based on clinical information obtained from all individuals, homozygous and heterozygous CCR5 Δ 32 were found to be associated with earlier age of onset. Disease-associated markers were measured using immune-based methods. When the authors adjusted the results for the ethnicity of the patients, the authors showed that ethnicity influenced autoimmune responses. However, CCR5 Δ 32 was not associated with disease activity [124]. Lupus nephritis (LN) refers to kidney inflammation and is often a result of SLE. The role of CCR5 in kidney inflammation is established (i.e., CCR5⁺T-cells involved in immune responses cause scarring or thickening of interstitial tissues known as fibrosis) [125]. A meta-analysis conducted by Zhou and co-workers [126] revealed no association of CCR5 Δ 32 with LN susceptibility in Asians and Caucasians. This result is based on five studies included in the meta-analysis. An increased risk of developing LN disease in African patients was reported in one study included in the meta-analysis. Overall, Zhou and co-workers [126] found no difference in CCR5 Δ 32 gene distribution among SLE patients and LN patients, suggesting that CCR5 Δ 32 cannot predict whether SLE patients will develop LN.

2.4 Infectious Diseases

Infectious diseases are caused, amongst others, by viral and parasitic organisms. Clearance of the pathogen is dependent on an effective immune response. Given the role of chemokines and chemokine receptors in the immune response, CCR5 is implicated in many infectious diseases.

2.4.1 Acquired Immunodeficiency Syndrome

In 1995 a paper was published describing the role of CCR5 in HIV-1 infection. Given their competitive binding to the CCR5 receptor, the chemokines MIP-1 α , MIP-1 β and CCL5 (RANTES) were shown to inhibit HIV-1 infection in cells [127].

The presence of a virus is generally detected by measuring the antibodies present in the blood against that specific virus. In the case of HIV-1, the virus can be present in the blood of individuals carrying the homozygous CCR5 Δ 32 genotype resulting in a seropositive test. However, given the role of CCR5 as a coreceptor for HIV-1 to enter the cell, decreased levels of CCR5 on the cell's surface limit viral entry. Therefore, seropositive individuals with the homozygous CCR5 Δ 32 genotype are still considered protected. In a meta-analysis conducted by Dean and co-workers [30], the homozygous CCR5 Δ 32 genotype was present exclusively in a group of HIV-exposed, but uninfected individuals. The authors reported increased frequencies of the heterozygous CCR5 Δ 32 genotypes in patients that survived HIV-1 infection for more than ten years. Survival analysis showed slower disease progression in heterozygous CCR5 Δ 32 patients. Therefore, homozygous CCR5 Δ 32 was associated with protection against HIV-1 infection, whereas the heterozygous CCR5 Δ 32 genotype was associated with slower HIV-1 disease progression [30]. Samson et al. [31] showed the CCR5 Δ 32 genotype to occur more frequently in Caucasian populations while being absent in African and Japanese populations. The homozygous CCR5 Δ 32 genotype was absent in Caucasian patients, while the frequency of heterozygous CCR5 Δ 32 genotypes was lower in HIV-infected patients. One homozygous CCR5 Δ 32 carrier donated white blood cells and the authors showed that HIV-1 entry was inhibited which confirmed CCR5 to be the main coreceptor for HIV-1. The authors concluded that the decreased frequencies of heterozygous CCR5 Δ 32 in seropositive patients suggest partial resistance against HIV-1 infection thus affording protection [31]. In a South African-based study done by Jaumdally et al. [33], the immune profiles of

samples carrying protective and non-protective haplotypes were investigated. The authors investigated the interaction between systemic inflammation, immune activation of T-cells and CCR5 genotype in context to HIV-1 resistance. PCR, immune assays and flow cytometry were applied to measure genetic and immune parameters. The authors showed CCR5Δ32 frequency to be similar among patients and controls. Patients bearing the protective haplotype presented with decreased levels of immune activation, CCR5 expression and plasma cytokines [33]. Marmor and co-workers [37] investigated HIV resistance in exposed but uninfected and infected cohorts, respectively. The cohort consisted of patients carrying either the homozygous or heterozygous CCR5Δ32 genotype. The individuals carrying the homozygous CCR5Δ32 genotype were exposed but not infected, while the heterozygous CCR5Δ32 group was infected and showed slow disease progression. The authors documented both genotypes to be associated with protection against HIV-1 infection [37]. Liu and co-authors [128] isolated CD4⁺ T-cells from two individuals that were exposed to HIV, but who remained uninfected. The authors showed that these cells remained resistant to HIV infection, despite being infected with T-cell line-adapted viruses. Subsequent analysis revealed both patients' cells to carry the homozygous CCR5Δ32 mutation. The authors concluded CCR5 to be implicated in HIV infection and disease progression, more specifically, that homozygous CCR5Δ32 genotypes confer resistance to HIV infection [128]. Rana and co-workers [129] used human primary blood cells to show that the homozygous CCR5Δ32 mutation results in the failed expression of functional CCR5 protein on the cell surface and that CCR5 is essential for HIV entry into the cell [129].

Balotta et al. [130] obtained blood samples from a cohort consisting of HIV-infected and uninfected individuals. PCR was used to determine CCR5Δ32 genotypes. Surprisingly, the authors observed one homozygous CCR5Δ32 genotype among HIV-infected patients which was confirmed through sequencing. In this instance the homozygous CCR5Δ32 genotype was found to not confer absolute protection against HIV infection [130]. Several independent studies conducted on the blood samples of males confirmed that the CCR5Δ32 homozygous genotype provides incomplete protection against HIV infection [129–135]. In all these studies genotyping was done using PCR and/or sequencing. In all of the samples HIV infection was also confirmed whether through Western blotting techniques [131], immunoassays (e.g., p24 assay), or measuring viral load [132], to name a few. Non-protection was ascribed to the fact that patients were infected with a dual-tropic virus, using receptors other than CCR5 [130–136]. A mutation of the stromal cell-derived factor 1 (SDF-1) gene, which generally serves to protect against HIV-infection, was also documented [136]. Collectively, these studies showed that CCR5Δ32 offers protection only in the case of macrophage-tropic HIV-1 infection. T-cell tropic HIV primarily uses the CXCR4 receptor, whereas dual-tropic viruses can use both CXCR4 and CCR5. Where patients are infected with T-cell and/or dual-tropic viruses as in the case of several studies reported here [129–135], CCR5Δ32 offers no protection. In fact, risk for infection is increased.

With respect to HIV-1 infection, the protective role of CCR5Δ32 in context to macrophage-tropic HIV infection, which makes use of CCR5 to gain entry into the cell, is thus clear and well established.

2.4.2 West Nile Fever

The West Nile virus (WNV) is a single stranded RNA virus and is considered the causative agent of West Nile fever (WNF). In general, WNV infection is asymptomatic while severe cases can result in meningitis or encephalitis. A study conducted by Glass et al. [137] showed that mice with a homozygous WT CCR5^{+/+} genotype, which is similar to the CCR5 WT genotype in humans, had a better survival rate than CCR5-deficient mice. In these mice there was increased expression of CCR5 which is required for lymphocyte recruitment into the central nervous system [137]. The same group conducted a study in 2006 using the blood and cerebrospinal fluid of Caucasians in which they established that the CCR5Δ32 homozygous genotype (generally associated with low CCR5 levels) is a risk factor for symptomatic WNV infection (minimal CCR5 for lymphocyte recruitment) [138]. Similar conclusions were made in the meta-analysis of Lim and co-workers in 2008 [139]. The findings for this disease are thus consistent, independent of the model used.

