
# Investigating the effects of oral contraceptives on thrombotic-risk markers.

By

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## PREFACE

The study described in this dissertation was carried out by Mr Mthokozisi Mahlobo and has not been submitted in any other form to another University. This study was carried out in the Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa, under the supervision of Dr Vuyolwethu Mxinwa and Prof. Bongani B. Nkambule.

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Prof. Bongani B. Nkambule: _____ [redacted] _____ Date:18 July 2024

## DECLARATION

I, Mr, Mthokozisi Mahlobo declare that:

i. The research reported in this thesis, except where otherwise indicated, is my own original work.

ii. This thesis has not been submitted for any degree or examination at any other university.

iii. This thesis does not contain other person's data, pictures, graphs, or other information, unless specifically acknowledged as being sourced from other persons.

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Signed:  Date: 18 July 2024

## DEDICATION

I would like to dedicate this work to:

- God, you have been with me through thick and thin, thank you for listening to my prayers and without you I would not have made it this far.
- My family as a whole. From both my mom and dad, my little sister, and my little brother. You have been nothing but supportive and kind to me. You believed in me and kept me going even on my worst days. I could not ask for a better family than you guys. Thank you so much.

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### CHAPTER 2: LITERATURE REVIEW



### CHAPTER 3: MANUSCRIPT – EXPERIMENTAL PAPER

**ABBREVIATIONS**

LFD - low fat diet

HFD - high fat diet

OC - oral contraceptive

IFN-γ - interferon gamma

TNF-α tumor necrosis factor

IL - interleukin

DVT - deep vein thrombosis

CVD - cardiovascular disease

TF - tissue factor

vWF - Willebrand factor

PAI - Plasminogen inhibitor

uPA - urokinase-type plasminogen activator

BMI - body mass index

CRP - C reactive protein

COX - cyclo-oxygenase

VTE - venous thromboembolism

Factor VII - proconvertin

Factor IX - mononine

Factor II - thrombin

BRU - Biomedical Research Unit

LDA - low dose aspirin

ELISA - enzyme linked immunosorbent assay

LDL - low density lipoprotein

HDL - high density lipoprotein

RBC - red blood cell

WBC - white blood cell

MPV - mean platelet volume

TC - total cholesterol

OGTT - oral glucose tolerance test

LOC - low dose oral contraceptive

HOC - high dose oral contraceptive

HOA - high dose oral contraceptive plus aspirin

LOA - low dose oral contraceptive plus aspirin

## ABSTRACT

**Background:** Oral contraceptives are one of the most commonly used birth control methods worldwide. However, some of them have been associated with increased risk of thrombosis. These include 2nd generation combined oral contraceptives. People at higher risk of developing the disease include women living with obesity. Therefore, the aim of this investigation was to assess and evaluate the effects of 2nd generation combined oral contraceptives on the development of thrombosis and inflammation.

**Methods:** In summary, the study involved 4 groups with the first group put under a low-fat diet (LFD) (n=5) with 10 kcal% derived from fat throughout the experimental procedure. The second group was put under a high-fat diet (HFD) (n=5) with 60 kcal% derived from fat for 8 weeks. After 8 weeks this group (2nd) further received low-dose oral contraceptives (LOC) (4.5 µg of levonorgestrel and 0.9 µg of ethinylestradiol) for 5 weeks and an additional low dose of aspirin (10 mg/day) for another 5 weeks on the last phase of the experiment. The third group was on HFD (n = 5) for 8 weeks. After 8 weeks this group (3rd) received high dose of oral contraceptives (HOC) (9 µg levonorgestrel and 1.8 µg of ethinylestradiol) for 5 weeks and later received a low dose of aspirin (10 mg/day) for another 5 weeks on the last phase. Lastly, the fourth group was put under a HFD group (n = 5) for 8 weeks and continued on experimental diet for an additional 5 weeks without oral contraceptives (OC) administration. During this phase this group only received normal saline for 5 weeks and was further put on low dose aspirin on the last additional 5 weeks of the experiment. The study used 2nd generation combined oral contraceptives, and all animals were euthanized at the end of the study. We then assessed metabolic parameters, haematological indices, coagulation factors, and inflammatory markers.

**Results:** There were significant changes in fasting blood glucose ($F_{(1.801, 9,005)} = 8.313$; $p = 0.01010$) and insulin ($F_{(2.530, 10.12)} = 5.039$; $p = 0.02510$) levels among the study groups, but no significant changes in body weights. Lipid profiles remained largely unchanged, except for total cholesterol levels ($p = 0.01530$), which showed a significant difference. We also observed a slight increase in platelet count with both high and low OC dosages. Clotting proteins, including thrombin ($F_{(1.931, 7.724)} = 5.361$; $p = 0.0354$) and D-dimers ($F_{(2.653, 10.61)} = 6.298$; $p = 0.0117$), were significantly altered across all groups. Additionally, there were significant changes in pro-inflammatory cytokines IFN-γ ($F_{(3.832, 34.49)} = 5.572$; $p = 0.0016$), TNF-α ($F_{(2.743, 24.69)} = 5.041$; $p = 0.0086$), IL-10 ($F_{(2.490, 22.41)} = 11.18$; $p = 0.0002$), IL-4 ($F_{(1.974, 17.76)} = 16.61$; $p = <0.0001$), and IL-6 ($F_{(2.342, 21.08)} = 14.40$; $p = <0.0001$) among all groups.

**Conclusion:** These effects of OCs on thrombosis and inflammation development vary depending on the dose and type of contraceptive. Second-generation combined oral contraceptives (OCs) increase the risk of thrombosis and inflammation due to elevated levels of pro-coagulant factors and inflammatory cytokines. 2nd generation combined oral contraceptives can also enhance platelet aggregation, which further indicates a potential for thrombosis and inflammation.

**Prologue**

The following chapter aims to introduce the background about combined oral contraceptives and their overall contribution towards the development of thrombosis. The background is based on existing literature and previous research. This chapter highlights the aims, objectives, research questions and the study significance. Combined oral contraceptives have been associated with thrombosis development in previous studies. These contraceptives are sensitive to some of the coagulation markers such as thrombin and as a result they stimulation coagulation/clotting in blood. This eventually increases the risk of thrombosis development. In a state of obesity, the use of oral contraceptives may result in increased risk of thrombosis which may be due to the interlink between inflammation and thrombosis in obesity. The risk of disease may be dependent on the amount of oestrogen used or the dosage of the oral contraceptive used.

# CHAPTER 1: INTRODUCTION

## 1. INTRODUCTION

Globally, approximately 150 million women between the age of 15-49 years use oral contraceptives as a birth control method (1). Previous research has reported increased risk of thrombosis in relation to oral contraceptives usage (2). Furthermore, research has also been conducted on individuals living with obesity on the effects of oral contraceptives on thrombosis development (3).

Venous thromboembolism and hyper coagulation are some of the most noticeable indications associated with thrombosis under oral contraceptive (OC) usage (2). First, second and third generation OCs have been associated with increased risk of thrombosis according to previous research (4). The use of OCs result in increased thrombin production and patients born with thrombophilia are at high risk of developing thrombosis when using OCs (5). Oral contraceptive usage has also been shown to decrease levels of antithrombin III, which is a regulatory coagulant protein responsible for inhibiting blood coagulation (6). This occurs due to increased expression of pro-coagulant factors under OC usage thereby causing suppressed expression of antithrombin III thus resulting in reduced antithrombin levels (6). Antithrombin III levels have also been found to be significantly reduced in individuals living with obesity (7).

The impact of OC usage on thrombosis risk can be severe when oral contraceptives and obesity coexist (3,8). Independently, each of these elements increase the risk of blood clots (6,9). The risk of thrombosis may be increased by the hormonal effects of oral contraceptives and obesity up to 10 or 24 fold (8,10).

Progestin-only contraceptives are recommended for premenopausal women who are most likely to develop thrombosis likewise for younger females with any inherited or acquired thrombophilia (2). They are also recommended for women who are affected by obesity since they are at high risk of developing venous thromboembolism (3).

Some women fear that contraceptive usage might lead to their weight gain, however there is no solid evidence that links contraceptive usage to weight gain (11). Evidence on the efficiency of oral contraceptives on obese women is contradictory as some reports suggest that obese women have a higher chance of getting pregnant while on oral contraceptives compared to non-obese women (12). Other reports have shown no significant difference on OC efficiency in relation to body weight, however, failure rates are slightly higher for obese women (13). In addition, the effects of these OCs on developing the risk of thrombosis is primarily dependant on the amount of progestogen and ethinylestradiol (oestrogen) used in their production (5).

This led to the speculation that the use or consumption of combined oral contraceptives will contribute to the development of thrombosis, which includes increased risk of venous thromboembolism and hypercoagulation in obesity.

