An *in vitro* study on the physiological actions of *Carbolicum acidum* 5CH, 9CH, 15CH, 30CH, 200CH on granulocytes and T-cells chemical mediators in cellular allergic responses.

BY

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DECLARATION

This is to certify that the work is entirely my own and not of any other person, unless explicitly acknowledged (including citation of published and unpublished sources). The work has not previously been submitted in any form to the Durban University of Technology or to any other institution for assessment or for any other purpose.

21 October 2025

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DEDICATION

To my mother, Veronica Mzimela, and siblings, Nhlanhla, Sabelo “Poni”, and Mpilonhle “Boy” Mzimela.

A special dedication to my father, Vusumuzi Collen Mzimela. Thank you for giving us the best chance at life. May you continue to rest in peace. Mnguni. Lwandle!

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ABSTRACT

With allergic diseases on the increase globally, alternative and complementary methods of treatment need to be investigated for the management of allergic conditions. Homoeopathy is an alternative system of medicine that works by stimulating the body's vital force, which is the unique energy of each individual, to cure illnesses.

This study investigated the physiological effects of a homoeopathic remedy, *Carbolicum acidum* 5CH, 9CH, 15CH, 30CH, and 200CH, on chemical mediators associated with allergic reactions by conducting an *in vitro* experimental study using granulocytes and T-cells isolated from human whole blood. The study was based on the hypothesis that higher potencies will have inhibitory effects on the expression of allergic chemical mediators, while lower potencies will have a stimulatory effect. This hypothesis was founded on the homoeopathic principle of infinitesimals which indicates that the higher the homoeopathic potency, the greater the curative power of the remedy . This study further observed the concept of hormesis, which states that the effects of an agent on biological systems will have stimulatory functions in low doses, whereas higher doses are inhibitory, thereby showing a dose-response relationship.

To evaluate the effects of this homoeopathic anti-allergy remedy, human whole blood was aliquoted with potencies of *Carbolicum acidum*. The cell suspensions were then stimulated with a BD leukocyte activation cocktail, which induced the activation of cells and chemical mediator release. The levels of chemical mediator release were quantified by measuring the upregulation of activation markers on the cell surfaces of granulocytes and T-cells using a BD FACSymphony A5 flow cytometer. The activation markers that were investigated included HLA-DR, CD203c, CD63, and CD69. The effects of treatment on the expression of these markers were also investigated using flow cytometric analysis.

The key findings of this study, first of its kind in South Africa, demonstrated that *Carbolicum acidum* had significant effects on the expression of activation markers HLA-DR ($p = 0.005$), CD203c/CD63 ($p < 0.001$), highlighting its physiological effect in modulating chemical mediator release.

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DEFINITION OF TERMS

Anaphylaxis: Severe allergic reaction characterised by symptoms of an immediate-type reaction response that can affect the entire organism and may cause death (Ring *et al.* 2021).

Chemical mediators: Class of small proteins that are produced by various cell types in response to stimuli, and these proteins are involved in mediating and regulating immune response (Appel and Jonsson, 2016).

Conventional medicine: System of medicine, also known as Western medicine, where diseases and symptoms are treated with drugs by medical practitioners and other healthcare professionals (National Cancer Institute, 2024).

Homoeopathy: complementary system of medicine that treats diseases based on the principle that “like cures like.” This is achieved by using pharmaceutical substances that when administered to healthy volunteers, produces the similar symptoms as those of the disease in question (Vithoulkas, 1979).

Potentisation: Process of diluting and succussion (shaking) of a substance to make it more potent, thereby increasing the medicinal power of the substance (Dubey and Dandwani, 2023; Sagar, 2007).

LIST OF ABBREVIATIONS

μl	Microliter
AHPCSA	Allied Health Professions Council of South Africa
AIDS	Acquired Immunodeficiency Syndrome
ANOVA	Analysis of Variance
BB	Brilliant blue
BSL-2	Biosafety Level 2
BUV	Brilliant Ultraviolet
BV	Brilliant violet
CD	Cluster of Differentiation
CH	Hahnemannian Centesimal Scale
CO2	Carbon Dioxide
DMSO	Dimethylsulfoxide
DUT	Durban University of Technology
EDTA	Ethylenediaminetetraacetic acid
FITC	Fluorescein Isothiocyanate
FMO	Fluorescence Minus One
FSC	Forward Scatter
HBV	Hepatitis B virus
HIV	Human Immunodeficiency Virus
HSD	Honestly Significant Difference (post hoc test)
IgE	Immunoglobulin E
LM	Millesimal Scale
MFI	Median Fluorescence Intensity
ml	Millilitre
PBMC	Peripheral Blood Mononuclear Cell
PMN	Polymorphonuclear leukocyte
SANBS	South African National Blood Services
SSC	Side Scatter
TPHA	Treponema Pallidum Hemagglutination Assay
WBCs	White Blood Cells
X	Decimal Scale

CHAPTER ONE

INTRODUCTION

1.1 Introduction to the study

Allergic diseases are an increasing cause of illnesses that affect about 20% of the global population at some time (Ralston *et al.* 2018; Dierick *et al.* 2020). Evidence shows that allergic diseases are very common and represent a notable burden of morbidity and health costs (Gupta *et al.* cited in Chippendale, 2018). A severe allergic reaction, also known as anaphylaxis, is a generalised multisystemic hypersensitivity reaction that is life-threatening (Sicherer and Simons, 2017). Allergic reactions are primarily triggered by chemical mediators from mast cells and basophils that depend on immunoglobulin E (IgE) (Simons *et al.* 2014; Barone and Castro, 2016). IgE is a plasma protein that is made by the plasma cells after they have been stimulated by an antigen (Kelly and Grayson, 2016). While IgE is mainly found on basophils and mast cells, Kim, Mayumi, and Mikawa (2012) reported that eosinophils and lymphocytes (T-cells and B-cells) also express IgE receptors and play a role in the development of allergic disorders by activating the cells and releasing active chemical mediators. These chemical mediators induce physiological responses and anatomical changes that are reported as allergic symptoms (Gauvreau, El-Gammal, and O'Byrne, 2015). Fatal anaphylactic reactions have been reported in countries including the United Kingdom, where approximately 8 deaths per 100 000 population were noted annually from anaphylaxis (Conrado *et al.* 2021). While in the United States of America (USA), an account of 500 deaths from anaphylactic reactions annually has been reported (Tanno *et al.* 2017). Furthermore, there have been reports of 200 000 annual emergency visits for anaphylaxis in the USA (Clark *et al.* 2011).

There is little information regarding anaphylaxis in Africa, as there are no register-based studies on severe anaphylactic reactions that have been reported (Chippendale, 2018). Gray (2017) indicated that the prevalence of severe allergic reactions in South Africa was still being studied. Notably, the World Allergy Organisation (2021) conducted an informal survey regarding the occurrence and

management of anaphylaxis with specialists from different countries and reported that South Africa could not respond to the survey due to a lack of data. Also, a recent report on paediatric anaphylaxis in South Africa showed that there was still a remarkable lack of data regarding anaphylaxis from the African continent (Chippendale *et al.* 2022). Despite the lack of data on the prevalence of allergic diseases in South Africa, the Allergy Foundation of South Africa (2018) indicated that allergies were on the increase worldwide, where 40% of the sufferers were children. Furthermore, Gupta *et al.* (2013) reported that childhood allergies contributed to significant medical costs for the healthcare system in the USA, estimated at around 24.8 billion dollars per annum. This was viewed as may mean that a similar trend may be observed in South Africa regarding the prevalence of allergic diseases and their associated medical costs if it were to be studied.

1.2 Problem statement

Conventional therapies against allergies are available in South Africa; however, they are not widely accessible due to limited healthcare resources (Richards *et al.* 2023) as well as cost constraints (Levin, 2012). The indicated conventional therapy for anaphylaxis is epinephrine; however, it has been reported that epinephrine auto-injectors are expensive (Levin, 2012). Moreover, even in curative doses, the administration of epinephrine has been observed to cause pharmacologic side effects including restlessness, palpitations, tremors, and anxiety in people of all ages (Levis, Ford, and Kuo, 2011). In some cases, more severe complications have been observed, with the reports indicating that epinephrine may result in various adverse effects including angina, ventricular dysrhythmias, myocardial infarction, and rapid increase in blood pressure (Levis, Ford, and Kuo, 2011). A study by Dami *et al.* (2022) showed that patients, and even healthcare practitioners, were reluctant to inject epinephrine in cases of allergic reaction; instead, they favoured the use of antihistamine drugs, which are not the recommended frontline treatment regimens for severe allergic reactions.

In addition, the available treatment guidelines for allergic diseases are usually written for upper-income countries, and most of the data on allergies comes from such countries (Mbugi and Chilongola, 2010); therefore, these treatment guidelines cannot

apply universally to most countries such as in Africa, where priority is designated to infectious diseases such as Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS) and Tuberculosis (TB), thus contributing to the inaccessibility of conventional therapy for allergies.

Due to the reported continual global increase of allergic diseases (Gutowska-Slesik, 2023), this prompts for alternative therapies including homoeopathy to be considered as possible modalities for the treatment of allergic diseases. Homoeopathy is reported to have benefits that include providing holistic treatment, use of natural remedies that have minimal adverse effects, and providing medication that is inexpensive and readily available (Majola, 2020). The availability and low cost of homoeopathic remedies may potentially mitigate the financial burden associated with managing allergic diseases, particularly those relating to cost and accessibility within the public health system. A pilot study by the AIDS Remedy Fund in Tanzania (ARF cited in Majola, 2020) also indicated that homoeopathic treatment that was provided through this study offered advantages that included the low cost of remedies, ease of administration, absence of resistance, and side effects. Further to this, homoeopathy needs to be considered as a treatment modality for allergic reactions within the broader context of public health management because research has shown that a substantial number of people in South Africa opt for complementary medicine for their primary healthcare needs (Ericksen-Pereira, Roman, and Swart, 2018).

1.3 Rationale of the study

As allergic reactions are on the increase, this study intended to contribute to public health by exploring an alternative treatment regimen for allergic diseases. This treatment was expected to be beneficial in cases where patients may experience adverse reactions from allopathic first-line treatment for allergies (Levis, Ford, and Kuo, 2011), or they may prefer to use a homoeopathic anti-allergy remedy.

The current study aimed to investigate the physiological effects of *Carbolicum acidum* 5CH, 9CH, 15CH, 30CH, and 200CH on granulocytes and T-cells chemical mediators in cellular allergic responses. The therapeutic indications of *Carbolicum acidum* have only been verified through homoeopathic provings and clinical observations in clinical

practice (Morrison, 2006; Paschmanns, 2013). In one such case report, Paschmanns (2013) documented treating successfully severe allergic reactions to an insect sting in clinical practice using *Carbolicum acidum* in potency M (1000CH), which was followed up with 200CH potency a year later for allergic rhinitis. Currently, there is a lack of published research data regarding the effects of *Carbolicum acidum* on the inflammatory mediators associated with allergic reactions. Which is what this current study sought to investigate.

This study was based on the hypothesis that higher potencies of *Carbolicum acidum* (15CH, 30CH, 200CH) will have an inhibitory effect on the chemical mediators involved in allergic reactions, while lower potencies (5CH, 9CH) will have a stimulatory effect. This was founded on the homoeopathic principle of infinitesimals, which says that a remedy's curative power increases with its dilution (Drofenik, 2022; Sagar, 2007).

Furthermore, while observing the law of infinitesimals, the current study aimed to investigate whether 5CH, 9CH, 15CH, 30CH, and 200CH potencies of *Carbolicum acidum* would exert varying effects on allergic chemical mediators in line with the concept of hormesis, which states that low doses of stimuli on biological systems can have stimulatory effects, and in higher doses can have inhibitory effects (Calabrese and Giordano, 2021; Henschler, 2006; Calabrese and Baldwin, 2001). The findings of this study were thus expected to contribute to cell culture research in homoeopathy.

1.4 Significance of the study

In vitro studies provide valuable insights into how various substances may influence specific cellular characteristics (Zeni and Scarfi, 2012; Romeo *et al.* 2022). While the prevalence was not part of the study objectives, this *in vitro* study was viewed as the first flow cytometric study to look at the effects of a homoeopathic remedy on cells involved in allergic pathology in South Africa, providing an insight into how immune cells respond to a remedy in a controlled, isolated environment.

Ethical considerations have highlighted the need to reduce the requirements of animal testing used to evaluate the pharmacological action of chemicals and drugs, especially in immunological studies where participants may be at risk of death from anaphylactic

reactions (Pourahmad and Salimi, 2015). Therefore, this study sought to conduct a safer scientific *in vitro* study using biotechnological methods to observe the response of cells to a homoeopathic remedy at the cellular level. The expected benefit of conducting this *in vitro* cellular study was that, ethically, it posed no risk to humans, animals, and the environment.

This *in vitro* study will also contribute to the body of knowledge of human cell culture research in homoeopathy, as research has shown that there is a lack of substantial scientific laboratory evidence on the effectiveness of homoeopathic medicines (Warner, 2021). Therefore, based on the findings of the current study, future in vivo and human studies could be developed to explore this phenomenon further.

1.5 Aim and Objectives

1.5.1 Aim of the study

The aim of this study was to evaluate the effects of the homoeopathic remedy *Carbolicum acidum* in potencies 5CH, 9CH, 15CH, 30CH, and 200CH on human granulocytes and T-cells in an *in vitro* study.

1.5.2 Objectives

1. To investigate the anti-allergy action of homoeopathic *Carbolicum acidum* potency of 5CH on human granulocytes and T-cells in an *in vitro* study.
2. To investigate the anti-allergy action of homoeopathic *Carbolicum acidum* potency of 9CH on human granulocytes and T-cells in an *in vitro* study.
3. To investigate the anti-allergy action of homoeopathic *Carbolicum acidum* potency of 15CH on human granulocytes and T-cells in an *in vitro* study.
4. To investigate the anti-allergy action of homoeopathic *Carbolicum acidum* potency of 30CH on human granulocytes and T-cells in an *in vitro* study.
5. To investigate the anti-allergy action of homoeopathic *Carbolicum acidum* potency of 200CH on human granulocytes and T-cells in an *in vitro* study.
6. To compare the action of low (5CH, 9CH) and high (15CH, 30CH, 200CH) dilutions of homoeopathic *Carbolicum acidum* potencies against each other on allergy induced human granulocytes and T-cells.

1.6 Outline of dissertation

The dissertation consists of Chapter 1 which covers the introduction to the study, the problem statement, the rationale of the study, as well as the aim and objectives. In Chapter 2 the literature that was reviewed was IgE-dependant allergic diseases, looking at the epidemiology of allergic diseases and cellular allergic reactions. Background on homoeopathy and homoeopathic medicines will be reviewed, including the homoeopathic remedy of this study, *Carbolicum acidum*. Cell culture, flow cytometry (measurement tool), and cellular studies in homoeopathy will be reviewed. Lastly, this chapter will also highlight the conceptual framework of this study. Chapter 3 will address the methodology and materials that were used in this study. Chapter 4 will present the study results and analysis of data. Chapter 5 will discuss the results of the study, and Chapter 6 will consist of the conclusion of the study and recommendations for further research.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

A comprehensive literature search was conducted to identify relevant studies on allergic diseases in Africa, with a specific focus on South Africa, as well as allergic diseases in the context of homoeopathy. Literature search was also conducted on in vitro studies related to homoeopathy, and research involving *Carbolicum acidum*. The search was carried out using multiple online databases, including African Journals, CINAHL Complete, HOMBREX, HOMIS, CAM-QUEST, Cochrane Library, Elsevier Science Direct, and PubMed. Additional searches were done in the Durban University of Technology and the University of Johannesburg's repositories. A combination of targeted keywords and Boolean operators was used to optimise the retrieval of focused literature, namely: allergy, allergic diseases, Africa, South Africa, homoeopathy, homeopathy, epidemiology, *Carbolicum acidum*, and in vitro. Other articles that were discovered through reference lists were also reviewed based on their titles to be included in this literature review.

2.2 Allergic diseases epidemiology

Allergic diseases are an increasingly common cause of illnesses that have contributed to a global increase in hospitalisations for anaphylaxis in the United Kingdom, Europe, the United States of America, Australia, and New Zealand (Turner *et al.* 2020), affecting between 15% and 20% of the global population at a given time (Ralston et al, 2018; Dierick *et al.* 2020). Evidence shows that allergic diseases are very common and affect both men and women; thus, they represent a notable burden in terms of morbidity and healthcare costs (Gupta *et al.* cited in Chippendale, 2018). In the United States of America, patients report to the emergency department every three minutes for food allergic reactions and every six minutes for anaphylaxis; this has resulted in 200 000 emergency visits yearly in the United States of America (Clark *et al.* 2011). Anaphylaxis is a severe, generalised multisystemic hypersensitivity reaction that may be life-threatening (Sicherer and Simons, 2017).

The Allergy Foundation of South Africa (2018) stated that allergies were on the increase worldwide from lower- to upper-income countries, where 40% of the sufferers are children. A cohort study of severe allergic reactions that was carried out by Chippendale (2018) at the Red Cross War Memorial Children's Hospital in Cape Town, South Africa, between January 2014 and August 2016 showed that males and young children were more frequently affected by severe allergic reactions. Kim *et al.* (2012) showed that younger children were mostly affected because the increased percentage of IgE receptors was easily detected in younger children compared to older children and adults. Furthermore, the literature presents that the percentage of IgE receptors on lymphocytes was shown to be high in patients with atopy and allergic rhinitis (Spiegelberg and Simon, cited in Harris, 2012).

The World Allergy Organization (2021) conducted an informal survey on the occurrence and management of anaphylaxis among experts in different countries. South Africa was unable to respond due to a lack of information, as there were only nine allergy specialists in South Africa for a population of approximately 43 million people (World Allergy Organization, 2021). Gray (2017) showed that the prevalence of severe allergic reactions in South Africa was still being studied, and a recent study on paediatric anaphylaxis in South Africa showed that there is still a remarkable lack of data regarding anaphylaxis from the African continent (Chippendale *et al.* 2022). It has also been reported that childhood allergies have resulted in a significant medical cost for the healthcare system, and even larger for the families that had children that suffered from allergic conditions (Gupta *et al.* 2013). It was estimated that the overall cost was 24.8 billion dollars per annum in direct and indirect medical costs in America. Therefore, allergic diseases represent a significant burden of medical costs (Gupta *et al.* cited in Chippendale, 2018).

2.3 Cellular allergic reactions

Anaphylaxis is a severe allergic reaction that is characterised by manifestations of an immediate-type response that can affect the entire body and may cause death (Ring *et al.* 2021), whereas chemical mediators refer to a class of small proteins that are produced by various cell types in response to a stimulus, and these proteins play a

crucial role in mediating and regulating immune responses and inflammatory reactions (Appel and Jonsson, 2016).

The anaphylactic reactions are generally caused by immunoglobulin E (IgE) dependent immunological mediators from mast cells and basophils (Simons *et al.* 2014; Barone and Castro, 2016). IgE is a plasma protein that is made by the plasma cells after they have been stimulated by an antigen. Once IgE has been produced, it is released into the blood (Kelly and Grayson, 2016), and it comprises less than 0.1% of other plasma proteins in blood; it is found predominantly tightly bound on the surfaces of mast cells and basophils during allergic reactions (Tortora and Derrickson, 2015). Although IgE is predominantly expressed on basophils and mast cells, Kim, Mayumi, and Mikawa (2012) reported that eosinophils and lymphocytes (T-cells and B-cells) also have low-affinity IgE receptors expressed on their surfaces and that they are also involved in the development of allergic disorders by participating in the activation of cells and the release of active chemical mediators.

Basophils and mast cells store their chemical mediators in membrane-bound vesicles and release them by exocytosis given an appropriate stimulus (Dvorak, 2013). Degranulation occurs when the release of chemical mediators is triggered from the basophils and mast cells. This happens when the IgE antibody that is bound to an antigen cross-links with the IgE receptors of mast cells and basophils. Dvorak (2013) also termed this process as ‘anaphylactic degranulation’. The chemical mediators that are released include histamine and heparin (Barone and Castro, 2016). Histamine is the major preformed mediator in both basophils and mast cells (Dvorak, 2013).

Eosinophils are primarily known to increase in allergic inflammation and play a role in the development of allergic diseases (Bui *et al.* 2024; Davoine and Lacy, 2014). This process is mostly controlled by a network of chemical mediators from T-cells that regulate IgE responses (Davoine and Lacy, 2014). Upon activation, eosinophils undergo degranulation, releasing a range of mediators including various cytokines and major basic proteins. These mediators of allergic reactions have been shown to contribute to the stimulation of eosinophil degranulation (Davoine and Lacy, 2014).

T-cells are also involved in allergic reactions; earlier research demonstrates that T-cells produce Th2-type cytokines (interleukins -3, -4, -5, -6, -10) and granulocytes/macrophage colony-stimulating factor (Robinson, 1992 and Robinson, 1993 cited in Kallinich *et al.* 2007) which account for the allergic reactions, including recruitment of eosinophils, production of IgE, and activation of mast cells (Kallinich *et al.* 2007).

The release of chemical mediators from granulocytes, particularly histamine, induces the physiological responses and anatomical changes that are reported as allergic symptoms. The physiological responses include increased vascular permeability, contraction of smooth muscle, airway mucus production, and gastric secretions (Gauvreau, El-Gammal, and O'Byrne, 2015). The effects that are caused by histamine may be systemic when the body undergoes anaphylaxis (for example, from drugs or a bee sting) or may be localised on the gastrointestinal tract, skin, or bronchioles as seen in food allergies, atopy, and asthma (Barone and Castro, 2016). Allergic diseases may therefore be defined as the clinical manifestation of this inappropriate immune response (Ralston *et al.* 2018). The most common clinical presentations of anaphylaxis include urticaria, swelling of lips and tongue (angioedema), erythema, and itching (Webb and Lieberman, cited in Fischer *et al.* 2018). The common triggers for allergic reactions are food, drugs, insect stings, and latex (Campbell *et al.* 2015). Death from anaphylaxis occurs because of cardiovascular shock or obstruction of the respiratory system, or both (Fischer *et al.* 2018).

2.4 Treatment of severe allergic reactions

The only first-line allopathic treatment for severe allergic reactions is epinephrine, and a delayed administration has been associated with fatal anaphylaxis and increased risk of hospitalisation (Sampson cited in Lee *et al.* 2018). The use of epinephrine is widely accepted globally, including in South Africa's primary healthcare settings and hospitals (Levin, 2012). Epinephrine is, however, underused in emergency departments for anaphylaxis due to frequent misdiagnosis and fear of epinephrine side effects (Asai *et al.* 2014), and the supporting evidence for its use is lacking; the meta-analysis of epinephrine studies notes that such research has not been conducted (Levin, 2012).

The administration of epinephrine to any person, even at curative doses, can lead to pharmacological side effects such as restlessness, palpitations, tremors, and anxiety (Levis, Ford, and Kuo, 2011). In some cases, more severe complications have been observed with the reports indicating that epinephrine may result in various adverse effects, including angina, ventricular dysrhythmias, myocardial infarction, and rapid increase in blood pressure (Levis, Ford, and Kuo, 2011). Therefore, healthcare practitioners approach the use of epinephrine with caution (Prince, Mikhail, and Stukus, 2018). Additionally, a recent study also showed that patients, and even healthcare practitioners, were reluctant to inject epinephrine in cases of allergic reactions; instead, they favoured the use of antihistamine drugs even though they are not the recommended frontline treatment for allergic reactions (Dami *et al.* 2022). Fear of harm and being unsure if epinephrine was necessary were some of the factors that contributed to the underuse of epinephrine.

Epinephrine often comes in the form of an auto-injector; however, it also comes in pre-filled syringes that require training on how to use them. The pre-filled syringes have been associated with handling and dosing errors (Prince, Mikhail, and Stukus, 2018); this was evident in experimental settings that did not involve anaphylaxis, where parents tended to be slower at preparing epinephrine with a needle and syringe, resulting in a dose that was often inaccurate, varying as much as 40 times from the intended amount (Simons *et al.* 2001). Pre-filled syringes have also been shown to be sensitive to temperature, and they need to be replaced every 2-3 months (Prince, Mikhail, and Stukus, 2018); this causes a financial burden of medical costs to people who require it.

Turner *et al.* (2020) reported that many episodes of severe allergic reactions happen in the community without appearing at a medical facility and that most countries have not developed reliable systems to monitor anaphylactic events. Experience also reveals that few patients, if any, may report allergies to medical practitioners unless the consequences were severe, particularly in rural Africa (Mbugi and Chilongola, 2010). In South Africa, these challenges can be attributed to the lack of information about allergic conditions (Chippendale *et al.* 2022; Levin, 2012) and financial limitations (Levin, 2012). All the medication surveyed by the World Allergy

Organization can be found in South Africa; however, the cost constraints hinder access to essential epinephrine auto-injector therapy (Levin, 2012).

2.5 Homoeopathic Treatment

Considering the mentioned challenges with epinephrine, homoeopathic intervention may be of importance in providing an alternative treatment plan for allergies because it has been shown that homoeopathic remedies cost less to use, can be easily administered, and are more readily available (Roberts cited in Majola, 2020).

Homoeopathy has been shown to be an effective system of medicine (Majola, 2020). The AIDS Remedy Fund (ARF) is an initiative that has assisted individuals living with AIDS in Tanzania, Kenya, with a reported positive response rate of over 90% among patients. The ARF Foundation aims to conduct research on the efficacy of homoeopathic therapy for HIV/AIDS and provide a remedy at an affordable price to large populations. One significant outcome of this pilot study was that more than 90% of patients showed improvement in their overall health, recovering from opportunistic infections, disappearance of diarrhoea, respiratory infections, and skin problems. The homoeopathic treatment provided through this initiative offered advantages that include low cost of remedies, ease of administration, absence of resistance and side effects, and restoration of independence for patients (Majola, 2020).

Similar initiatives exist in South Africa; an example includes the community health centres in the greater areas of Durban as part of the community outreach programmes run by the Durban University of Technology (DUT Community Engagement, 2024), offering free homoeopathic consultations and treatment to the communities it serves. Previous students have evaluated some of these clinics, and their findings consistently demonstrated positive outcomes (Majola, 2020). Therefore, this current study sought to offer an alternative homoeopathic regimen for allergic reactions that would complement conventional medicine in addressing challenging issues experienced with the use of epinephrine, as previously stated.

2.6 Background on Homoeopathy

Homoeopathy is a therapeutic system of medicine that works by stimulating the body's vital force to cure illnesses. It was founded by the German doctor Samuel Christian F. Hahnemann (Vithoulkas, 1979). The word homoeopathy contains two Greek words: "omios" meaning "similar" and "pathos" meaning "disease" (Mukherjee *et al.* 2016). Therefore, this therapeutic system of treating diseases is based on the principle that "like cures like." This is achieved using pharmaceutical substances that when administered to healthy volunteers, it produces similar symptoms as those of the disease in question (Vithoulkas, 1979).

Homoeopathy is legally practised in South Africa, and it is regulated by the Allied Health Professions Council of South Africa (AHPCSA). Thus, homoeopathic practitioners function as primary healthcare providers in the private sector in South Africa. As such, literature shows that there is a substantial number of people who opt for using complementary and alternative medicine in South Africa (Ericksen-Pereira, Roman, and Swart, 2018). As there is an increasing number of people who rely on alternative medicine for their primary healthcare needs in Sub-Saharan Africa (James *et al.* 2018) and with allergic reactions on the increase, especially in paediatric cases, more research on this phenomenon needs to be explored.

2.7 Homoeopathic medicines

2.7.1 Sources of homoeopathic medicines

Homoeopathic medicines are prepared from natural substances (Rehman and Ahmad, 2017). These natural substances come from the three major sources referred to as kingdoms in homoeopathy: the plant, animal, and mineral kingdoms. Other additional sources of homoeopathic medicines include a) sarcodes, which are made from healthy tissue; b) nosodes, which are made from diseased tissue; and c) imponderables, made from intangible materials (Rehman and Ahmad, 2017). There is another class of medicine found in homoeopathy called tautopathy that deals with removing the side effects of other medications; here, the same medicinal drug is utilised to prepare the homoeopathic medicine through a process of potentisation (Banerjee, 2006). Potentisation is the process of diluting and succussing (shaking) a

substance to make it more potent, thereby increasing the medicinal power of the substance (Dubey and Dandwani, 2023; Sagar, 2007).

2.7.2 Preparation of homoeopathic medicines

Homoeopathic medicines are prepared according to the homoeopathic pharmacopoeia, an authoritative book that is officially published by the government or authority of any country that contains rules, regulations, and standards on the preparation of drugs. The pharmacopoeia explicitly provides the source material of each drug and specifies the exact method of extraction from the source material, their preservation, and standards that determine their strength and purity (Lipinski *et al.* 2014). Countries that do not have an official pharmacopoeia adopt those of other countries for standard reference (Das, 2016), and South Africa is one of those countries that does not have an official pharmacopoeia; as such, South Africa primarily uses the German Homoeopathic Pharmacopoeia and the United States Homoeopathic Pharmacopoeia.