2.4.3 Hepatitis B Infection

Hepatitis B virus (HBV) infection is the causative agent of severe liver infection which may be fatal. Chronic HBV infection can progress and result in cirrhosis as well as hepatocarcinoma. Activation of Th1 lymphocytes is necessary during viral infections. The main immune cells involved in eliminating viral infections are the CD8⁺ T-cells [140]. Chemokines play a pivotal role in leukocyte migration and viral eradication. Nevertheless, constant expression of certain chemokines affects immune activation. Persistent immune activation may in turn result in tissue damage while varying levels of CCR5 expression can influence immune responses [141,142]. Immune cells migrate to the HBV-infected liver due to the presence of CCR5 receptors on the surface of cells [143]. In a study performed by TrehanPati and co-workers [144], blood samples were obtained from healthy controls, acute HBV infection and chronic HBV infection patients. The authors did not mention the ethnic group(s) used for their study. RT-PCR was used to analyse CCR5 expression profiles while flow cytometry was used to type CD4⁺ T-cells. Various antibodies related to HBV disease were detected using ELISA. The authors showed that T regulatory cells were more abundant during the acute phase of HBV disease when compared to chronic HBV patients and healthy controls. In addition, the authors showed increased CCR5 expression levels on CD4⁺ cells during acute HBV infection which might be implicated in viral clearance and offering protection against disease. [144]. A group of researchers investigated CCR5 expression on the T lymphocytes of Iranians in two respective studies. In the first study conducted by Arababadi et al. [145], the rate of CCR5 expression on natural killer (NK) cells was determined. The authors tested for various antibodies related to HBV infection and flow cytometry analysis was performed to evaluate CCR5 levels. Low levels of CCR5 on NK cells of HBV patients were associated with reduced viral clearance signifying non-protection against the disease [145]. Arababadi et al. [146] investigated the expression of CCR5 on CD8⁺ T-cells in HBV patients. The CCR5Δ32 mutation was not detected in any patients. The authors found reduced CCR5 levels on CD8⁺ T-cells, unrelated to the CCR5Δ32 mutation, suggesting that HBV has an influence on CCR5 expression. Therefore, the authors concluded that viral and host genetics can impact CCR5 expression resulting in reduced viral clearance [146]. Ahmadabadi et al. [147] (not summarised in Table 1) investigated CCR5 expression levels on CD8⁺ T-cells in chronic HBV patients. Various immune markers related to liver function were measured using ELISA-based assays. CCR5 levels were determined using flow cytometry analysis. The authors reported a reduced percentage of CD8⁺ T-cells in the chronic HBV patients. Surprisingly, the amount of CCR5 on CCR5⁺/CD8⁺ T-cells was increased in the chronic HBV group. The chronic HBV group also presented reduced levels of total lymphocytes. Similar to the findings of Arababadi [145], the lower frequency of CCR5⁺/CD8⁺ cells in chronic HBV patients was found insufficient to eradicate HBV from the hepatocytes and was therefore characterised as non-protective [147]. These results however contrast with an earlier study performed by Thio and co-workers [148] which showed that the homozygous CCR5Δ32 polymorphism offers genetic protection for patients recovering from HBV infection. Eight out of nine CCR5Δ32 homozygotes in this cohort recovered from infection. In addition, CCR5Δ32 was frequent in the recovery group and could thus be associated with protection regarding clinical outcome [148].

Ellwanger and co-workers [149] investigated the role of CCR5Δ32 in HBV mono-infection and HBV/HIV-1 co-infection. Blood was obtained from a Brazilian cohort. Genotypic analysis was performed using PCR. Patients were grouped according to HBV, HIV-1 and HBV/HIV-1 infection and CCR5Δ32 allelic frequencies were compared to that of healthy controls. Similar genotypic frequencies were observed among groups. The authors found that the CCR5Δ32 allele was not associated with HBV infection susceptibility, but that it did offer some protection against HBV/HIV-1 co-infection. The authors noted that the protective effect observed might be due to the known protective effect of CCR5Δ32 against HIV-1 infection [149].

.4.4 Hepatitis C Infection

Hepatitis C virus (HCV) infection leads to inflammation of the liver, which ultimately results in chronic liver disease as well as liver damage. It is established that chemokines play a pivotal role in chronic HCV infection. More specifically, CCR5 is involved in promoting cellular responses such as inflammation in the liver and during fibrogenesis. During the acute phase of infection, chemokines are involved in chemoattraction of effector T-cells to the site of infection in the liver in order to aid viral clearance. During the chronic phase of infection, the inflammatory cells present at the site of infection aggravate liver cell injury which ultimately results in the progression of chronic HCV to fibrosis. CCL5 is involved in the regulation of the migration of immune cells and also serves as a ligand for several other C-C chemokine receptors besides CCR5. In an *in vitro* study, CCL5 expression was upregulated by the presence of full-length HCV [150] or the viral envelope protein [151]. The secreted chemokines required interaction with CCR5 in order to exert an inflammatory response. Studies have however found that the interaction between CCR5 and CCL5 may result in reduced expression of CCR5 on the cell surface as a result of receptor internalization, an endocytotic process in which the receptors are relocated from the cell surface to the inside of the cell [151,152]. Lichterfeld and co-workers [153] obtained blood samples from chronically-infected HCV patients and healthy controls, respectively. The authors sought to determine whether CCR5 levels may be affected by HCV disease instead of CCR5 Δ 32 genotypes. The authors confirmed CCR5 Δ 32 genotypes using PCR and determined CCR5 expression on T-cells using flow cytometry. Chemokine levels were measured using ELISA. In addition, the levels of mRNA for the CCR5 protein were detected using RT-PCR. All patients carried a homozygous WT CCR5 genotype. The authors showed that the CD8 T-cells of healthy controls presented with increased levels of CCR5 compared to HCV patients. Levels of CCR5 mRNA were not altered and therefore did not influence CCR5 expression on the cell surface. Due to the absence of CCR5 Δ 32 genotypes in patient samples, the authors suggest that receptor internalization best explains the reduced amount of peripheral CCR5⁺ cells in chronic HCV patients. [153]. Hellier et al. [154] sampled liver biopsies from a group of patients to investigate several CCR5-related polymorphisms and their association with persistent HCV carriage, liver disease severity, hepatic inflammation and response to treatment. The authors confirmed that patients were free of liver disease that derived from HCV infection. The authors reported that, among patients, homozygous CCR5 Δ 32 genotypes were found to be associated with reduced portal inflammation and less severe fibrosis, and suggested CCR5 Δ 32 to be associated with HCV infection clinical outcomes [154]. Promrat and co-authors [155] investigated several polymorphisms linked to the chemokine system using the blood of liver disease patients and healthy controls, respectively. Most liver disease patients suffered from HCV infection. The authors reported the frequencies of homozygous CCR5 Δ 32 genotypes to be similar among patients and controls and therefore not associated with HCV disease susceptibility. Furthermore, CCR5 Δ 32 was not associated with clinical parameters related to HCV infection [155]. Wald et al. [156] investigated the role of CCR5 Δ 32 genotypes and inflammation during the early stages of HCV disease. Blood and liver biopsies were obtained from all study participants. In addition, inflammatory activity was assessed by a hematopathologist during liver biopsy collection. CCR5 Δ 32 genotypes were determined using PCR. The authors identified the heterozygous CCR5 Δ 32 and homozygous WT genotypes. Based on similar frequencies of CCR5 Δ 32 genotypes among patients and healthy controls, the authors reported that CCR5 Δ 32 was not associated with HCV infection susceptibility. Based on inflammatory activity scores, CCR5 Δ 32 genotypes do not influence disease progression towards end-stage liver disease. However, when the authors stratified patients according to CCR5 genotypes, the heterozygous CCR5 Δ 32 genotypes were associated with reduced inflammation during early stages of HCV disease [156]. Ruiz-Ferrer et al. [157] used blood and liver biopsies of a Spanish population to investigate the involvement of chemokine system-related polymorphisms in the response to HCV infection. PCR was used to determine CCR5 Δ 32 genotypes. None of the patients carried the homozygous CCR5 Δ 32 genotype. The authors found no significant difference in the CCR5 Δ 32 allelic frequencies of patients when compared with controls, hence no association of

CCR5Δ32 to HCV infection, liver damage or clinical outcome [157]. Goulding and co-workers [158] investigated the role of CCR5Δ32 to HCV in a British population. The authors obtained blood samples for genotyping and liver biopsies to determine histological changes. Genotyping was performed using PCR, while HCV antibody status was analysed using an ELISA. The authors compared the frequencies of several chemokine and chemokine receptor polymorphisms with disease severity in HCV patients and healthy controls. The frequency of the heterozygous CCR5Δ32 genotype in both HCV patients and controls was similar [158]. The heterozygous CCR5Δ32 genotype was associated with spontaneous elimination of HCV. In addition, individuals with the heterozygous genotype presented with decreased hepatic inflammation. Reduced inflammation and elimination of HCV suggested protection against HCV disease.