**Study significance**

Understanding the effects of 2nd generation combined oral contraceptives on thrombosis in women affected by obesity is vital for choosing the safest birth control method and preventing the risk of developing venous thromboembolism among women affected by obesity. Venous thromboembolism is a life-threatening medical condition as it may result in many cardiac abnormalities such as stroke and heart attack. Thus, its prevention is vital, especially for individuals that already have an underlying condition such as obesity. Obesity and 2nd generation combined oral contraceptives have both been linked as risk factors for increased risk of thrombosis individually. Therefore, their coexistence may further exacerbate the chances of thrombosis development. Therefore, this study will reveal the associated risk of combined oral contraceptives on thrombosis development in obesity.

## 1.1 Aim

- To investigate the effects of 2nd generation combined oral contraceptives on biomarkers of thrombosis using a diet induced obesity rat model.

## 1.2 Objectives

- To determine changes in coagulation proteins (fibrinogen, thrombin, d-dimers and vWF) in a diet-induced obesity rat model following oral contraceptive use.
- To determine the effects of oral contraceptives on T-helper 1 cytokines (TNF-α, IFN-*y*) and T-Helper 2 cytokines (IL-4, IL-6, IL-10) using a diet induced obesity rat model.
- To evaluate the effects of low-dose aspirin on coagulation makers following oral contraceptive use in a diet induced obesity rat model.

## 1.3 Research questions

- How are coagulation factors altered following oral contraception use in diet induced obesity?
- What are the effects of oral contraceptives on inflammation in diet induced obesity?
- What are the effects of low dose aspirin on thrombosis risk markers?

## 1.4 Hypothesis

We hypothesised that combined oral contraceptive usage will lead to an increased risk of thrombosis and inflammation. We further hypothesized that the presence of an underlying condition such as obesity will synergistically increase the risk of thrombosis in individuals using combined oral contraceptives.

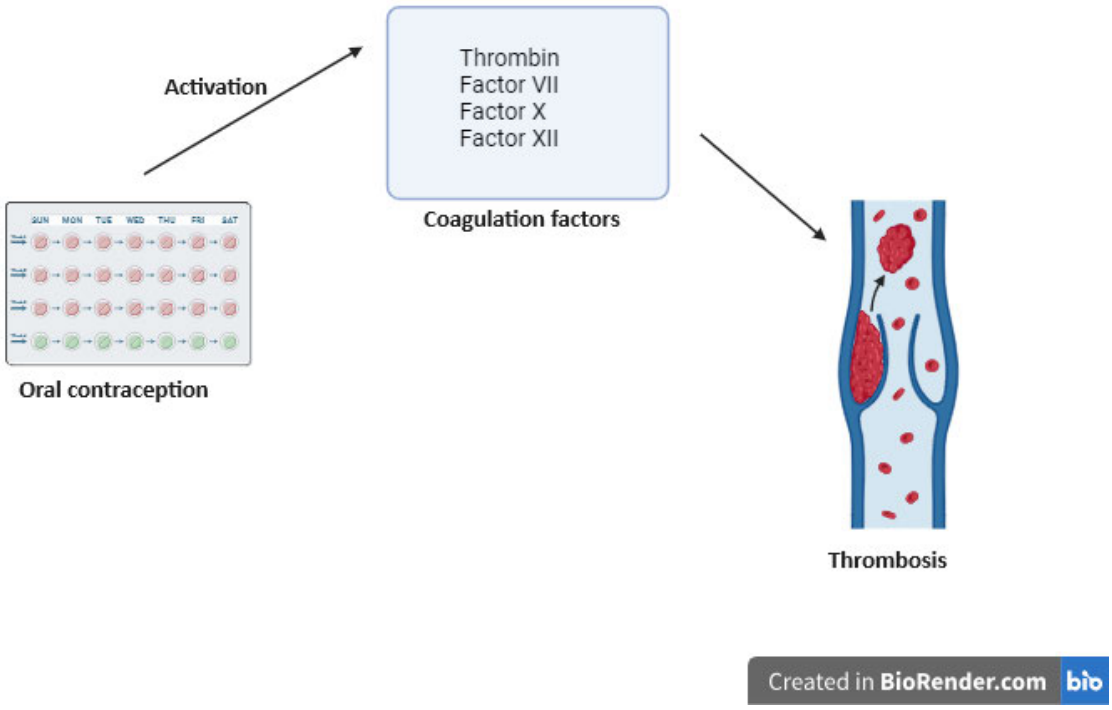


**Figure 1:** The above gives a summary of the impact of 2nd generation combined oral contraceptives on thrombosis development. Created with BioRender.com

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**Prologue**

The following chapter synthesises the current literature on the effects of oral contraceptive on thrombosis, the pathogenesis of thrombosis, the associations of oral contraceptive usage with increased cardiovascular risk. Additionally, it highlights the links between the immune response and thrombosis and/or cardiovascular risk.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 INTRODUCTION

Oral contraceptives are one the most commonly used birth control method (1), and are used by approximately 150 million women worldwide (2). The oral contraceptive (OC) pill serves to prevent unwanted pregnancy in women between the ages of 15 and 44 (1,3). OCs usually contain synthetic oestrogen and progesterone thereby preventing ovulation as the body gets deceived that ovulation has already happened due to high oestrogen and progesterone levels (4).

Even though OC has been proven to prevent unwanted pregnancy, it has also been associated with negative health implication. Studies have shown that OCs are associated with increased risk of thrombotic events and cardiovascular risks (5,6), such as stroke, heart attacks, haemorrhage, deep vein thrombosis (DVT) and arterial thrombosis (7). The relative risk of thrombosis in women using oral contraceptives is approximately 3-7 times compared non-use (8). It has been discovered that oral contraceptive usage causes elevation of coagulant factors especially pro-coagulant factors such as factors VII and X thereby suppressing the expression of anti-coagulant factors such as antithrombin III and causing increased risk of thrombosis (9,10). Fibrinogen formation and platelet aggregation also get elevated under oral contraceptive usage further implying the increased risk of thrombosis development (9).

Another condition that has been associated with increased risk of thrombosis is obesity (11). Obesity has also been associated with a state of hypercoagulation (11). Therefore, in the case whereby a women affected by obesity chooses to use combined oral contraceptives as her birth control method, the relative risk of thrombosis further increases (12). Obesity and oral contraceptive usage have been associated with a ten-fold increased risk of thrombosis according to previous research (13). Previous studies also recommend that women living with obesity should at least use progestin-only contraceptives than combined oral contraceptives to reduce the risk of thrombosis development (12,14).

Therefore, this review aims to discuss the role of oral contraceptive use in inflammation and development of thrombosis in obesity. Furthermore, this review also highlights the role of current anti-inflammatory drugs in preventing cardiovascular disease (CVD) risks and thrombosis.

## 2.2 The pathogenesis for thrombosis

Thrombosis is a medical condition characterized by abnormal blood clot formation thereby causing disruption in normal blood flow (15). During an injury, blood clot formation is essential for preventing blood loss on the damaged area and reducing blood flow across the damaged area (16). However, the blood clot eventually dissolves during recovery (16). Certain medical abnormalities such as thrombosis may cause abnormal blood clot formation within blood vessels (15). This may occur due to an imbalance in the homeostatic coagulation pathway (17). As a result, there might be increased activation of procoagulant factors such as prothrombin, fibrinogen and tissue factor (TF) (17).

This might cause suppression of anticoagulant factors such as antithrombin III thereby causing dysregulation of blood clot formation and failure to break down blood clot formation (15). Uncontrolled formation of blood clots might disrupt blood flow and cause blockages within blood vessels (figure 1) thus causing thus increasing thrombosis risk (17). Some of the risk factors for thrombosis include conditions such as diabetes, obesity, surgery, pregnancy, aging and oral contraceptive usage (18). Thrombosis development may also result in cardiac abnormalities thereby causing increased risk of stroke and heart attack (19). It is also responsible for cerebrovascular accidents which is one the leading cause of deaths associated with thrombosis (20).