The preparation of homoeopathic medicines involves the preparation of mother tincture, which is an extract that is prepared directly from the corresponding source of raw material, as an initial step. Then the preparation of further potencies from the mother tincture begins by following a series of dilutions and succussions. The homoeopathic potency is the scale of the degree of how much a remedy has been potentised, that when given in minimum dosage at specified intervals, depending on the nature of the disease and susceptibility of the patient, would cure sickness (Rehman and Ahmad, 2017).

2.7.3 Potency scales of homoeopathic medicines

There are two potency scales that are commonly used in homoeopathy: the decimal scale that is diluted in a 1:10 ratio (X) and the centesimal scale that is diluted in a 1:100 ratio (CH). Hahnemann also introduced a third scale, the 50 millesimal scale, 1:50 000 ratio (LM) (Mondal, 2006 cited in Rehman and Ahmad, 2017). A centesimal scale (CH) of potencies was used in this present study.

2.8 Carbolicum acidum

2.8.1 Chemistry

Carbolic acid was discovered by a German chemist, Runge, in 1834. It was first used in medicine as an antiseptic, and it was also used as a topical anaesthetic, seeing it used in surgery, which resulted in accidental poisoning of patients and doctors. These accidental experiences with carbolic acid provided symptoms that later indicated its medicinal use in homoeopathy. To date, carbolic acid is still added to many medications and vaccines as a preservative (Murphy, 2000; Morrison, 2006).

2.8.2 Proving

Homoeopathic drug proving, also known as homoeopathic pathogenetic trials, is a double-blind clinical trial of testing and establishing the efficacy of homoeopathic remedies on healthy individuals (volunteers) commonly referred to as provers (Hahnemann, 2004). The prepared remedy under study produces mental, emotional, and physical symptoms that can be observed in disease when given to healthy participants. The provers then record a report of the symptoms that they experienced while on the remedy. All the symptoms that arise due to the remedy under study can be reversed with an antidote remedy (Rehman and Ahmad, 2017).

Carbolicum acidum was first proved by Dr Hoyne in 1869; he proved it up to the 30th CH potency (Morrison, 2006).

2.8.3 Homoeopathic indication of *Carbolicum acidum*

Carbolicum acidum has especially been used in conditions that are associated with shock and severe collapse, sinusitis, and other nasal conditions. Clinically, it is strongly indicated for allergic reactions, anaphylactic shock, and bee stings (Murphy, 2006). It is a potent irritant, antiseptic, and anaesthetic. It acts primarily on the central nervous system. It affects the mucous membranes, heart, blood, and respiration (Morrison, 2006; Phatak, 1999). It is the only high-grading remedy in the Synthesis repertorium (homoeopathic repertory) that is indicated for anaphylaxis (Schroyens, 2016).

Morrison (2006) showed that James Tyler Kent specifically recommended the use of *Carbolicum acidum* in anaphylactic reactions following bee stings because this remedy has many allergic symptoms in its picture. There is also great sensitivity to chemicals, fumes, and perfumes. This remedy has also been mentioned by many authors in the treatment of chemical hypersensitivity syndrome (Morrison, 2006), a multisystem disease that is caused by exposure to subtoxic doses (concentrations that are below those that are considered toxic to most people) of environmental contaminants such as chemicals, solvents, organophosphates, fumes, and heavy metals (Rossi and Pitidis, 2018). Furthermore, Klein (2003) noted that *Carbolicum acidum* should be the primary remedy to consider for severe allergic reactions resulting in anaphylactic shock. *Carbolicum acidum* also contributes to the treatment of both chronic and acute conditions (Klein, 2003).

The therapeutic indications of this homoeopathic medicine have only been predominantly verified through homoeopathic clinical observations in clinical practice and through homoeopathic provings. No research data has been published to date on the effects of *Carbolicum acidum* on the inflammatory mediators associated with severe allergic reactions.

2.9 Cell culture

The cells that were used in this study were peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leukocytes (PMNs). PBMCs include T-cells, B-cells, natural killer cells, and monocytes. While PMNs consist of granulocytes such as basophils, neutrophils, and eosinophils (Pourahmad and Salimi, 2015; Schaller *et al.* 2004). Granulocytes originate from haematopoietic stem cells which develop in the bone marrow into basophils, neutrophils, and eosinophils before entering the peripheral blood to circulate freely (Stone, Prussin and Metcalfe, 2010).

This research study focused on T-cells and granulocytes, which can be isolated or studied from human whole blood for *in vitro* immunological studies.

2.10 Cellular studies in Homoeopathy

Homoeopathy has limited cellular studies (Warner, 2021), and Bellavite *et al.* (2006) reviewed that there were very few groups of researchers that worked on laboratory models for homoeopathy; hence, there is a gap in cellular studies in homoeopathy. However, a small number of studies have employed immunological cell models in homoeopathic research. Manchanda (2018) then demonstrated that the association between homoeopathy and immunology dated back to 1906, where analogies had been drawn between homoeopathy and immunology based on the homoeopathic principle of regulating the endogenous system of healing, which is known as the immune system and its neuroendocrine integrations.

Bellavite *et al.* (2006) described the results of laboratory experiments that were aimed at verifying the efficacy of high dilutions and homoeopathic remedies in models of immunity and inflammation. In one such study, the granulation of basophils was investigated using highly diluted IgE, and the results showed that human blood basophils underwent degranulation not only at the usual anti-IgE antibody doses but also at extremely high dilutions.
In the studies carried out by Poitevin (1998), *Apis mellifica* and *Lung Histaminum* in potencies 1CH to 15CH were used on basophils. They found that lower potencies (5CH) activated the basophils and that higher potencies (9CH and 15CH) inhibited the basophils from releasing histamine, thus preventing an allergic reaction. Ive (2010) also performed an *in vitro* study to evaluate how *Arsenicum album* at potencies 6CH, 30CH, and 200CH affects white blood cells that had been previously antagonised with arsenic trioxide and found that potency 200CH promoted greater cell recovery compared to lower potencies.

Previous studies have only focused on basophils alone (Poitevin, 1988, 1998; Benveniste, 1994; Lorenz *et al.* 2003; Belon, 2004; Guggisberg *et al.* 2005) and other immune cells separately (Bellavite *et al.* 2006). The current study aimed to investigate the actions of *Carbolicum acidum* on other granulocytes, such as eosinophils, as well as T-cells, due to their role as effector cells in severe allergic reactions (Metcalfe *et al.* 2016). Basophils have been shown to release chemical mediators that are chemotactic to eosinophils (Kruger-Krasagakes and Henz, 1998), while T-cell

chemoattractant factors have been shown to attract both monocytes and eosinophils to the target organs involved in allergic reactions (Goetzl and Austen, 1975). Eosinophils are major regulatory leukocytes of anaphylaxis (Metcalfe *et al.* 2016). Thus, this study investigated the effects of *Carbolicum acidum* on granulocytes and T-cells *in vitro,* as there is no known data that has been published.

2.11 Measurement tool

2.11.1 Flow cytometry

A flow cytometer is an instrument that is used in biotechnology to assess the physical and chemical properties of single cells at the whole cell level (Bushnell, 2015). Flow cytometry enables the measurement and analysis of cell populations individually as they flow in a fluid stream through a beam of light while simultaneously quantifying several parameters such as cellular size and several membrane markers (BD Biosciences, 2024). It has been shown that it is exceptionally fast; the routine sample analysis rates can range up to 10 000 cells per second (Sanz *et al.* 2002; BD Biosciences, 2024). The use of this instrument has expanded over the years from basic cell counting and sorting to solving complex mysteries in health sciences (Bushnell, 2015).

The assessment of immune cells through density gradient centrifugation is a process that has been demonstrated to be both labour-intensive and time-consuming due to the need for precise physical separation of different cell populations based on their density (Alsved *et al.* 2024). This procedure involves layering blood onto a gradient medium, followed by centrifugation at specific speeds (Alsved *et al.* 2024; Yu *et al.* 2018). The process often requires multiple washing steps, which, if not carefully executed, may lead to reduced yields of quantifiable analytes (Yu *et al.* 2018). In contrast, the adoption of multicolour flow cytometry for the identification of cells, such as basophils, has simplified their study in whole blood, eliminating the need for the aforementioned labour-intensive separation techniques (Metcalfe *et al.* 2016).

A review of the literature shows that flow cytometric methods have been used *in vitro* to diagnose IgE-mediated allergic reactions (Goetz, Hammerbeck, and Bonnevier, 2018). Moreover, flow cytometry can identify infrequent occurrences such as the

detection of CD34-positive haematopoietic stem cells in peripheral blood when in a resting state (Delmonte and Fleisher, 2019). This has made flow cytometry a prevalent technique that is used nearly in every aspect of cell biology and clinical patient cell analyses (Goetz, Hammerbeck, and Bonnevier, 2018). This method of testing has been proven to be reliable and has been used in numerous studies. The effects of *Carbolicum acidum* on allergic chemical mediators were analysed by flow cytometry using antibodies that were conjugated with fluorochromes to identify the cluster of differentiation (CD) markers on the surfaces of granulocytes and T-cells.

2.11.2 Flow cytometry markers

CD45 is an antigen that is found on the surfaces of blood cells except platelets and mature erythrocytes (Rheinländer, Schraven, and Bommhardt, 2018). CD45 is also known as the common leukocyte antigen (Hemmings *et al.* 2018), and in humans, its expression is found on all leukocytes (Pilling *et al.* 2009), including lymphocytes, monocytes, and granulocytes (basophils, neutrophils, eosinophils) in peripheral blood. Utilising CD45 for the identification of leukocytes has been a long-known technique in flow cytometry.

The antigen CD203c has been defined as an identification marker on the cell surface of basophils among other haematopoietic cells (Li, He, and Tong, 2023; Eberlein *et al.* 2015; Metcalfe *et al.* 2016; MacGlashan, 2010). CD203c is present at low levels in resting basophils with some specificity, but its expression is quickly upregulated upon activation (MacGlashan, 2010) in reaction to the cross-linking of the IgE receptor (Sadovnik *et al.* 2023). As a result, CD203c is a valuable marker for the identification of basophil activation and for detecting type I allergic reactions (BD Biosciences, 2024).

Activation markers are cellular antigens that are usually expressed minimally or even absent in resting cells whose expression can be upregulated when the cells are activated (Rea, McNerlan, and Alexander, 1999) and can be used to study immune responses. These activation markers are easily evidenced by flow cytometric analysis using antibodies (Caruso *et al.* 1997). There is evidence linking the expression of

activation markers to clinical findings, such as the severity of illness (Metcalfe *et al.* 2016).

CD63 is a widely used activation marker of basophils, and it is an accurate marker of anaphylactic degranulation; it parallels histamine release (Hemmings *et al.* 2018; He *et al.* 2013; MacGlashan, 2010). The release of histamine from blood basophils, among other chemical mediators, is more commonly assessed by measuring the cell membrane expression of CD63 by flow cytometry (Uyttebroek *et al.* 2014; Hengel *et al.* 2007). Research has indicated a strong relationship between the upregulation of CD63 and histamine release following basophil stimulation, suggesting that CD63 serves as a reliable indicator of the expression associated with the release of chemical mediators (Knol *et al.* 1991; Hengel *et al.* 2007).

On the other hand, activation of eosinophils is detected by the molecule CD69 (Thurau *et al.* 1996). In a study by Hartnell (1993), CD69 was not detected in unstimulated eosinophils as determined by flow cytometry and immunofluorescence. However, upon stimulation, it was detected in eosinophils in significant concentrations (Hartnell *et al.* 1993). CD69 is therefore expressed on activated eosinophils and neutrophils (BD Biosciences, 2024). Eosinophils contribute to the development of allergic reactions (Bui *et al.* 2024; Johansson, 2014) by releasing chemical mediators. During this process, eosinophils express the activation marker CD69 (Yun *et al.* 2020).

The human leukocyte antigen-DR (HLA-DR) has been documented as an activation marker of T-cells (Sun *et al.* 2023; Mousset, 2019; Hemmings *et al.* 2018). While this marker is also present in antigen-presenting cells, it has been shown that it is normally expressed on activated T-cells (Sun *et al.* 2023). This molecule plays a major role in mediating cellular interactions during antigen presentation to T-cells (Dieckmann *et al.* 2001).

The activation markers discussed above (HLA-DR, CD63, and CD69) are stored intracellularly in resting granulocytes and T-cells (Sun *et al.* 2023; Hartnell, 1993; Knol *et al.* 1991; Uyttebroek *et al.* 2014). Upon cellular activation, leading to degranulation and the release of chemical mediators, these markers are translocated to the cell surface. The assessment of their surface expression *in vitro* is essential for evaluating

disease progression and response to therapy. Consequently, the present study aimed to investigate the effects of *Carbolicum acidum* on the regulation of chemical mediators involved in allergic reactions, using flow cytometry for detailed analysis.

The antibodies that were used in this study conjugated with fluorochromes (Table 2.1 below) were Brilliant ultraviolet 395 Anti-Human CD45 (CD45 BUV395), Allophycocyanin Anti-Human CD203c (CD203c APC), R-phycoerythrin Anti-Human CD63 (CD63 PE), Fluoresceine α-isothiocyanate Anti-Human CD69 (CD69 FITC/BB515), and Brilliant violet 421 Anti-Human HLA-DR (HLA-DR BV421) for identification and activation markers of cells of study.

Table 2.1 BD Biosciences Fluorochrome reference (BD Biosciences, 2024).

Excitation Laser Line	Filter	Relative Brightness	Ultraviolet Laser (355 nm)
355 nm 349 nm	379/28		**BD Horizon Brilliant™ Ultraviolet 395 (BUV395) (Ex_{max} 348 nm/Em_{max} 395 nm)** is a polymer-based dye with an emisson max at 395 nm. BUV395 is designed for instruments equipped with a 355 nm UV laser and a 379/28 filter. This dye is optimal for multicolor flow cytometry because it has minimal spillover into other detectors. BUV395 has been exclusively developed by BD Life Sciences.
Excitation Laser Line	**Filter**	**Relative Brightness**	**Violet Laser (405 nm)**
405 nm	450/40		**BD Horizon Brilliant Violet™ 421 (BV421) (Ex_{max} 407 nm/Em_{max} 421 nm)** is a polymer-based dye excited by the violet laser and is one of the brightest fluorochromes offered by BD Life Sciences. Conjugates are typically 10 times brighter than Pacific Blue™ conjugates and are often as bright as or brighter than PE conjugates. Due to similar excitation and emission properties, BD Horizon™ BV421 and BD Horizon™ V450 cannot be used simultaneously.
Excitation Laser Line	**Filter**	**Relative Brightness 488 / 561**	**Blue Laser (488 nm) / Yellow-Green Laser (561 nm)**
488 nm	530/30		**BD Horizon Brilliant™ Blue 515 (BB515) (Ex_{max} 490 nm/Em_{max} 515 nm)** is a dye that was exclusively developed by BD Life Sciences as an additional bright dye for the blue laser. This dye is significantly brighter than FITC and has less spillover into the PE channel. Due to similar excitation and emission properties, BD Horizon™ BB515 and FITC/Alexa Fluor® 488 cannot be used simultaneously.
488 nm	530/30		**FITC (Ex_{max} 494 nm/Em_{max} 520 nm)** Fluorescein isothiocyanate (FITC) is a fluorochrome with a molecular weight of 389 Da. FITC is sensitive to pH changes and photobleaching. Due to nearly identical excitation and emission properties, FITC and Alexa Fluor® 488 cannot be used simultaneously. FITC is relatively dim and should be reserved for highly expressed markers whenever possible.
488 nm 532 nm 561 nm	575/26		**PE (Ex_{max} 496 nm, 566 nm/Em_{max} 578 nm)** R-phycoerythrin (PE) is an accessory photosynthetic pigment found in red algae. It exists in vitro as a 240-kDa protein with 23 phycoerythrobilin chromophores per molecule. This makes PE the brightest fluorochrome for flow cytometry applications, but its photobleaching properties make it unsuitable for fluorescence microscopy.
488 nm 532 nm 561 nm	780/60		**PE-Cy™7 (Ex_{max} 496 nm, 566 nm/Em_{max} 778 nm)** is a tandem fluorochrome that combines PE and the cyanine dye Cy7. PE-Cy7 is sensitive to photo-induced degradation, resulting in loss of fluorescence and changes in spillover. Extreme caution must be taken to avoid light exposure and prolonged exposure to paraformaldehyde fixative. Fixed cells should be analyzed within 4 hours of fixation in paraformaldehyde or transferred to a paraformaldehyde-free buffer for overnight storage.
Excitation Laser Line	**Filter**	**Relative Brightness**	**Red Laser (640 nm)**
628 nm 633 nm 635 nm 640 nm	660/20		**APC (Ex_{max} 650 nm/Em_{max} 660 nm)** Allophycocyanin (APC) is an accessory photosynthetic pigment found in blue-green algae. Its molecular weight is approximately 105 kDa. Due to nearly identical excitation and emission properties, APC and Alexa Fluor® 647 cannot be used simultaneously.

2.12 Conceptual framework

This study sought to investigate the actions of an anti-allergy remedy, *Carbolicum acidum*, in potencies 5CH, 9CH, 15CH, 30CH, and 200CH on chemical mediators involved in allergic reactions in an *in vitro* experimental study using granulocytes and T-cells that have been isolated from human whole blood. This study was based on the hypothesis that higher potencies will have inhibition effects on the expression of chemical mediators, while lower potencies will have a stimulatory effect. The homoeopathic principle of infinitesimals served as the foundation for this (Sagar, 2007). Additionally, this study observed the concept termed Arndt-Schulz law or hormesis, which states that the effects of an agent on biological systems will have stimulatory functions in low doses, whereas higher doses are inhibiting (Henschler, 2006) as depicted by the adapted framework in Figure 2.1. Therefore, low potencies (5CH and 9CH) were expected to have stimulatory effects on the cellular model, while high potencies (15CH, 30CH, and 200CH) would have inhibiting effects.

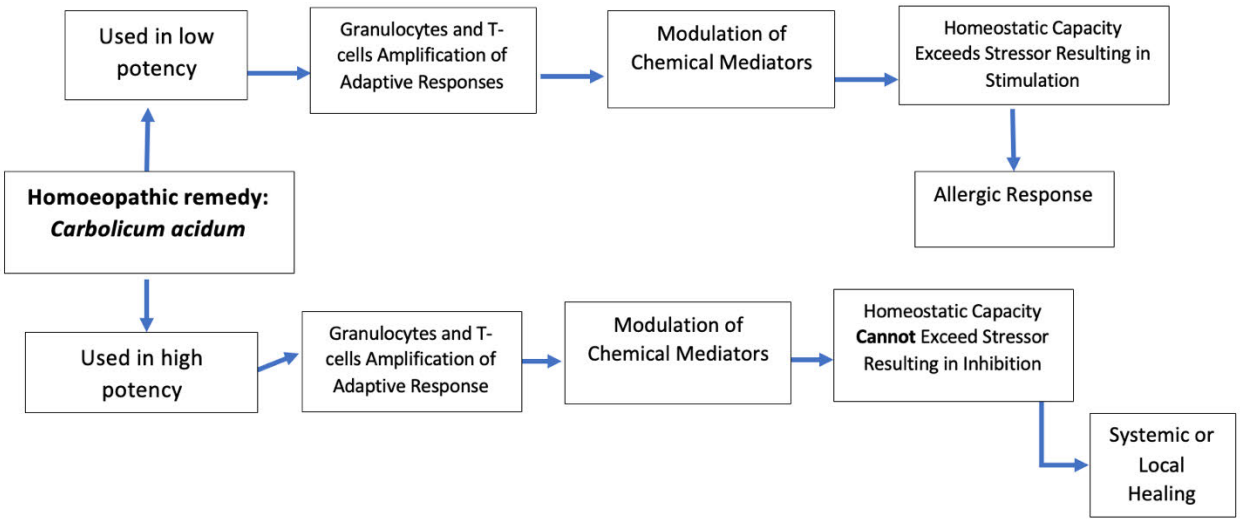

Figure 2-1 The conceptual framework that underpinned the current study, indicating that in a biological system low doses of an agent will have stimulatory function leading to an allergic response, whereas high doses will be inhibitory resulting in healing. Adapted from Bell and Koithan (2012).

Figure 2.2 shows that hormesis displays a biphasic dose-response relationship (Calabrese and Baldwin, 2001), which is a biological occurrence that is marked by stimulation at low doses and inhibition at high doses, as has been described. This dose-response relationship is usually represented as either an inverted U-shape or a J-shape on a graph (Calabrese, 2013), as seen in Figure 2.2. In pharmacological literature, the biphasic dose-response relationships resembling hormesis have been reported and described in the immune activation biological system (Calabrese, 2013; Calabrese and Giordano, 2022).

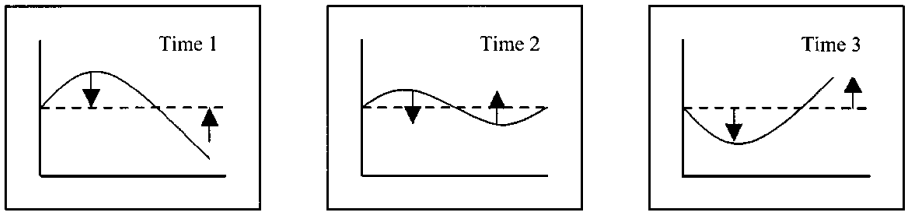

Figure 2-2 Dose-Response graphs showing patterns of direct stimulation in biological systems, indicating the biphasic dose-response relationship illustrated by the U- and J-shaped curves (Calabrese and Baldwin, 2001).

Evidence shows that allergic diseases are on the increase globally and have resulted in an increase in hospitalisations (Turner *et al.* 2020). Data on this phenomenon is lacking in South Africa and the African continent in general (Chippendale, 2018; Chippendale, 2022; World Allergy Organization, 2021). Furthermore, literature shows that there is a substantial number of South Africans who opt for using alternative and complementary medicine (Ericksen-Pereira, Roman, and Swart, 2018) and that there is also an increasing number of people who rely on alternative medicine for their primary healthcare needs in Sub-Saharan Africa (James *et al.* 2018). Thus, it is necessary to look into complementary approaches to treating allergic diseases because of the rise in these conditions and the growing use of alternative medicine. Which is what this study sought to do.

This study aimed to perform a safer scientific *in vitro* investigation because of the ethical concerns surrounding the disease process of severe allergic reactions, making it not feasible to conduct clinical trials. This approach helps to enhance the

understanding of human cell culture research in homoeopathy by linking homoeopathic principles to biomedical research by observing physiological responses at the cellular level. Thereby contributing to public health by offering an alternative treatment regimen for severe allergic reactions.

Furthermore, *Carbolicum acidum* is the only high-grading homoeopathic remedy that is clinically indicated for anaphylaxis (Schroyens, 2016). However, the therapeutic indications of this remedy have only been predominantly verified through homoeopathic clinical observations and homoeopathic provings. No research data has been published to date on the effects of *Carbolicum acidum* on granulocytes and T-cells chemical mediators involved in allergic reactions.

CHAPTER THREE

METHODOLOGY

3.1 Introduction

The design of this study was an *in vitro* experimental study. *In vitro* studies are experimental in nature in that they utilise standardised methods to keep all conditions constant except for the independent variable, looking at cause-and-effect (Ross and Morrison, 2014), offering a higher level of control over exposure and experimental conditions compared to live animals or human volunteers (Zeni and Scarfi, 2012). These studies can offer insights into the potential effects of agents on specific cell properties, providing a more efficient and cost-effective approach to conducting molecular studies (Zeni and Scarfi, 2012).

This *in vitro* experimental study used granulocytes and T-cells which were isolated from human whole blood by lysis of red blood cells. This study aimed to investigate the effects of *Carbolicum acidum* on chemical mediators involved in allergic responses to determine which potency would have effects on the release of allergic chemical mediators. Therefore, this *in vitro* experimental study investigated the effects of *Carbolicum acidum* 5CH, 9CH, 15CH, 30CH and 200CH on stimulated granulocytes and T-cells.

The study was performed in a Biosafety Level 2 accredited cell culture laboratory at a University in Durban, South Africa. The cell culture was prepared and acquired using the BD FACSymphony A5 flow cytometer. All tests were performed in duplicates to ensure validity and reliability.

3.2 Ethical approval and gatekeeper permission

This experiment received full approval on 09 June 2022 from the Faculty of Health Sciences Research Committee at the Durban University of Technology (DUT) (Appendix A). This research study was exempt from Institutional Research and Ethics Committee (IREC) review at DUT, as this study fell under Ethics Category 1. Ethics Category 1 studies are straightforward research without ethical problems. This research study had no risk to humans, animals, or the environment. The gatekeeper permission letter was granted by the Directorate for Research and Postgraduate Support at DUT on the 5th of June 2022 (Appendix B).

The blood sample utilised in this study was supplied by the South African National Blood Services (SANBS) post-testing from the SANBS Donation Testing department. An application for the blood sample was sent to SANBS, and the SANBS Scientific Review Committee and Human Research Ethics Committee approved the application on the 08th of April 2024 (Clearance Certificate Number 2024/0004) (Appendix C).

3.3 Biosafety

This study was carried out in a Biosafety Level 2 (BSL-2) laboratory. The airflow in the room was balanced to create a negative airflow into the room. The laboratory is designed in a way that it can be easily cleaned and decontaminated. The researcher used personal protective equipment for a BSL-2 laboratory, namely, a front-closure laboratory coat, gloves, and eye protection. Laboratory health and safety orientation was provided by the safety officer before the commencement of experimental work.

All cell culture work was performed under the cell culture cabinet (Baker SterilGARD II). For disinfection, 70% alcohol was used to clean the surfaces of the cell culture cabinet before and after use. Test tube holders, consumables, and materials were sprayed down with 70% alcohol before being placed in the cell culture hood to avoid contamination. All cell culture material was decontaminated and disposed of under laboratory protocols, following appropriate biohazard waste disposal procedures, so that it could not be reused for any purpose other than that of the current study.

3.4 Materials and methods

The antibodies and reagents that were used in this investigation were purchased from Becton Dickinson Biosciences (BD Biosciences) unless specified otherwise and were obtained from The Scientific Group and later from Obsidian Health, the sole suppliers of BD Biosciences in South Africa. Additional information about the reagents and instruments used in this study can be found in Appendix D.

3.5 Preparation of *Carbolicum acidum*

Carbolicum acidum stock potencies 4CH, 7CH, 13CH, 27CH, and 198CH in 20% alcohol were purchased from CoMed Health Pty Ltd, Waltloo, Pretoria. The remedy potencies were prepared according to the German Homoeopathic Pharmacopoeia (certificate of analysis in appendix E), where one part of the drug material was dissolved in 99 parts of the liquid vehicle and succussed. Ethanol, distilled water, and ethanol/water mixtures are usually used as vehicles as listed in the Pharmacopoeia (British Homoeopathic Association, 1993).

The potencies of study were prepared by the researcher at the Durban University of Technology Homoeopathic Dispensary under sterile conditions in the laminar flow (LABAIRE). *Carbolicum acidum* stock potencies were used to prepare 20ml of 5CH, 9CH, 15CH, 30CH, 200CH in aqua distillata (distilled water). The remedy was in a centesimal scale (CH) which is a 1:100 dilution.

<u>To make 20 ml *Carbolicum acidum* 5CH in distilled water base:</u>

Amount of *Carbolicum acidum* 4CH needed to make 20ml of *Carbolicum acidum* 5CH was determined by formula:

$$\text{Dilution scale} \times \text{Required volume} = \frac{1}{100} \times 20\text{ml} = 0.2\text{ ml 4CH.}$$

Then the amount of distilled water needed to make the final volume of *Carbolicum acidum* 5CH was determined by:

$$\text{Final volume} - 0.2\text{ml} = 19.8\text{ml distilled water.}$$

Using a 20ml graduated pipette, 19ml of distilled water was added to a 30ml amber glass bottle, and 0.8ml distilled water was added using a micropipette. Then 0.2ml of *Carbolicum acidum* 4CH was added to the amber glass bottle containing 19.8ml distilled water using a micropipette. The bottle was capped and succussed 100 times to make *Carbolicum acidum* 5CH. The micropipette tips were changed after each use.

To make 20ml of *Carbolicum acidum* 9CH in distilled water base:

An amount of *Carbolicum acidum* 7CH needed to make 20ml of *Carbolicum acidum* 8CH was determined using the same formula above to be 0.2 ml 7CH.

Then the amount of distilled water needed was determined to be 19.8ml as above. Using a 20ml graduated pipette, 19ml of distilled water was added to a 30ml amber glass bottle, and 0.8ml distilled water was added using a micropipette. Then 0.2ml of *Carbolicum acidum* 7CH was added to the amber glass bottle containing 19.8ml distilled water. The bottle was capped and succussed 100 times to make *Carbolicum acidum* 8CH.

To make *Carbolicum acidum* 9CH, 0.2ml of *Carbolicum acidum* 8CH was measured by micropipette and added to 30ml amber glass bottle containing 19.8ml distilled water, measured by means of a 20ml graduated pipette and micropipette as above. The bottle was capped and succussed 100 times to make *Carbolicum acidum* 9CH.