2.4.5 Chagas Disease

Chagas disease is an inflammatory infectious disease caused by the parasite *Trypanosoma cruzi*. Transmission occurs mainly via insects. The parasite is disseminated by the immune system and, in some cases, ultimately results in the development of potentially fatal CM [159]. The role of chemokines and chemokine receptors has been studied in Chagas disease in several populations. A recent study demonstrated several genes involved in the immune response to influence the severity of Chagas disease [160]. The role of chemokines in the early stages of Chagas-induced CM is well established. In a study conducted by Talvani et al. [161], blood samples were obtained from non-infected individuals, patients suffering from mild Chagas-induced CM and patients suffering from severe Chagas-induced CM. Flow cytometry was used to determine the levels of CCR5 on peripheral blood mononuclear cells. Patients suffering from mild Chagas-induced CM presented with elevated CCR5 expression on leukocytes compared to patients suffering from severe Chagas-induced CM and non-infected individuals. In addition, the amount of CCR5 correlated with disease severity (i.e., reduced CCR5 levels were found in patients suffering from severe Chagas-induced CM which related to decreased heart function). The authors concluded that CCR5 is involved during the early stages of Chagas-induced CM, highlighting the role of the chemokine system in Chagas-induced CM [161]. Fernandez-Mestre et al. [162] obtained blood from a Venezuelan cohort and determined CCR5Δ32 genotypes using PCR. The authors confirmed *T. cruzi* infection with immunofluorescence. The homozygous CCR5Δ32 genotype was absent. Allelic and genotypic frequencies were similar among asymptomatic and symptomatic patients. It was concluded that CCR5Δ32 was not associated with Chagas disease. However, further studies are required due to the low prevalence of CCR5Δ32 in Venezuelan individuals [162]. Due to the low prevalence of CCR5Δ32 within selected cohorts, other studies found polymorphisms (other than CCR5Δ32) within the CCR5 gene to influence Chagas disease susceptibility and progression [159,163–165]. Collectively, these results indicate that further studies are required to elucidate the role of CCR5 in the presence of Chagas disease.

2.4.6 Influenza A

Influenza, an acute viral respiratory infection, promotes a severe pro-inflammatory cytokine and chemokine response in lung tissue. Influenza A is the most common type of influenza, also known as “the flu”. It is caused by the influenza virus for which there are several strains. Following infection, an inflammatory response is initiated in order to limit viral replication. Pro-inflammatory mediators form the majority of the immune molecules present at the site of infection [166]. The inflammatory response results in recruitment of antigen-specific memory CD8⁺ T-cells and is essential to regulate infection and viral replication [167]. However, instead of offering protection, a variety of cell types can have a negative influence on patient outcome when present in large quantities [168]. Disease outcome can thus vary based on differential inflammatory response patterns. In a study conducted by Kohlmeier and co-workers [167] (not summarised in Table 1), the authors used a mouse model to investigate the role of certain chemokine receptors in the recruitment of virus-specific memory CD8⁺ cells. In addition, the influence of chemokine-receptor deficiency on an effective cellular response was also investigated.

The results suggested that CD8⁺ T-cell recruitment depends on CCR5 expression. CCR5 is therefore crucial for an effective immune response [167]. Swine flu is a respiratory disease caused by a specific strain of the influenza virus known as H1N1. Keynan and co-workers [169] investigated the role of CCR5Δ32 genotypes in H1N1-infected patients. Blood was obtained from patients comprising various ethnicities. In patients suffering from severe disease, an increase in the prevalence of heterozygous CCR5Δ32 genotypes was observed. The heterozygous CCR5Δ32 genotypes were associated with disease severity, but not disease susceptibility [169]. In a study conducted by Sironi et al. [170], blood was obtained from several H1N1-infected patients presenting with various clinical symptoms. The CCR5Δ32 genotypes were determined using PCR. One individual presented with the heterozygous CCR5Δ32 genotype and mild disease. CCR5Δ32 therefore did not influence influenza A disease severity in a significant way [170]. In an investigation by Maestri and co-workers [171] which comprised a Brazilian cohort, no significant difference was found in either allele or genetic frequencies of CCR5Δ32 in hospitalised patients compared to non-hospitalised patients. The homozygous and heterozygous CCR5Δ32 genotypes were thus not associated with influenza A (H1N1) [171]. Similar results were obtained by Matos et al. [172] who used the nasopharyngeal swabs and post mortem specimens of a Brazilian cohort (i.e., no significant difference in the distribution of the homozygous WT and heterozygous genotypes as well as CCR5Δ32 allelic frequencies in the different clinical groups were found). The findings highlight a lack of association of CCR5Δ32 with disease susceptibility, severity or mortality rates in a Brazilian cohort [172].

The aforementioned studies highlight the contrasting role(s) of CCR5Δ32 with regards to diseases. While several studies concluded the CCR5Δ32 genotype to be associated with increased susceptibility to disease due to impaired immune signalling, thus preventing effective viral clearance, further studies are required to clarify this.

2.5 Cancer

Cancer is a broad term used to describe a diseased state in which cells show an abnormal growth pattern. Certain changes result in immortal cells that undergo uncontrolled growth and division. There are various cancer types with some more severe than others. The type of cancer is characterised by the localization of the immortal cells. The tumour environment contains important elements such as chemokines and chemokine receptors [173]. The chemokines produced and secreted by cancer cells play a pivotal role in leukocyte infiltration [174]. In a study conducted by Lavergne and co-workers [175], the authors used a mouse model to determine whether CCL5 can control the growth of tumours. CCL5 expression was induced in these mice. In CCR5-deficient mice, decreased CCL5 levels were observed and anti-tumour effects were absent. The results of this study therefore support the idea that CCL5 together with CCR5 plays an important role in the recruitment of immune cells to tumour sites and ultimately delays tumour growth [175]. Systems lacking the CCR5-CCL5 interaction are thus at risk of cancer development and tumour growth. Gao and co-workers [22] used a mouse model to demonstrate the enhanced proliferation of breast cancer cells upon CCL5-CCR5 activation. Cell lines were used to show that the presence of CCL5 activates CCR5 and ultimately results in increased glucose uptake and ATP production. Ultimately, these metabolic changes resulted in increased proliferation of cancer cells [22]. In a later study, Gao et al. [176] again showed that CCL5-CCR5 interactions influence metabolic events during tumour onset which ultimately enhance the formation of new tumours, a process known as tumorigenesis. This conclusion was made after the authors showed that, in the absence of CCR5, tumour progression is delayed. The authors made use of advanced techniques, such as chromatography and mass spectrometry, to measure the altered metabolism [176].

The role of CCR5Δ32 in breast cancer in an Iranian cohort was investigated by Eskandari-Nasab and co-workers [177]. Blood samples were collected and genotyped. Homozygous and heterozygous CCR5Δ32 were not associated with breast cancer development [177]. A meta-analysis conducted by Li and co-workers [178] included six breast cancer studies. Three of these studies used Caucasian cohorts. Two studies used Asian cohorts, and one study used a Brazilian cohort. The authors reported CCR5Δ32

to not be associated with susceptibility to breast cancer. This finding was consistent even when the authors stratified cases according to ethnicity. The authors failed to specify whether CCR5Δ32 was present as homozygous or heterozygous genotypes. [178]. More recently, a study conducted by Jiao et al. [179] reported on a possible protective role of CCR5 in breast cancer as investigated in a mouse model. Flow cytometry was used to sort cells according to CCR5 expression. These cells were then implanted in mice; some mice received CCR5⁺ cells, whereas other mice received CCR5-negative (CCR5⁻) cells. CCR5 is involved in various cell signalling pathways. The regulation of pathways involved in DNA repair is altered in CCR5⁺ and CCR5⁻ cells. The authors showed mice harbouring CCR5⁺ cells to have an improved activity of pathways involved in DNA repair. Consequently, the authors concluded that DNA damage repair can be induced by the presence of functional CCR5 due to the cell signalling pathways affected [179]. In this context, the CCR5⁺ cells serve a protective role.