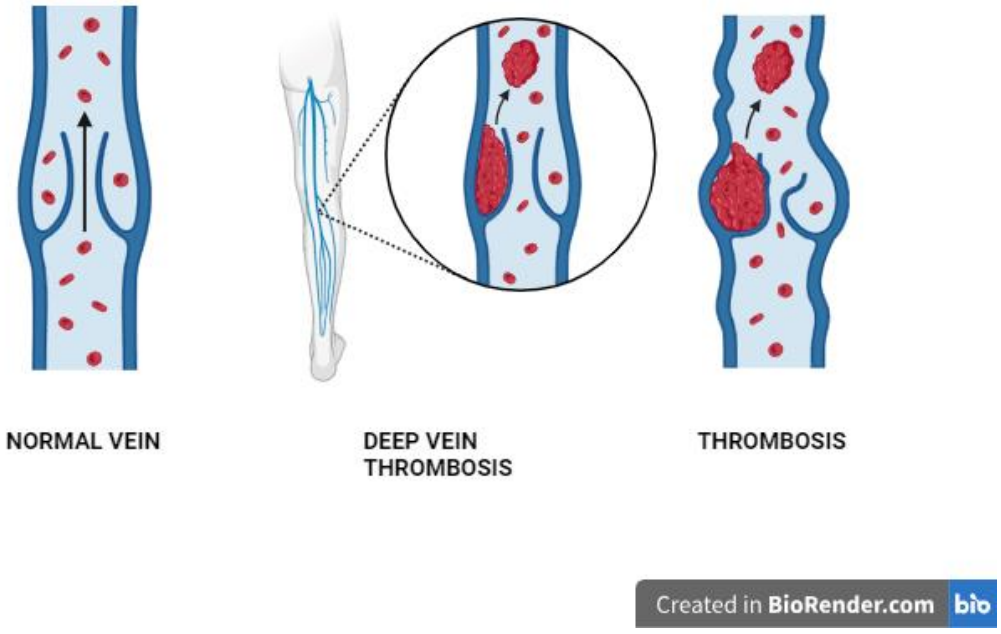


**Figure 1:** An illustration of normal blood vessel and blood vessel characterised by abnormal blood clot formation (thrombosis). Created with BioRender.com using work described by (15).

Another theory that was introduced to assume the development of vascular thrombosis is Virchow's triad (figure 2), which predicts thrombosis development in the presence of three factors which are: hypercoagulability of blood, endothelial or vessel wall injury, and changes in blood flow. A hypercoagulable state results due to increased activation of blood pro-coagulant factors thereby causing abnormal blood clot formations leading to the development of thrombosis (15). Endothelial damage also contributes to increased risk of thrombosis as a result of increased activation of tissue factor, von Willebrand factor (vWF) and platelet aggregation, which promote blood clot formation that can lead to thrombosis if not regulated (21). Another factor contributing to thrombosis according to Virchow's

triad, is the change in blood flow (22). Blockages by abnormal thrombus formations may result in turbulent flow (22). Abnormal blow flow may also be as a result of damaged blood vessels that cause rapid blood flow towards the area of injury (23).

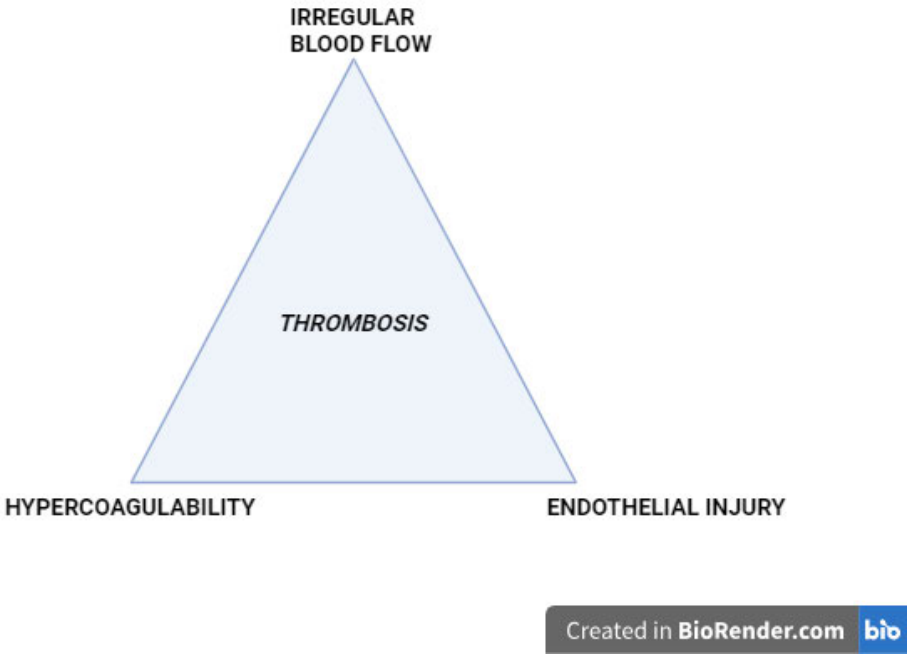


**Figure 2:** Illustration of Virchow's triad showing the three factors that contribute to the development of thrombosis i.e hypercoagulability, irregular blood flow and endothelial injury. Created with BioRender.com using work by (15).

### 2.3 The association of oral contraceptive usage with increased risk of thrombosis and cardiovascular risk

Oral contraceptives are one of the most popular birth control methods used by women to prevent unwanted pregnancy (24). The pills have been thoroughly studied over the years and have been highly associated with increased risk of thrombosis which includes venous thromboembolism, arterial thrombosis and pulmonary embolism with an increased risk of cardiac abnormalities (25). This may be attributed to increased blood pro-coagulant factors due to their oestrogen and progesterone composition being highly sensitive to some of the coagulation proteins such as factors II, VII, VIII, IX, and X (9).

The composition of oestrogen present primarily determines the effect the pill will have on the development of thrombosis (26). A higher dosage is associated with a higher risk of thrombosis

development. OC usage has been linked with hypercoagulation in the blood due to elevated pro-coagulant blood factors such as factors II, VII, IX, X, von Willebrand factor, d-dimer and fibrinogen hence why its presence results in increased blood coagulation (27). OCs are also responsible for the suppression of antithrombin III which is responsible for coagulation inhibition therefore resulting in uncontrolled coagulation (26). Activation of these coagulant proteins by OCs activates a series of enzymatic reactions that lead to abnormal blood clot formation (9,27). This abnormal blood clot formation therefore increases the risk of thrombosis development (26).

Combined oral contraceptives have also been linked to heart related abnormalities such as stroke and myocardial infarctions (7,28,29). This is due to blockage in blood vessels by the abnormally formed clots (9). The blockages therefore disrupt the normal blood flow and cause cardiac abnormalities such as stroke and heart attack (9). Previous studies have shown that progestin only oral contraceptives have no association with increased risk of thrombosis and cardiac abnormalities as compared to combined oral contraceptives (27). This makes progestin-only OCs the safest birth control OCs for people with increased risk of thrombosis and cardiovascular abnormalities (12).

**2.4 Development of thrombosis in obesity**

Obesity may be responsible for the development of many thrombotic disorders such as venous thromboembolism, stroke, heart attack and other cardiac abnormalities (30). Some of the main pathways that have been linked to thrombosis in obesity include a state of chronic inflammation and dysregulated fibrinolysis (11). This is due to continued secretion of pro-inflammatory cytokines secreted by adipocytes as a result of fat deposition (31,32). An increase in Reactive Oxygen Species (ROS) has also been associated with chronic inflammation as a result of continued fat deposition, hyperglycaemia, insulin resistance and impaired glucose tolerance (33). This stimulates recruitment of macrophages to adipose tissue resulting in more macrophages that reside in adipose tissue (34). TNF-α, IL-6, and IL-1β are among the inflammatory cytokines that are secreted and circulated throughout the body by activated macrophages in interaction with preadipocytes and adipocytes (34). These cytokines also promote an inflammatory state in the liver and other locations such as vascular cells thus further contributing to the continued pro-inflammatory state (35).

Due to increased platelet activation through inflammation, the amount of circulating reticulated platelets is increased in obesity therefore leading to increased synthesis of thrombopoietin (30). This also causes increased production of procoagulant factors and suppression of anti-coagulant factors therefore resulting in increased thrombin synthesis (36). In addition, the increased expression of tissue factor and plasma levels of pro-coagulant factors such as fibrinogen are likely caused by increased secretion of proinflammatory cytokine such as TNF-α, IL-1β and IL-6 which further attributes to the development of thrombosis (32). Dysregulation of endogenous anticoagulant systems, such as antithrombin, tissue factor pathway inhibitor, and the protein C anticoagulation system, is also linked to development of

chronic inflammation (35). Unbalanced haemostasis and a higher risk of thrombosis result from these changes (35).

Obesity has also been associated with impaired fibrinolysis through suppression of plasmin activator proteins to degrade fibrin clots (30). Plasminogen activator inhibitor-1 (PAI-1) is a key regulator for fibrinolysis inhibition through inhibition of plasmin activator proteins such as tissue plasminogen activator also known as tissue plasminogen activator (tPA), and urokinase-type plasminogen activator also known as urokinase-type plasminogen activator (uPA) (30). In patients who are classified as being obese or those who have a higher Body Mass Index (BMI), the levels of PAI-1 are increased (37). This means that there is less degradation of fibrin clots and more formation thus increasing the risk of thrombosis development. A recent study demonstrated that with reduced PAI-1 levels, the prothrombotic state associated with obesity significantly decreased (38). TNF-α has also been linked to the increased production of PAI-1, which indicates that the chronic inflammation associated with obesity might be influential to the antifibrinolytic process linked with obesity that results in prothrombotic events (30).