The same method mentioned above was used to obtain *Carbolicum acidum* 15CH, 30CH, and 200CH in distilled water from the *Carbolicum acidum* stock potencies 13CH, 27CH, and 198CH respectively.

3.6 Cell culture

In vitro cell culture is a technique that is employed to study the behaviour of animal cells in a controlled setting, free from systemic variations (Arango et al., in Anaya et al., 2013). The cell culture that was used in this study was from fresh human whole blood in anticoagulant ethylenediaminetetraacetic acid (EDTA) that was obtained from the South African National Blood Services (SANBS) post-testing from the Donation Testing Department.

The blood samples were tested independently by SANBS and found to be negative for HIV, Hepatitis B virus (HBV), syphilis, and Treponema Pallidum Hemagglutination Assay (TPHA) (Certificate of analysis attached in Appendix F). Therefore, the blood samples were deemed safe for use in research. The blood used in this study was pooled blood collected as residual samples from different donors by SANBS (Clearance Certificate Number 2024/0004). The blood was used the same day after collection from SANBS.

3.7 Optimisation study: flow cytometry assay

3.7.1 Antibody titration experiment

To obtain a good antibody resolution, an antibody titration was performed to optimise the concentration that would yield the best antibody resolution for the identification of cells and their levels of expression. The antibody amounts were tested at the recommended concentration by the manufacturer (Table 3.1), at half the recommended concentration (Table 3.2), and at a quarter of the recommended concentration (Table 3.3). The antibodies that were titrated were CD45 BUV395 (Cat. No. 563791), CD203c APC (Cat. No. 562973), CD63 PE (Cat. No. 561925), CD69 BB515 (Cat. No. 560969), and HLA-DR BV421 (Cat. No. 562805). During titration, each tube contained only one antibody. Each antibody had three titrations; therefore, a total of 15 tubes were prepared for the titrations.

Table 3.1: Neat titer. Testing antibodies at recommended concentration.

	CD45	**CD203c**	**CD69**	**CD63**	**HLA-DR**
Blood sample (µl)	50	50	50	50	50
Antibody volume (µl)	5	5	20	20	5
Buffer (µl)	15	15	0	0	15
Final volume (µl)	70	70	70	70	70

Table 3.2: 1/2 dilution. Testing antibodies at half the recommended concentration.

	CD45	CD203c	CD69	CD63	HLA-DR
Blood sample (µl)	50	50	50	50	50
Antibody volume (µl)	2.5	2.5	10	10	2.5
Buffer (µl)	12.5	12.5	10	10	12.5
Final volume (µl)	70	70	70	70	70

Table 3.3: 1/4 dilution. Testing antibodies at quarter the recommended concentration.

	CD45	CD203c	CD69	CD63	HLA-DR
Blood sample (µl)	50	50	50	50	50
Antibody volume (µl)	1.25	1.25	5	5	1.25
Buffer (µl)	18.75	18.75	15	15	18.75
Final volume (µl)	70	70	70	70	70

To prepare the sample for titrations, 2ml of pooled blood was added to a FACS tube. Then 2µl of BD Leukocyte Activation cocktail (Cat. No. 550583) was added to the same FACS tube. The blood with the activation cocktail was then incubated in a 5% CO2 humidified incubator at 37 degrees Celsius for 4 hours.

After the activation period, 50µl of the activated blood was aliquoted into 15 tubes. Each antibody titration volume was added to each tube, followed by the sheath fluid to make the final volume of 70µl as per titration tables (Tables 3.1, 3.2, and 3.3).

The samples were incubated for 30 minutes in the dark at room temperature. After the incubation period, the red blood cells were lysed using 1X BD FACS Lyse solution (Cat No. 349202). The samples were then acquired in BD FACSymphony A5 for detecting optimal antibody concentrations to be used in the main experimental study.

3.7.2 Antibody staining indexes

From the titration experiment, FlowJo version 10.10 software was used to determine the staining index of the antibodies (Table 3.4). The staining index is the difference between the medians of the positive and negative populations, divided by two times the robust standard deviation of the negative population (Bonilla *et al.* 2024). The antibody dilution that achieved optimal staining resolution with minimal background noise was identified from the titration experiments.

Table 3.4 Antibody Staining Indexes.

	Titrations	*Antibody Volume*	*Antibody+ Median*	*Antibody- Median*	*Antibody- Robust SD*	*Stain Index*
CD45 BUV395	Titrations_CD45_1.fcs	5 µl	20369.54	135.2	280.13	36.12
	Titrations_CD45_2.fcs	2.5 µl	18239.75	117.17	234.84	38.58
	Titrations_CD45_3.fcs	1.25 µl	16096.86	77.32	160.49	49.91
CD63 PE	Titrations_CD63_1.fcs	20 µl	33921.26	1246.38	1383.98	11.8
	Titrations_CD63_2.fcs	10 µl	18243.08	510.34	808.93	10.96
	Titrations_CD63_3.fcs	5 µl	9928.64	240.94	372.24	13.01
CD203c APC	Titrations_CD203_1.fcs	5 µl	9448.54	1056.84	946.74	4.43
	Titrations_CD203_2.fcs	2.5 µl	5912.44	557.78	678.27	3.95
	Titrations_CD203_3.fcs	1.25 µl	2854.13	258.14	337.82	3.84
CD69 BB515	Titrations_CD69_1.fcs	20 µl	2354.59	93.81	217.39	5.2
	Titrations_CD69_2.fcs	10 µl	1927.68	146.8	178.39	4.99
	Titrations_CD69_3.fcs	5 µl	1394.25	145.5	142.19	4.39
HLA-DR BV421	Titrations_HLA-DR_1.fcs	5 µl	6559.02	464.02	412.13	7.39
	Titrations_HLA-DR_2.fcs	2.5 µl	5824.19	325.09	496.25	5.54
	Titrations_HLA-DR_3.fcs	1.25 µl	5313.16	295.47	496.14	5.06

3.7.3 Compensation setting for multi-flow cytometric analysis.

When performing a simultaneous multicolour analysis on a flow cytometer, the different fluorochromes used tend to overlap if not corrected, resulting in fluorochromes being emitted into inappropriate detectors (BD Biosciences, 2024). The spectral overlap can lead to misinterpretation of data if this is not compensated. Compensation is a way to correct this by electronically subtracting unwanted signals to remove the effects of spectral spillover (BD Biosciences, 2024).

The flow cytometer was calibrated using Rainbow Particles (Cat. No. 559123) to determine the relative voltage range for detectors in the flow cytometer. Then compensation was set for the panel design for the study (Figure 3.1) based on running each antibody conjugated with fluorochrome individually (single stain); namely, CD45 BUV395, CD203c APC, CD63 PE, CD69 BB515, and HLA-DR BV421. Compensation settings were adjusted so that positively stained cells were in line with unstained cells. All the samples in this study were run under the same conditions and instrument configurations.

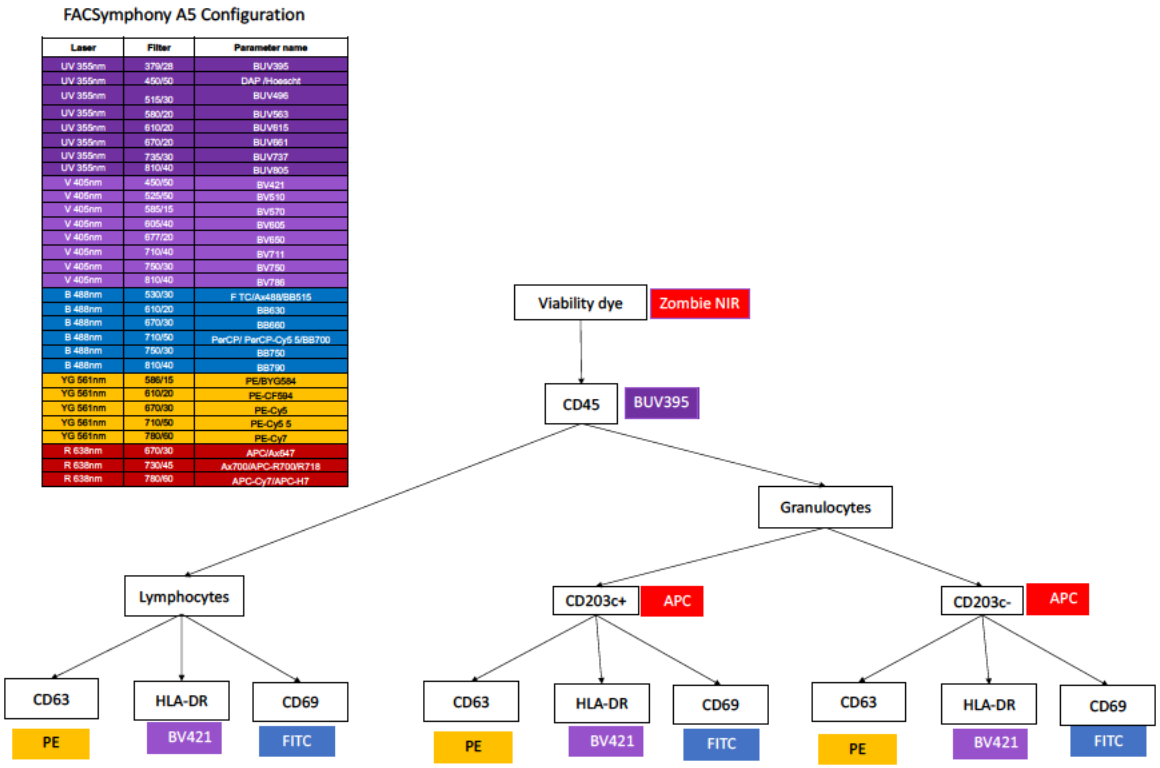

Figure 3-1 BD FACSymphony A5 Flow Cytometer laser configurations and 6 colour panel design of antibody-conjugates for the study.

3.7.4 Fluorescence Minus One Control

Fluorescence minus one (FMO) control contains all the markers in the panel of antibodies except one of interest to assist with determining the gating strategy for identifying the positive and negative populations for markers, especially for activation markers or markers with no clear positive and negative population distinctions.

For this study, the FMOs for activation markers HLA-DR, CD63, and CD69 were prepared, as well as for CD203c, which is a marker with low expression. The FMOs were prepared as described in Table 3.5 below. 50µl of pooled blood was then aliquoted into each of the tubes containing 50µl of FMOs to make a 100µl cell suspension. The FMOs were then acquired in the BD FACSymphony A5 flow cytometer, and data was analysed using FlowJo V10.10 software.

Table 3.5 Fluorescence Minus One Controls.

ANTIBODIES	CD69 FMO	HLA-DR FMO	CD63 FMO	CD203c FMO	FULLY STAINED
CD45 BUV395	1.25	1.25	1.25	1.25	1.25
CD63 PE	1.25	1.25	0	1.25	1.25
CD203c APC	2.5	2.5	2.5	0	2.5
CD69 BB515	0	2.5	2.5	2.5	2.5
HLA-DR BV421	2.5	0	2.5	2.5	2.5
Zombie NIR	0.5	0.5	0.5	0.5	0.5
TOTAL AB VOLUME	*8*	*8*	*9.25*	8	*10.5*
STAIN BUFFER VOLUME	*42*	*42*	*40.75*	42	*39.5*
FINAL ANTIBODY VOLUME	**50**	**50**	**50**	**50**	**50**

3.7.5 Cell viability control

A water bath was heated to 65 degrees Celsius. 500μl of blood was aliquoted into a FACS tube labelled “dead cells.” The cells were left in a heated water bath for 8 minutes, then set aside.

100μl of room temperature tissue culture grade DMSO was added to the Zombie NIR Fixable Viability dye powder (BioLegend Cat. No. 423105) and vortexed until it was completely dissolved to make a stock solution. To make a working solution, 1μl stock solution was added to an Eppendorf tube and diluted with 9μl sheath fluid. The rest of the viability dye stock solution was aliquoted into Eppendorf tubes and stored in a -20 degrees Celsius freezer protected from light.

To make cell viability control, 25μl of the dead cells and 25μl of the live blood cells were aliquoted in a FACS tube with 69μl sheath fluid. Then 1μl working solution of Zombie NIR was added. The FACS tube was capped and mixed gently, followed by 15 minutes of incubation at room temperature in the dark.

After incubation, the cell suspension was run in the flow cytometer for analysis.

3.8 Experimental study

3.8.1 Preparation of treatment groups

Under a cell culture cabinet (Baker SterilGARD II), 1 ml of pooled blood was aliquoted with 2μl of each treatment potency into appropriately labelled FACS tubes (Control, 5CH, 9CH, 15CH, 30CH, 200CH). The tubes were gently mixed and incubated at 37 degrees Celsius in a 5% CO2 humidified incubator for 30 minutes. Each test group had 6 samples.

After incubation, the samples were activated using 2μl of leukocyte activation cocktail. The cell suspensions were gently mixed and incubated at 37 degrees Celsius in a 5% CO2 humidified incubator for 4 hours. After the activation period, from each tube containing 1ml of activated blood, 50μl of activated blood was aliquoted into newly labelled tubes for staining.

This process was performed in duplicates to ensure reliability, having two distinct test groups, each conducted in duplicates. Each test group was assigned an "N-of" designation, used solely as a group label, not to indicate the number of samples within the group. Each test group consisted of five treatments and one control, totalling six samples per group. Accordingly, the first test group was labelled N-of-1, and its replicate was labelled N-of-2. The second test group was designated N-of-3, with its replicate labelled N-of-4. In total, twenty-four samples were acquired across all groups.

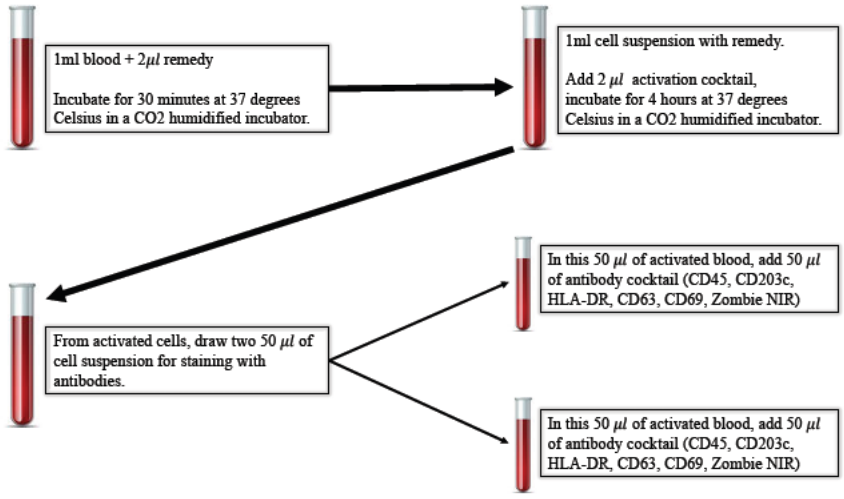

Figure 3-2 Schematic diagram overview showing preparation of treatment groups.

3.8.2 Antibody Staining

The antibody cocktail was prepared according to Table 3.6 using optimised antibody concentrations determined in the titration experiment.

For staining, 50µl of antibody cocktail was added to tubes containing 50µl activated blood. The tubes were gently mixed and incubated in the dark for 30 minutes at room temperature.

After staining incubation, 450µl 1X BD FACS lysing solution was added to each of the labelled tubes to lyse the red blood cells and fix the leukocytes. This aids with efficient detection of leukocytes by eliminating interfering cells without losing leukocyte subsets. The tubes were incubated for a further 15 minutes in the dark at room temperature for the lysing process.

The samples were then acquired on the BD FACSymphony A5 flow cytometer and analysed using FlowJo v10.10 software. Data was exported to Microsoft Excel for further analysis in IBM SPSS Statistics Version 30.0.

Table 3.6 Antibody cocktail.

Reagents	**Antibody Volume (µl) for one sample**	**Antibody Volume (µl) for seven samples + 10% for pipetting**
CD45 BUV395	1.25	10
CD63 PE	1.25	10
CD203c APC	2.5	20
CD69 BB515	2.5	20
HLA-DR BV421	2.5	20
Zombie NIR Fixable Viability APC-Cy7	0.5	4
TOTAL ANTIBODY VOLUME	10.5	84
STAIN BUFFER VOLUME	39.5	316
FINAL ANTIBODY COCKTAIL VOLUME	**50**	**400**
* To use 50 µl whole blood per sample. * add 50 µl of this antibody master-mix to the samples.		

3.8.3 Flow Cytometry Gating Strategy

For the analysis of cells, the gating strategy was done in FlowJo v10.10 software. The white blood cells (WBC) were initially identified based on the side scatter (SCC) versus forward scatter (FSC), and a gate was applied to this population (Figure 3.3). Single cells were then selected from the WBC population, followed by the identification of CD45+ leukocytes using SSC vs CD45 in the single-cell population. From the CD45+ leukocyte population, viable cells were selected to eliminate any debris and dead cells from analysis using the Zombie NIR parameter. The leukocyte subsets, including lymphocytes, monocytes, and granulocytes, were then gated from the viable cell population. Granulocytes were further distinguished using the CD203c marker, with CD203c-positive and CD203c-negative granulocytes identified; activation markers CD63 and CD69 were subsequently identified within these subsets. Lymphocytes and monocytes were gated for their activation marker HLA-DR from their respective SSC/CD45 gates. Activation markers were used to assess the cellular activation status of these cells.

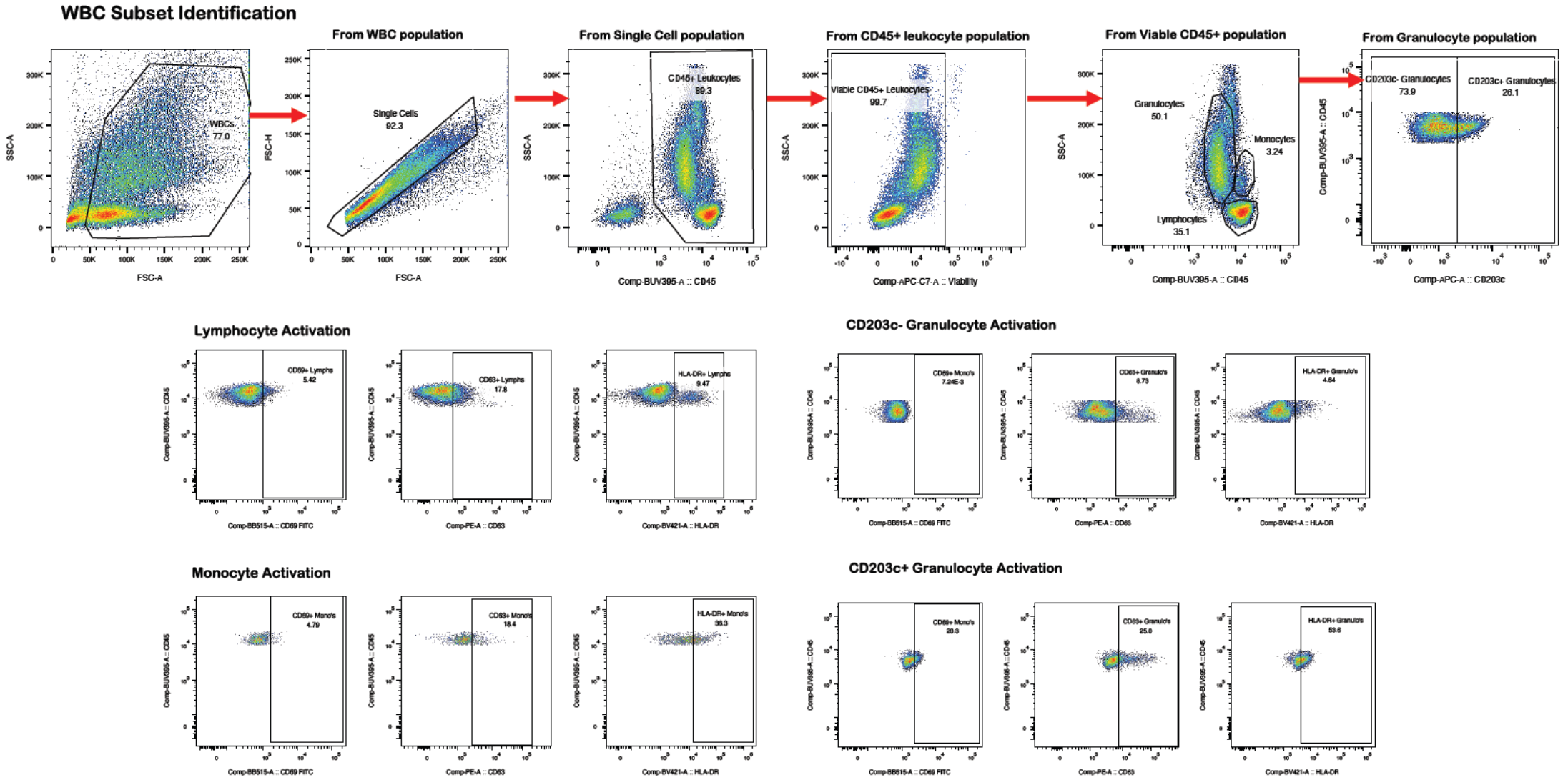

Figure 3-3 Flow cytometry gating strategy.

3.9 Statistical analysis

A statistical analysis will be performed to evaluate the significance of the empirical data that will be gathered from this experiment, with a p-value of < 0.05 deemed statistically significant. A One-Way Analysis of Variance (ANOVA) will be conducted in IBM Statistics (Version 30.0) to assess the effects of different treatments (5CH, 9CH, 15CH, 30CH, and 200CH) on the expression of activation markers, comparing them to the control groups. Additionally, a Tukey Honestly Significant Difference (HSD) post hoc analysis will be conducted for pairwise comparisons among treatment groups where statistical significance will be found. The statistical analysis is presented in Chapter 4.

CHAPTER FOUR

RESULTS

4.1 Introduction

This study aimed to investigate the effects of *Carbolicum acidum* on T-cells and granulocytes, specifically focusing on the chemical mediators involved in allergic responses, which are indicated by the expression of activation markers on the surfaces of these cells.

The upregulation and downregulation of these activation markers were quantified using median fluorescence intensity (MFI), a measure that assesses the changes in protein expression of cells in response to various treatments or stimuli (Li, He, and Tong, 2023). The MFI was extracted from FlowJo for further analysis in SPSS v30.0.

Each flow cytometric analysis consisted of six samples per test group comprising five treatments (5CH, 9CH, 15CH, 30CH, 200CH) and a control (0CH). The study had two test groups that were conducted in duplicates, N-of-2 being a replicate of N-of-1 and N-of-4 being a replicate of N-of-3. A total of twenty-four samples were acquired across the test groups (Table 4.1) and analysed. The control groups (stimulated without treatment, 0CH) provided the baseline for comparing the effects of the treatment potencies (5CH, 9CH, 15CH, 30CH, 200CH) on the expression of activation markers.

Table 4.1 FlowJo generated frequency statistics and MFIs of the cell populations.

	Frequency statistics of cell populations						Median Fluorescence Intensity (MFI)			
Treatment Groups	% Live Leukocytes	% CD45+ Lymphocytes	% CD45+ Monocytes	% CD45+ Granulocytes	% CD203c+ Granulocytes	% CD203c- Granulocytes	Lymphocytes HLA-DR+ MFI	Monocytes HLA-DR+ MFI	CD203c+ Granulocytes CD63+ MFI	CD203c- Granulocytes CD69+ MFI
N1 Control	87.30%	34.70%	3.28%	52.20%	26.90%	69.70%	8063	8562	5213	1302
N1 5 CH	87.50%	33.30%	4.27%	52.70%	30.90%	66.00%	7430	7939	5580	1310
N1 9 CH	90.70%	37.20%	3.47%	49.10%	29.60%	67.30%	7365	7612	5824	1302
N1 15 CH	90.80%	35.40%	3.74%	50.00%	29.00%	67.70%	7765	8448	5762	1295
N1 30 CH	91.50%	35.20%	3.11%	50.50%	29.90%	66.80%	7253	7546	5713	1322
N1 200 CH	91.40%	35.50%	3.20%	50.20%	27.40%	69.00%	7003	7939	5521	1332
N2 Control	99.00%	40.20%	2.89%	47.00%	33.50%	63.00%	7080	7496	5125	1297
N2 5 CH	98.30%	40.50%	2.83%	46.70%	35.70%	60.80%	6689	7018	5404	1305
N2 9 CH	99.00%	39.80%	2.95%	47.20%	33.50%	63.30%	6660	7714	5257	1300
N2 15 CH	98.70%	40.30%	3.16%	46.80%	33.60%	63.20%	6718	7579	5180	1300
N2 30 CH	98.90%	40.00%	2.83%	46.90%	33.20%	63.50%	6488	7349	5347	1307
N2 200 CH	99.00%	41.20%	2.90%	45.50%	33.40%	63.20%	7018	7003	5191	1310
N3 Control	93.90%	38.40%	3.21%	48.90%	32.20%	64.60%	6762	7663	5750	1312
N3 5 CH	94.10%	37.30%	3.15%	49.80%	32.30%	64.40%	7333	7939	5964	1312
N3 9 CH	93.90%	37.30%	3.21%	49.50%	28.80%	68.20%	6792	8373	5824	1292
N3 15 CH	93.90%	37.40%	3.14%	49.60%	27.90%	69.00%	7284	7957	5640	1287
N3 30 CH	94.10%	38.00%	3.09%	49.00%	31.90%	65.00%	6545	7562	5887	1305
N3 200 CH	94.10%	38.90%	3.32%	47.60%	30.90%	66.10%	6602	7596	5701	1307
N4 Control	89.60%	38.30%	3.43%	48.20%	27.30%	69.70%	7349	8281	5862	1312
N4 5 CH	90.50%	35.90%	3.67%	50.40%	29.50%	67.80%	7189	7869	6015	1307
N4 9 CH	89.40%	36.60%	3.38%	49.50%	23.10%	74.10%	7680	8153	5964	1310
N4 15 CH	90.30%	36.20%	3.48%	50.10%	25.60%	71.80%	7158	8226	5900	1300
N4_30 CH	90.10%	37.40%	3.62%	48.70%	27.80%	69.50%	7189	8081	6107	1305
N4 200 CH	89.90%	38.10%	3.53%	48.10%	25.60%	71.60%	7189	8189	6093	1320
Mean	**93.20%**	**37.60%**	**3.29%**	**48.90%**	**30.00%**	**66.90%**	**7108**	**7837**	**5659**	**1306**
SD	**3.86%**	**2.06%**	**0.33%**	**1.75%**	**3.16%**	**3.32%**	**404**	**415**	**310**	**9.75**

To ensure the reliability of the data, samples from two different test groups (N-of-1 and N-of-3) were each analysed in duplicate, allowing for a strong comparison between experimental and control variables. The effects of *Carbolicum acidum* were tested on pooled blood samples collected from different donors. These blood samples were randomised into FACS tubes, and the tubes were then randomised to treatments. Before starting the analysis, several important control steps were performed to optimise the experimental conditions to minimise potential sources of technical variation.

First, the antibody titration experiments were performed to determine the optimal concentrations for each antibody to ensure accurate detection and prevent nonspecific binding. This process was important to minimise false-positive and false-negative signals that may affect the interpretation of flow cytometric data. After antibody titrations, Fluorescence Minus One (FMO) controls were prepared to determine gating strategies. These controls allowed for accurate identification of positive and negative populations of markers, especially for activation markers and markers with no distinction between positive and negative populations. Thirdly, the pre-analytical setup included compensation settings for spectral overlap between fluorochromes conjugated to antibodies. Compensation ensured that signals from multiple fluorochromes were accurately assigned to their channels, preventing spillover and improving the specificity of analysis. The same compensation settings were applied uniformly to all samples, ensuring consistency of data acquisition.

The reliability of the flow cytometric analysis was ensured by strict adherence to these control protocols. To reduce technical variability, it was essential to use consistent parameters for all samples, from antibody titrations to gating strategies and compensation settings. This method made it easier to accurately evaluate the effects of *Carbolicum acidum* on granulocyte and T-cell activation markers and guaranteed that the observed changes were brought about by the remedy and not by technical inconsistencies.

Furthermore, by systematically using established flow cytometric protocols and controlling for technical variability, the study minimised bias and allowed for a clear

interpretation of the potential effects of *Carbolicum acidum* on the expression of chemical mediators in allergic reactions.

4.2 Dose-response pattern of *Carbolicum acidum* on activation markers.

The dose-response effects of *Carbolicum acidum* 5CH, 9CH, 15CH, 30CH, and 200CH are presented next for each activation marker, followed by the statistical analysis of these findings.

4.2.1 Dose-response pattern of *Carbolicum acidum* on activation marker HLA-DR

The control sample in each treatment group (N-of-1 = 8063 MFI; N-of-2 = 7080 MFI; N-of-3 = 6762 MFI; N-of-4 = 7349 MFI) served as a baseline reference for the expression of the HLA-DR marker on T-cells in the absence of treatment (Table 4.1).

5CH Treatment Group:

A decrease in MFI was observed in N-of-1, N-of-2, and N-of-4 when compared to their respective controls. While an increase in MFI was seen in N-of-3.

9CH Treatment Group:

N-of-1, N-of-2, and N-of-3 further demonstrated a decrease in MFI, while sample N-of-4 displayed a marked increase in MFI in this treatment group (Figure 4.1 below).