Degerli and co-workers [180] conducted an investigation regarding the role of the heterozygous CCR5Δ32 genotype in various carcinomas in a Turkish population. Carcinomas typically originate from tissues lining the body's outer and inner surfaces. In this study, the various carcinomas studied included: breast, laryngeal, thyroid and brain. The study also included healthy individuals as controls. The heterozygous CCR5Δ32 genotype was more prevalent in breast cancer patients, but it was not statistically significant when compared to the prevalence of CCR5Δ32 in other carcinomas studied. The authors concluded that CCR5Δ32 was not associated with carcinomas, but may be a risk factor for the development of breast cancer [180]. Duell and co-workers [181] investigated the role of various mutations in pro-inflammatory genes as well as the role of CCR5 in the development of pancreatic cancer. With regards to CCR5, no association was found between CCR5Δ32 and pancreatic cancer risk. However, the authors did report a possible interaction between CCR5Δ32 and active smoking.

The data obtained suggested pancreatic cells to be partially protected against the effects of tobacco smoke by intact CCR5. Mutations such as the 32 bp deletion within the CCR5 gene generally result in non-functional receptors, reduce the secretion of tobacco-induced inflammatory mediators and can therefore be considered as a risk factor for pancreatic cancer [181].

Singh and co-authors [182] investigated the role of CCR5Δ32 in the development of cervical cancer. The authors used cervical carcinoma biopsies and found a non-significant higher frequency of heterozygous CCR5Δ32 in patients when compared to controls. Homozygous CCR5Δ32 genotypes were absent among patients. CCR5Δ32 was found to not be a risk factor for the overall development of cervical cancer. Nevertheless, the risk for early stage cervical cancer may be influenced by this mutation, but further studies are necessary [182]. In a study conducted by Zheng and co-workers [183], the possible association of CCR5Δ32 with cervical cancer and human papillomavirus (HPV) was investigated. Cancer induces an immune response which is exacerbated by the presence of HPV. The overactive and long-term immune response may influence cells to become cancerous due to constant cell signalling and the inflammatory mediators secreted. In the Swedish cohort studied, the frequency

of CCR5Δ32 was increased among HPV⁺ cervical cancer patients compared to HPV⁻ cervical cancer patients. CCR5Δ32 may therefore be a risk factor for HPV infection and thus increase the risk for cervical cancer [183].

Srivastava and co-authors [184] investigated the influence of CCR5Δ32 on the development of gallbladder cancer. Blood samples were used to investigate the CCR5 genotype distribution in patients and healthy controls. The heterozygous CCR5Δ32 genotype was more frequent in patients compared to controls. When the authors stratified the cases according to the presence of gallstones, they showed that both homozygous and heterozygous CCR5Δ32 genotypes are associated with a risk for gallbladder cancer irrespective of the presence of gallstones. CCR5Δ32 did not have an influence on disease susceptibility based on gender, but may be implicated in the early development of gallbladder cancer [184].

The role of CCL5 in the progression of gastric cancer was investigated by Sugawara and co-workers [185]. The data revealed that gastric cancer cells may have an influence on the level of CCL5 secretion by CD4⁺ T-cells. Cancer cells express receptors for CCL5 and increased levels of CCL5 are

present in cancerous tissue. The increased expression of CCL5 activates CCR5 and ultimately results in tumour progression by means of increased cancer cell proliferation, while the elimination of tumours by CD8⁺ T-cells is inhibited. The authors thus showed that CCR5 expression is associated with a worse prognosis [185]. A study regarding mutations in chemokine and chemokine receptor genes and their influence on gastric cancer development and survival was conducted by Gawron and co-workers [186]. Blood samples were used from a cohort consisting of individuals from Poland. The authors found no association between CCR5Δ32 and gastric cancer risk or survival outcome [186]. In contrast, a study conducted by Cao and co-workers [187] revealed that CCR5 may contribute to gastric cancer metastasis, a process in which some cancer cells separate from the tumour to form secondary tumours at a different location. The authors showed that metastatic gastric cancer tissues present with increased CCR5 levels compared to gastric cancer without metastasis. Immunohistochemical staining was used to determine the levels of chemokines and chemokine receptors. Th1 and Th2 cytokines were measured using RT-PCR and the authors concluded that CCR5 may influence the Th1/Th2 balance [187].

The role of various chemokines and chemokine receptors in bladder cancer was investigated by Kucukgergin and co-workers [188] using a Turkish population. Bladder tumour biopsies were used. The authors reported an association of the heterozygous CCR5Δ32 genotype with increased risk of bladder cancer development [188].

In another investigation conducted by Kucukgergin and co-workers [189], the role of CCR5Δ32 in prostate cancer was investigated. Blood samples from a male Turkish population were collected and PCR-RFLP was used to determine sample genotypes. The cohort did not contain individuals carrying a homozygous CCR5Δ32 genotype. The authors reported that the heterozygous CCR5Δ32 genotype was associated with an increased risk of developing prostate cancer [189]. In contrast, Zambra and co-workers [190] investigated the role of CCR5Δ32 in a Brazilian population using DNA samples derived from blood. The authors reported no association of either homozygous or heterozygous CCR5Δ32 genotype with prostate cancer development, nor with the clinicopathologic status [190]. Similar results were obtained in an earlier case-control study by Petersen and co-workers [191] in a larger Australian cohort. Blood samples were obtained from participants and subjected to PCR analysis to determine CCR5Δ32 genotypes. Similar allelic frequencies were observed among patients and healthy controls. The authors thus concluded CCR5Δ32 to not be associated with susceptibility to prostate cancer [191].

Acute myeloid leukemia (AML) is a type of cancer which affects the bone marrow and normal formation of various blood cells. Khorramdelazad and co-workers [192] investigated the prevalence of CCR5Δ32 within Iranian patients suffering from AML. Neither the homozygous nor heterozygous CCR5Δ32 genotypes were present in the patient group, while three individuals in the control group presented with the heterozygous CCR5Δ32 genotype. The authors concluded a lack of association of CCR5Δ32 with AML susceptibility in an Iranian cohort [192]. To date, the role of CCR5 in various cancers remains unclear.

Table 1. The contrasting role(s) of C-C chemokine receptor 5 (CCR5) in different diseases possibly explained by CCR5 status (frequency, genotype, levels, haplotype), ethnicity, disease type and/or model.

(A)										
Reference	[61]	[62]	[63]	[64]	[65]	[66]	[67]	[68]	[69]	[70]
Category of Disease	CVD	CVD	CVD	CVD	CVD	CVD	CVD	CVD	CVD	CVD
Disease	AS	CHD	CAD	CAD	CAD	CAD	CAD	CM	CAD	CAD
Population	Mixed	Caucasian	Caucasian	Greek	German; Caucasian	Indian	Bruneck	German	Hungarian	Mixed
Model	Meta-analysis	Blood	Blood	Blood	Blood	Blood	Blood	Blood	Blood	Meta-analysis
CCR5A32 frequency	Patients No difference Controls	↑ Asian X Mixed	X	X	X	X	↑	X		X
CCR5 levels	Patients/test subject/sample No difference Controls									
Genotype	Homozygous CCR5A32	X Asian	X	X		X		X	X	
	Heterozygous CCR5A32	X Asian	X	X		X		X	X	X
	Absent CCR5A32 Homozygous WT		X ^{Px}		X ^a		X ^a			X ^{Px,a}
Association/ effect of CCR5A32	Protective			X ^a				X	X ^M	X ^a
	No association/effect	X ^{S, Mixed}		X ^b	X	X ^S				X ^{hS}
	Risk factor/non-protective	X ^{S, Asian}	X ^O				X ^S			
Assay(s) used	PCR		X	X	X	X	X	X	X	X
	RFLP				X	X				
	Sequencing									
	Immune-based		X		X			X		X
	Microscopy									
	Spectroscopy				X	X				
	Flow Cytometry									
	Chromatography									
	MS									
	Other	Meta-analysis								Meta-analysis
Parameters	Immune		X			X		X		
	Metabolic		X	X		X	X	X	X	

Table 1. Cont.