**2.5 The link between immune response and thrombosis/cardiovascular risk**

Thrombosis has been associated with impaired immunity as a result of uncontrolled inflammation associated with it (35,39). The key regulatory pathways for pro-coagulation include the tissue factor pathway inhibitor, protein C and antithrombin III which act as anticoagulants thus maintaining homeostasis in coagulation (35). Antithrombin III production is disturbed in a state of chronic inflammation and continued neutrophil activation (35). This further enhances the production of pro-coagulant factors which result in increased thrombin synthesis thus increasing the risk of thrombosis and reducing antithrombin III levels (figure 3) (19,35).

Another important factor linking the development of thrombosis with inflammation is the excessive expression of tissue factor (figure 3) which results in high thrombin generation (35,40). Tissue factor is a coagulant protein that is responsible for the initiation of thrombin synthesis in blood clot formation (41)**.** Tissue factor takes part in both inflammation and the coagulation pathway (35). Tissue factor expression mostly occurs in tissues and not in direct contact with blood (35). However, in the case that a vessel is damaged and direct contact occurs between tissue factor and the blood, it will bind and cause activation of a series of pro-coagulant proteins such as factor VIIa thus leading to activation of factors IX and X via the extrinsic pathway (41). A series of coagulation factors will be activated on the extrinsic coagulation cascade up until formation of thrombin (40,41). This will result in enhanced fibrin clot formation as a result of increased thrombin production converting fibrinogen into fibrin thus causing more platelet aggregation and an end result of blood clot formation (42). Platelets also play a key role in the development of thrombosis as they are highly associated with both inflammation and coagulation (43).

Elevated levels of mean platelet volume are also linked with an increased cardiovascular risk which is accompanied by a state of low-grade chronic inflammation (44). Platelets have been also found to store some chemokines and pro-inflammatory cytokines such as IL-1β which are over secreted in a state of chronic inflammation further increasing the risk of thrombosis development (35).

C reactive protein (CRP), a protein produced by the liver, has been identified as a protein that is involved in both the immune response and the development of thrombosis (45). This protein also plays a crucial role in the regulation of inflammation (figure 3) (46). C reactive protein typically reveals the intensity of systemic inflammation and disease activity, contributing to immune defence against various pathogens (35). CRP binds to foreign cells or pathogens making them more susceptible to phagocytosis by binding to Fc receptors thus triggers the complement cascade (45,46). Moreover, CRP assists in the removal of apoptotic and necrotic cells, which is vital for preventing autoimmunity (47). Plasma CRP levels are great markers for inflammation as they reveal the intensity of an inflammatory state at a given time (48). However, there may be exceptions in inflammatory diseases such as inflammatory myositis, where plasma CRP levels may not correlate to the present inflammatory state (48).

Atherosclerosis is primarily caused by inflammation, and in acute coronary syndromes, elevated CRP levels may exacerbate prothrombotic and proinflammatory events (45). Through many mechanisms involved in primary and secondary haemostasis, CRP may trigger the onset of thrombosis (figure 3) (35). CRP initiates primary haemostasis through an increase in platelet count, adhesiveness, and also platelet aggregation (35). Additionally, prostacyclin release from human endothelial cells is notably reduced by CRP (45). Conversely, CRP decreases tissue factor pathway inhibitor and increases tissue factor, tissue plasminogen activator, and Von Willebrand factor to initiate secondary haemostasis (49). When combined, CRP may cause increase blood coagulation, reduce the body's natural fibrinolytic ability, and stimulate platelets, among other prothrombotic effects (45).

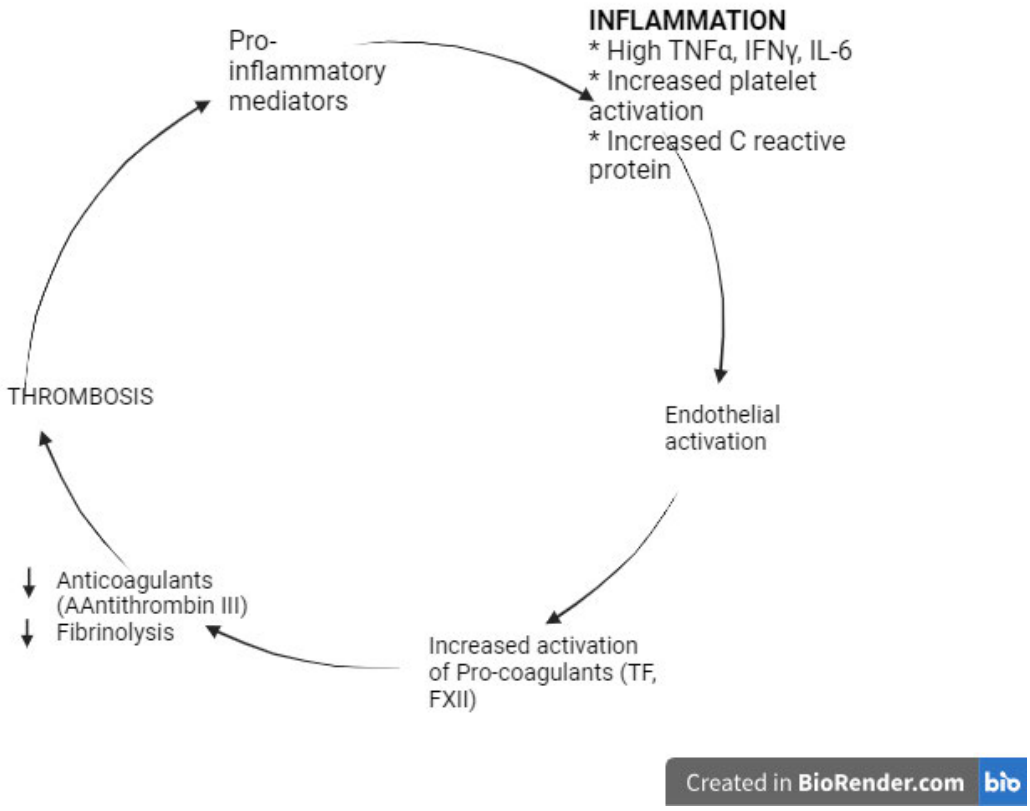


**Figure 3:** Illustration of the associations between activation of the immune system (inflammation), endothelial tissue, pro and anti-coagulant factors towards the development of thrombosis. In a pro-thrombotic state, there is an increased expression of pro-inflammatory cytokines thereby causing inflammation and endothelial activation. These result in increased platelet activation and aggregation. In addition, there is increased activation of pro-coagulant and suppression of anti-coagulants. Therefore, the end result is the development of thrombosis. Created with BioRender.com using work by (35).

### 2.6 Clinical diagnosis of thrombosis

There is no specific test that is used for the diagnosis of thrombosis, however there are some tests that help to detect if there is development of thrombosis in the anatomical system (50). These tests include duplex ultrasound D-dimer blood test, MRI scans, ultrasound and venography (the test is rarely done because it is invasive) (50). With improving medical techniques, several clinical models have been introduced for the prediction of thrombosis development.

One of the most popular and widely used model is the Wells clinical model (Table 1) (51). It is used to predict the probability of deep vein thrombosis and classify patients as either low or high-risk populations (51).

**Table 1.** Wells clinical model for predicting deep vein thrombosis (51)**.**

| **Clinical feature** | **Score** |
|---|---|
| Active cancer (treatment ongoing or within previous 6 months or palliative) | 1 |
| Paralysis, paresis, or recent plaster immobilisation of the legs | 1 |
| Recently bedridden for more than 3 days or major surgery within 4 weeks | 1 |
| Localised tenderness along the distribution of the deep venous system | 1 |
| Entire leg swollen | 1 |
| Calf swelling by more than 3 cm compared with the asymptomatic leg (measured 10 cm below the tibial tuberosity) | 1 |
| Pitting oedema (greater in the symptomatic leg) | 1 |
| Collateral superficial veins (non-varicose) | 1 |
| Alternative diagnosis as likely or wider than that of deep vein thrombosis | -2 |
| | |
| Low probability 0 or less, moderate probability 1-2, high probability 3 or more. | |
| | |

The risk scores in this table are interpreted as follows: a score of 3 or more is regarded as high risk; a score of 1-2 is regarded as moderate/intermediate risk; and a score less than 1 is regarded as low risk.