15CH Treatment Group:

N-of-1, N-of-2, and N-of-3 exhibited an elevation in MFI, suggesting an increase in the expression of HLA-DR marker. However, N-of-4 in this treatment group demonstrated a decrease in MFI.

30CH and 200CH Treatment Groups:

In both the 30CH and 200CH groups, a further reduction in MFI was observed across the treatment groups in both these potencies, indicating a more pronounced suppression of HLA-DR expression at very high potencies of *Carbolicum acidum*.

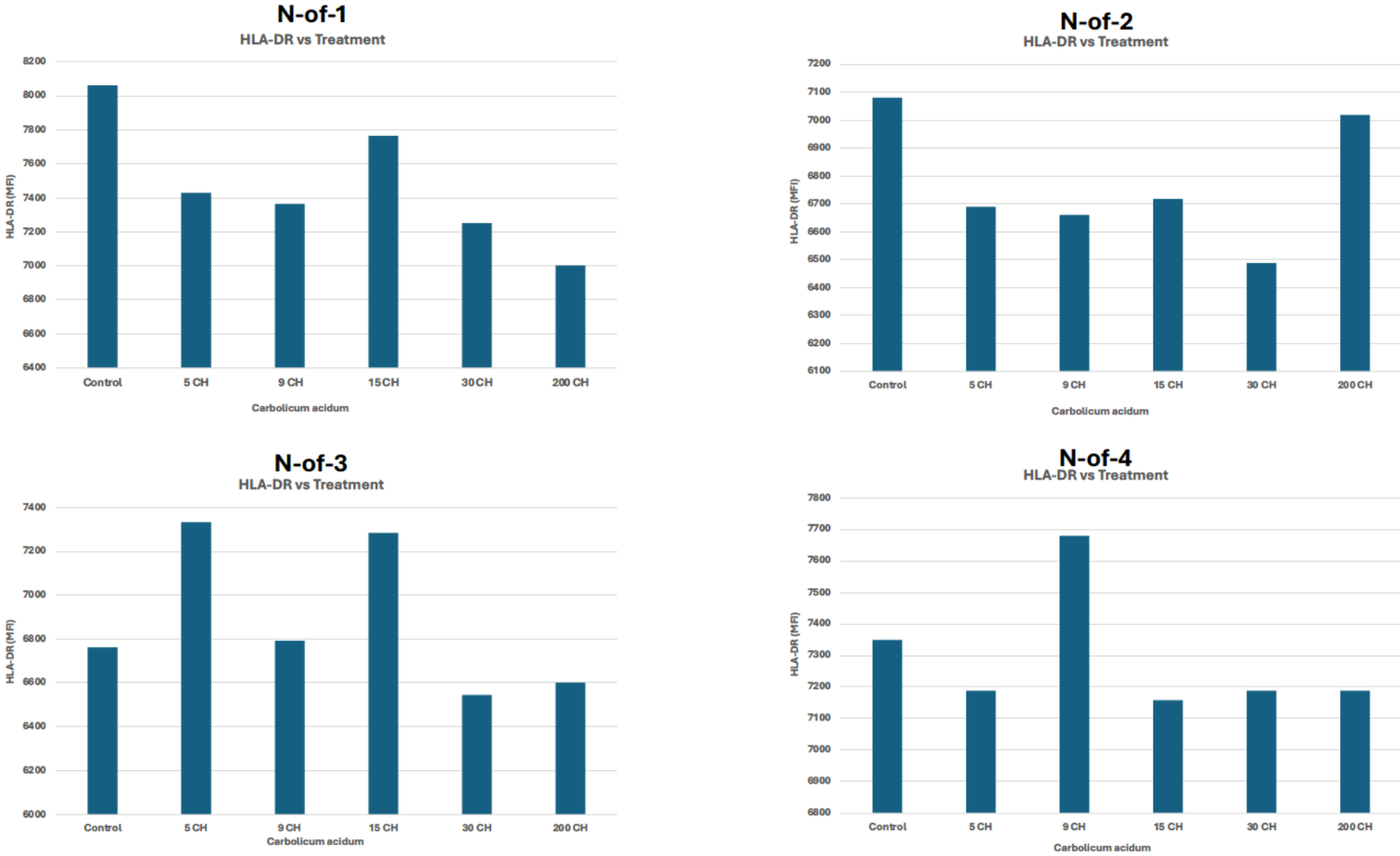

Figure 4-1 Bar graphs illustrating effects of *Carbolicum acidum* on activation marker HLA-DR, demonstrating a dose-response relationship across the test groups (N-of-1 to N-of-4). Antibody MFI on the vertical axis is plotted as a function of *Carbolicum acidum* potencies on the horizontal axis.

Differences in expression of HLA-DR were detected between the control group and the treatment groups at all tested potencies, highlighting changes in the activation marker HLA-DR in response to treatment. Figure 4.2 presents the density plot of flow cytometric data related to Figure 4.1

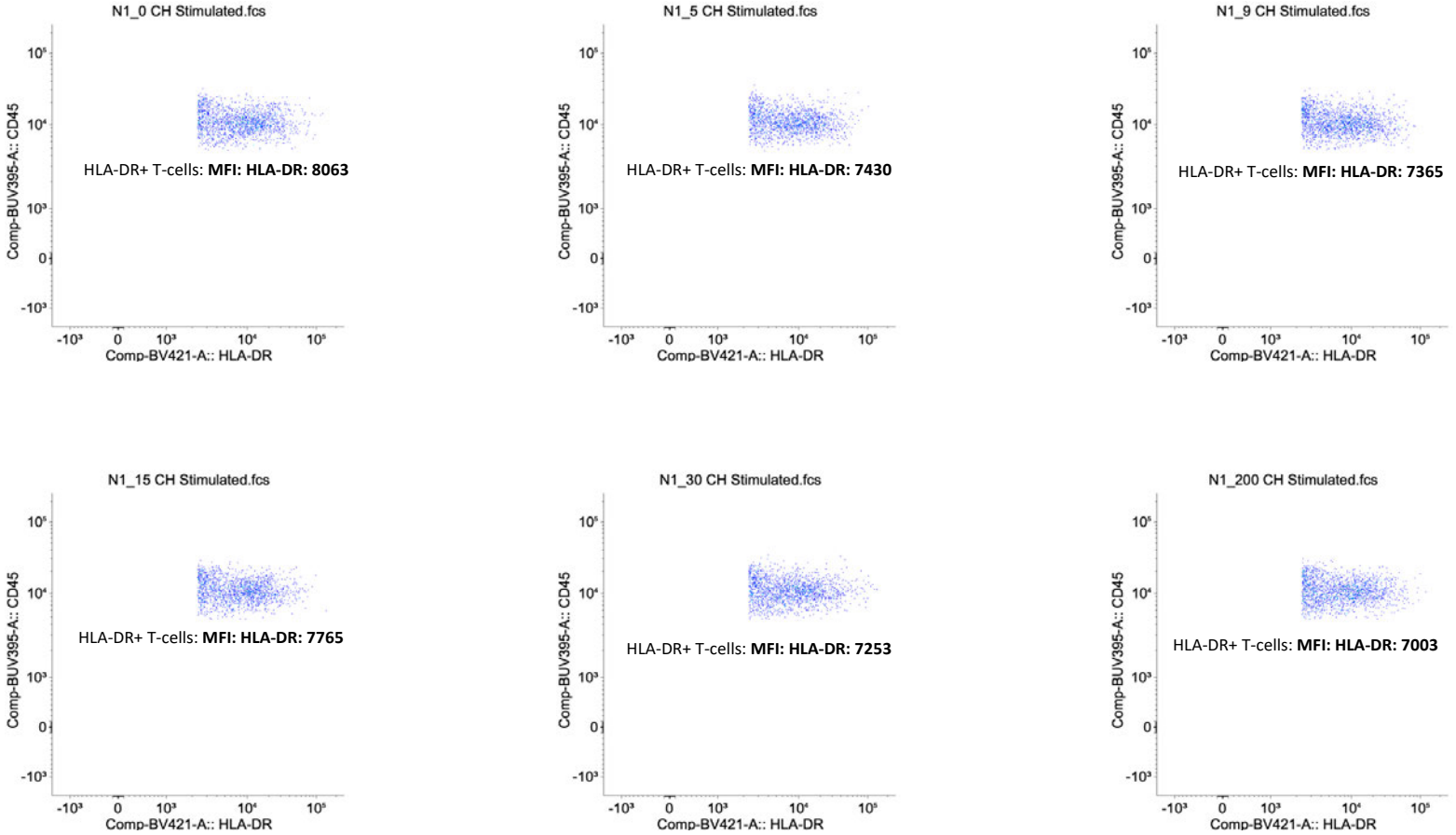

Figure 4-2 Density plot showing MFI of HLA-DR in N-of-1 samples in response to treatment. The density plots illustrating the MFI of HLA-DR in other test groups in response to treatment are presented in Appendix G.

4.2.2 Dose-response pattern of *Carbolicum acidum* on activation marker CD63.

The control samples in the gated population of CD203c+ granulocytes served as a baseline for the expression of the activation marker CD63 in the absence of treatment. The observed MFI baseline for CD63 across replications of the study was N-of-1 = 5213, N-of-2 = 5125, N-of-3 = 5750, and N-of-4 = 5862.

5CH Treatment Group:

When compared to their respective controls, the samples (N-of-1 to N-of-4) exhibited a similar increasing pattern in MFI (Figure 4.3). This suggests that this potency had a stimulatory effect on the expression of CD63.

9CH Treatment Group:

This increasing pattern persisted in the N-of-1 sample. While N-of-2, N-of-3, and N-of-4 had a decline in expression of CD63, starting to show suppressive effects.

15CH Treatment Group:

The expression of CD63 continued to decline at this potency. The decrease of MFI at was most pronounced in samples N-of-2, N-of-3, and N-of-4.

30CH Treatment Group:

At 30CH, an increase in MFI was observed across all samples.

200CH Treatment Group:

This was followed by a decline in MFI at 200CH across all samples (Figure 4.3).

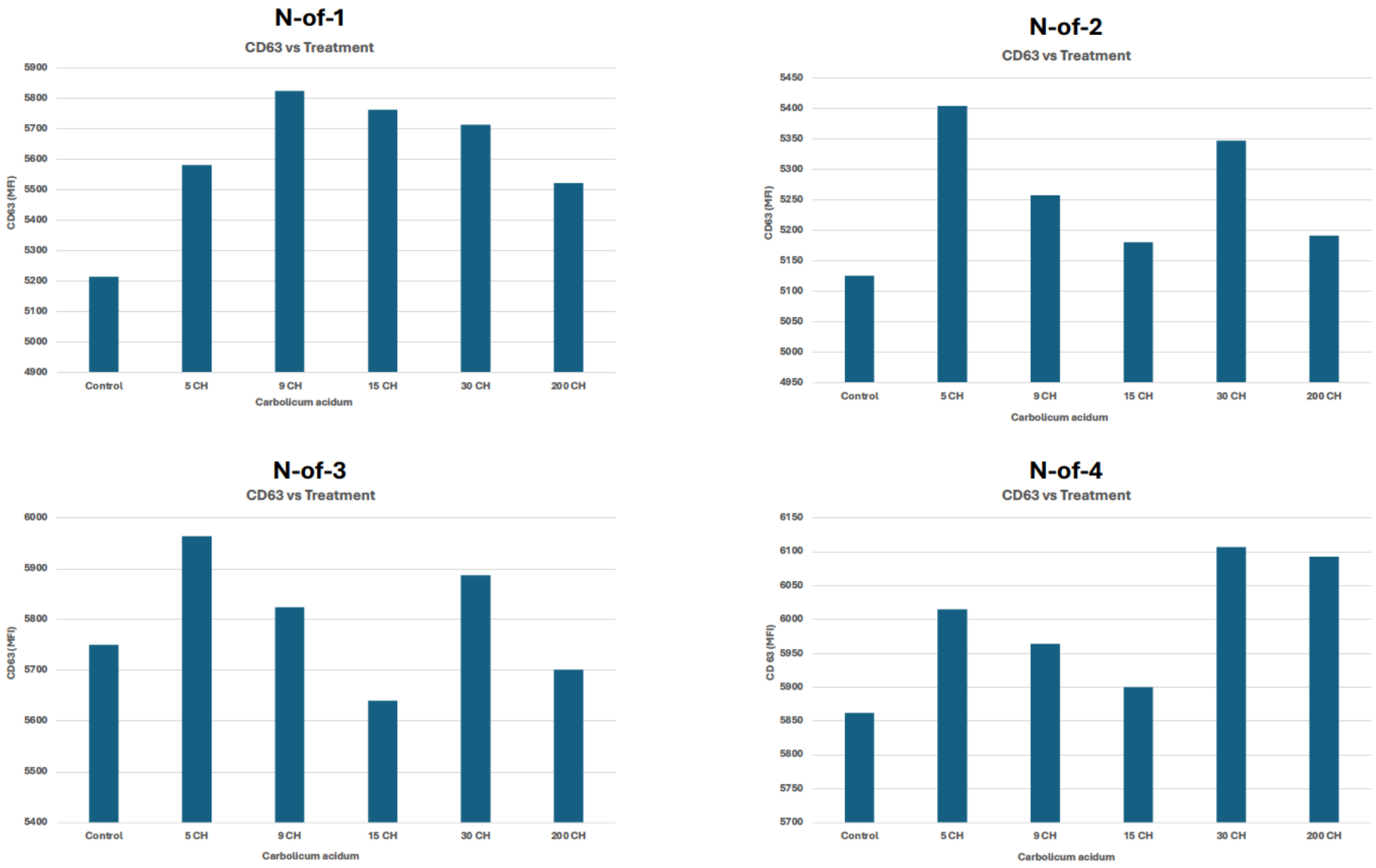

Figure 4-3 Bar graphs illustrating effects of *Carbolicum acidum* on activation marker CD63, demonstrating a dose-response relationship across the test groups (N-of-1 to N-of-4). Antibody MFI on the vertical axis is plotted as a function of *Carbolicum acidum* potencies on the horizontal axis.

Notable differences in CD63 expression were observed between the control group (0CH) and the treatment groups across all tested potencies, indicating variation in the activation marker CD63 expression in response to treatment. Figure 4.4 below shows the density plot of flow cytometric data corresponding to Figure 4.3.

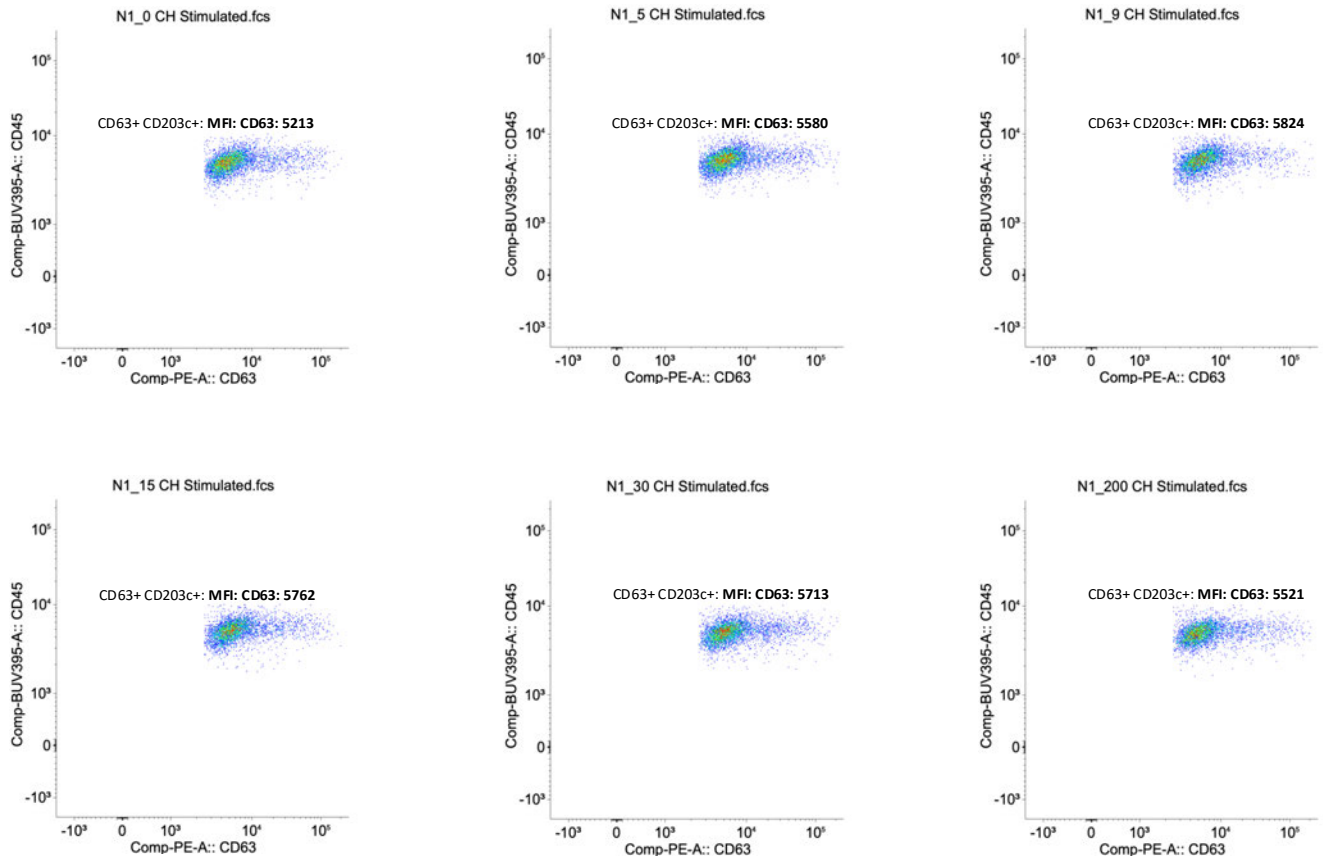

Figure 4-4 A density plot illustrating the median fluorescence intensity (MFI) of CD63 in N-of-1 samples in response to treatment. The density plots depicting the MFI of CD63 in other test groups in response to treatment are presented in Appendix H.

4.2.3 Dose-response pattern of *Carbolicum acidum* on activation marker CD69.

CD69 is an activation marker found in CD203c- granulocytes, and the controls of MFI for this marker were N-of-1 = 1302, N-of-2 =1297, N-of-3 = 1312, and N-of-4 = 1312.

5CH Treatment Group:

In this treatment group, when compared to their respective controls, there was an increase in MFI in the samples N-of-1 and N-of-2, while in N-of-3 the MFI remained constant. N-of-4 exhibited a slight decline in MFI relative to the control (Figure 4.5).

9CH Treatment Group:

A decline in the expression of CD69 was noted in all samples, falling below the levels observed in the 5CH group.

15CH Treatment Group:

This downward trend persisted into this treatment group, which demonstrated the greatest reduction in CD69 expression.

30CH and 200CH Treatment Groups:

At 30CH, the expression of CD69 was upregulated. An increase in MFI was observed. This increase continued to climb in the 200CH where MFI exceeded the control levels across all samples.

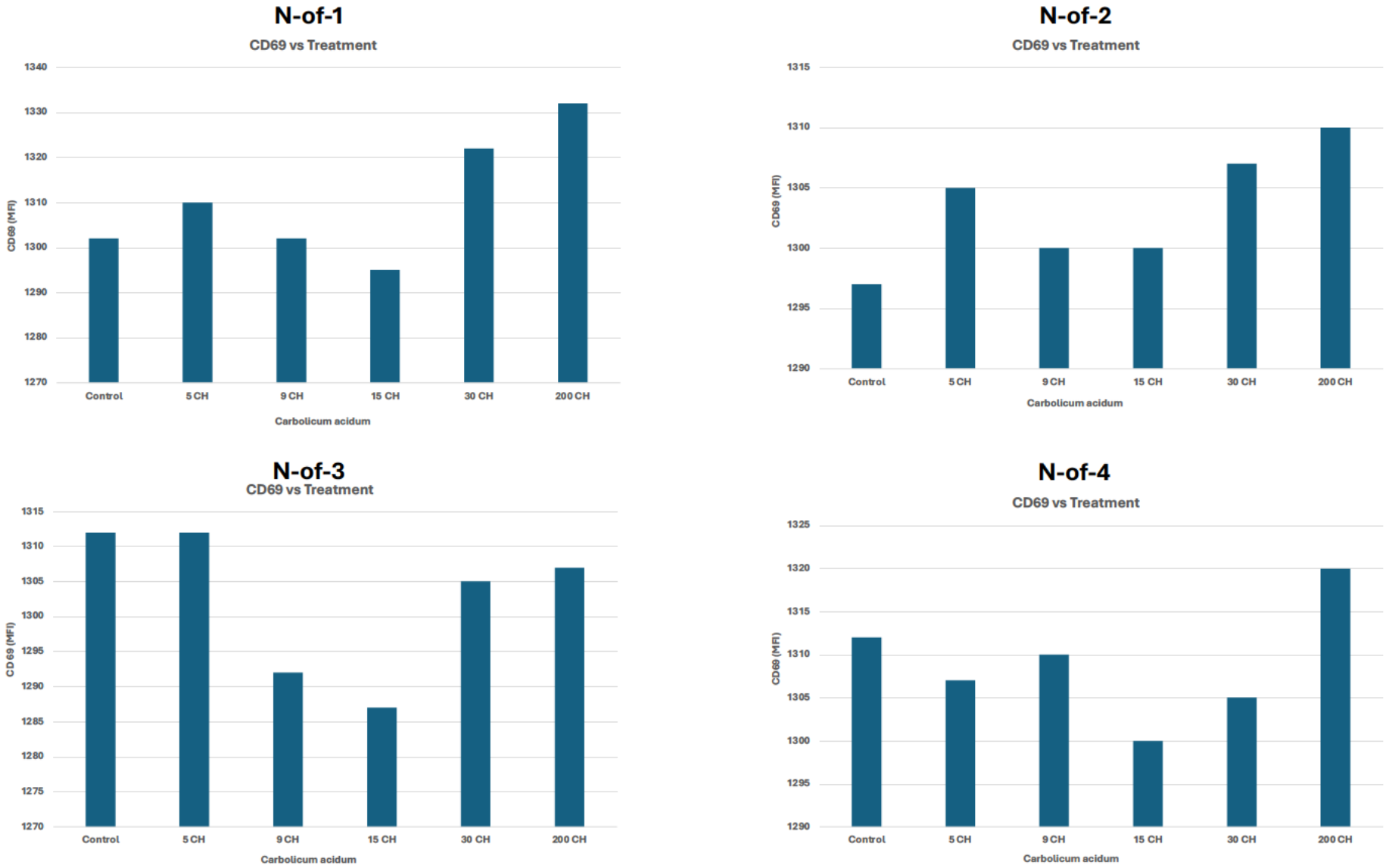

Figure 4-5 Bar graphs illustrating effects of *Carbolicum acidum* on activation marker CD69, demonstrating a dose-response relationship across the test groups (N-of-1 to N-of-4). Antibody MFI on the vertical axis is plotted as a function of *Carbolicum acidum* potencies on the horizontal axis.

Slight differences in CD69 expression were observed between the control (0CH) and treatment groups for all tested potency levels, indicating a change in activation marker CD69 expression in response to treatment. Figure 4.6 shows the density plot of flow cytometric data that corresponds to Figure 4.5.

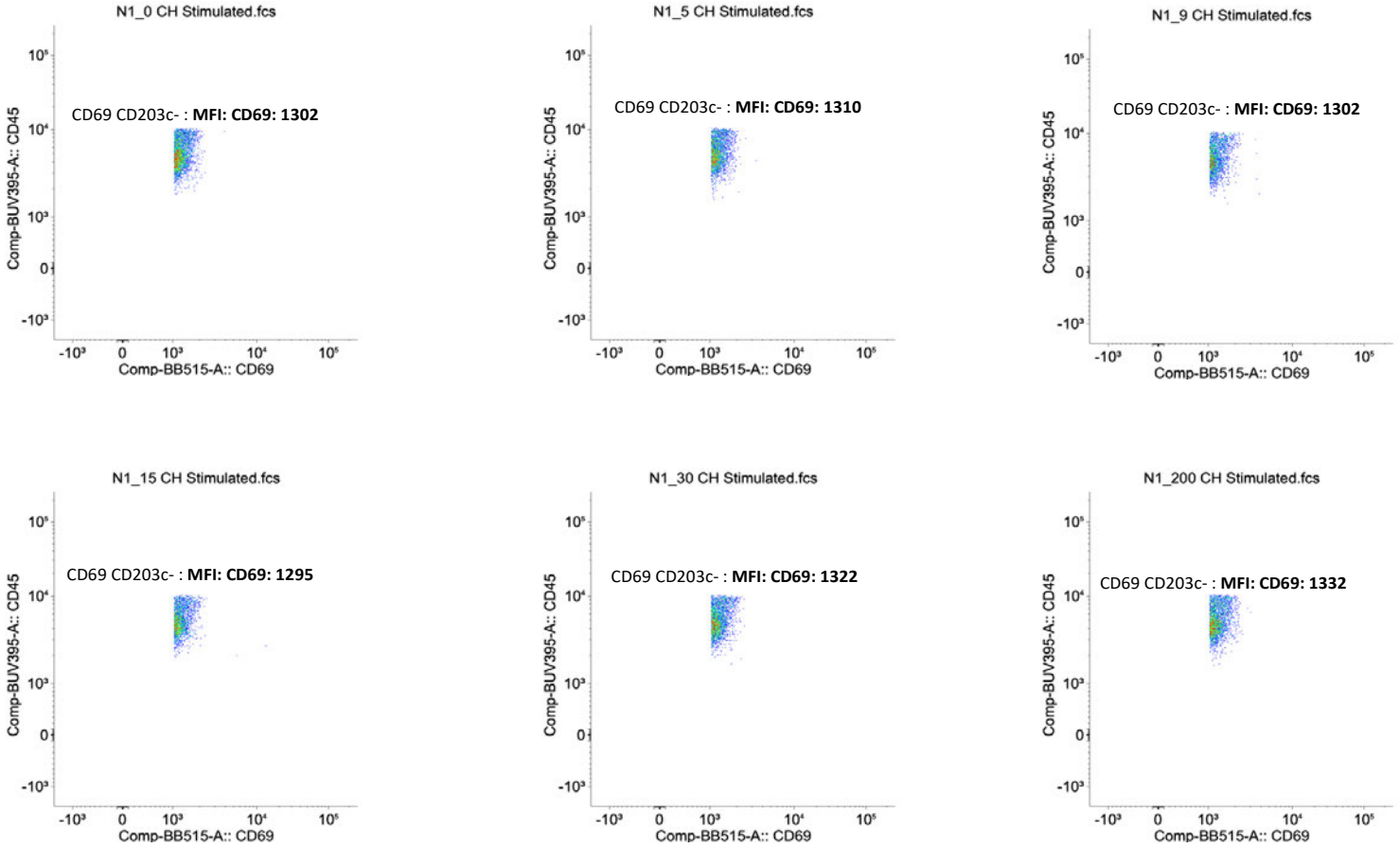

Figure 4-6 Density plot illustrating the MFI of CD69 in N-of-1 samples in response to treatment. The density plots showing the MFI of CD69 in other test groups in response to treatment are presented in Appendix I.

4.3 Summary of findings

The findings from this experiment demonstrated the therapeutic effects of different potencies of *Carbolicum acidum* (5CH, 9CH, 15CH, 30CH, 200CH) on chemical mediators of T-cells and granulocytes after they were stimulated with a leukocyte activation cocktail, resulting in both inhibitory and stimulatory actions on the expression of activation markers (MFI) at different potencies. This empirical evidence is considered reliable due to its consistency and reproducible protocol. As a result, it is regarded as useful in the scientific and homoeopathic domains.

4.4 Statistical analysis

A statistical analysis was conducted to assess the significance of the empirical data obtained from this experiment. A p-value of < 0.05 was considered to be statistically significant. One-way Analysis of Variance (ANOVA) was conducted in IBM SPSS Statistics (Version 30.0) to assess the effects of various treatments (5CH, 9CH, 15CH, 30CH, and 200CH) on activation marker expression, with comparison to the control group. A Tukey Honestly Significant Difference (HSD) post hoc was conducted for pairwise comparisons between treatment groups where statistical significance was observed.

4.4.1 One-way ANOVA for HLA-DR expression by Treatment Group.

A one-way ANOVA (Table 4.2 below) was conducted to assess the differences in HLA-DR expression across treatment groups. The analysis revealed a significant effect of treatment on HLA-DR expression, $F(3, 16) = 6.187$, $p = 0.005$, indicating that the treatment groups significantly differed from each other in terms of HLA-DR expression levels.

Table 4.2 ANOVA results for HLA-DR expression.

ANOVA					
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1409944.200	3	469981.400	6.187	0.005
Within Groups	1215376.800	16	75961.050		
Total	2625321.000	19			

The effect sizes for HLA-DR expression were calculated in Table 4.3. The Eta-squared statistic showed a moderate effect size (53.7%) suggesting a substantial proportion of the variance in HLA-DR expression can be attributed to the treatment effect. While Epsilon-squared showed a more variable estimate with a negative lower bound, and the Omega-squared estimate provided a more conservative effect size.