(B)										
Reference	[72]	[73]	[74]	[75]	[76]	[77]	[84]	[85]	[86]	[87]
Category of Disease	CVD	CVD	CVD	CVD	CVD	CVD	NS	NS	NS	NS
Disease	MI	MI	MI	ICAS	HT	HT	AD	AD	AD	AD
Population	Sicilian	Caucasian	Turkish	Italian	Caucasian	Caucasian	Spanish	Italian	Italian	Iranian
Model	Blood	Blood	Blood	Blood	Blood	Blood	Blood	Blood	Blood	Blood
CCR5Δ32 frequency	Patients	↑ >60 years	↑ ^{a,b}		↑ ^{a,b}					
	No difference Controls	↑	↑ <55 years	X		X	X	X	X	X
CCR5 levels	Patients/test subject/sample									
	No difference Controls									
Genotype	Homozygous CCR5Δ32	X		X	X	X	X			
	Heterozygous CCR5Δ32	X	X	X	X	X	X			
	Absent CCR5Δ32		X ^a							
	Homozygous WT									
Association/ effect of CCR5Δ32	Protective	X	X <55 years							
	No association/effect Risk factor/non-protective		X >60 years	X ^{a,b}	X	X ^{a,b}	X	X ^P	X	X
Assay(s) used	PCR	X	X	X	X	X	X	X	X	X
	RFLP			X		X			X	X
	Sequencing									
	Immune-based									
	Microscopy									
	Spectroscopy									
Parameters	Flow Cytometry									
	Chromatography									
	MS									
Parameters	Other									
	Immune									
	Metabolic			X						

Table 1. *Cont.*

(C)											
Reference	[88]	[90]	[91]	[92]	[93]	[94]	[95]	[96]	[97]	[100]	[101]
Category of Disease	NS	NS	NS	NS	NS	NS	NS	NS	NS	IS	IS
Disease	AD	PD	ms	ms	ms	ms	ms	ms	ms	Asthma	Asthma
Population	N/A	Spanish	Caucasian	Finland	Israeli	Dutch	Australian	Brazilian	Irish	Scottish	Scottish
Model	Mice	Blood	Tissue; Blood	Blood	Blood	Blood; Brain Tissue	Blood	Blood	Blood	Cells	Blood
CCR5Δ32 frequency	Patients No difference Controls	X		↑ ^a		X	X	X	↑ X		
CCR5 levels	Patients/test subject/sample No difference Controls	↓		↓							
Genotype	Homozygous CCR5Δ32		X	X	X						
	Heterozygous CCR5Δ32		X		X			X		X	
	Absent CCR5Δ32							X ^a		X ^{Px}	X ^{Px,ACA}
	Homozygous WT										
Association/ effect of CCR5Δ32	Protective	X				X ^{BC}	X	X ^{O,b}		X	X ^{CA}
	No association/effect Risk factor/non-protective		X ^{SC}	X ^S		X ^S	X ^{SC}	X ^S	X ^{SC}	X ^{SC,P}	
				X ^{C,M}	X ^{SC}				X ^O		
Assay(s) used	PCR	X	X	X	X	X	X	X	X	X	X
	RFLP						X				
	Sequencing										
	Immune-based	X								X	
	Microscopy										
	Spectroscopy										
	Flow Cytometry				X						
	Chromatography										
	MS										
	Other										
Parameters	Immune Metabolic	X									

Table 1. Cont.

(D)												
Reference	[103]	[104]	[105]	[106]	[24]	[110]	[111]	[112]	[113]	[114]	[115]	
Category of Disease	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS	
Disease	Asthma	Asthma	Asthma	T2DM	T2DM	T1DM	T1DM	T1DM	T1DM; T2DM	Asthma; T1DM	RA	
Population	Mixed	Iranian	Polish	Dutch; Swedish	N/A	Italian	Hungarian	British	Estonian	Mixed	Brazilian	
Model	Blood	Blood	Blood	Blood; Urine	Rats; Blood; Tissue	Blood	Blood	Blood	Blood	Meta- Analysis	Blood; Synovial fluid	
CCR5A32 frequency	Patients No difference Controls	X ↑ ^b	X			X	X		X		X	
CCR5 levels	Patients/test subject/sample No difference Controls				↓						↑ CCR5+ cells	
Genotype	Homozygous CCR5A32 Heterozygous CCR5A32 Absent CCR5A32 Homozygous WT		X X ^{Px,a}			X X	X X	X X	X		X ^{Px} X ^a	
Association /effect of CCR5A32	Protective No association/effect Risk factor/non-protective	X	X	X	X ^M	X ^C	X ^S	X	X X ^a	X ^{C,b} X ^S	X ^{T1DM} X ^{Asthma}	X
Assay(s) used	PCR RFLP Sequencing Immune-based Microscopy Spectroscopy Flow Cytometry Chromatography MS Other	X	X	X	X	X X X	X	X	X		X	
Parameters	Immune Metabolic				X X	X				Meta-analysis		

Table 1. Cont.

		(E)									
Reference		[116]	[117]	[118]	[119]	[120]	[122]	[123]	[124]	[126]	[30]
Category of Disease		IS	IS	IS	IS	IS	IS	IS	IS	IS	ID
Disease		RA; JIA	RA	RA; SLE	RA	RA	SLE	SLE; LN	SLE	LN	AIDS
Population		Norwegian	Danish	Caucasian	New Zealand	German	Portuguese	Brazilian	Brazilian	Mixed	Mixed
Model		Blood	Blood	Blood	Blood	Blood	Blood	Blood	Blood	Meta-analysis	Meta-analysis
CCR5Δ32 frequency	Patients				↓ ^b	↑ ^a	↓ ^b	↓ ^a European; ↑ ^a African	↑		↑ ^{a,b} EU
	No difference Controls	X	X	X ^{SLE}						X	
CCR5 levels	Patients/test subject/sample										
	No difference Controls										
Genotype	Homozygous CCR5Δ32	X	X ^C		X	X			X		X
	Heterozygous CCR5Δ32	X	X ^C	X	X	X	X	X	X		X
	Absent CCR5Δ32			X ^{Px (RA),a}	X ^{Px,a}		X ^{Px,a}	X ^{Px,a} (European)			
	Homozygous WT			X							
Association /effect of CCR5Δ32	Protective		X ^C	X ^{RA,a}	X ^a	X ^{S,C}	X	X ^{SLE-European}			X ^{S,P}
	No association/effect Risk factor/non-protective	X		X ^{SLE}	X ^C			X ^{LN African}	X ^C	X ^{S,Caucasian,Asian}	X ^{African}
Assay(s) used	PCR	X	X	X	X	X	X	X	X		
	RFLP										
	Sequencing										
	Immune-based					X			X		
	Microscopy										
	Spectroscopy										
Parameters	Flow Cytometry										
	Chromatography										
	MS										
	Other					X				Meta-analysis	Meta-analysis
	Immune					X					
	Metabolic										

Table 1. *Cont.*

(F)										
Reference	[31]	[33]	[37]	[128]	[129]	[130]	[131]	[132]	[133]	[134]
Category of Disease	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID
Disease	AIDS	AIDS	AIDS	AIDS	AIDS	AIDS	AIDS	AIDS	AIDS	AIDS
Population	Caucasian	Black South African	Caucasian	European descent	Human	Italian	European descent	American	Caucasian	N/A
Model	Cells	Blood	Blood	Cells	Cells	Blood; cells	Blood	Blood	Blood	Blood
CCR5Δ32 frequency	Patients No difference Controls	↓ ^b	X							
CCR5 levels	Patients/test subject/sample No difference Controls		↓			X ^{NH}				
Genotype	Homozygous CCR5Δ32	X		X	X	X	X	X	X	X
	Heterozygous CCR5Δ32			X			X			
	Absent CCR5Δ32 Homozygous WT	X ^{Px,a}								
Association /effect of CCR5Δ32	Protective	X	X	X ^{S,P}	X	X				
	No association/effect Risk factor/non-protective						X	X	X	X
Assay(s) used	PCR	X	X	X	X	X	X	X	X	X
	RFLP									
	Sequencing	X			X		X			X
	Immune-based		X			X		X		
	Microscopy					X				
	Spectroscopy				X					
	Flow Cytometry		X					X		
Parameters	Chromatography									
	MS									
	Other									
Parameters	Immune		X							
	Metabolic									

Table 1. Cont.