### 2.7 Treatment for thrombosis

#### 2.7.1 Aspirin

Aspirin is commonly known as an anti-inflammatory drug that is usually used to treat fever, inflammation and pain (52). It is sometimes referred to as a blood thinner due to its ability to inhibit platelet activation and reduce blood clotting (53). Aspirin has also been associated with increased prothrombin time and fibrinolysis. During inflammation, membrane phospholipids are converted to arachidonic acid by the enzyme phospholipase A2 (figure 4). This arachidonic acid is then converted to prostaglandin and prostacyclin by enzymes cyclo-oxygenase 1 (COX-1) and cyclo-oxygenase 2 (COX-2) (figure 4) and then released into the blood stream thus causing vasodilation and inflammation through the attraction of immune cells (52). Aspirin therefore acts by targeting enzymes COX-1 and COX-2 thus inhibiting their activation which prevents arachidonic acid from being converted to prostaglandin and prostacyclin (53). Reduced production of these prostaglandins and thromboxane result in lowered platelet aggregation and prevents increased activation of immune cells thus reducing blood clot formation (54). This assists in reducing thrombosis associated abnormalities such as stroke and heart attack and ensures optimal blood circulation (54).

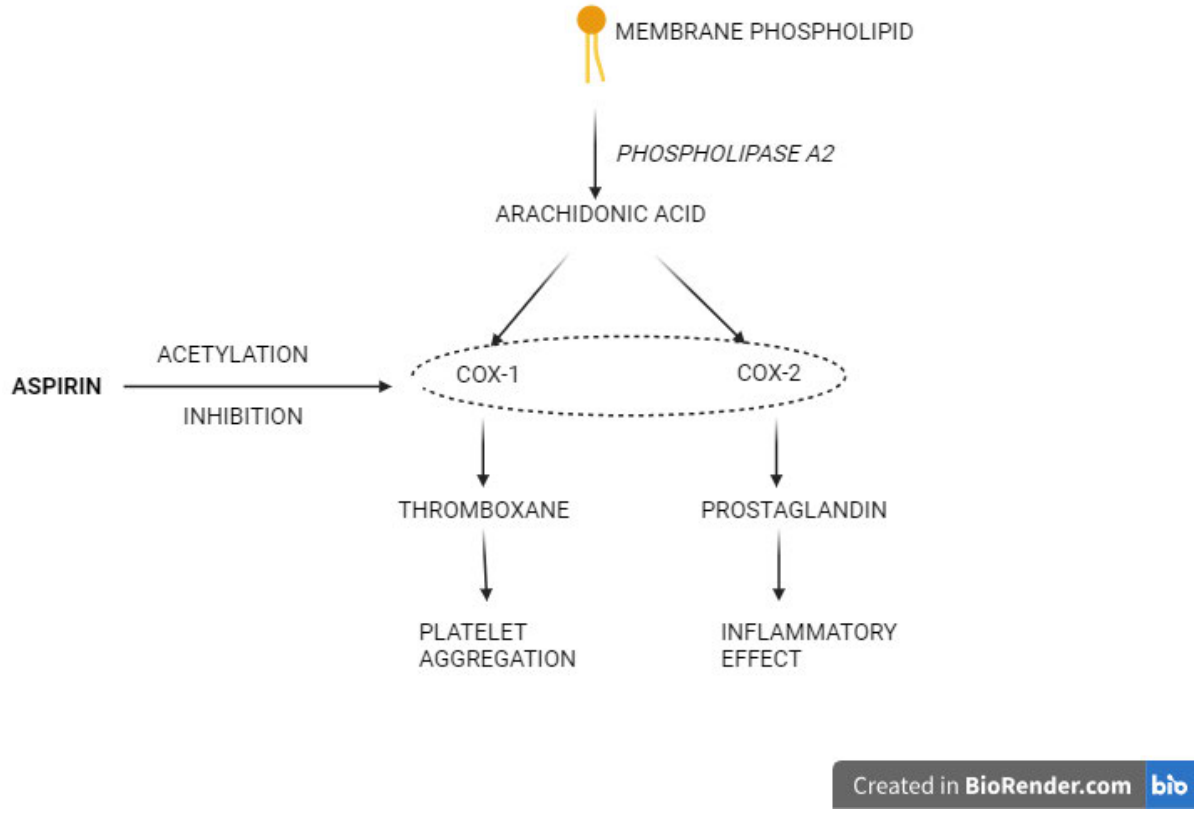


**Figure 4: mechanism of action for aspirin.** Aspirin acts through acetylation of the cyclo-oxygenase enzymes thereby causing inactivation of the enzymes. Inactivation of the COX enzymes inhibit thromboxane and prostaglandin production. This reduces pro-inflammation and platelet aggregation. Created with BioRender.com using work by (53).

### 2.7.2 Heparins

Heparins are another form of anticoagulants that have been used to treat deep vein thrombosis (50). The most preferred type of heparins have been low molecular weight heparins over unfractionated heparins (50). Low molecular weight heparins are the fragments of unfractionated heparins and have been found to be most effective and have less side effects compared to fractionated heparins (55). Low molecular weight heparins have been most recommended for patients with contraindications for certain oral anticoagulants (50). The includes pregnant women, patients who have recently undergone surgery or those with bleeding disorders such as haemophilia.

There are many anticoagulant medicines that may be prescribed by health care professionals to aid in dissolving abnormal blood clots in thrombosis. In some cases, a patient may have to undergo surgery to remove blood clots that may have caused blockage inside veins and arteries (56). Another method recommended by previous research is wearing compression stockings to try and relieve pain and swelling in deep venous thrombosis (57).

## 2.8 Clinical implications

Understanding the associated risk of oral contraceptives in thrombosis development on obesity is vital for the well-being of women living with obesity. This might assist health care workers in choosing other alternatives for the safest method of contraception for women affected by obesity thereby reducing the risk of thrombosis development. It might also help develop public awareness thereby revealing the potential risk associated with oral contraceptives for women affected by obesity. Therefore, this study is of high significance on the health of women living with obesity who wish to undergo contraception.

## 2.9 Concluding remarks

Over the years, oral contraceptives have been highly associated with increased thrombosis risk. However, with advancing research, this has led to a variety of oral contraceptive generation types which have been associated with less to none thrombosis risk. Moreover, obesity may be another factor associated with the development of thrombosis. If a patient living with obesity uses combined oral contraceptives as their preferred birth control method, it might further elevate their risk of developing thrombosis. Hence why scientists recommend progestin-only contraceptives for women living with obesity to try and reduce the risk of further increasing their risk of thrombosis development. Aspirin and heparins may be used as both anti-coagulant/anti-inflammatory drugs for the treatment of thrombosis.

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**Prologue**

This chapter forms part of the experimental paper in which we discuss the findings of our study design. The experiment used an animal model which included diet phase (HFD and LFD), an 2nd generation oral contraceptive phase (high and low dose), and a low dose aspirin phase. The aim was to assess 2nd generation oral contraceptive effects in diet induced obesity on the development of thrombosis, and to determine the effect of low dose aspirin in a thrombotic state. Interestingly, the usage of 2nd generation combined oral contraceptives resulted in increased procoagulant factors in both high and low OC usage. Furthermore, low dose aspirin usage helped lower some of the thrombosis markers such as d-dimers in a thrombotic state. The findings in this paper concluded that 2nd generation OC usage further increases the risk of thrombosis.

**CHAPTER 3: MANUSCRIPT – EXPERIMENTAL PAPER**

# High-dose 2nd generation combined oral contraceptives promote thromboinflammation in obesity.

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***This paper has been submitted for publication***

**Abstract**

***Background:*** Oral contraceptives are one of the most commonly used birth control methods used by women worldwide. Second generation combined oral contraceptives have been associated with increased risk of thrombosis. Therefore, the aim of our study was to determine the effects of second generation combined oral contraceptive usage on the development of thrombosis.

***Methods***: In this experiment, 20 female Sprague Dawley rats were randomised into 4 groups with an n = 5 each group. The LFD group (n = 5) remained under LFD throughout the experiment. Furthermore, 3 individual groups of rats were put on HFD for 8 weeks. After 8 weeks of HFD-feeding, the first group (I) (n = 5) received low dose oral contraceptive (4.5 µg of levonorgestrel and 0.9 µg ethinylestradiol) plus HFD, the second group (II) (n = 5) received high dose oral contraceptive (9 µg levonorgestrel and 1.8 µg ethinylestradiol) plus HFD, both daily for a 5-week period. The third group (III) (n = 5) was kept on the experimental diet without any OC administration and just received the vector (saline) on daily basis. After OC administration, the two groups of OC administration were further put on a low dose aspirin (10 mg/day) treatment for a 5-week period. The group under HFD that did not receive OC administration was also put under low dose aspirin treatment for a period of 5 weeks. Animals were sacrificed using halothane inhalation after the at the end of the study. Measurements of metabolic profiles, lipid profiles, haematological parameters, coagulation factors, and pro-inflammatory cytokines were then assessed.