Table 4.3 Effect Size Estimates for HLA-DR expression.

ANOVA Effect Sizes [a,b]				
			95% Confidence Interval	
		Point Estimate	Lower	Upper
HLADR	Eta-squared	0.537	0.082	0.682
	Epsilon-squared	0.450	-0.090	0.622
	Omega-squared Fixed-effect	0.438	-0.085	0.610
	Omega-squared Random-effect	0.206	-0.027	0.343

a. Eta-squared and Epsilon-squared are estimated based on the fixed-effect model.
b. Negative but less biased estimates are retained, not rounded to zero.

In Table 4.4, the Tukey HSD post hoc test was performed to investigate pairwise differences in HLA-DR expression between the various treatment groups. Statistically significant differences were observed between the following pairs ($p < 0.05$):

- Control 7080 vs. 7349: Mean difference of -566.40, $p = 0.023$
- Control 7080 vs. 8063: Mean difference of -648.60, $p = 0.009$

These results suggested that specific treatment groups differed significantly in terms of HLA-DR expression, with the 7080-group showing a notably lower expression

compared to the other groups. Thus, the treatments associated with control 7080 seemed to produce the lowest HLA-DR scores, while control 7349 and 8063 groups were higher, potentially indicating differences in how these treatments affect HLA-DR expression.

Table 4.4 Tukey HSD Post-Hoc Comparisons for HLA-DR Expression.

Multiple Comparisons						
Dependent Variable: HLADR						
Tukey HSD						
		Mean			95% Confidence Interval	
(I) Control	(J) Control	Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
6762	7080	196.600	174.311	0.678	-302.11	695.31
	7349	-369.800	174.311	0.188	-868.51	128.91
	8063	-452.000	174.311	0.083	-950.71	46.71
7080	6762	-196.600	174.311	0.678	-695.31	302.11
	7349	-566.400*	174.311	0.023	-1065.11	-67.69
	8063	-648.600*	174.311	0.009	-1147.31	-149.89
7349	6762	369.800	174.311	0.188	-128.91	868.51
	7080	566.400*	174.311	0.023	67.69	1065.11
	8063	-82.200	174.311	0.964	-580.91	416.51
8063	6762	452.000	174.311	0.083	-46.71	950.71
	7080	648.600*	174.311	0.009	149.89	1147.31
	7349	82.200	174.311	0.964	-416.51	580.91
*. The mean difference is significant at the 0.05 level.						

Potency 30CH showed significant suppression of HLA-DR expression when compared to 15CH ($p = 0.023$) and demonstrated the most significant inhibition when compared to 200CH ($p = 0.009$). These findings suggest that all three potencies (15CH, 30CH, and 200CH) had inhibitory effects on HLA-DR expression, however, 30CH was the most effective potency in achieving this therapeutic effect. This indicates that 30CH had the highest potential for inhibiting chemical mediators associated with the activation marker HLA-DR.

Conversely, 9CH showed a stimulatory effect on the expression of HLA-DR marker ($p = 0.023$), highlighting its potential for promoting immune cell activation.

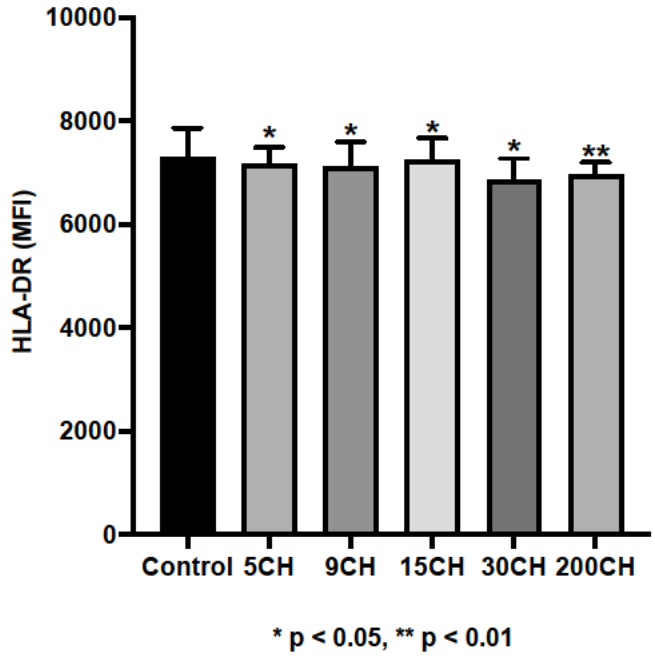

Figure 4-7 Effects of *Carbolicum acidum* on HLA-DR.

4.4.2 One-way ANOVA for CD63 expression by Treatment Group.

In Table 4.5 below, one-way ANOVA was performed to evaluate the differences in CD63 expression across treatment groups. The results indicated a statistically significant difference between the groups, F (3, 16) = 38.204, p <0.001, suggesting that the treatment groups differed significantly from one another in terms of CD63 expression levels.

Table 4.5 ANOVA results for CD63 expression.

ANOVA					
Source of Variance	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	1452833.800	3	484277.933	38.204	<0.001
Within Groups	202818.400	16	12676.150		
Total	1655652.200	19			

Effect sizes in Table 4.6 for CD63 were calculated using Eta-squared, Epsilon-squared, and Omega-squared statistics. The Eta-squared value suggested a very large effect size (approximately 87.7%) indicating that a significant portion of the

variance in CD63 expression is explained by the treatment effect. Epsilon-squared and Omega-squared estimates also supported the conclusion that the treatment groups substantially differed in terms of CD63 expression.

Table 4.6 Effect Sizes Estimates for CD63 Expression.

ANOVA Effect Sizes [a]				
			95% Confidence Interval	
		Point Estimate	Lower	Upper
CD63	Eta-squared	0.877	0.669	0.916
	Epsilon-squared	0.855	0.607	0.900
	Omega-squared Fixed-effect	0.848	0.595	0.895
	Omega-squared Random-effect	0.650	0.328	0.741
a. Eta-squared and Epsilon-squared are estimated based on the fixed-effect model.				

The Tukey HSD post-hoc test (Table 4.7 below) was conducted to assess the pairwise differences in CD63 expression between various treatment groups. Significant differences were observed in most pairs with $p < 0.05$, except for Control 5213 vs. 5750 ($p = 0.341$). The pairwise results in table 4.8 indicated significant differences in CD63 expression between multiple pairs of treatment groups, with several comparisons showing large mean differences, particularly between control 5125 and 5862 group. Overall, treatments in control group 5125 consistently showed lower CD63 levels when compared to the other groups, and the differences were statistically significant.

Table 4.7 Tukey HSD Post-Hoc Comparisons for CD63 Expression.

Multiple Comparisons						
Dependent Variable: CD63						
Tukey HSD						
		Mean			95% Confidence Interval	
(I) Control	(J) Control	Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
5125	5213	-404.200*	71.207	<0.001	-607.93	-200.47
	5750	-527.400*	71.207	<0.001	-731.13	-323.67
	5862	-740.000*	71.207	<0.001	-943.73	-536.27
5213	5125	404.200*	71.207	<0.001	200.47	607.93
	5750	-123.200	71.207	0.341	-326.93	80.53
	5862	-335.800*	71.207	0.001	-539.53	-132.07
5750	5125	527.400*	71.207	<0.001	323.67	731.13
	5213	123.200	71.207	0.341	-80.53	326.93
	5862	-212.600*	71.207	0.039	-416.33	-8.87
5862	5125	740.000*	71.207	<0.001	536.27	943.73
	5213	335.800*	71.207	0.001	132.07	539.53
	5750	212.600*	71.207	0.039	8.87	416.33
*. The mean difference is significant at the 0.05 level.						

Potency 15CH significantly inhibited the expression of activation marker CD63 ($p < 0.001$). 200CH also demonstrated inhibitory effects on CD63 expression ($p < 0.001$). In contrast, potency 5CH showed the most stimulatory effect on the expression of CD63, also displaying *Carbolicum acidum*'s potential for promoting cellular immune response at lower potencies.

The expression of CD63 in $CD203c^{+}$ granulocytes was slightly elevated in the test group N-of-1, N-of-2, and N-of-4 compared to their respective controls following the stimulatory phase induced by the 5CH potency. The expression of CD63 remained consistently above the control level in these groups though a decreasing trend in the expression of chemical mediators was noted as the potencies increased. This therapeutic trend was however evident in N-of-3, which demonstrated a reduction in chemical mediator expression below control levels as the potency increased.

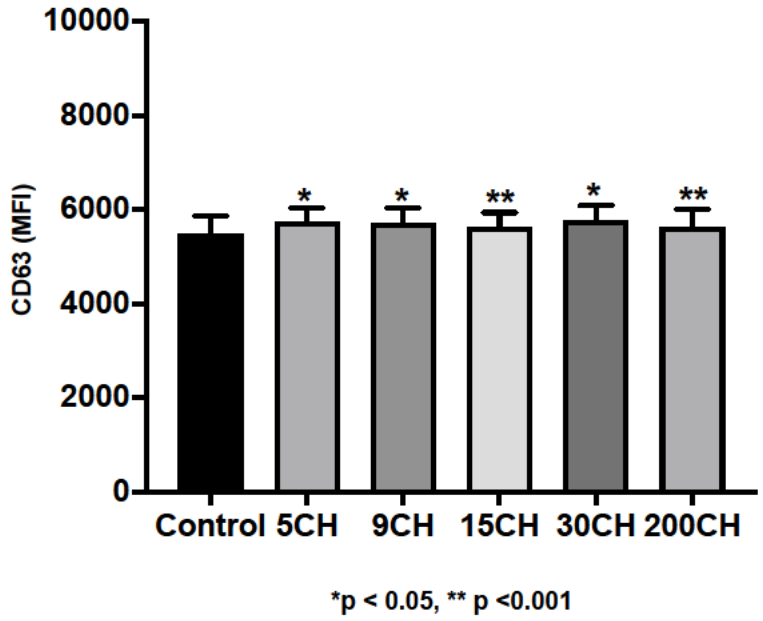

Figure 4-8 Effects of *Carbolicum acidum* on CD63.

4.4.3 One-way ANOVA for CD69 expression by Treatment Group.

A one-way analysis of variance (Table 4.8) was conducted to assess the differences in CD69 expression across treatment groups. The results showed no statistically significant differences between the groups (F (2, 17) = 1.064, p = 0.367), indicating that the treatment groups did not differ in terms of CD69 expression levels.

Table 4.8 ANOVA results for CD69 expression.

ANOVA					
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	224.300	2	112.150	1.064	.367
Within Groups	1792.500	17	105.441		
Total	2016.800	19			

The effect sizes (Table 4.9) for CD69 expression were indicated to be small. Eta-squared was 11.1%, suggesting that only a small proportion of variance in expression of CD69 was explained by the treatment effect. Epsilon-squared and Omega-squared were both extremely small, further confirming that the treatment effect was too small to explain the variance in CD69 expression. Overall, the effect size estimates

suggested that the treatment had minimal influence on CD69 expression, and the results were not practically significant, aligning with the findings from the ANOVA ($p = 0.367$). The mean differences between the groups were small, CD69 expression appeared to be similar across the groups tested. The post hoc test was not performed due to the insignificant ANOVA results.

Table 4.9 Effect Size Estimates for CD69 Expression.

ANOVA Effect Sizes [a,b]				
			95% Confidence Interval	
		Point Estimate	Lower	Upper
CD69	Eta-squared	0.111	0.000	0.343
	Epsilon-squared	0.007	-0.118	0.265
	Omega-squared Fixed-effect	0.006	-0.111	0.255
	Omega-squared Random-effect	0.003	-0.053	0.146

a. Eta-squared and Epsilon-squared are estimated based on the fixed-effect model.
b. Negative but less biased estimates are retained, not rounded to zero.

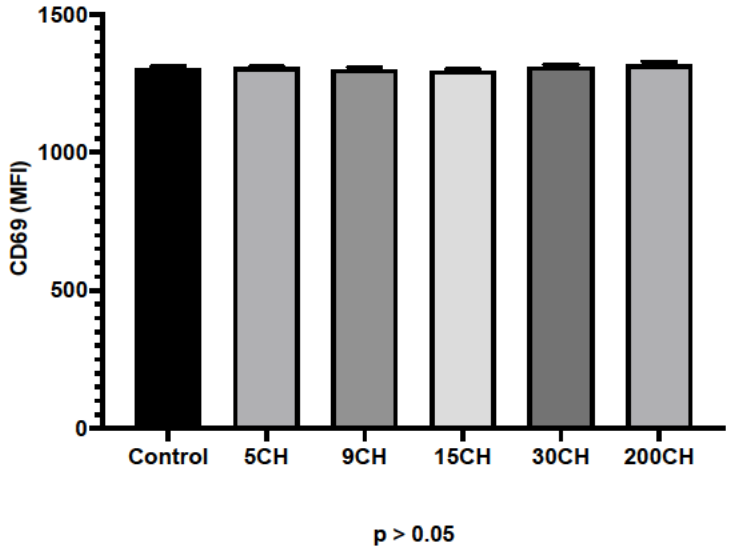

Figure 4-9 The effects of *Carbolicum acidum* on CD69.

4.5 Supplementary results on monocytes

While monocytes were not the primary focus of this study, an interesting observation was made on the effect of *Carbolicum acidum* on their activation marker. Monocytes accounted for 3.29% (SD = 0.33%) of the live leukocytes that were gated for analysis. These cells also displayed the monocytic HLA-DR marker (to be denoted mHLA-DR) on their surfaces when stimulated, but this occurs quite early in their activation process and the expression of this marker in monocytes is quite low when compared to the lymphocytes in this study (T-cells) and it is tightly regulated (Hammad et al., 2022). While mHLA-DR in monocytes is expressed to a variable extent, it has been shown that on peripheral blood monocytes, mHLA-DR is expressed at high levels.

One-way ANOVA analysis (Table 4.10) produced significant results when assessing the differences in the expression of mHLA-DR on monocytes across the treatment groups (p = 0.006). About 53.2% of the variance in mHLA-DR expression could be explained by the treatment effect according to the Eta-squared statistic (Table 4.11).

Table 4.10 ANOVA Results for Monocytes mHLA-DR.

ANOVA					
mHLADR					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	1636072.400	3	545357.467	6.060	0.006
Within Groups	1439968.400	16	89998.025		
Total	3076040.800	19			

Table 4.11 Effect Size Estimates for Monocytes mHLA-DR Expression.

ANOVA Effect Sizes[a,b]				
			95% Confidence Interval	
		Point Estimate	Lower	Upper
mHLADR	Eta-squared	0.532	0.077	0.678
	Epsilon-squared	0.444	-0.096	0.618
	Omega-squared Fixed-effect	0.431	-0.091	0.606
	Omega-squared Random-effect	0.202	-0.029	0.339

a. Eta-squared and Epsilon-squared are estimated based on the fixed-effect model.
b. Negative but less biased estimates are retained, not rounded to zero.

The Tukey HSD post hoc comparisons were used to identify the specific group that differed significantly in terms of mHLA-DR expression in monocytes (Table 4.12).

Table 4.12 Tukey HSD Post-Hoc Comparisons for Monocytes mHLA-DR Expression.

Multiple Comparisons						
Dependent Variable: mHLADR						
Tukey HSD						
		Mean			95% Confidence Interval	
(I) Control	(J) Control	Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
7496	7663	-552.800*	189.735	0.045	-1095.63	-9.97
	7869	-771.000*	189.735	0.005	-1313.83	-228.17
	8562	-564.200*	189.735	0.040	-1107.03	-21.37
7663	7496	552.800*	189.735	0.045	9.97	1095.63
	7869	-218.200	189.735	0.665	-761.03	324.63
	8562	-11.400	189.735	1.000	-554.23	531.43
7869	7496	771.000*	189.735	0.005	228.17	1313.83
	7663	218.200	189.735	0.665	-324.63	761.03
	8562	206.800	189.735	0.700	-336.03	749.63
8562	7496	564.200*	189.735	0.040	21.37	1107.03
	7663	11.400	189.735	1.000	-531.43	554.23
	7869	-206.800	189.735	0.700	-749.63	336.03
*. The mean difference is significant at the 0.05 level.						

The post hoc test compared the mHLA-DR expression levels across the various control groups. Statistically significant differences were observed between the treatment potencies in control group 7496 and other control groups (7663, 7869, and 8562), with control group 7496 consistently showing lower mHLA-DR expression. In contrast, no significant differences were found between control group 7663 and control groups 7869 and 8562, nor between control groups 7869 and 8562.

CHAPTER FIVE

DISCUSSION

5.1 Introduction

An *in vitro* experimental study design was used to investigate the effects of different potencies of *Carbolicum acidum* on the release of chemical mediators from human blood T-cells and granulocytes. Since T-cells and granulocytes are involved in several autoimmune, inflammatory, and allergic diseases, it was of great interest to investigate the level of activation of these cells *in vitro* to evaluate their response to homoeopathic treatment. The BD leukocyte activation cocktail was used to stimulate the cells, resulting in cellular activation and the release of chemical mediators. Flow cytometry was then used to assess the upregulation of activation markers on cell surfaces to determine the level of chemical mediator expression. This approach allowed for the identification and quantification of cell surface markers HLA-DR, CD63, and CD69, which are associated with cell activation and release of chemical mediators during an allergic response (Sun *et al.* 2023; Hartnell, 1993; Knol *et al.* 1991; Uyttebroek *et al.* 2014). The treatment effect on these markers was investigated using a flow cytometer, which also allowed for precise monitoring of changes in marker expression levels in response to stimulation and treatment with *Carbolicum acidum*. An increase in levels of HLA-DR, CD63, and CD69 surface markers meant that *Carbolicum acidum* was able to activate their expression on the surfaces of T-cells and granulocytes, while decreased levels of these activation markers meant that *Carbolicum acidum* inhibited their expression. This chapter will be based on the findings that were presented in Chapter 4.

5.2 Evaluation of the study hypothesis

This study was based on the hypothesis that higher potencies will have inhibition effects on the expression of chemical mediators, while lower potencies will have a stimulatory effect. The homoeopathic principle of infinitesimals served as the foundation for this, stating that higher remedy potencies have higher curative powers (Sagar, 2007). Additionally, the results were interpreted in the context of the Arndt-Schulz law, or hormesis, which states that the effects of an agent on biological systems

will exhibit a dose-response relationship (Henschler, 2006). According to this principle, low doses of an agent can have stimulatory effects, promoting activation and chemical mediator release, while higher doses may exert inhibitory effects, lessening these responses (Henschler, 2006). This was highlighted by how *Carbolicum acidum* modulated the activation markers in a dose-dependent manner, indicating that the potencies tested in this study had different effects on the activation markers. Heightening of these activation markers has been linked to symptomatic manifestation of allergic diseases (Wang *et al*., 2023) and may be severe in some cases, resulting in anaphylaxis (Wang *et al*., 2023). Therefore, the use of *Carbolicum acidum* in high potencies could be beneficial in modulating allergic reactions since the findings suggest that high potencies have suppressing effects on the chemical mediators associated with activation markers HLA-DR, CD63, and CD69.

Furthermore, the objectives of this study were to investigate the effects of *Carbolicum acidum* 5CH, 9CH, 15CH, 30CH, and 200CH on granulocytes and T-cells chemical mediators and then compare the effects of low potencies (5CH, 9CH) to high potencies (15CH, 30CH, 200CH). The study findings showed that *Carbolicum acidum* had significant effects ($p < 0.05$) on the expression of activation markers which are associated with chemical mediator release and demonstrated a dose-response relationship of *Carbolicum acidum* to the release of chemical mediators. Where high potencies of *Carbolicum acidum* exerted a greater inhibitory effect on chemical mediator release while low potencies had a stimulatory effect on the release of chemical mediators, proving the hypothesis that underpinned this study. Thus, this suggested the action of *Carbolicum acidum* on modulating the allergic responses by stimulating the release of chemical mediators in low potencies (5CH and 9CH) and inhibiting them in high potencies (15CH, 30CH, and 200CH). These findings support the conceptual framework outlined in Chapter 2, which indicates that in a biological system, low doses of an agent will have stimulatory function leading to an allergic reaction response, whereas high doses will be inhibitory, resulting in healing (Bell and Khoithan, 2012). In addition, these findings are similar to previous research where high homoeopathic potencies were shown to exhibit an inhibitory action on cellular models, while lower potencies were stimulatory (Poitevin, 1988; Bellavite *et al.* 2006; Ive, 2010). Based on the findings, it can be postulated that the selection of *Carbolicum acidum* potencies in therapeutic applications can be made according to the desired

therapeutic outcome. This implies that higher potencies may be selected and used when a suppression of granulocytes and T-cells is needed to inhibit the allergic reactions.

5.3 Effects of *Carbolicum acidum* 5CH, 9CH, 15CH, 30CH, and 200CH

According to the ANOVA and Tukey HSD analyses, the treatment that showed the largest statistically significant difference from the control group would be the most inhibiting. The 30CH potency exhibited statistically significant inhibitory effect ($p < 0.05$) on the expression of chemical mediators associated with HLA-DR, followed by 15CH and 200CH. Additionally, both 15CH and 200CH showed a statistically significant inhibition ($p < 0.001$) of CD63 expression compared to their respective control groups. This finding suggests that *Carbolicum acidum* 15CH, 30CH, and 200CH can be used to inhibit the expression of chemical mediators involved in allergic reactions, thus preventing the symptoms associated with allergic diseases. This is a desired outcome, as research has shown that treatment against allergic diseases is targeted at preventing degranulation and further release of chemical mediators from immunological cells (Brown, Simons, and Rudders, 2020). Mannaioni, Mastroianni, and Mastrangelo's study (2010) also showed that activation of basophils was significantly decreased by epinephrine at ultra-diluted doses by blocking the expression of CD63. Similar effects were achieved by the current study using high dilutions of *Carbolicum acidum*. When *Carbolicum acidum* was tested on CD69, it did not yield statistically significant results ($p > 0.05$) because the mean effects of potencies tested were closely aligned, indicating that very little variance in chemical mediator expression of CD69 could be attributed to the treatment effect. A trend toward efficacy was observed in the empirical data, with 15CH, 30CH, and 200CH displaying inhibitory effects; however, these effects were not statistically significant.

Previous research (Poitevin, 1988; Bellavite *et al.* 2006) investigated the impact of homoeopathic remedies *Apis mellifica* and *Lung Histaminum* on basophil activity using potencies 1CH to 15CH. The study revealed that 5CH induced basophil activation, whereas 9CH and 15CH inhibited basophils from degranulating (Poitevin, 1988), highlighting the effects of low and high potencies of homoeopathic remedies on cells.

These findings were similar to the results of the current study, where *Carbolicum acidum* 15CH, 30CH, and 200CH demonstrated statically significant inhibition of activation marker expression on T-cells and granulocytes. Additionally, while Poitevin observed a consistent stimulatory effect with 5CH potency, the present study produced more heterogeneous results where 5CH resulted in both inhibitory and stimulatory effects, with stimulatory effects observed in approximately 67% of the samples. Furthermore, 9CH potency also yielded both stimulatory and inhibitory effects, with stimulatory effects observed in approximately 70% of the samples. The variations observed between these studies may be due to methodological differences since Poitevin (1988) used purified cell populations, while this current study investigated cells in whole blood. The latter closely reflects the physiological environment of a living organism where other elements present in whole blood may have influenced these minor differences. Despite these variations, both studies had comparable low and high potency effects.

In a study conducted by Ive (2010), 200CH of *Arsenicum album* was shown to increase cell viability after the cells were exposed to arsenic *in vitro*, whereas 6CH potency resulted in the lowest absorbance readings. This suggests that higher potencies promoted greater cell recovery compared to lower potencies. In the present study, potencies 200CH, 30CH, and 15CH exhibited curative effects in terms of inhibiting the expression of activation markers associated with allergic reactions, further supporting the principle observed in this study that high potencies have inhibitory properties in biological systems.

Furthermore, previous studies on the effect of homoeopathic remedies have largely focused only on basophils (Poitevin, 1988; Poitevin, 1998; Benveniste, 1994; Lorenz *et al.* 2003; Belon, 2004; Guggisberg *et al.* 2005) and other immune cells (eosinophils, neutrophils, and mast cells) separately (Bellavite *et al.* 2006). In contrast, this current study investigated a subset of leukocytes, specifically granulocytes (including basophils as $CD203c^{+}$, eosinophils and neutrophils as $CD203c^{-}$) as well as T-cells in human whole blood under the same homoeopathic remedy *Carbolicum acidum*. By investigating both granulocyte and T-cell populations within a homoeopathic cell model, this study considered the interplay between the immune cells during allergic reactions, as evidence suggests that an interaction between these cells plays a crucial

role in immune responses (Metcalfe *et al.* 2016). An inhibition of activation markers in both granulocytes and T-cells, as observed in this current study, suggested the prevention of chemotactic effects between these cells, thereby arresting the allergic reaction.

5.4 Clinical application of the study findings

Clinically, this suggests that *Carbolicum acidum* 15CH, 30CH, and 200CH may be effective in treating allergic reactions by preventing the manifestation of symptoms that are typically associated with the activation of immune cells and the release of chemical mediators, as these potencies have been shown to inhibit the expression of chemical mediators from the immunological cells. This is regarded as beneficial because medications such as epinephrine act on immunological cells to inhibit the secretion of chemical mediators from allergy effector cells, thus treating allergic reactions (Brown, Simons, and Rudders, 2020). In documented clinical cases in homoeopathic practice, anaphylactic reactions to insect bites were successfully treated with *Carbolicum acidum* M (1000CH), resulting in symptom relief within minutes of administration. In one of these cases, subsequent treatment with potency 200CH was prescribed one year later to address mild allergic rhinitis, leading to the complete resolution of symptoms with this potency (Paschmanns, 2013).

With the increasing prevalence of allergic diseases worldwide, there has been a growing interest in alternative therapies such as homoeopathy (Bonizzoni *et al*. 2019; Yonekura *et al*. 2017; Grundling, Schimetta, and Frass, 2012), driven in part by the high costs of conventional treatment options which are too expensive for many individuals (Levin, 2012). Homoeopathy has been shown to be inexpensive and accessible (Majola, 2020) and should be taken into consideration as a viable alternative for allergic diseases within the broader context of public health management.

While current therapies for severe allergic conditions remain effective and irreplaceable, *Carbolicum acidum* may offer a valuable alternative for individuals who are either unable to afford conventional treatments or who experience adverse reactions to them. In such cases, *Carbolicum acidum* may provide symptom relief and

therapeutic benefit, thereby improving quality of life for these individuals and assisting in reducing the medical cost burden associated with conventional treatment options.

5.5 Limitations of study

The pooled blood sample that was used in this study was collected from different donors; therefore, the results of this study reflected the average response of the pooled immune cells to *Carbolicum acidum*. They did not fully capture the biological variability between individual donors. Nevertheless, the pooled blood provided useful insights into the overall collective behaviour of granulocytes and T-cells in response to *Carbolicum acidum*. Its dose-dependent effects were still meaningful, in that it was stimulatory at low potencies and inhibitory at high potencies. The observed treatment patterns thus suggested a clear therapeutic effect of *Carbolicum acidum* which was likely to be consistent across the individual donors included in the blood pool.

CHAPTER SIX

CONCLUDING REMARKS AND RECOMMENDATIONS

6.1 Concluding remarks

The key finding in this current study was that *Carbolicum acidum* 200CH, 30CH, and 15CH were found to inhibit the expression of chemical mediators of granulocytes and T-cells ($p < 0.05$). In contrast, 9CH and 5CH had mixed effects, primarily showing stimulatory responses and inhibitory effects in some samples. This biphasic response pattern highlights the importance of selecting the appropriate potency of *Carbolicum acidum* for therapeutic applications, depending on whether a stimulating or inhibiting outcome on immune cells is desired.

No published data exists to date regarding the influence of *Carbolicum acidum* on the chemical mediators involved in allergic reactions at cellular level. This gap in literature was addressed by this present study. The therapeutic effects of *Carbolicum acidum* observed in this study were consistent with its primary clinical indications, specifically anaphylactic reactions, as documented in the homoeopathic Materia Medica, repertories and supported by prior drug provings (Murphy, 2000; Morrison, 2006; Phatak, 1999; Klein, 2003; Schroyens, 2016; Paschmanns, 2013).

Furthermore, this research marks an important step in confirming the efficacy of homoeopathic remedies in cellular models. Being the first study of its kind in South Africa, it established a foundation for future research and highlights the potential of flow cytometry as a powerful tool for advancing homoeopathic research in the country.

The World Health Organization has recognised allergic diseases as one of the top three conditions requiring attention and control (World Health Organization, 2024), especially as they are becoming a growing health burden in developing countries (Gupta *et al.* cited in Chippendale, 2018). The findings of this current study highlighted that the therapeutic effects of a homoeopathic remedy can help to inhibit the chemical mediators that are involved in allergic diseases. Therefore, encouraging the use of alternative medical therapies like homoeopathy for managing allergic diseases within the broader context of public health.