(G)											
Reference	[135]	[136]	[137]	[138]	[139]	[144]	[145]	[146]	[148]	[149]	[153]
Category of Disease	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID
Disease	AIDS	AIDS	WNF	WNF	WNF	HBV	HBV	HBV	HBV	HBV/HIV-1	HCV
Population	Caucasian	Caucasian	N/A	Caucasians	American	N/A	Iranian	Iranian	Caucasian	Brazilian	Mixed
Model	Blood	Blood	Mice	Blood; CSF	Meta- analysis	Blood; CD4 ⁺ cells	Blood; NK cells	Blood; CD8 ⁺ cells	Blood	Blood	Blood
CCR5A32 frequency	Patients No difference Controls								↑ Recovery	X	
CCR5 levels	Patients/test subject/sample No difference Controls		↑	↓		↑ ^A	↓	↓			↓ CCR5+cells ↑
Genotype	Homozygous CCR5A32 Heterozygous CCR5A32 Absent CCR5A32 Homozygous WT	X	X		X				X		
Association/ effect of CCR5A32	Protective No association/effect Risk factor/ non-protective		X ^M			X			X ^C	X ^{HBV/HIV} X ^{SHIV}	X ^{Ps} X ^{Ps}
Assay(s) used	PCR RFLP Sequencing Immune-based Microscopy Spectroscopy Flow Cytometry Chromatography MS Other	X X X	X X	X X	X X	X X	X X	X X	X X	X X	X X
Parameters	Immune Metabolic		X			X					X

Table 1. Cont.

(H)										
Reference	[154]	[155]	[156]	[157]	[158]	[161]	[162]	[169]	[170]	[171]
Category of Disease	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID
Disease	HCV	HCV	HCV	HCV	HCV	CD	CD	H1N1	H1N1	H1N1
Population	European	Caucasian	Jewish Israeli	Spanish	British	Mixed	Venezuelan	Mixed	Southern Europe	Brazilian admixed
Model	Tissue	Blood	Blood; Tissue	Blood; Tissue	Blood	Blood	Blood	Blood	Blood	Blood
CCR5Δ32 frequency	Patients No difference Controls	X	X	X	X		X	↑ ^b	↓	X
CCR5 levels	Patients/test subject/sample No difference Controls					↑ Mild disease, ↓ Severe disease				
Genotype	Homozygous CCR5Δ32 Heterozygous CCR5Δ32 Absent CCR5Δ32 Homozygous WT	X		X	X ^{Pa}	X		X	X	X
Association/ effect of CCR5Δ32	Protective No association/effect Risk factor/non-protective	X ^C X ^S	X ^{S,C}	X ^b X ^{S,P}	X ^C	X ^C	X ^C	X	X ^S X ^C	X X
Assay(s) used	PCR RFLP Sequencing Immune-based Microscopy Spectroscopy Flow Cytometry Chromatography MS Other	X	X X	X	X	X		X	X	X
Parameters	Immune Metabolic									

Table 1. *Cont.*

(I)										
Reference	[172]	[175]	[22]	[176]	[177]	[178]	[179]	[180]	[181]	[182]
Category of Disease	ID	Cancer	Cancer	Cancer	Cancer	Cancer	Cancer	Cancer	Cancer	Cancer
Disease	H1N1	General	General	General	BC	BC	BC	Carcinomas	PanC	CC
Population	Brazilian	N/A	Breast; tumour	Breast; tumour	Iranian	Mixed	N/A	Turkish	Mixed	Indian
Model	Tissue; cells	Mice	Cells	Cells	Blood	Meta-analysis	Human cells; Mice	Blood	Blood	Tissue
CCR5Δ32 frequency	Patients No difference Controls	X						† ^b BC		† ^b
CCR5 levels	Patients/ test subject/ sample No difference Controls		↓	↑	↓		↑			
Genotype	Homozygous CCR5Δ32				X					
	Heterozygous CCR5Δ32	X			X			X		X
	Absent CCR5Δ32 Homozygous WT	X	X ^{KO}							X ^{P_{4,6}}
Association/ effect of CCR5Δ32	Protective No association/effect	X ^{S,M}			X ^{O,P}		X			
	Risk factor/ non-protective		X	X		X	X ^S	X ^{Carcinoma}	X ^{BC}	
Assay(s) used	PCR	X	X			X		X	X	X
	RFLP	X							X	
	Sequencing							X		
	Immune-based		X		X			X		
	Microscopy							X		
	Spectroscopy		X	X	X					
	Flow Cytometry		X	X	X			X		
Parameters	Chromatography			X						
	MS			X	X				X	
Parameters	Other					Meta-analysis				
	Immune Metabolic			X	X					