***Results:*** There were significant changes in glucose ($F_{(1.801,\ 9.005)} = 8.313$; $p = 0.01010$) and insulin ($F_{(2.530,\ 10.12)} = 5.039$, $p = 0.02510$) levels across the groups, but no significant changes occurred in body weights. No significant changes occurred on lipid profiles except for total cholesterol levels ($p = 0.01530$). A slight increase in platelet count was also noticed under both high and low OC dosages. Clotting proteins such as thrombin ($F_{(1.931,\ 7.724)} = 5.361$; $p = 0.0354$), D-dimers ($F_{(2.653,\ 10.61} = 6.298$; $p = 0.0117$) were significantly altered across all groups. Lastly, there were significant changes in pro-inflammatory cytokines IFN-γ ($F_{(3.832,\ 34.49)} = 5.572$; $p = 0.0016$), TNF-α ($F_{(2.743,\ 24.69)} = 5.041$; $p = 0.0086$), IL-10 ($F_{(2.490,\ 22.41)} = 11.81$; $p = 0.0002$), IL-4 ($F_{(1.974,\ 17.76)} = 16.61$; $p = <0.0001$), IL-6 ($F_{(2.342,\ 21.08)} = 14.40$; $p = <0.0001$) across all groups.

***Conclusion:*** The 2nd generation combined OC increased risk thrombosis and inflammation as a shown by increased levels of pro-coagulant factors and inflammatory cytokines. The OC usage also contributes to increased platelet aggregation further implying the development of thrombosis and inflammation.

**Keywords**: Oral contraceptives, thrombosis, aspirin, inflammation, coagulation

### 3.1 Introduction

The most common type of thrombosis is venous thromboembolism (VTE) which results in pulmonary embolism in approximately 33% of patients with VTE. Deep vein thrombosis occurs in approximately 64% of patients with VTE (1). This disorder can both be acquired or genetic (2). Thrombosis is more prevalent in males than in females (3). Some of the risk factors associated with this disorder include pregnancy, hormonal contraception, obesity, surgery, history of thrombosis and pro-thrombotic mutations (4). Obesity, as one of the risk factors for thrombosis, is also associated with elevated thrombosis risk (5). It has also been linked with hypercoagulation due to increased activation of pro-coagulation factors (5). Obesity and oral contraceptive use have been associated with a 10-24 fold increased risk of thrombosis development (6,7).

Oral contraceptives (OCs), are a widely used method for birth control, and have been associated with increased risk for thrombosis (8). This may be attributed to an increase in pro-coagulation factors such as factors proconvertin (VII), mononine (IX), thrombin (II), fibrinogen (I) and also an increase in D-dimer levels in the blood (6). An increased expression of pro-inflammatory cytokines has also been linked to oral contraceptive usage (9). This links OC usage with a pro-inflammatory state and further risk of thrombosis development due to an increased activation of platelets and tissue factor (10,11).

OCs alter platelet activation indirect through Von Willebrand Factor (vWF) mediated pathways that induce platelet aggregation (12,13). The levels of platelet activation are OC dose-dependent (14). Von Willebrand factor, which plays a key role in platelet tethering , has is elevated following administration of OC (13). Infact, endothelial cells are directly stimulated by estrogen and this promotes the synthesis of vWF (13). This further contributes to increased risk for thrombosis (13). Notably, progestin-only oral contraceptives have not been associated with increased thrombotic-risk and this makes them most suitable for patients with increased risk of thrombosis (15).

Therefore, the aim of our study was to determine the effects of 2$^{nd}$ generation combined oral contraceptive usage on the development of thrombosis.

### 3.2 METHODOLOGY

#### 3.2.1 Animals and animal handling

Twenty-five-weeks-old Sprague Dawley rats were purchased and housed at the Biomedical Research Unit (BRU) at the of University KwaZulu-Natal. Animal handling followed guidelines and principles, as published by the Committee for the Care and Use of Laboratory Animals (16). The animals were allowed to acclimatize for 2 weeks, with unrestricted access to food and water throughout the study. The animals were kept in a, temperature-controlled environment (22±2°C), humidity (55±5%), an a 12-h light cycle (6:00–18:00) and dark cycle (18:00–6:00). Ethical clearance for this study was granted by the UKZN animal research ethics committee, ethics registration number AREC/00003067/2021. To maintain a clean environment, the cage bedding in was cleaned twice a week.

### 3.2.2 Study design

The study included 20 rats in total for the experimental procedure. Briefly, five rats were kept on a low-fat diet (LFD) with 10 kcal% derived from fat throughout the experimental procedure. In addition, 3 individual groups of rats were put on a high fat diet (HFD) with 60 kcal% derived from fat for a period of 8 weeks with each group having an $n$ = 5 rats per group. After 8 weeks of HFD-feeding, the HFD rats were further monitored as follows (Figure 1). Group I: the low dose oral contraceptive group (LOC) received 4.5 µg of levonorgestrel and 0.9 µg ethinylestradiol; Group II: the high dose oral contraceptive group (HOC) received 9 µg levonorgestrel and 1.8 µg (ethinylestradiol) daily for a 5-week period. Group III the last HFD group was kept on the experimental diet without any OC administration and just received the vector (saline) on daily basis. 2$^{nd}$ generation combined OCs were used in this experiment and were supplied by Aspen pharmaceuticals. After OC administration, the two groups of OC administration were further put on a low dose aspirin (10 mg/day) treatment for a 5-week period. The group under HFD that did not receive OC administration was also put under low dose aspirin treatment for a period of 5 weeks. Samples were collected after each phase via tail vein and cardiac puncture during the terminal end. There were no wash out phases in between. Animals were sacrificed using halothane inhalation after the at the end of the study.

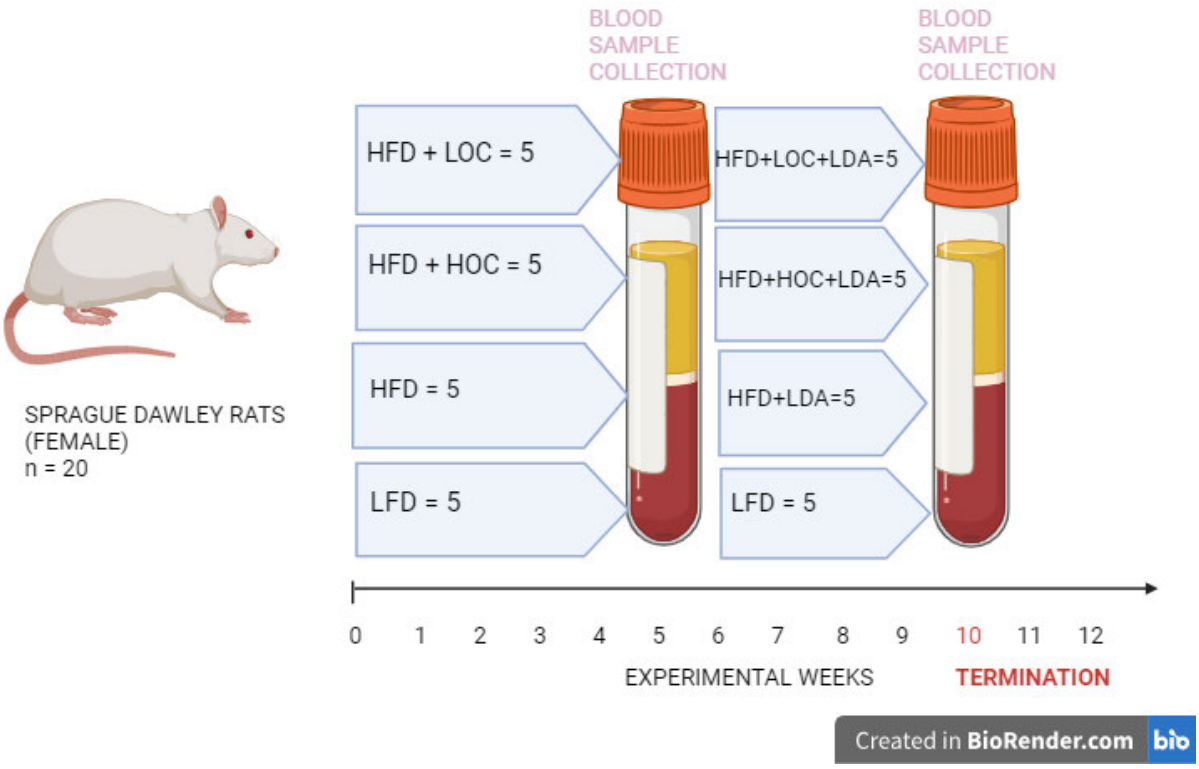


Figure 1: Study design. Thirty Sprague Dawley rats were randomised into two diet groups, the LFD and the HFD group for 8 weeks. After 8 weeks the animals on the HFD were further randomised into low

dose oral contraceptive (LOC) and High dose oral contraceptives for 5 weeks. Lastly, after 5 weeks on contraceptives, the animals were given low dose aspirin (LDA) for further 5 weeks.