6.2 Recommendations

The incidence of allergic diseases has been reported to be constantly increasing in the world, with a marked increase seen among children and young adults, particularly in developing countries (Ndjindji *et al.* 2023; Hossny *et al.* 2019). This prompts for alternative therapies, including homoeopathy, to be considered as possible modalities for the treatment of allergic diseases while supporting existing conventional therapies. The actions of *Carbolicum acidum* on chemical mediators of allergy have been highlighted by this *in vitro* study. Further research should involve:

- Exploring the inclusion of homoeopathic treatments in the national treatment guidelines for the management of allergic diseases.

- An epidemiological investigation on the prevalence of allergies in South Africa, exploring how they are managed by homoeopathic practitioners and at the community level.

REFERENCES

Allied Health Professions Council of South Africa. 2024. (Online) Available: https://ahpcsa.co.za

Alsved, J., Rezayati Charan, M., Ohlsson, P., Urbansky, A., and Augustsson, P. 2024. Label-free separation of peripheral blood mononuclear cells from whole blood by gradient acoustic focusing. *Sci Rep.* 14:8748.

Appel, S., and Jonsson, R. 2016. Chapter 15 - Cytokines, Chemokines, and the Innate Immune System. In: Gerli, R., Bartoloni, E., and Alunno, A. *Sjögren's Syndrome: Novel Insights in Pathogenic, Clinical and Therapeutic Aspects.* Academic Press. 229-239. Available:https://www.sciencedirect.com/science/article/abs/pii/B9780128036044000150#preview-section-abstract

Arango, M., Quintero-Ronderos, P., Castiblanco, J., and Montoya-Ortiz, G. 2013. Section V: Basic Principles of Laboratory: Cell Culture and Cell Analysis. In: Anaya, J.M., Shoenfield, Y., Rojas-Villarraga, A., Levy, R., and Cervera, R. Autoimmunity: From Bench to Bedside. Colombia: El Rosario University Press.

Asai, Y., Yanishevsky, Y., Clarke, A., La Vieille, S., Delaney, J., Alizadehgar, R., Joseph, L., Mill, C., Morris, J., and Ben-Shoshan, M. 2014. Triggers, Severity and Management of Anaphylaxis in Adults Treated in a Canadian Emergency Department. *Int Arch Allergy Immunol*. 164:246-252.

Banerjee, D. 2006. *Augmented Textbook of Homoeopathic Pharmacy*. India: B. Jan Publishers (P) Ltd.

Barone, J., and Castro, M.A. 2016. *USMLE Step 1: Pathology*. New York: Kaplan Medical.

BD Biosciences. 2024. Protocol Library. Available: https://www.bdbiosciences.com/en-es/resources/protocols

BD Biosciences. 2024. Applications & Techniques: Multicolor Flow Cytometry. Available: https://www.bdbiosciences.com/en-eu/learn/applications/multicolor-flow-cytometry?tabs=Overview

BD Biosciences. 2023. Technical Data Sheet. Leukocyte Activation Cocktail 550583.

Bell, I.R., and Koithan, M. 2012. A model for homeopathic remedy effects: low dose nanoparticles, allostatic cross-adaptation, and time dependent sensitization in a complex adaptive system. *BMC Complementary & Alternative Medicine*. 12(191):1-21.

Bellavite, P., Conforti, A., and Ortolani, R. 2006. Immunology and Homeopathy: 2. Cells of the Immune System and Inflammation. *Evidence-based Complementary and Alternative Medicine*. 3(1):13-24.

Belon, P., Cumps, J., Ennis, M., Mannaioni, P.F., Roberfroid, M., Sainte-Laudy, J., and Wiegant, F.A. 2004. Histamine dilutions modulate basophil activation. *Inflamm Res.* 53(5):181-8.

Bonizzoni, G., Caminati, M., Ridolo, E., Landi, M., Ventura, M.T., Lombardi, C., Senna, G., Crivellaro, M., and Gani, F. 2019. Use of complementary medicine among patients with allergic rhinitis: an Italian nationwide survey. *Clin Mol Allergy*. 17:1-3.

British Association of Homoeopathic Manufacturers. 1993. *British Homoeopathic Pharmacopoeia: (B. Hom. P.).* United Kingdom. British Ass of Homoeopathic Manufacturers.

Bui, D. V., Nguyen, L. M., Kanda, A., Chu, H. H., Thi Le, N. K., Yun, Y., Kobayashi, Y., Suzuki, K., Mitani, A., Shimamura, A., Fukui, K., Sawada, S., Dombrowicz, D., and Iwai, H. 2024. CD69 Signaling in Eosinophils Induces IL-10 Production and Apoptosis via the Erk1/2 and JNK Pathways, Respectively. *Biomolecules*, *14*(3): 360.

Bushnell, T. 2015. *Modern Flow Cytometry.* Excyte. Available: www.ExpertCytometry.com

Calabrese, E.J. 2013. Biphasic dose responses in biology, toxicology and medicine: Accounting for their generalizability and quantitative features. *Environmental Pollution*. 182: 452-460.

Calabrese, E.J., and Baldwin, L.A. 2001. U-shaped dose-responses in biology, Toxicology, and public health. *Annu. Rev. Public Health.* 22:15–33.

Calabrese, E.J., and Giordano, J. 2021. Ultra-low doses and biological amplification: Approaching Avogadro's number. *Pharmacol Res*. 170:105738.

Campbell, R.L., Park, M.A., Keuber, M.AA., Lee, S., and Hagan, J.B. 2015. Outcomes of allergy/immunology follow-up after an emergency department evaluation for anaphylaxis. *J. Allergy Clin Immunol Pract*. 3(1):88-93

Caruso, A., Licenziati, S., Corulli, M., Canaris, A.D., De Francesco, M.A., Fiorentini, S., Peroni, L., Fallacara, F., Dima, F., Balsari, A., Turano, A. 1997. Flow cytometric analysis of activation markers on stimulated T cells and their correlation with cell proliferation. *Cytometry.* 27(1):71-6.

Chippendale, S. 2018. Severe Allergic Reactions at a Tertiary Paediatric Service 2014-2016. MMed (Paediatrics), University of Cape Town.

Chippendale, S., Reichmuth, K., Worm, M., and Levin, M. 2022. Paediatric anaphylaxis in South Africa. *World Allergy Organization Journal*. 15:100666.

Clark, S., Espinola, J., Rudders, S.A., Banerji, A., and Camargo, C.A. 2011. Frequency of US emergency department visits for food-related acute allergic reactions. *J Allergy Clin Immunol*. 127(3):682-683.

Conrado, A.B., Ierodiakonou, D., Gowland, M., Boyle, R., and Turner, P. 2021. Food anaphylaxis in the United Kingdom: analysis of national data 1998-2018. *BMJ.* 372:n251.

Dagur, P.K., and McCoy, J.P. 2016. Collection, Storage, and Preparation of Human Blood Cells. *Current Protocols in Cytometry*. 73(1): 5.1.1 – 5.1.16

Dami, F., Enggist, R., Comte, D., and Pasquier, M. 2022. Underuse of Epinephrine for the Treatment of Anaphylaxis in the Prehospital Setting. *Emergency Medicine International*. Volume 2022.

Das, E. 2016. Introduction to homeopathic pharmacopeia. (online). Available: https://www.nhp.gov.in/introduction-to-homeopathic-pharmacopeia_mtl (Accessed 03 July 2020).

Davoine, F. and Lacy, P. 2014. Eosinophil cytokines, chemokines, and growth factors: emerging roles in immunity. *Frontiers in Immunology*. 5(570).

Delmonte, O.M., and Fleisher, T.A. 2019. Flow cytometry: Surface markers and beyond. *Journal of Allergy and Clinical Immunology*. 143(2): 528 – 537.

Dieckmann, D., Plottner, H., Berchtold, S., Berger, T., and Schuler, G. 2001. Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. *J Exp Med*. 193(11):1303-1310.

Dierick, B.J.H., van der Molen, T., Flokstra-de Blok, B.M.J., et al. 2020. Burden and socioeconomics of asthma, allergic rhinitis, atopic dermatitis and food allergy. *Pharmacoeconomic Outcomes.* 20: 437-53.

Drofenik, M. 2022. A New Explanation of Homeopathy. *Prensa Med Argent*. 108:1

Dvorak, A.M. 2013. *Blood Cell Biochemistry: Basophil and Mast Cell Degranulation and Recovery*. New York: Springer-verlag New York Inc.

Durban University of Technology. 2024. Community Engagement Projects. (Online) Available at https://www.dut.ac.za/engagement/dce-projects/

Eberlein, B., Hann, R., Eyerich, S., Pennino, D., Ring, J., Schmidt-Weber, C.B. and Buters, J. 2015. Optimizing of the Basophil Activation Test: Comparison of Different Basophil Identification Markers. *Cytometry Part B: Clinical Cytometry*. 88B:183–189.

Ericksen-Pereira, W.G., Roman, N.V., and Swart, R. 2018. An overview of the history and development of naturopathy in South Africa. *Health SA Gesondheid.* 23: 1-8.

Fischer, D., van der Leek, T.K., Ellis, A.K., and Kim, H. 2018. Anaphylaxis. *Allergy, Asthma & Clinical Immunology*. 14(54):63-70.

Gauvreau, G.M., El-Gammal,A.I., and O'Byrne, P.M. 2015. Allergen-induced airway response. *European Respiratory Journal*. 46:819-831.

Goetz, C., Hammerbeck, C. and Bonnevier, J. 2018. Flow Cytometry Basics for the Non-Expert. Switzerland. Springer Nature.

Goetzl, E.J., and Austen, K.F. 1975. Purification and synthesis of eosinophilotactic tetrapeptides of human lung tissue: Identification as eosinophil chemotactic factor of anaphylaxis. *Proc Natl Acad Sci*. 72:4123-4127.

Gray, C.L. 2017. Food Allergy in South Africa. *Curr Allergy Asthma Rep*.

Gründling C, Schimetta W, Frass M. 2012. Real-life effect of classical homeopathy in the treatment of allergies: A multicenter prospective observational study. *Wien Klin Wochenschr*. 124(1–2):11–7.

Guggisberg, A.G., Baumgartner, S.M., Tschopp, C.M., and Heusser, P. 2005. Replication study concerning the effects of homeopathic dilutions of histamine on human basophil degranulation *in vitro*. *Complement Ther Med*. 13(2):91-100.

Gupta, R., Holdford, D., Bilaver, L., Dyer, A., Holl, J.L., and Meltzer, D. 2013. The Economic Impact of Childhood Food Allergy in the United States. *JAMA Pediatrics* 167(11).

Gutowska-Ślesik, J., Samoliński, B., Krzych-Fałta, E. 2023. The increase in allergic conditions based on a review of literature. *Advances in Dermatology and Allergology*.

Hahnemann, S. 2004. *Organon of Medicine*. 6th Edition. India: B. Jan Publishers (P) Ltd.

Hammad, R., Kotb, H., Eldesoky, G.A., Mosaad, A.M., El-Nasser, A.M., El Hakam, F., Eldesoky, N., Mashaal, A., and Farhoud, H. 2022. Utility of Monocyte Expression of HLA-DR versus T Lymphocyte Frequency in the Assessment of COVID-19 Outcome. *International Journal of General Medicine*. 15: 5073 – 5087.

Hartnell, A., Robinson, D. S., Kay, A. B., and Wardlaw, A. J. 1993. CD69 is expressed by human eosinophils activated in vivo in asthma and *in vitro* by cytokines. *Immunology*, 80(2): 281–286.

Harris, J.R. 2012. *Blood Cell Biochemistry: Lymphocytes and Granulocytes*. England: Springer Science + Business Media, LLC.

Hemmings, O., Kwok, M., McKendry, R. and Santos, A.F. 2018. Basophil Activation Test: Old and New Applications in Allergy. *Current Allergy and Asthma Reports*. 18:77.

Hengel, A.V., Anklam, E., Taylor, S.L., Hefle, W. 2007. Chapter 7: Analysis of Food Allergens. *Practical Applications*.

Henschler, D. 2006. The origin of hormesis: historical background and driving forces. *Human & Experimental Toxicology*. 25: 347-351.

Hossny E, Ebisawa M, El-Gamal Y, Arasi S, Dahdah L, El-Owaidy R, et al. 2019. Challenges of managing food allergy in the developing world. *World Allergy Organ J* 12:100089.

Ive, E. 2010. An in-vitro assessment of the effects of *Arsenicum album* (30CH and 200CH) on leukocytes previously antagonised by arsenic trioxide. M.Tech: Homoeopathy, Durban University of Technology.

James, P.B., Wardle, J., Steel, A., and Adams, J. 2018. Traditional, Complementary, and Alternative Medicine use in Sub-Saharan Africa: a systematic review. *BMJ Global Health.* 3: 1-18.

Johansson MW. 2014. Activation states of blood eosinophils in asthma. Clin Exp Allergy. 2014;44(4):482–98.

Kallinich, T., Beier, K.C., Wahn, U., Stock, P., and Hamelmann, E. 2007. T-cell co-stimulatory molecules: their role in allergic immune reactions. *European Respiratory Journal*. 29: 804–812.

Kelly, B.T., and Grayson, M.H. 2016. IgE, what is it good for? *Ann Allergy Asthma Immunology*. 116(3):183-187

Khajuria, A. 2015. Total Immunoglobin E: Clinical Utility and Measurement. *MOJ Immunology*. 2(3):49

Kim, K., Mayumi, M., and Mikawa, H. 2012. IgE Receptors on Lymphocytes and IgE-Binding Factors. In: Harris, J.R. *Blood Cell Biochemistry: Lymphocytes and Granulocytes*. England: Springer Science + Business Media, LLC, 115.

Klein, L. 2003. Clinical Focus Guide to Homeopathic Remedies. Volume 1. Luminos Homeopathic Courses Ltd. Canada.

Knol EF, Mulf FPJ, Jansen H, Calafat J, Roos D. 1991. Monitoring human basophil activation via CD63 monoclonal antibody 435. *J Allergy Clin Immunol*. 328-338.

Kruger-Krasagakes, S., and Henz, B.M. 1998. Eosinophil Chemotactic Factors. In: Delves, P.J., and Roitt, I.M. *Encyclopedia of Immunology*. 2nd Edition. London, UK.

Lee, J., Rodio, B., Lavelle, J., Lewis, M., English, R., Hadley, S., Molnar, J., Jacobstein, C., Cianferoni, A., Speigel, J., Zielinski, L., Tsarouhas, N., and Brown-Whitehorn, T. 2018. Improving Anaphylactic Care: The Impact of a Clinical Pathway. *Pediatrics*. 141(5).

Levin, M. 2012. Adrenaline for anaphylaxis what is the evidence? *Current Allergy & Clinical Immunology*. 25(3):117-122.

Levis, J.T., Ford, J.B., and Kuo, A.M. 2011. Intracranial hemorrhage after prehospital administration of Intramuscular epinephrine. *The Journal of Emergency Medicine.* 40(6):e107– e110.

Li, X., He, S., & Tong, Q. 2023. Expression Changes of CD63, CD203c and FcεRIα in Blood Basophil of Patients with Drug Allergy. *Journal of Biological Regulators and Homeostatic Agents*. 37(5):2679–2689.

Lipinski, A., Beck-Dreschel, A., Wehausen, R., Hering, J., Orth, H., Striebel, P., and Steinhoff, B. 2014. Manufacture of Homoeopathic Preparations. *Pharmazeutische Industrie (Pharmind)*. *76*(3): 388–394.

Lorenz, I., Schneider, E.M., Stolz, P., Brack, A., and Strube, J. 2003. Influence of the diluent on the effect of highly diluted histamine on basophil activation. *Homeopathy*. 92(1):11-8.

MacGlashan, D. 2010. Expression of CD203c and CD63 in human basophils: relationship to differential regulation of piecemeal and anaphylactic degranulation processes. *Clin Exp Allergy*. 40(9):1365–77.

Majola, S.F. 2020. A model for the integration of Homoeopathy into the public healthcare System in the province of KwaZulu-Natal, South Africa. Durban University of Technology.

Manchanda, R.K. 2018. Experimentation in Homoeopathy: History and Prospects. *Indian Journal of Research in Homoeopathy*. 12(2):61-63.

Mbugi EV, Chilongola JO. 2010. Allergic Disorders in Africa and Africans: Is it primarily a priority? *World Allergy Organisation Journal.* 3:175–181.

Morrison, R. 2006. *Carbon: Organic and Hydrocarbon Remedies in Homeopathy*. United States of America: Hahnemann Clinic Publishing.

Mousset, C.M., Hobo, W., Woestenenk, R., et al. 2019. Comprehensive Phenotyping of T Cells Using Flow Cytometry. *Cytometry* Part A. 95:647-654

Mukherjee, P.K., Harwansh, R.K., Bahadur, S., Banerjee, S., and Kar, A. 2016. Evidence Based Validation of Indian Traditional Medicine – Way Forward. *World Journal of Traditional Chinese Medicine*. 2(1):48-61.

Mullins, R.J., Wainstein, B.K., Barnes, E.H., Lew, W.K., and Campbell, D.E. 2016. Increases in anaphylaxis fatalities in Australia from 1997 to 2013. *Clinical & Experimental Allergy*. 46:1099-1110.

Murphy, R. 2000. *Homeopathic Remedy Guide: 1 200 Homeopathic and Herbal Remedies*. India: Indian Book & Periodicals Publishers.

National Cancer Institute. 2024. Complementary and Alternative Medicine. (Online) Available: https://www.cancer.gov/about-cancer/treatment/cam

Ndjindji O, Rogombe S, MD, Bissiengou P, Mveang-Nzoghe A, Leboueny M, Mbina O, et al. 2023. Allergen sensitization and polysensitization pattern of adults and children in an urban Sub-Saharan African setting (Libreville, Gabon). *J allergy clin immunol global* 2(1): 23-29.

Palmo, E., Galllucci, M., Cipriani, F., Bertelli, L. Giannetti, A., and Ricci, G. 2019. Asthma and Food Allergy: which risks? *Medicina (Kaunas)*. 55(9):509.

Paschmanns, R. 2013. Shock reactions: Acute prescription with *Carbolicum acidum* for an insect bite also solves chronic allergy. *Spectrum of Homeopathy: Allergies*.

Phatak, S.R., 1999. *Materia Medica of Homoeopathic Medicines*. 2nd Edition. India: B. Jan Publishers (P) Ltd.

Pilling, D., Fan, T., Huang, D., Kaul, B., and Gomer, RH. 2009 Identification of Markers that Distinguish Monocyte-Derived Fibrocytes from Monocytes, Macrophages, and Fibroblasts. *PLoS ONE* 4(10): e7475.

Poitevin, B. 1998. Experimental Study of Homoeopathy in Allergology: 2. Biological Studies. *British Homoeopathic Journal*. 87:154-164

Pourahmad, J., and Salimi, A. 2015. Isolated Human Peripheral Blood Mononuclear Cell (PBMC), a Cost Effective Tool for Predicting Immunosuppressive Effects of Drugs and Xenobiotics. *Iranian Journal of Pharmaceutical Research*. 14(4):679-980.

Prince, B.T., Mikhail, I., and Stukus, D.R. 2018. Underuse of epinephrine for the treatment of anaphylaxis: missed opportunities. *Journal of Asthma and Allergy.* 11:143-151.

Ralston, S.H., Penman, I.A., Strachan, M., and Hobson, R. 2018. *Davidson's Principles and Practice of Medicine*. 23rd Edition. New York: Elsevier.

Rea, I. M., McNerlan, S. E., & Alexander, H. D. 1999. CD69, CD25, and HLA-DR activation antigen expression on CD3+ lymphocytes and relationship to serum TNF-alpha, IFN-gamma, and sIL-2R levels in aging. *Experimental gerontology*, *34*(1):79–93.

Rehman, T., and Ahmad, S. 2017. Introduction of Homeopathy and Homeopathic medicines: A review. *International Journal of Homoeopathic Sciences*. 1(1):21-25.

Richards, G., McDonald, M., Gray, C., de Waal, P., Friedman, R., Hockman, M., Karabus, S. et al. 2023. Allergic rhinitis: Review of the diagnosis and management: South African Allergic Rhinitis Working Group. *S Afr Fam Pract.* 65(1).

Ring, J., Beyer, K., Biedermann, T., Bircher, A., Fischer, M., Fuchs, T., Heller, A., Hoffmann, F., Huttegger, I., Jakob, T., Klimek, L., Kopp, M.V., Kugler, C., Lange, L., Pfaar, O., Rietschel, E., Rueff, F., Schnadt, S., Seifert, R., Stöcker, B., Treudler, R., Vogelberg, C., Werfel, T.,Worm, M., Sitter, H., and Brockow, K. 2021. Guideline (S2k) on acute therapy and management of anaphylaxis: 2021 update. *Allergo J Int* 30:1–25.

Romeo, S., Zeni, O., Scarfi, M., Poeta, L., Lioi, M.B., and Sannino, A. 2022. Radiofrequency Electromagnetic Field Exposure and Apoptosis: A Scoping Review of *In vitro* Studies on Mammalian Cells**.** *Int. J. Mol. Sci. 23*(4): 2322

Ross, S.M., and Morrison, G.R. 2014. Experimental Research Methods. In: Jonassen, D., and Driscoll, M. *Handbook of Research on Educational Communications and Technology*, 1021.

Rossi, S., and Pitidis, A. 2018. Multiple Chemical Sensitivity: Review of the State of Art in Epidemiology, Diagnosis, and Future Perspectives. *Journal of Occupational and Environmental Medicine*. 60(2):138-146.

Sadovnik, I., Ivanov, D., Smiljkovic, D., Stefanzl, G., Degenfeld-Schonburg, L., Herndlhofer, S., Eisenwort, G., Hauswirth, A. W., Sliwa, T., Keil, F., Sperr, W. R., and Valent, P. 2023. Identification of CD203c as a New Basophil-Specific Flow-Marker in Ph + Chronic Myeloid Leukaemia. *Cells.* 12(1).

Sagar, S.M. 2007. Homeopathy: does a teaspoon of honey help the medicine go down? *Current Oncology*. 14(4): 126-127.

Sanz, M. L., Gamboa, P. M., Apara, I., Uasuf, C., Vila, L., Garcia-Avile, C., Chazot, M. and De Weck, A. L. 2002. Flow cytometric basophil activation test by detection of

CD63 expression in patients with immediate-type reactions to betalactam antibiotics. *Clinical and Experimental Allergy*. 32:277-286.

Schaller, M., Boeld, U., Oberbauer, S., Hamm, G., Hube, B., and Korting, H. 2004. Polymorphonuclear leukocytes (PMNs) induce protective Th1-type cytokine epithelial responses in an *in vitro* model of oral candidosis. *Microbiology*. 150:2807-2813.

Schroyens, F. 2020. *Synthesis.* 9.1 Ed. B. Jain Publishers.

Sicherer, S.H., and Simons, F.E.R. 2017. Epinephrine for First-aid Management of Anaphylaxis. *Pediatrics*. 139(3): e2-e9.

Simons, F.E.R., Ardusso, L., Bilo, M., Cardona, V., Ebisawa, M., El-Gamel, Y., Lieberman, P., Lockey, R., Muraro, A., Roberts, G., Sanchez-Borges, M., Sheikh, A., Shek, L., Wallace, D., and Worm, M. 2014. International Consensus on Anaphylaxis. *World Allergy Organ J*. 7(9):1-9

Stone, K.D., Prussin, C., and Metcalfe, D.D. 2010. IgE, Mast Cells, Basophils, and Eosinophils. *J Allergy Clin Immunol*. 125:73-80.

Sun, J., You, R., Lyu, B., Li, X., Gao, Y., Wen, Y., Qu, C., and Wang, Y. 2023. HLA-DR Helps to Differentiate Erythrodermic Cutaneous T-cell Lymphoma from Erythrodermic Inflammatory Dermatoses in Flow Cytometry. *Acta Dermato-Venereologica*. 103(5668).

Simons FE, Chan ES, Gu X, Simons KJ. 2001. Epinephrine for the out-of-hospital (first-aid) treatment of anaphylaxis in infants: is the ampule/syringe/needle method practical? *J Allergy Clin Immunol*. 108(6):1040-1044.

Tanno, L.K., Simons, F.E., Annesi-Maesano, I., Calderon, M.A., and Demoly, S.A. 2017. Fatal anaphylaxis registries data support changes in the who anaphylaxis mortality coding rules. *Orphanet Journal of Rare Diseases*. 12:8

Thurau, A.M., Schulz, U., Wolf, V., Krug, N. and Schauer, U. 1996. Identification of Eosinophils by Flow Cytometry. *Cytometry*. 23:150-158.

Tortora, G.J., and Derrickson, B. 2015. *Introduction to Human Body: The essentials of Anatomy and Physiology*. 10th Edition. United States of America: John Wiley & Sons, Inc.

Turner, P.J., Campbell, D.E., Motusue, M.S., and Campbell, R.L. 2020. Global Trends in Anaphylaxis Epidemiology and Clinical Implication. *J. Allergy Clin Immunol Pract.* 8: 1169-1176.

Uyttebroek AP, Sabato V, Faber MA, Cop N, Bridts CH, Lapeere H, et al. 2014. Basophil activation tests: time for a reconsideration. *Clin Immunol.* 10(10):1325–35.

Van der Veeken, J., Gonzalez, A.J., Cho, H., Arvey, A., Hemmers, S., Leslie, C.S., and Rudensky, A.Y. 2016. Memory of Inflammation in Regulatory T cells. *Cell*. 166(4):977-990.

Vithoulkas, G. 1979. *Homoeopathy: Medicine of the New Man*. Touchstone.

Wang, J., Zhou, Y., Zhang, H., Hu, L., Liu, J., Wang, L., Wang, T., Zhang, T., Cong, L., and Wang, Q. 2023. Pathogenesis of allergic diseases and implications for therapeutic interventions. *Signal Transduction and Targeted Therapy*. 8: 138

Warner, J.M. 2021. Mathematical Interpretation of the Pharmacodynamics of Homoeopathic Medicines. *Homœopathic Links*.34(1):3–16

World Allergy Organisation. 2021. Anaphylaxis: Global Overview (online). Available: www.worldallergy.org/education-and-programs/education/allergic-disease-resource-center/professionals/anaphylaxis-global-overview accessed 17 January 2021.

World Health Organization. 2024. Noncommunicable Diseases (online). Available: https://www.who.int?news-room/fact-sheet/detail/noncommunicable-diseases#cms

Yonekura S, *et al*. 2017. Complementary and alternative medicine for allergic rhinitis in Japan. *Allergol Int*. 66(3):425–31.

Yun, Y., Kanda, A., Kobayashi, Y., Van Bui, D., Suzuki, K., Sawada, S., Baba, K., Yagi, M., Asako, M., Okazaki, H., Ikeda, H., Kawamura, S., Nakamura, A., Dombrowicz, D., Tomoda, K., and Iwai, H. 2020. Increased CD69 expression on activated eosinophils in eosinophilic chronic rhinosinusitis correlates with clinical findings. *Allergology international : official journal of the Japanese Society of Allergology*. 69(2): 232–238.

Zeni, O. and Scarfi, M. 2012. Chapter 7 - Experimental Requirements for *in vitro* Studies Aimed to Evaluate the Biological Effects of Radiofrequency Radiation. In: Costanzo, S. *Microwave Materials Characterization*. InTech. Available at: http://dx.doi.org/10.5772/51421

APPENDICES

Appendix A

9 June, 2022

Mr MVK Mzimela
Student No: 21106811

13 Chestnut Lane
Doonheights
4126

Dear Mr Mzimela

MASTER OF HEALTH SCIENCES: HOMOEOPATHY

I am pleased to advise that:

1. The Faculty Research Committee approved the following:

(i) Your research proposal and dissertation title, being:

An *in vitro* study on the physiological actions of *Carbolicum acidum* 5CH, 9CH, 15CH, 30CH, 200CH on granulocytes and T-Cell chemical mediators in cellular allergic responses.

Please note: ANY PROPOSED CHANGES in the DISSERTATION TITLE require the approval of your supervisor and the Faculty Research Committee.

(ii) Supervisor – **Dr BT Mkhize**
Co-supervisor – **Dr NH Mavela**

2. Your request for funding totalling R 5 000.00 subject to any literature referred to in Section A of the PG 4a form being accessioned by this University, and any equipment purchased shall become the property of the department.

NOTE: - This funding is not paid directly to you but is controlled by the Faculty Office. Any proposed changes to this funding allocation needs the approval of your supervisor, and Faculty Research Committee

The University Research Committee has stipulated that:

(a) Ownership of any patent registered in respect of the results of your Master's studies is retained by you as the initiator of the project;

(b) Should you make any drift from the results of your Master's studies, you will be required to repay pro rata, the R 5 000.00 investment which the University Research Committee has made in approving your request for funding;

(c) If the Durban University of Technology provided the equipment/materials for the creation of artefacts, this cost would be refunded to the University if such artefacts were sold and

(d) Durban University of Technology is given first refusal in respect of any possible future sale by you of any patent that may be registered in respect of your said project.

(e) All journal articles, referenced in your dissertation, are to accompany your ring-bound copies when submitting for examination purposes.

Should you experience any problems relating to your research studies, your supervisor must be informed as soon as possible. If the difficulty persists, you must then approach your Head of Department and thereafter the Executive Dean of the Faculty.