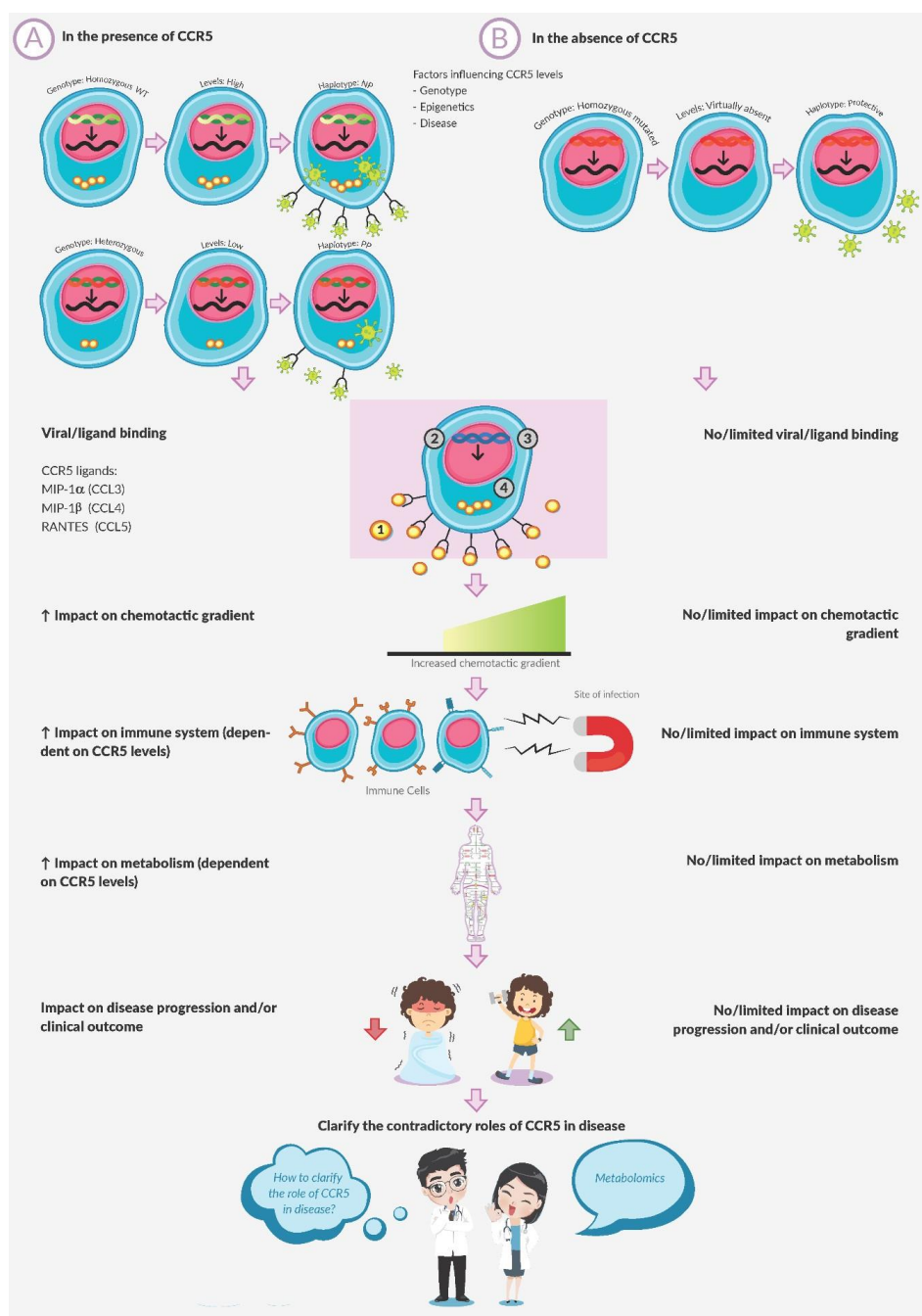
Table 1. *Cont.*

(j)										
Reference	[183]	[184]	[185]	[186]	[187]	[188]	[189]	[190]	[191]	[192]
Category of Disease	Cancer	Cancer	Cancer	Cancer	Cancer	Cancer	Cancer	Cancer	Cancer	Cancer
Disease	CC; HPV	GallC	GasC	GasC	GasC	BIC	PC	PC	PC	AML
Population	Swedish	Indian	Japanese	Polish	Chinese	Turkish	Turkish	Brazilian	Australian	Iranian
Model	Tissue	Blood	Blood	Blood	Tissue; Cell line	Tissue	Blood	Blood	Blood	Blood
CCR5Δ32 frequency	Patients No difference Controls	↑ ^(HIV+)	↑ ^b						X	↑ ^b
CCR5 levels	Patients/test subject/sample No difference Controls			↑		↑				
Genotype	Homozygous CCR5Δ32		X		X				X	
	Heterozygous CCR5Δ32		X		X	X	X	X		
	Absent CCR5Δ32 Homozygous WT						X			X ^{Px}
Association/ effect of CCR5Δ32	Protective									
	No association/effect Risk factor/non-protective	X	X ^S X ^O		X			X	X ^S	X ^S
			X ^C		X	X	X			

Table 1. *Cont.*

Assay(s) used	PCR	X	X		X	X	X	X	X	X
	RFLP		X				X	X	X	
	Sequencing	X								
	Immune-based			X	X	X				
	Microscopy									
	Spectroscopy									
	Flow Cytometry			X						
Parameters	Chromatography									
	MS									
Parameters	Other									
	Immune					X				
Parameters	Metabolic									

Table 1: The contrasting role of CCR5 in various diseases. ↑ increased; ↓ decreased; ^{Mixed} Cohort contains individuals from various ethnicities; ^H Homozygous genotype; ^H heterozygous genotype; ^{Px} patient group; ^S susceptibility; ^O disease onset; ^P disease progression; ^C clinical course/severity of disease; ^M mortality; ^A acute; ^{Ch} chronic; ^{BC} breast cancer; ^{KO} CCR5 knockout mice; ^{NH} not expressed; ^{WT} wild type; ^{CA} childhood asthma; * sum effect, other CCR5 polymorphisms; ^{EU} exposed but uninfected; ^{PC} pancreatic cancer. Abbreviations: PCR—polymerase chain reaction; RFLP—restriction fragment length polymorphism; MS—mass spectrometry; CVD—cardiovascular disease; AS—atherosclerosis; CHD—coronary heart disease; CAD—coronary artery disease; CM—cardiomyopathy; MI—myocardial infarction; ICAS—ischemic carotid artery stenosis; HT—hypertension; NS—nervous system disease; AD—Alzheimer's disease; PD—Parkinson's disease; ms—multiple sclerosis; IS—immune system disease; T2DM—type II diabetes mellitus; T1DM—type I diabetes mellitus; RA—rheumatoid arthritis; JIA—juvenile idiopathic arthritis; SLE—systemic lupus erythematosus; LN—lupus nephritis; IID—infectious disease; AIDS—acquired immunodeficiency syndrome; WNF—West Nile fever; HBV—hepatitis B virus; HCV—hepatitis C virus; CD—Chagas disease; PanC—pancreatic cancer; CC—cervical cancer; GallC—gallbladder cancer; GasC—gastric cancer; PC—prostate cancer; AML—acute myeloid leukemia.



1.

Figure 1. Graphical display of the cascading effect of CCR5 on signal transduction, chemotaxis, immune function and metabolism, all of which ultimately impact disease progression and/or clinical outcome. The cascading effect presents an opportunity to specifically measure the immuno-metabolic changes through metabolomics. Several factors influence CCR5 status. We depict scenarios for when (A) CCR5 is present (high and low levels) and (B) absent. In the presence of CCR5, (1) viral/ligand binding occurs and (2) induces a cell signaling cascade that causes (3) gene activation leading to (4) a protein product and/or cellular response. (B) Lack of viral/ligand binding due to the absence of CCR5 limits the signaling cascade and its subsequent processes. Abbreviations: CCR5 – C-C chemokine receptor 5; CCL3 – C-C chemokine ligand 3; CCL4 – C-C chemokine ligand 4; CCL5 – C-C chemokine ligand 5; MIP1- α – macrophage inflammatory protein one alpha; MIP1- β – macrophage inflammatory protein one beta; NP – non-protective; PP – partially protective; RANTES – regulated upon activation, normal T-cell expressed and secreted; WT – wild type.

3. Conclusions

In this article, the main focus was to review the role of CCR5 particularly in disease since genotype influences the expression and/or the amount of CCR5 present on the cell surface, susceptibility to infection and disease pathogenesis. Taken together, CCR5 is crucial for mediating signal transduction and activating immune responses which ultimately impact the cell's metabolism. Upon reviewing the role of CCR5, frequency of the receptor, genotype and/or levels of CCR5 were frequent terminologies encountered in the literature. While the CCR5 Δ 32 genotype is synonymous with CCR5 levels, this relationship does not hold for allelic frequency. While the role of CCR5 in some diseases such as HIV-1/AIDS is clearer, it is contradictory in others. The different findings may be attributed to various factors such as differences in study design (experimental vs. meta-analysis) as well as sample size. Most often, meta-analysis yielded no association between CCR5 Δ 32 and disease. Given that the information from varied studies are "pooled" as part of a meta-analysis, some of the associations that may exist in individual experiments are possibly overshadowed when reported in this way.

In the case of cardiovascular diseases, central nervous system diseases and cancer for example, the sample matrix and/or model depicts different roles for CCR5. Blood is often the choice of sample since it is acquired easily using minimally invasive techniques. Furthermore, genomic DNA can easily be extracted for genetic analysis. However, while blood was the more common matrix analysed (Table 1), it mostly showed no association to CCR5 while a localised sample such as brain tissue often defined CCR5's protective vs. non-protective roles better. Similarly, protective and non-protective roles were often better characterised in animal (mice) models. Contrasting roles for the receptor were also evident when the samples of different ethnic groups and/or from different geographical regions as well as different genders or age were compared. The type of immune response, number of cells, type of immune cells and biological processes such as internalization all have an impact on CCR5 levels and function and may ultimately influence disease pathogenesis and outcome. Depending on whether an adult or paediatric model was used, the role of CCR5 in the disease was different. With respect to cancer, CCR5 is associated with different outcomes depending on the type of cancer and/or tumour investigated and its associated microenvironment.

From Table 1 it is also evident that most of the assays used to measure CCR5 status and/or its effects comprised of molecular-type assays. The PCR and RFLP methods were the most common methods used. Both methods are easy to do, time-efficient and require low sample volumes relative to sequencing. Immune analysis was limited as was the recording of metabolic parameters. Where immune and metabolic data were recorded, investigators mostly reported on select molecules using older conventional assays.