### 3.2.3 Oral glucose tolerance test and metabolic profiles

An oral glucose tolerance test was performed after 8 weeks of HFD, 5 weeks of OCs and 5 weeks of low dose aspirin. The rats were fasted for 8 hours and have 3mg/kg of glucose administered orally to measure glucose levels from mouse tail blood using the OneTouch®Select® handheld glucometer (LifeScan Inc., Milpitas, CA, USA) in each of the groups. A glucometer was used to take readings at intervals of 0, 15, 30, 60, 90 and 120 minutes (17).

Insulin levels were also assessed using an ELISA kit as per manufacturer's protocol (Elabscience®, Houston Texas, USA).

### 3.2.4 Lipid Profiles and Hematological indices

We collected 200µl of venous blood through the tail bleeding method (18). A mouse specific enzyme-linked immunosorbent assay kit was used to monitor lipid profiles such as Low-Density Lipoprotein and High-Density Lipoprotein and the total cholesterol levels as per manufacture's protocol (Sigma-Aldrich®, St Louis, USA).

Baseline hematological indices were measured using a hemo analyser (Beckman Coulter, Miami, USA). This enabled us to monitor RBC count, WBC count, lymphocytes and platelets count.

### 3.2.5 Coagulation factors

An enzyme-linked immunosorbent assay (ELISA) was used to assess the following coagulation factors: thrombin (Elabscience®, Houston Texas, USA), fibrinogen (Elabscience®, Houston Texas, USA), Von Willebrand factor (vWF) (Elabscience®, Houston Texas, USA) and D-dimers (Elabscience®, Houston Texas, USA) as per manufacture's protocol.

### 3.2.6 Measurement and analysis of pro-inflammatory cytokines using flow cytometry.

A flow cytometer was used to monitor circulating blood IL-6, IFN-γ, TNF-α, IL-10 and IL-4 cytokines using a rat Th Legend plex 5 Multi Analyte Flow Assay kit as per the manufacture's protocol. A DxFLEX flow cytometer was used to conduct flow cytometry (Beckman Coulter, Miami, USA). Data analysis was performed using a BioLegend online software (legendplex.qognit.com).

### 3.2.7 Data analysis

For all data analysis, a GraphPad Prism 8 software was employed. The one-way ANOVA repeated measures test was used for data analysis. A normality test was performed and for parametric data, a Tukey post hoc test was conducted; however, for non-parametric results, a Kruskal Wallis test was used. Data was presented as mean +/- standard deviation. A $p$ value less than 0.05 was considered statistically significant.

## 3.3 RESULTS

### 3.3.1 Metabolic changes following oral contraceptive administration.

Metabolic changes such as body weight, insulin and oral glucose tolerance were assessed to determine the impact done on cardiovascular risk and metabolism after oral contraceptive administration following high and low OC dosages. Notably, Rats under high OC and low OC dosages did not show any significant changes during and after oral contraceptive administration compared to the LFD group with regards to body weights. Furthermore, insulin levels were compared across all groups to assess any impact done on the development of insulin resistance, Briefly, there were significant changes in the insulin levels across the groups ($F_{(2.530,\ 10.12)}$ = 5.039, $p$ = 0.0251). Insulin levels were significantly elevated in the LOC (35.76 ± 1.814) group when compared to the LFD group (24.09 ± 3.968), $p$ = 0.0042. Insulin levels were slightly increased by HOC compared to the LFD group, Table 1. Oral Glucose Tolerance Test levels were also compared across all groups, high OC dosage caused elevation of OGTT in the HOC group (5.860 ± 1.447) whereas low OC dosage reduced OGTT levels in LOC group (5.503 ± 1.128) when compared to the LFD group (5.613 ± 1.140), $p$ = 0.0101, Table 1.

### 3.3.2 Metabolic changes following 5 weeks of aspirin administration.

Metabolic changes were also reported after 5 weeks of aspirin administration. There were no significant changes that occurred on body weights under aspirin treatment. Insulin levels were significantly altered during the aspirin phase, $p$ = 0.0251, in the LDA (25.55 ± 4.424), HOA (27.79 ± 5.182) and LOA (32.10 ± 3.901) and LFD (24.09 ± 3.968) groups, Table 1. Low dose aspirin usage slightly reduced insulin levels across all groups, however, the changes were not significant per individual group comparison.

### 3.3.3 Impact of oral contraceptives in on lipid profiles

Assessing alterations in lipid profiles was essential to determine the potential risk of cardiovascular disease. There were no significant changes with the HDL and LDL parameters during both oral contraceptive and aspirin administration. However, the levels of Tc were significantly altered by OC and low dose aspirin administration ($p$ = 0.0153, Table 1). Briefly, the levels of Tc were slightly increased by oral HOC (0.3770 ± 0.000) when compared to LFD (0.3043 ± 0.1626). Similarly, the levels of Tc were slightly elevated in the LOC (0.3452 ± 0.01681) group when compared to LFD (0.3043 ± 0.1626), Table 1.

Lipid profiles were also measured after treatment with low dose aspirin. Noticeably, TC levels were also slightly increased following aspirin treatment in the LDA (0.3770 ± 0.000), HOA (0.3136 ± 0.09470) and LOA (0.3770 ± 0.000) groups compared to the LFD group (0.3043 ± 0.1626), $p$ = 0.01530, Table 1. Therefore, low dose aspirin did not help in reducing elevated insulin levels caused by OC usage and HFD.

### 3.3.4 Impact of oral contraceptives and aspirin on haematological indices

A full blood count was conducted to assess the alterations caused by oral contraceptive and low dose aspirin administration in Sprague Dawley rats. With the present results, there were no significant changes on all the haematological indices assessed i.e WBC, RBC, lymphocytes, platelets and MPVs.

**Table 1.** Metabolic changes under oral contraceptive and aspirin administration in Sprague Dawley rats

| | **LFD** | **HOC** | **LOC** | **HOA** | **LOA** | **LDA** | ***p*-value** |
|---|---|---|---|---|---|---|---|
| Body weights (g) | 290.2±10.45 | 291.2±22.35 | 310.8±16.77 | 288.8±21.46 | 288.4±13.90 | 307.8±14.48 | 0.2340 |
| Insulin (µU/L) | 24.09±3.968 | 29.0 ± 5.479 | 35.76±1.814 | 27.79±5.182 | 32.10±3.901 | 25.55±4.424 | **0.02510** |
| OGTT (AUC) (mmol/L X120 min) | 5.613±1.140 | 5.860±1.447 | 5.503±1.128 | 4.737±0.7749 | 5.633±1.081 | 4.763±1.032 | **0.01010** |
| ***Lipid profiles*** | | | | | | | |
| LDL *(µg/µl)* | 0.6814±0.04561 | 0.09960±0.04122 | 0.1317±0.05819 | 0.08344±0.05261 | 0.1211±0.01514 | 0.1260±0.05535 | 0.3173 |
| HDL *(µg/µl)* | 0.2156±0.1041 | 0.1054±0.07817 | 0.2199±0.09761 | 0.1785± 0.1362 | 0.1255±0.07973 | 0.1894±0.06907 | 0.4100 |
| TC *(µg/µl)* | 0.3043±0.1626 | 0.3770±0.000 | 0.3452±0.01681 | 0.3136±0.09470 | 0.3770±0.000 | 0.3770 ± 0.000 | **0.01530** |

The results are reported as mean ± SD. Significance shown in bold (P<0.05). OGTT: Oral Glucose Tolerance Test. LDL: Low-Density Lipoprotein. HDL: High-Density Lipoprotein. TC: Total Cholesterol. LFD: Low Fat Diet. HOC: High Oral Contraceptive dosage. LOC: Low Oral Contraceptive dosage. HOA: High Oral Contraceptive dosage + Low Dose Aspirin. LOA: Low Oral Contraceptive dosage + Low Dose Aspirin. LDA: Low Dose Aspirin.

**Table 2.** Haematological indices following six weeks of oral contraceptive and aspirin administration in Sprague Dawley rats

| | **LFD** | **HOC** | **LOC** | **HOA** | **LOA** | **LDA** | **P - value** |
|---|---|---|---|---|---|---|---|
| WBC (10^3/µL) | 2.960±1.2220 | 2.960±0.9182 | 2.700±1.389 | 2.820±0.4207 | 2.500±0.5612 | 1.820±0.3493 | 0.3337 |
| RBC (10^6/µL) | 4.300±0.6892 | 4.600±0.2449 | 4.920±0.2168 | 4.780±0.1789 | 4.840±0.7861 | 4.600±0.8660 | 0.5269 |
| Platelets (10^3/µL) | 400.0±37.38 | 467.4±60.14 | 460.2±23.03 | 449.6±31.25 | 460.4±41.39 | 415.2±56.86 | 0.2162 |
| Lymphocyte (%) | 88.44±4.251 | 90.28±2.524 | 89.04±2.492 | 90.40±1.523 | 87.74±2.580 | 87.14±2.789 | 0.3407 |
| MPV (fL) | 5.460±0.2510 | 5.620±0.3564 | 5.540±0.1673 | 5.340±0.2408 | 5.320±0.2168 | 5.380±0.2683 | 0.4326 |

The results are reported as mean ± SD. Significance shown in bold (P<0.05). WBC: White Blood Count. RBC: Red Blood Count. MPV: Mean Platelet Volume. LFD: Low Fat Diet. HOC: High Oral Contraceptive dosage. LOC: Low Oral Contraceptive dosage. HOA: High Oral Contraceptive dosage + Low Dose Aspirin. LOA: Low Oral Contraceptive dosage + Low Dose Aspirin. LDA: Low Dose Aspirin.