Yours sincerely

Ms S Perumal
FACULTY RESEARCH OFFICER
FACULTY OF HEALTH SCIENCES

Student's signature in acceptance
of the conditions contained herein.

Date: _____

Appendix B

Directorate for Research and Postgraduate Support
Durban University of Technology
Tromso Annexe, Steve Biko Campus
P.O. Box 1334, Durban 4000
Tel.: 031-3732576/7
Fax: 031-3732946

5th June 2022
Mr Mnqobi Mzimela
c/o Department of Homoeopathy
Faculty of Health Sciences
Durban University of Technology

Dear Mr Mzimela

PERMISSION TO CONDUCT RESEARCH AT THE DUT

Your email correspondence in respect of the above refers. I am pleased to inform you that the Institutional Research and Innovation Committee (IRIC) has granted **Gatekeeper Permission** for you to conduct your research "An *in vitro* study on the physiological actions of Carbolicum acidum 5CH, 9CH, 15CH, 30CH, 200CH on granulocytes and T-Cell chemical mediators in cellular allergic responses." at the Durban University of Technology.

The DUT may impose any other condition it deems appropriate in the circumstances having regard to nature and extent of access to and use of information requested.

We would be grateful if a summary of your key research findings would be submitted to the IRIC on completion of your studies.

Kind regards.
Yours sincerely

PROF. KEO MOTAUNG
ACTING-DIRECTOR: RESEARCH AND POSTGRADUATE SUPPORT DIRECTORATE

Appendix C

Head Office or Zone
1 Constantia Boulevard
Constantia Kloof Ext 22, 1709

Postal Address: Private Bag X14, Weltevreden Park, 1715
Tel: 011 761 9000 **Email:** customerservice@sanbs.org.za
www.sanbs.org.za

SOUTH AFRICAN NATIONAL BLOOD SERVICE NPC
Human Research Ethics Committee

OHRP Number : IORG0006278
FWA Registration Number : IRB00007553
SA NHREC Registration Number : REC-270606-013

Secretariat: Tel: 011 761 9347 : Lerato.Mautlane@sanbs.org.za

To: : Mr. Mnqobi Mzimela

DATE OF FINAL REVIEW: **: 13 March 2024**
PROJECT TITLE : An in vitro study on the physiological actions of Carbonium acidum 5CH, 9CH, 15CH, 30CH, 200CH on granulocytes and T-cell chemical mediators in cellular allergic responses.
DECISION OF THE COMMITTEE **: Approved**
CLEARANCE CERTIFICATE NUMBER **: 2024/0004**
RISK CATEGORY **: Minimal Risk**

Ethics approval is subject to the following:

The ethics approval is conditional on the research being conducted as stipulated in the details of all the documents submitted to and approved by the SANBS HREC. The research may commence as of 08 April 2024.

This approval is valid for one year, from **(08 April 2024).** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to the SANBS HREC on the appropriate SANBS form 2-3 months before the expiry date. Failure to do so will lead to an automatic lapse of ethics approval, which will need to be reported to study sponsors and relevant stakeholders.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by SANBS HREC prior to implementation. If a need arises to revise the investigator's role, the methods, or any other aspect, such changes must be submitted to the committee as an amendment for approval.

A report or link to the report must be submitted to the SANBS HREC within three months of the study being completed.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2020) (if applicable) and with

Appendix D

Figure 7-1 BD FACSymphony A5 Flow Cytometer (Special Order Research Product).

Figure 7-2 Cell Culture Cabinet (Baker SterilGARD II).

Figure 7-3 CO2 Humidified Incubator (RWD Life Sciences).

MATERIALS:

Antibodies	Source	Cat. No.
BUV395 Mouse Anti-Human CD45	BD Biosciences	563791
APC Mouse Anti-Human CD203c	BD Biosciences	562973
PE Mouse Anti-Human CD63	BD Biosciences	561925
FITC Mouse Anti-Human CD69	BD Biosciences	560969
BV421 Mouse Anti-Human HLA-DR	BD Biosciences	562805
Biological Sample	**Source**	
Residual Blood Samples	South African National Blood Services	
Reagents	**Source**	**Cat. No.**
BD Leukocyte Activation Cocktail	BD Biosciences	550583
BD FACS Lysing Solution	BD Biosciences	349202
BD Rainbow Calibration Particles	BD Biosciences	559123
Zombie NIR Fixable Viability Dye	BioLegend	423105
BD FACSFlow Sheath Fluid	BD Biosciences	-
Dimethylsulfoxide (DMSO) for Molecular Biology	Sigma-Aldrich	-

Software	Source
FlowJo version 10.10	BD Biosciences
SPSS version 30.0	IBM

Other	Source	Cat. No.
Carbolicum acidum 4CH, 7CH, 13CH, 27CH, 198CH	CoMED Health	09SI20L0025
Examination gloves	Lasec Group	-
Falcon 5ml Round Bottom Polystyrene Test Tube, Sterile. 12 X 75mm style (FACS TUBES)	Corning	352054
Eppendorf tubes	Sigma	030119460
0.5µl - 10µl, 10µl - 100µl, and 100µl - 1000µl Micropipettes	BioPette Plus	-
Zap Aerosol Filter Pipette Tips, Racked, Sterile	Lasec Group	-
10x 30ml Amber Glass Bottles	DUT Homoeopathic Dispensary	-
Lamina Flow	DUT Homoeopathic Dispensary	-
200ml Distilled Water	DUT Homoeopathic Dispensary	-
20ml Graduated Pipette	DUT Homoeopathic Dispensary	-

Appendix E

CoMED HEALTH

CoMED Health (Pty) Ltd
313 Kuit St, Waltloo, Pretoria, 0184
P O Box 659, Silverton, 0127
T: 012 813 9400 • F: 012 813 9698
www.comedhealth.co.za
SA Pharmacy Council No. 0095
[Vat. No. 4010253583] [Reg. No. 2008/005773/07]
Directors: C. Dillon (B.Sc, MBA); G. Shayne (B.Com; CA)*; M. Levien (HD.; ND.; DO.);
W. Jonker (B.Com (Hons); B.Compt.; MBA); P. Kreft (B.Sc. (Hons))**
*British **Managing
Bank: Nedbank Business Banking
Branch No: 198765
Acc.No: 1497218365

Release certificate

Name of Product: **Acidum carbolicum 4CH (20%)**
Starting substance batch number: **23R01074**
Potency batch number: **232363**
Reference date: **24/06/2024**
Expiry date: **06/2027**
Order number: **SO232363**

The Head of the Dispensing Laboratory certifies:

- that the homoeopathic starting substances have been manufactured according to standards of German Pharmacopoeia,
- that the excipients used have been tested and released for quality,
- that manufacturing process for the potency was carried out according to the rules as stipulated in the Pharmacopoeia,
- that the packaging materials have been tested and released for quality,
- that the labelling on the finished product has been controlled,

In view of the inspection performed, the Head of the Dispensing Laboratory authorises release for shipment.

Dr. R. Mazibuko
Dispensing Laboratory Manager

CoMED Health (Pty) Ltd
313 Kuit St, Waltloo, Pretoria, 0184
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*British **Managing
Bank: Nedbank Business Banking
Branch No: 198765
Acc.No: 1497218365

Release certificate

Name of Product: **Acidum carbolicum 7CH (20%)**
Starting substance batch number: **23R01074**
Potency batch number: **232363**
Reference date: **24/06/2024**
Expiry date: **06/2027**
Order number: **SO232363**

The Head of the Dispensing Laboratory certifies:

- that the homoeopathic starting substances have been manufactured according to standards of German Pharmacopoeia,
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W. Jonker (B.Com (Hons), B.Compt.; MBA); P. Kreft (B.SC. (Hons))**
*British **Managing
Bank: Nedbank Business Banking
Branch No: 198765
Acc.No: 1497218365

Release certificate

Name of Product: **Acidum carbolicum 13CH (20%)**
Starting substance batch number: **23R01074**
Potency batch number: **232363**
Reference date: **24/06/2024**
Expiry date: **06/2027**
Order number: **SO232363**

The Head of the Dispensing Laboratory certifies:

- that the homoeopathic starting substances have been manufactured according to standards of German Pharmacopoeia,
- that the excipients used have been tested and released for quality,
- that manufacturing process for the potency was carried out according to the rules as stipulated in the Pharmacopoeia,
- that the packaging materials have been tested and released for quality,
- that the labelling on the finished product has been controlled,

In view of the inspection performed, the Head of the Dispensing Laboratory authorises release for shipment.

Dr. R. Mazibuko
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Directors: C. Dillon (B.Sc, MBA); G. Shayne (B.Com, CA)*; M. Levien (HD.; ND.; DO.);
W. Jonker (B.Com (Hons); B.Compt.; MBA); P. Kreft (B.SC. (Hons))**
*British **Managing
Bank: Nedbank Business Banking
Branch No: 198765
Acc.No: 1497218365

Release certificate

Name of Product: **Acidum carbolicum 27CH (20%)**
Starting substance batch number: **23R01074**
Potency batch number: **232363**
Reference date: **24/06/2024**
Expiry date: **06/2027**
Order number: **SO232363**

The Head of the Dispensing Laboratory certifies:

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- that the excipients used have been tested and released for quality,
- that manufacturing process for the potency was carried out according to the rules as stipulated in the Pharmacopoeia,
- that the packaging materials have been tested and released for quality,
- that the labelling on the finished product has been controlled,

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Dr. R. Mazibuko
Dispensing Laboratory Manager

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W. Jonker (B.Com (Hons); B.Compt.; MBA); P. Kreft (B.SC. (Hons))**
*British **Managing
Bank: Nedbank Business Banking
Branch No: 198765
Acc.No: 1497218365

Release certificate

Name of Product: **Acidum carbolicum 198CH (20%)**
Starting substance batch number: **23R01074**
Potency batch number: **232363**
Reference date: **24/06/2024**
Expiry date: **06/2027**
Order number: **SO232363**

The Head of the Dispensing Laboratory certifies:

- that the homoeopathic starting substances have been manufactured according to standards of German Pharmacopoeia,
- that the excipients used have been tested and released for quality,
- that manufacturing process for the potency was carried out according to the rules as stipulated in the Pharmacopoeia,
- that the packaging materials have been tested and released for quality,
- that the labelling on the finished product has been controlled,

In view of the inspection performed, the Head of the Dispensing Laboratory authorises release for shipment.

Dr. R. Mazibuko
Dispensing Laboratory Manager

Appendix F

NPO Number: 049066NPO
NPC Registration No. 2000/026390/08

Head Office or Zone
1 Constantia Boulevard
Constantia Kloof Ext 22, 1709

Postal Address: Private Bag X14, Weltevreden Park, 1715
Tel: 011 761 9000 **Email:** customerservice@sanbs.org.za
www.sanbs.org.za

TO WHOM IT MAY CONCERN

Virologically positive human plasma, Single donation frozen product for 'in vitro' use only

This is to certify that the material specified below, was collected from voluntary, non-remunerated blood donors at the South African National Blood Services donor clinics during the normal operational procedures and fully complies with the National Legal regulations. The donation was collected as a whole blood donation and separated using the Opti pack processing system into a red cell, buffy coat and plasma component. The blood samples were tested in SANBS Donation Testing department and were found to be negative for HIV, HBV, HCV, TPHA and Syphilis, and stored between 2 and 6˚C

Collection: Human blood is collected strictly according to the USA FDA regulations.

Product name: HIV, HBV, HCV, TPHA and Syphilis Negative residual blood samples

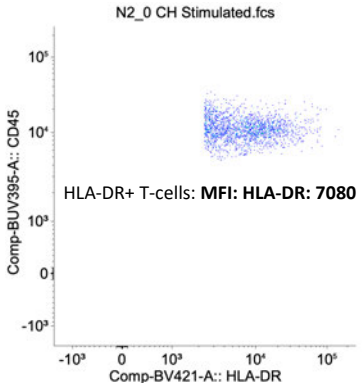
Material Identification	Code	Genotype	Volume
Negative whole blood (residual samples)	PRU000	N/A	25ml

Testing: This material has tested negative for HIV, HBV, HCV and Syphilis testing in the Donation Testing Laboratory of the South African National Blood Service. The following assay are performed for each marker:

Marker	Trade name	Manufacturer
HIV Anti-HIV HIV RNA	 Alinity S HIV O Plus Procleix Ultrio Elite (Individual donation)	 Abbott Hologic/Grifols
HCV Anti-HCV HCV RNA	 Alinity S anti-HCV Procleix Ultrio Elite (Individual donation)	 Abbott Hologic/Grifols
HBV HBsAg HBV DNA	 Alinity S HBsAg Procleix Ultrio Elite (Individual donation)	 Abbott Hologic/Grifols
Syphilis	TPHA OC 2000	Biorad

Universal Blood Type
O-
Donates to Everyone
Receives from O-

Appendix G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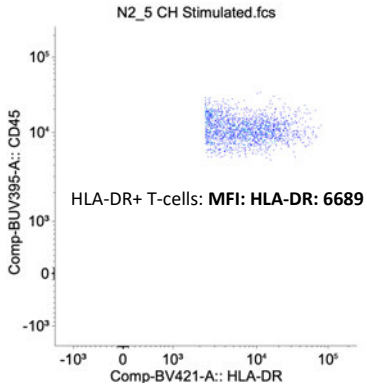
HLA-DR+ T-cells: **MFI: HLA-DR: 7080**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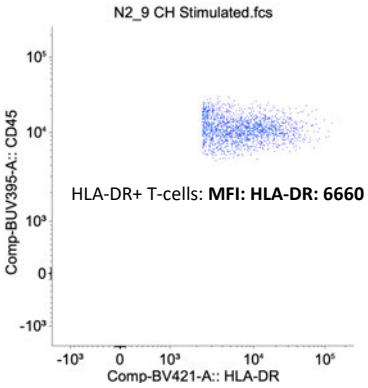
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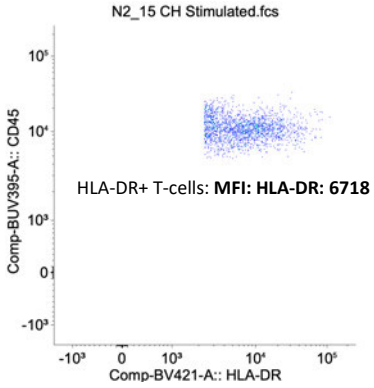
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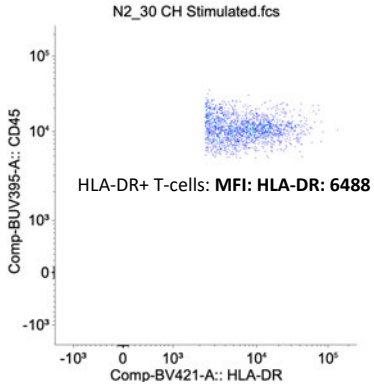
HLA-DR+ T-cells: **MFI: HLA-DR: 6718**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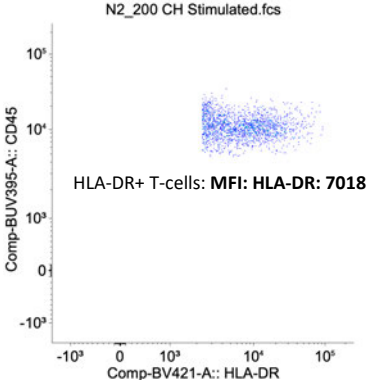
Comp-BV421-A:: HLA-DR
HLA-DR+ T-cells: **MFI: HLA-DR: 6488**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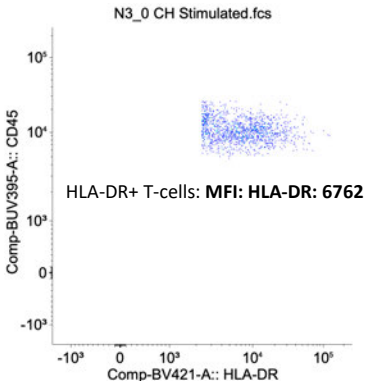
HLA-DR+ T-cells: **MFI: HLA-DR: 7018**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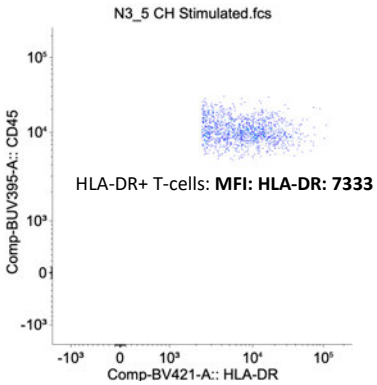
Comp-BV421-A:: HLA-DR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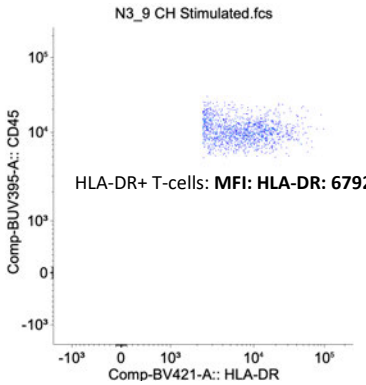
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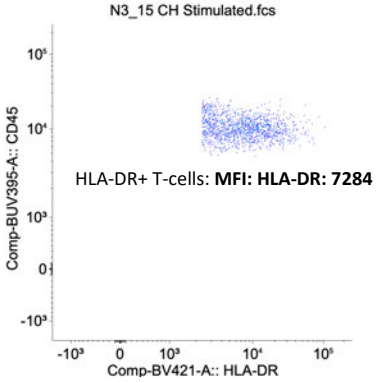
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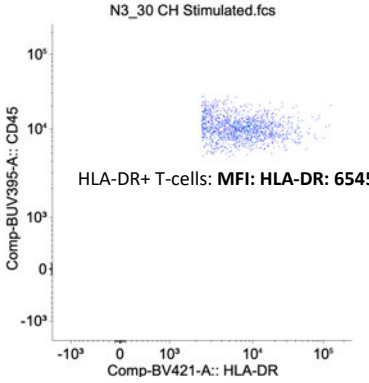
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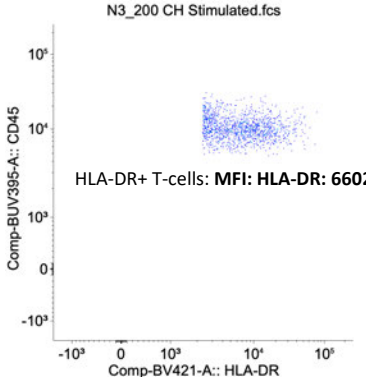
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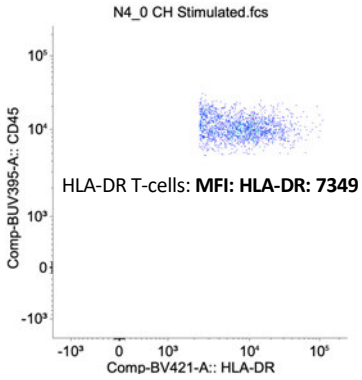

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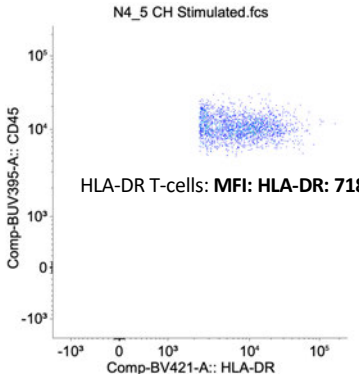
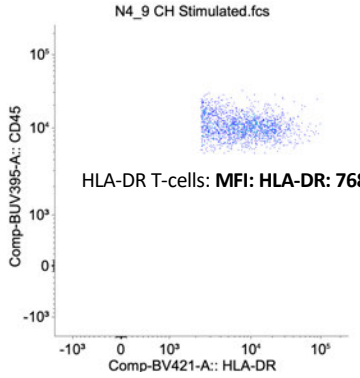


HLA-DR T-cells: **MFI: HLA-DR: 7189**



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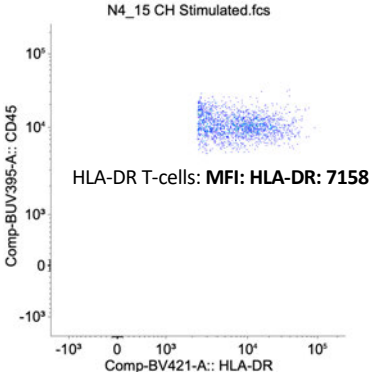

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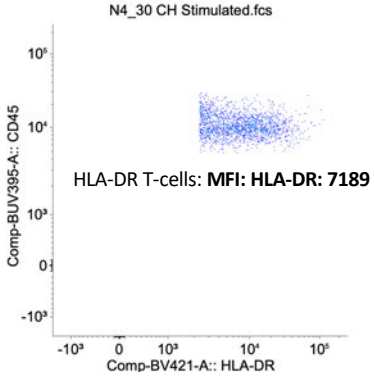

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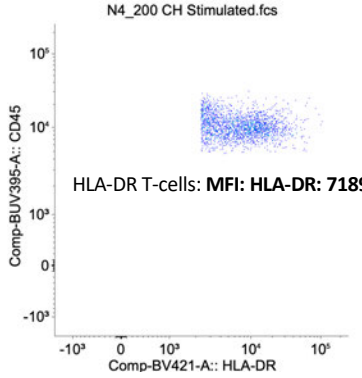
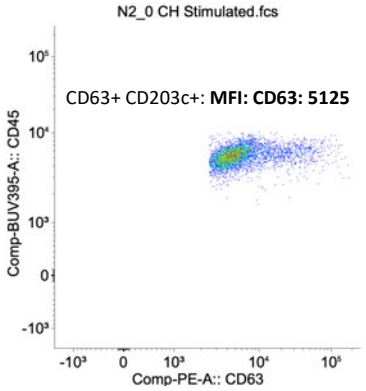


HLA-DR T-cells: **MFI: HLA-DR: 7189**

Appendix H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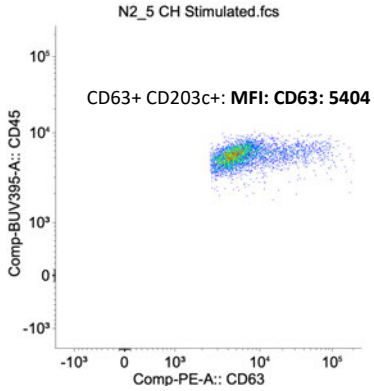
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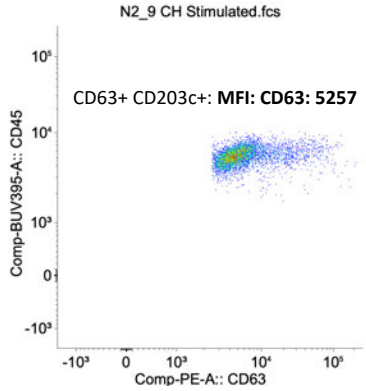
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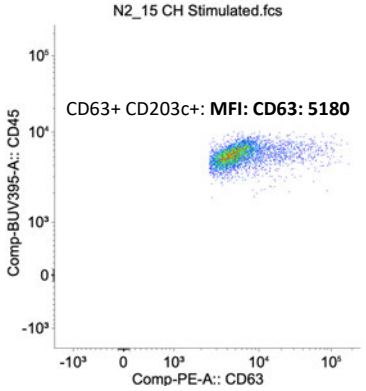
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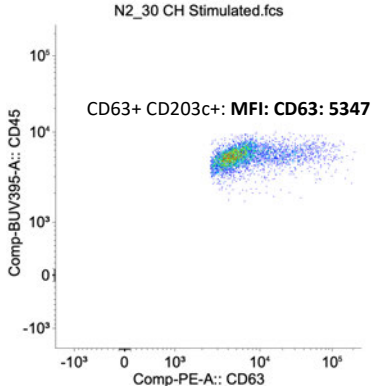
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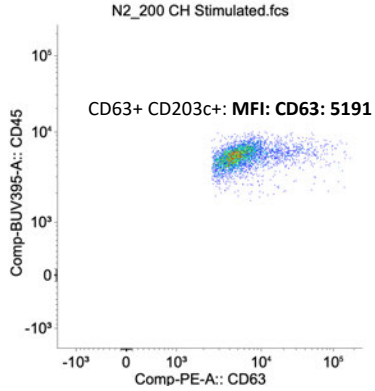
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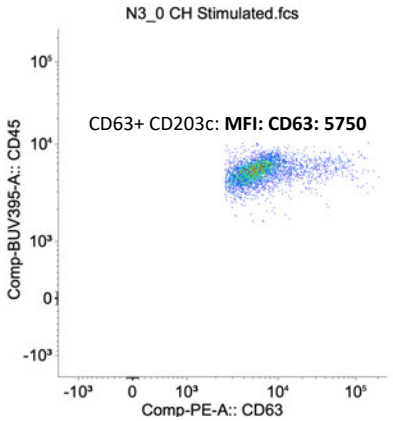
CD63+ CD203c+: MFI: CD63: 5191
Comp-BUV395-A:: CD45
Comp-PE-A:: CD63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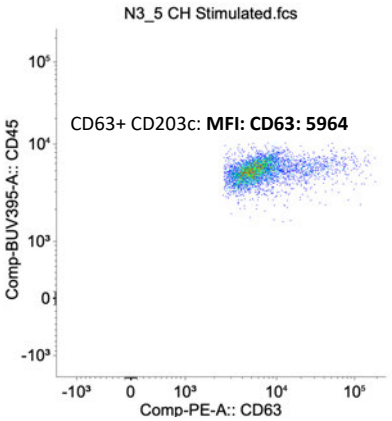
CD63+ CD203c: **MFI: CD63: 5750**
Comp-BUV395-A:: CD45
Comp-PE-A:: CD63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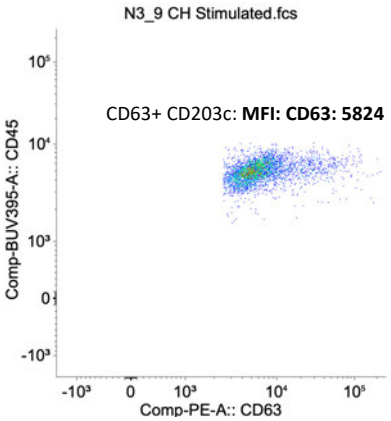
CD63+ CD203c: **MFI: CD63: 5964**
Comp-BUV395-A:: CD45
Comp-PE-A:: CD63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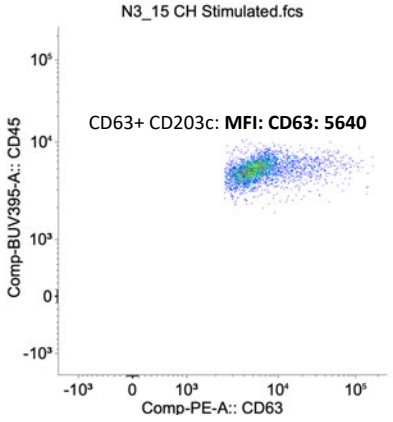
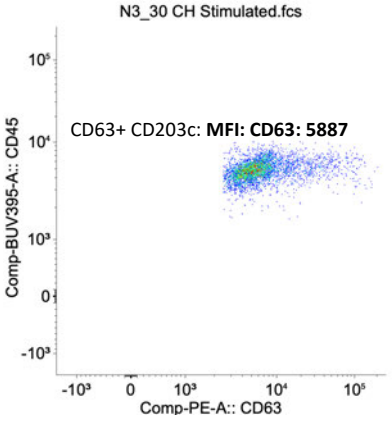
CD63+ CD203c: **MFI: CD63: 5824**
Comp-BUV395-A:: CD45
Comp-PE-A:: CD63


N3_15 CH Stimulated.fcs
CD63+ CD203c: **MFI: CD63: 5640**
Comp-BUV395-A:: CD45
Comp-PE-A:: CD63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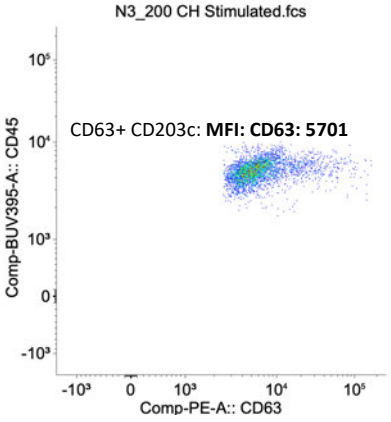
CD63+ CD203c: **MFI: CD63: 5887**
Comp-BUV395-A:: CD45
Comp-PE-A:: CD63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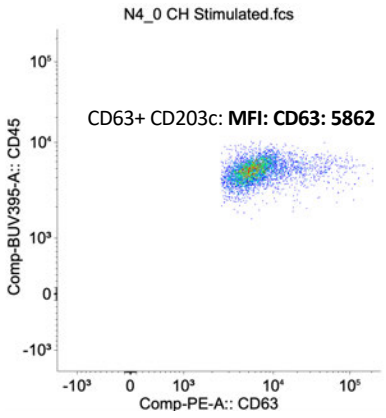
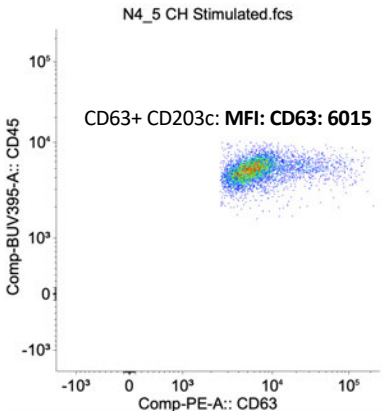
CD63+ CD203c: **MFI: CD63: 5701**
Comp-BUV395-A:: CD45
Comp-PE-A:: CD63