Variable phenotypic presentation is common in most diseased states (i.e., patients with the same disease often present with different symptoms). This review and the research of others [40,53] highlights the role of CCR5 in various diseases. The increased biological characterisation of CCR5 may however clarify the contrasting roles of this molecule. Gaining knowledge regarding the altered immune and metabolic profiles due to differential CCR5 status of uninfected and infected models will ultimately improve our understanding of the receptor.

Metabolomics is defined as the study of metabolites [193] and offers the ability to link CCR5 homozygous WT, homozygous mutated and/or heterozygous genotypes with phenotype (e.g., CCR5 levels, frequency, etc.). In living organisms, small molecules known as metabolites are continuously chemically transformed in various metabolic pathways. Measuring metabolite concentrations can thus inform on the biochemical activity of cells and its associated phenotype(s) [194]. Using a metabolomics approach, metabolites can be detected in samples displaying differential CCR5 status. These metabolites can be identified and quantified in an unbiased manner in several different biofluids. Indeed, CCR5 has been shown in a CCR5-deficient mouse model during *Toxoplasma gondii* infection to modulate host metabolism (i.e., an increase in serum triglycerides and liver metabolic dysfunction was measured) [23]. In cancer cells, the interaction between CCL5 and CCR5 increased the expression of glucose transporters, glucose uptake, glycolysis and subsequent ATP production [21]. In diabetic mice, blocking of the CCR5

receptor increased LDL and blood triglyceride levels [24]. These CCR5-influenced metabolic changes justify a metabolomics-based investigation of the chemokine receptor.

One of the requirements of metabolomics-based analysis is that a homogenous sample set be analysed so as to make conclusive remarks about the test variable. As part of future work, a metabolomics approach can therefore be used to study those parameters identified in this review to skew CCR5-based findings. By including or excluding these parameters in a metabolomics investigation, CCR5-specific conclusions can be made. Metabolomics makes use of highly sensitive and selective techniques [195]. To date, several different analytical platforms exist, yet there is no one analytical platform which can detect and measure all of the metabolites present within a sample. This is thought to be due to the diversity of the molecules as well as the different concentrations in which they are present. Nonetheless, several metabolomics approaches exist. Untargeted metabolomics approaches investigate the metabolic profile of samples in a global, unbiased manner and the data obtained can give a comprehensive overview of the metabolism. This approach is followed when there is no prior knowledge regarding the metabolites present in the sample and the method is therefore not focused on a specific molecule or class of molecules [196,197]. In contrast, a targeted approach focuses on a specific class of compounds to inform on biochemical pathways of interest. Using any of the aforementioned metabolomics approaches, new insights can be discovered regarding the altered metabolism, as effected through CCR5-induced immune changes.

From the articles reviewed here which report on metabolic parameters [21–24,61–64,66,72,73,103,173], a general trend regarding CCR5 in disease is that it impacts mainly lipid levels, cholesterol and the glycolysis pathway. Future work should therefore analyse samples displaying differential CCR5 status, using untargeted and targeted metabolomics approaches. Untargeted analysis will uncover metabolic changes not previously associated with CCR5 whilst targeted approaches will expand on the affected pathways already known to be impacted by CCR5 and further uncover mechanisms regarding the functioning of the molecule.

The metabolomics approach followed will be guided by the aim of the study which will in turn influence the choice of the biological sample and analytical platform. Sample choice is generally made based on ease of access. Biofluids that can be obtained non-invasively are typically the first choice to use and often includes urine. Furthermore, the use of blood is also a popular choice since its collection is minimally invasive. Muscle or tissue biopsies are generally hard to come by, which makes them less popular for research purposes. In addition to clinical samples which may be scarce, primary cell cultures or established cell lines can also be used for analysis. Cell lines are more homogenous in make-up and response and their environment is more controlled, hence less variation will be built into models used to better characterise CCR5. More conclusive remarks can thus be made regarding the role of CCR5 in the absence and/or presence of disease.

Preparation of the sample will ensure that it is compatible with a specific analytical platform. This may include but is not limited to: adding an internal standard; performing metabolite extraction and derivatization; or adding a buffer and after which the sample is subjected to the analytical platform for metabolomics analysis.

To conclude, chemokines and chemokine receptors such as CCR5 play a pivotal role in health and disease. Mutations present in genes encoding for these protein molecules can impact on immune and metabolic profiles and therefore influence disease susceptibility and progression rates. The metabolic changes can be particularly measured through metabolomics. This cascading impact of CCR5 on various biosystems is summarised in Figure 1. While the role of CCR5 in the context of HIV-1 infection is more defined, its role in other diseases remains contradictory. In an article published by Klein [53], the author also highlights the contrasting role(s) of CCR5 in diseases. However, our knowledge of CCR5 has increased in the last ten years. In addition, technological advancements make it possible to investigate CCR5 in more depth. This review therefore provides a more in-depth explanation for the role(s) of CCR5 in disease, and highlights metabolomics as a tool with which to clarify the contrasting role(s) of CCR5. Metabolic and immune profiling of samples presenting differential CCR5 status stand to further improve our understanding of this molecule.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

CCR5	C-C chemokine receptor 5
kDa	kilodalton
CMI	Cell-mediated immunity
MIP1- α	Macrophage inflammatory protein one alpha
MIP1- β	Macrophage inflammatory protein one beta
RANTES	Regulated upon activation, normal T-cell expressed and secreted
CCL5	C-C chemokine ligand 5
MCP-2	Monocyte chemotactic protein two
Th1	T helper type 1
Th2	T helper type 2
ATP	Adenosine triphosphate
LDL	Low-density lipoprotein
HDL	High-density lipoprotein
$\Delta 32$	32-base pair deletion
bp	Base pairs
WT	Wild type
HIV-1	Human immunodeficiency virus
AIDS	Acquired immunodeficiency syndrome
CCR5 ⁺	CCR5-positive
CRISPR	Clustered regularly interspaced short palindromic repeats
DNA	Deoxyribonucleic acid
UK	United Kingdom
CVD	Cardiovascular disease
MCP-1	Monocyte chemoattractant protein-1
CAD	Coronary artery disease
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
PCR	Polymerase chain reaction
HDL-C	High-density lipoprotein cholesterol
TG	Triglycerides
LDL-C	Low-density lipoprotein cholesterol
RFLP	Restriction fragment length polymorphism
MI	Myocardial infarction
ICAS	Ischemic carotid artery stenosis
AD	Alzheimer's disease
PD	Parkinson's disease
ms	Multiple sclerosis
RT-PCR	Reverse-transcription polymerase chain reaction
LOAD	Late-onset Alzheimer's disease
mRNA	Messenger ribonucleic acid
IgE	Immunoglobulin E

T2DM	Type 2 diabetes mellitus
HbA1c	Haemoglobin A1c
T1DM	Type 1 diabetes mellitus
RA	Rheumatoid arthritis
RF	Rheumatoid factor
JIA	Juvenile idiopathic arthritis
SLE	Systemic lupus erythematosus
LN	Lupus nephritis
SDF-1	Stromal cell-derived factor one
WNV	West Nile virus
WNF	West Nile fever
HBV	Hepatitis B virus
NK	Natural killer
HCV	Hepatitis C virus
CCR5	CCR5-negative
HPV	Human papillomavirus
HPV+	Human papillomavirus-positive
AML	Acute myeloid leukaemia
MS	Mass spectrometry
AS	Atherosclerosis
CHD	Coronary heart disease
CM	Cardiomyopathy
HT	Hypertension
NS	Nervous system
IS	Immune system
ID	Infectious disease
CD	Chagas disease
BC	Breast cancer
PanC	Pancreatic cancer
CC	Cervical cancer
GallC	Gallbladder cancer
GasC	Gastric cancer
PC	Prostate cancer
CCL3	C-C chemokine ligand 3
CCL4	C-C chemokine ligand 4
NP	Non-protective
PP	Partially protective

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