N4_0 CH Stimulated.fcs
CD63+ CD203c: **MFI: CD63: 5862**
Comp-BUV395-A:: CD45
Comp-PE-A:: CD63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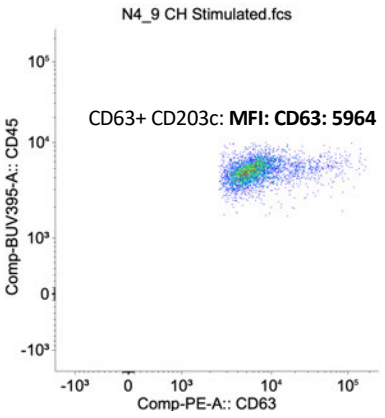
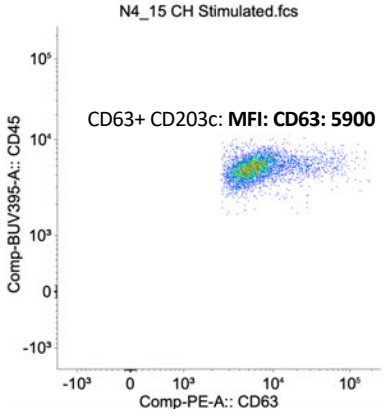
CD63+ CD203c: **MFI: CD63: 6015**
Comp-BUV395-A:: CD45
Comp-PE-A:: CD63


N4_9 CH Stimulated.fcs
CD63+ CD203c: **MFI: CD63: 5964**
Comp-BUV395-A:: CD45
Comp-PE-A:: CD63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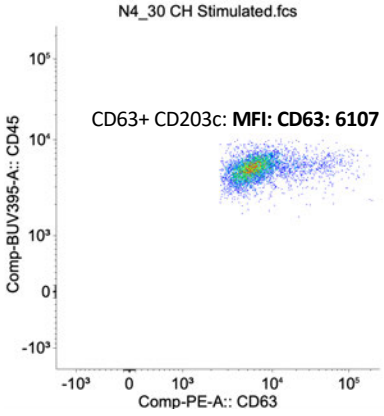
CD63+ CD203c: **MFI: CD63: 5900**
Comp-BUV395-A:: CD45
Comp-PE-A:: CD63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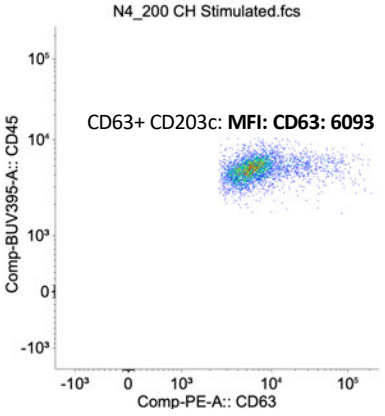
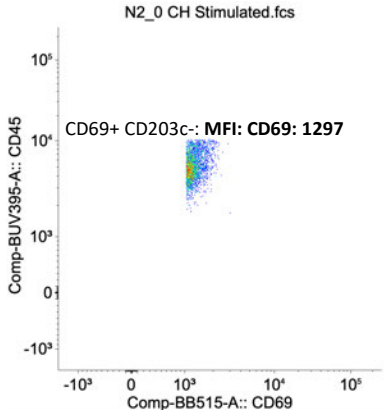
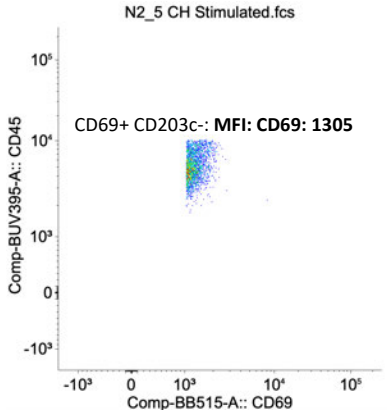
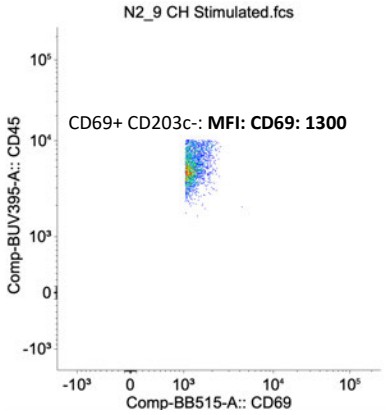
CD63+ CD203c: **MFI: CD63: 6107**
Comp-BUV395-A:: CD45
Comp-PE-A:: CD63

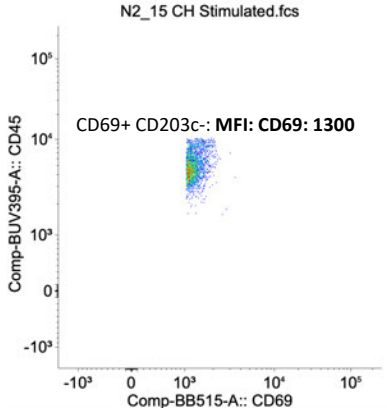

N4_200 CH Stimulated.fcs
CD63+ CD203c: **MFI: CD63: 6093**
Comp-BUV395-A:: CD45
Comp-PE-A:: CD63

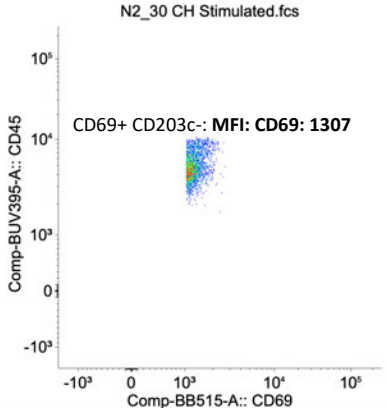
Appendix I


N2_0 CH Stimulated.fcs
CD69+ CD203c-: MFI: CD69: 1297
Comp-BUV395-A:: CD45
Comp-BB515-A:: CD69


N2_5 CH Stimulated.fcs
CD69+ CD203c-: MFI: CD69: 1305
Comp-BUV395-A:: CD45
Comp-BB515-A:: CD69


N2_9 CH Stimulated.fcs
CD69+ CD203c-: MFI: CD69: 1300
Comp-BUV395-A:: CD45
Comp-BB515-A:: CD69


N2_15 CH Stimulated.fcs
CD69+ CD203c-: MFI: CD69: 1300
Comp-BUV395-A:: CD45
Comp-BB515-A:: CD69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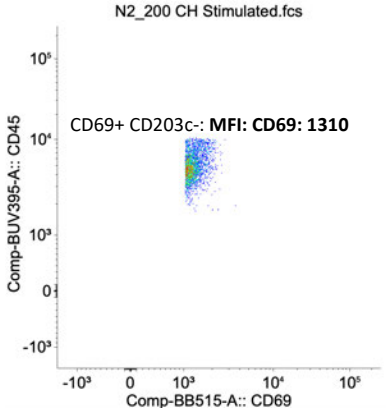
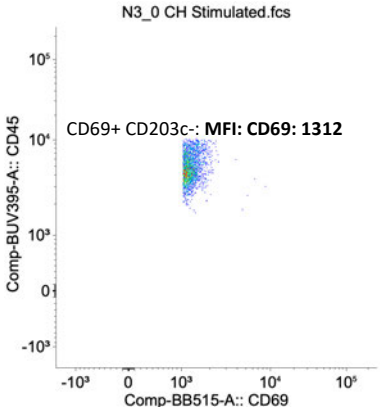
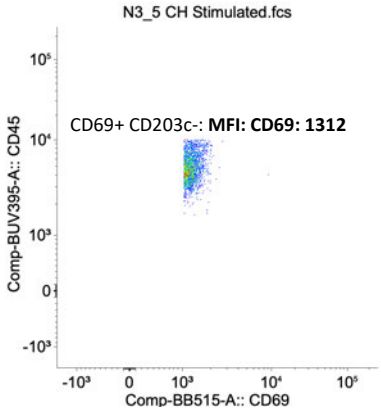
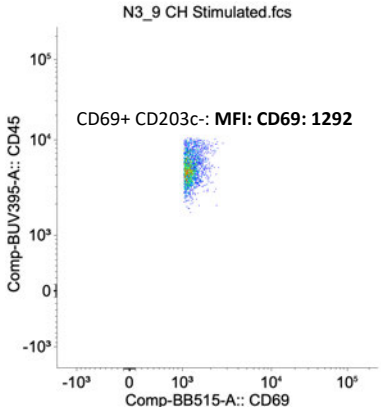
Comp-BUV395-A:: CD45
Comp-BB515-A:: CD69


N2_200 CH Stimulated.fcs
CD69+ CD203c-: MFI: CD69: 1310
Comp-BUV395-A:: CD45
Comp-BB515-A:: CD69


N3_0 CH Stimulated.fcs
CD69+ CD203c-: MFI: CD69: 1312
Comp-BUV395-A:: CD45
Comp-BB515-A:: CD69


N3_5 CH Stimulated.fcs
CD69+ CD203c-: MFI: CD69: 1312
Comp-BUV395-A:: CD45
Comp-BB515-A:: CD69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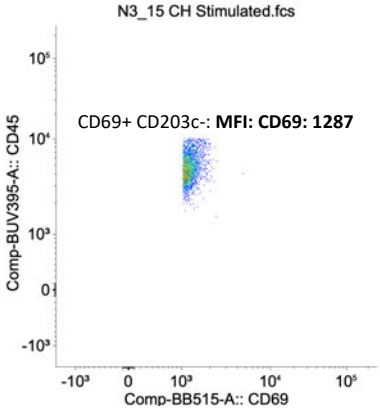
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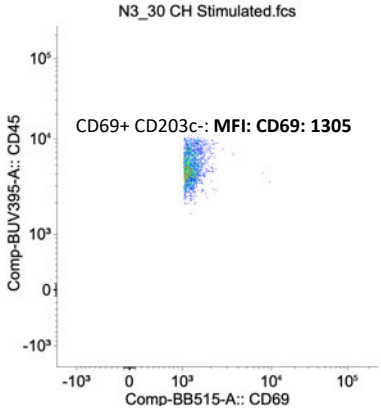
CD69+ CD203c-: MFI: CD69: 1287
Comp-BUV395-A:: CD45
Comp-BB515-A:: CD69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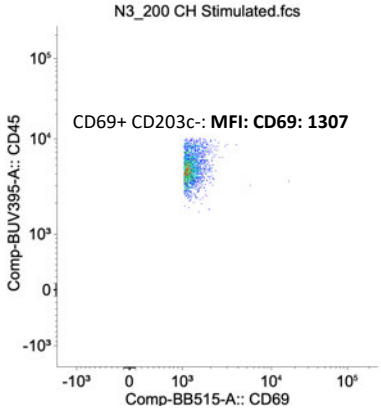
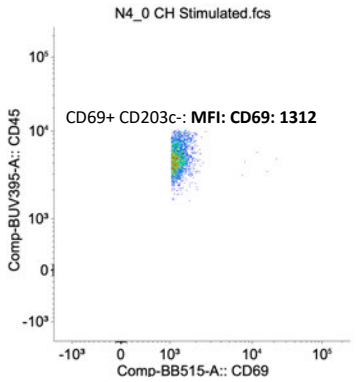
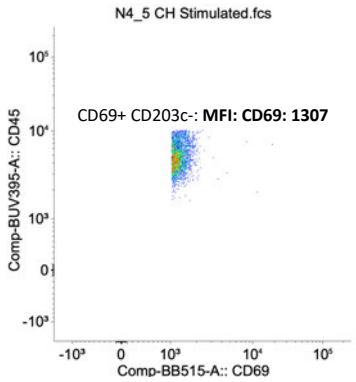
CD69+ CD203c-: MFI: CD69: 1305
Comp-BUV395-A:: CD45
Comp-BB515-A:: CD69


N3_200 CH Stimulated.fcs
CD69+ CD203c-: MFI: CD69: 1307
Comp-BUV395-A:: CD45
Comp-BB515-A:: CD69


N4_0 CH Stimulated.fcs
CD69+ CD203c-: MFI: CD69: 1312
Comp-BUV395-A:: CD45
Comp-BB515-A:: CD69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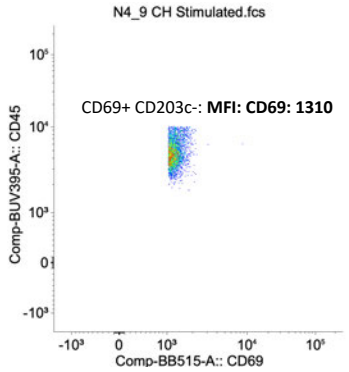
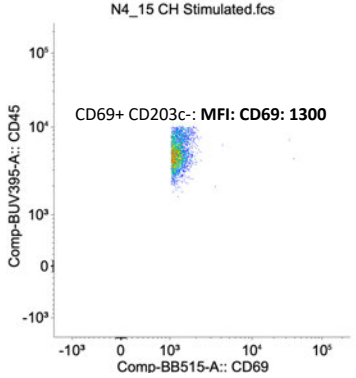
CD69+ CD203c-: MFI: CD69: 1307
Comp-BUV395-A:: CD45
Comp-BB515-A:: CD69


N4_9 CH Stimulated.fcs
CD69+ CD203c-: MFI: CD69: 1310
Comp-BUV395-A:: CD45
Comp-BB515-A:: CD69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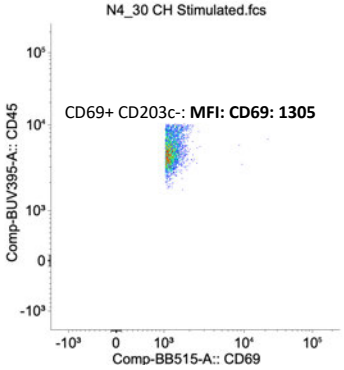
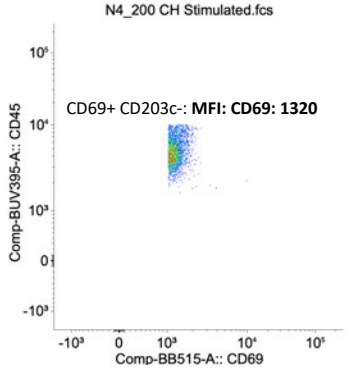
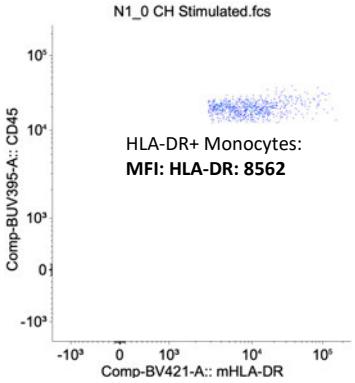
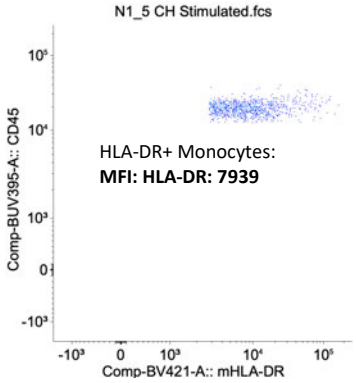
CD69+ CD203c-: MFI: CD69: 1300
Comp-BUV395-A:: CD45
Comp-BB515-A:: CD69

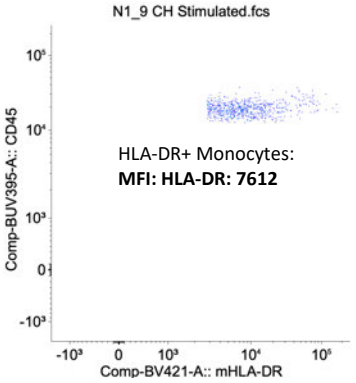

N4_30 CH Stimulated.fcs
CD69+ CD203c-: MFI: CD69: 1305
Comp-BUV395-A:: CD45
Comp-BB515-A:: CD69

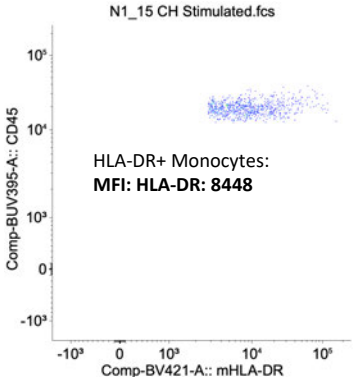

N4_200 CH Stimulated.fcs
CD69+ CD203c-: MFI: CD69: 1320
Comp-BUV395-A:: CD45
Comp-BB515-A:: CD69

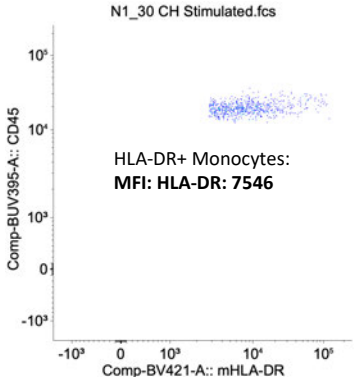
Supplementary Density Plots of Monocytes

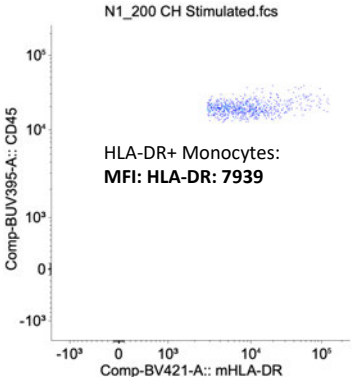


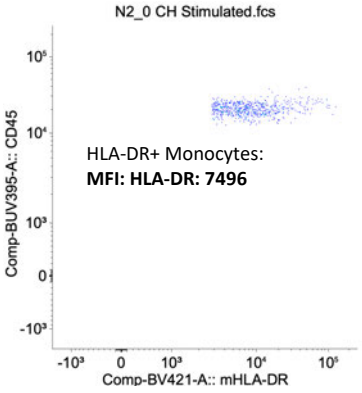


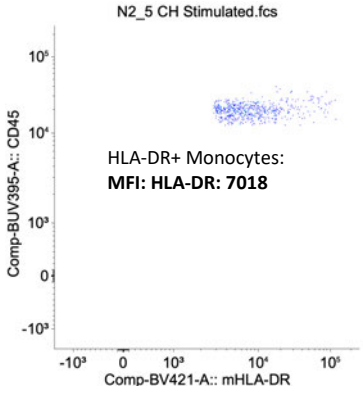


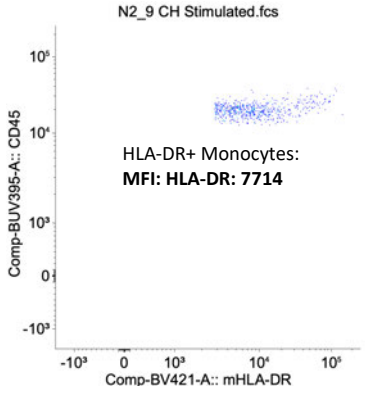


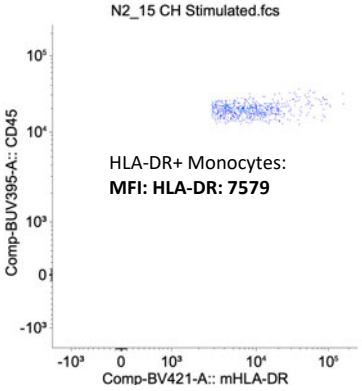


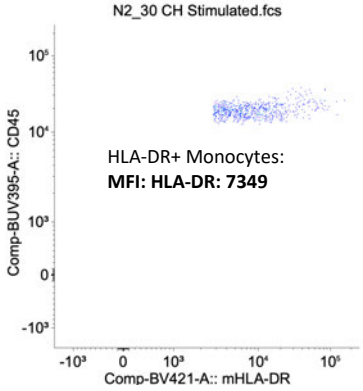


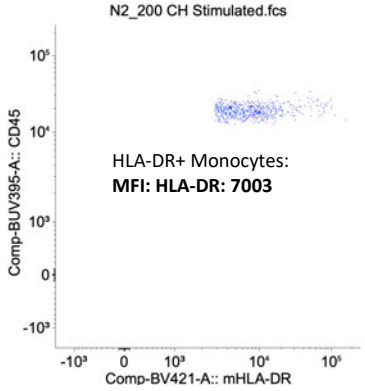

N2_0 CH Stimulated.fcs
HLA-DR+ Monocytes:
MFI: HLA-DR: 7496
Comp-BUV395-A:: CD45
Comp-BV421-A:: mHLA-DR

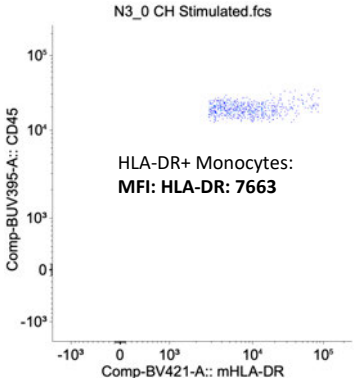

N2_5 CH Stimulated.fcs
HLA-DR+ Monocytes:
MFI: HLA-DR: 7018
Comp-BUV395-A:: CD45
Comp-BV421-A:: mHLA-DR


N2_9 CH Stimulated.fcs
HLA-DR+ Monocytes:
MFI: HLA-DR: 7714
Comp-BUV395-A:: CD45
Comp-BV421-A:: mHLA-DR


N2_15 CH Stimulated.fcs
HLA-DR+ Monocytes:
MFI: HLA-DR: 7579
Comp-BUV395-A:: CD45
Comp-BV421-A:: mHLA-DR


N2_30 CH Stimulated.fcs
HLA-DR+ Monocytes:
MFI: HLA-DR: 7349
Comp-BUV395-A:: CD45
Comp-BV421-A:: mHLA-DR


N2_200 CH Stimulated.fcs
HLA-DR+ Monocytes:
MFI: HLA-DR: 7003
Comp-BUV395-A:: CD45
Comp-BV421-A:: mHLA-DR


N3_0 CH Stimulated.fcs
HLA-DR+ Monocytes:
MFI: HLA-DR: 7663
Comp-BUV395-A:: CD45
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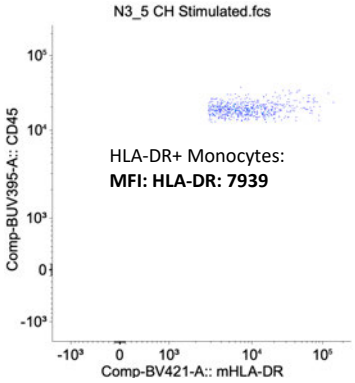
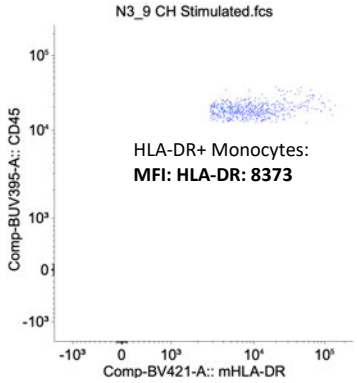
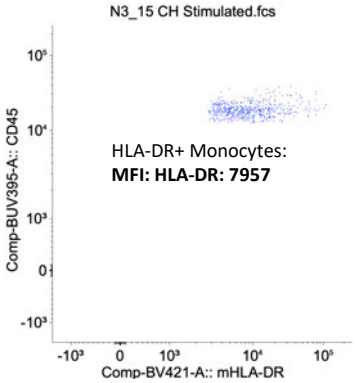
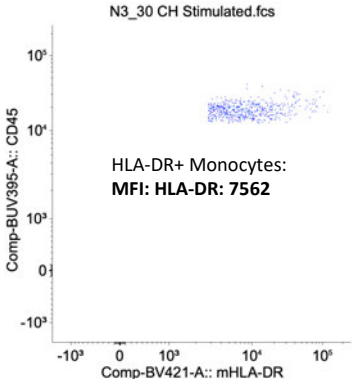
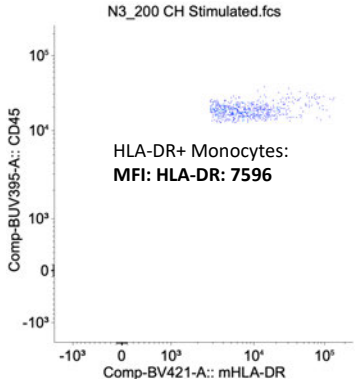
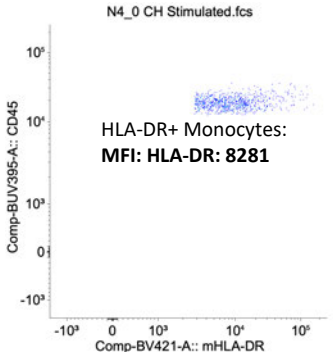

N3_5 CH Stimulated.fcs
HLA-DR+ Monocytes:
MFI: HLA-DR: 7939
Comp-BUV395-A:: CD45
Comp-BV421-A:: mHLA-DR

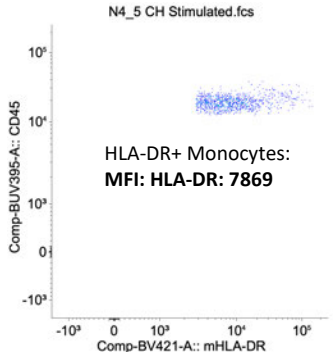

N3_9 CH Stimulated.fcs
HLA-DR+ Monocytes:
MFI: HLA-DR: 8373
Comp-BUV395-A:: CD45
Comp-BV421-A:: mHLA-DR

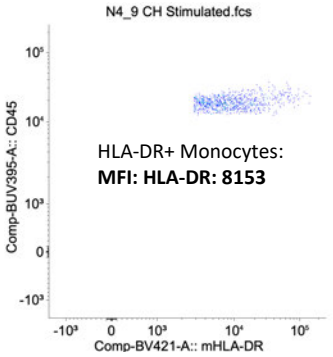

N3_15 CH Stimulated.fcs
HLA-DR+ Monocytes:
MFI: HLA-DR: 7957
Comp-BUV395-A:: CD45
Comp-BV421-A:: mHLA-DR

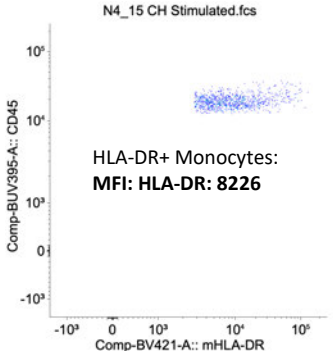

N3_30 CH Stimulated.fcs
HLA-DR+ Monocytes:
MFI: HLA-DR: 7562
Comp-BUV395-A:: CD45
Comp-BV421-A:: mHLA-DR

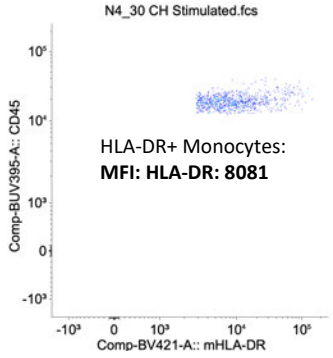

N3_200 CH Stimulated.fcs
HLA-DR+ Monocytes:
MFI: HLA-DR: 7596
Comp-BUV395-A:: CD45
Comp-BV421-A:: mHLA-DR

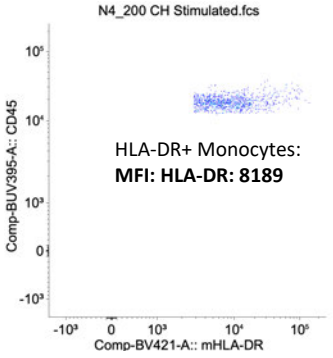

N4_0 CH Stimulated.fcs
HLA-DR+ Monocytes:
MFI: HLA-DR: 8281
Comp-BUV395-A:: CD45
Comp-BV421-A:: mHLA-DR


N4_5 CH Stimulated.fcs
HLA-DR+ Monocytes:
MFI: HLA-DR: 7869
Comp-BUV395-A:: CD45
Comp-BV421-A:: mHLA-DR


N4_9 CH Stimulated.fcs
HLA-DR+ Monocytes:
MFI: HLA-DR: 8153
Comp-BUV395-A:: CD45
Comp-BV421-A:: mHLA-DR


N4_15 CH Stimulated.fcs
HLA-DR+ Monocytes:
MFI: HLA-DR: 8226
Comp-BUV395-A:: CD45
Comp-BV421-A:: mHLA-DR


N4_30 CH Stimulated.fcs
HLA-DR+ Monocytes:
MFI: HLA-DR: 8081
Comp-BUV395-A:: CD45
Comp-BV421-A:: mHLA-DR


N4_200 CH Stimulated.fcs
HLA-DR+ Monocytes:
MFI: HLA-DR: 8189
Comp-BUV395-A:: CD45
Comp-BV421-A:: mHLA-DR