### 3.3.5 Impact of high and low dose oral contraceptive on coagulation markers

In order to determine the effect of oral contraceptives on the development of thrombosis, levels of coagulation markers such as (thrombin, fibrinogen, d-dimer and vWF) were measured. The results showed that there were significant changes in d-dimer levels across the groups ($F_{(2.653, 10.61)} = 6.298$, $p = 0.01170$). Briefly, low-dose administration of OCs caused a significant increase in d-dimer levels on the LOC (354.2 ± 75.90) group compared to the LFD (193.2 ± 63.53), $p = 0.0414$. High dose OC caused a slight increase in the HOC (242.1 ± 64.45) group compared to LFD (193.2 ± 63.53). However, d-dimer levels were slightly reduced by low dose aspirin treatment in the LDA (210.1 ± 49.28), HOA (206.0 ± 57.32) and LOA (300.0 ± 64.54) groups, Table 2.

We also looked at changes in thrombin levels following OC and low dose aspirin administration. Thrombin levels were significantly altered in both oral OC and aspirin administration ($F_{(1.931, 7.724)} = 5.361$, $p = 0.0354$). The HOC (20.12 ± 3.896) treatment caused a slight elevation of thrombin levels when compared to the LFD (13.80 ± 2.525) group, Table 2. Similarly, the levels of thrombin were slightly elevated in the LOC (15.64 ± 5.375) groups when compared to the LFD (13.80 ± 2.525) group, Table 2. Additionally, aspirin administration further elevated thrombin levels in the groups under aspirin treatment i.e A (16.48 ± 5.228), HOA (27.02 ± 6.081) and LOA (25.00 ± 5.520), $p = 0.0354$, Table 2.

There were no significant differences that occurred on the fibrinogen and vWF parameters both under oral contraceptive and aspirin administration. However, OC usage caused a slight reduction in fibrinogen levels both under HOC (10459±3503) and LOC (9413±2974) groups compared to LFD (11575±3383) group, Table 2. Low dose aspirin usage resulted in a slight increase in fibrinogen levels after OC usage, Table 2. Furthermore, OC usage caused a slight increase in vWF levels both under HOC (0.6848±0.2805) and LOC (1.136±1813±0.1813) groups compared to LFD (0.6485±2933), Table 2. Treatment with low dose aspirin slightly decreased vWF levels in all groups, Table 2.

**Table 3.** Alterations in coagulation factors under oral contraceptive and aspirin administration in Sprague Dawley rats.

| **Coagulation markers** | **LFD** | **HOC** | **LOC** | **HOA** | **LOA** | **LDA** | **P – value** |
|---|---|---|---|---|---|---|---|
| D-dimer (ng/mL) | 193.2±63.53 | 242.1±64.45 | 354.2±75.90 | 206.0±57.32 | 300.0±64.54 | 210.1±49.28 | **0.0117** |
| Thrombin (ng/mL) | 13.80±2.525 | 20.12±3.896 | 15.64±5.375 | 27.02±6.081 | 25.00±5.520 | 16.48±5.228 | **0.0354** |
| Fibrinogen (ng/mL) | 11575±3383 | 10459±3503 | 9413±2974 | 10679±4050 | 10857±4497 | 7270±5385 | 0.5731 |
| vWF (ng/mL) | 0.6485±0.2933 | 0.6848±0.2805 | 1.136±0.1813 | 0.5912±0.1090 | 0.9603±0.2727 | 0.6349±0.2572 | 0.0576 |

The results are reported as mean ± SD. Significance shown in bold ($P<0.05$). vWF: Von Willebrand factor. LFD: Low Fat Diet. HOC: High Oral Contraceptive dosage. LOC: Low Oral Contraceptive dosage. HOA: High Oral Contraceptive dosage + Low Dose Aspirin. LOA: Low Oral Contraceptive dosage + Low Dose Aspirin. LDA: Low Dose Aspirin.

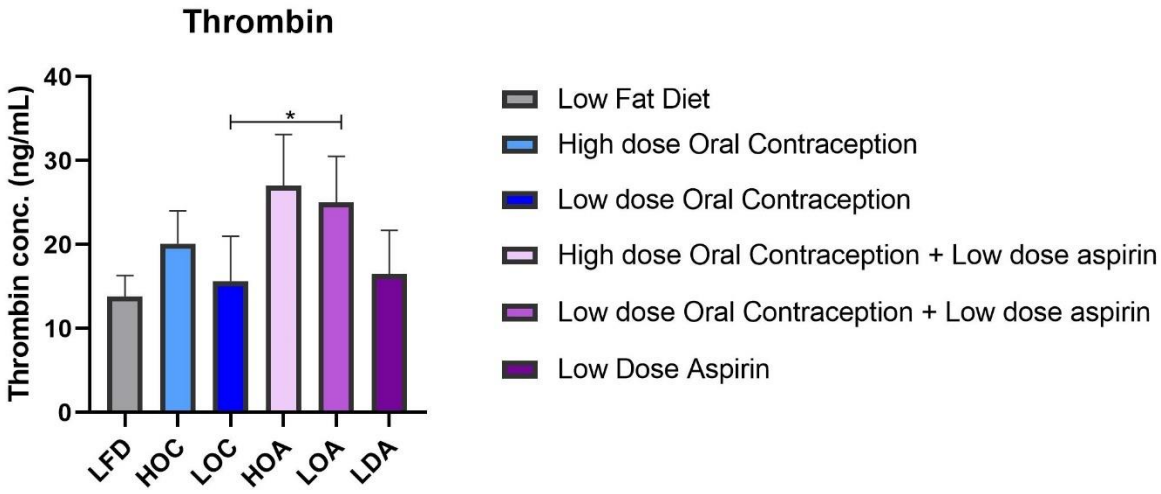


Figure 2: Changes in blood coagulation marker thrombin following 8 weeks of LFD and HFD, 5 weeks of high and low dose OC, and 5 weeks of low dose aspirin treatment. * indicates the significance between LOC and LOA groups, $p = 0.0447$.

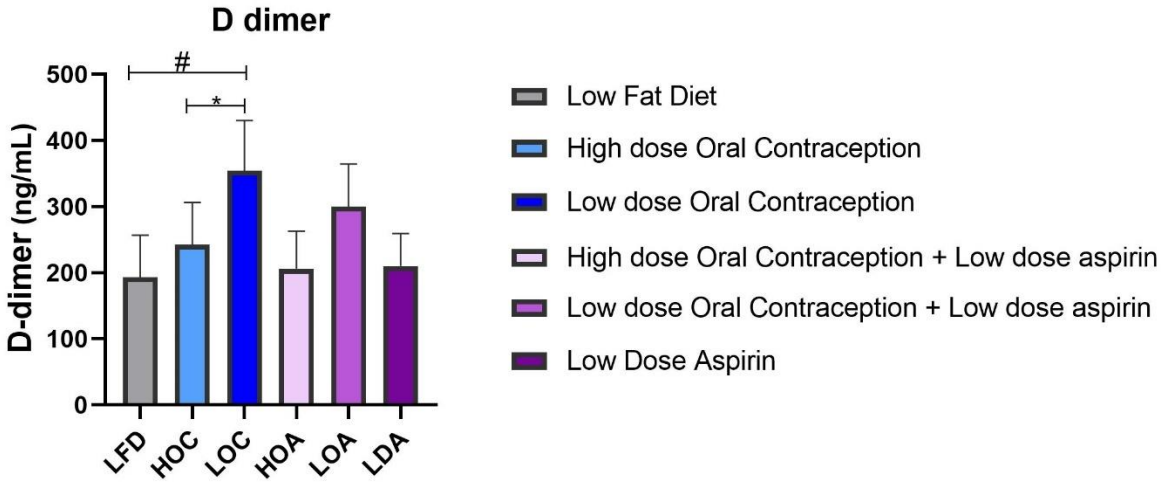


Figure 3: Illustrates D-dimer levels across all groups. OC administration caused an increase in both LOC and HOC groups. # represents the significance between the LFD and LOC groups, $p = 0.0414$. * represents the significance between the HOC and LOC groups, $p = 0.0050$. The data is presented as the mean, ± standard deviation

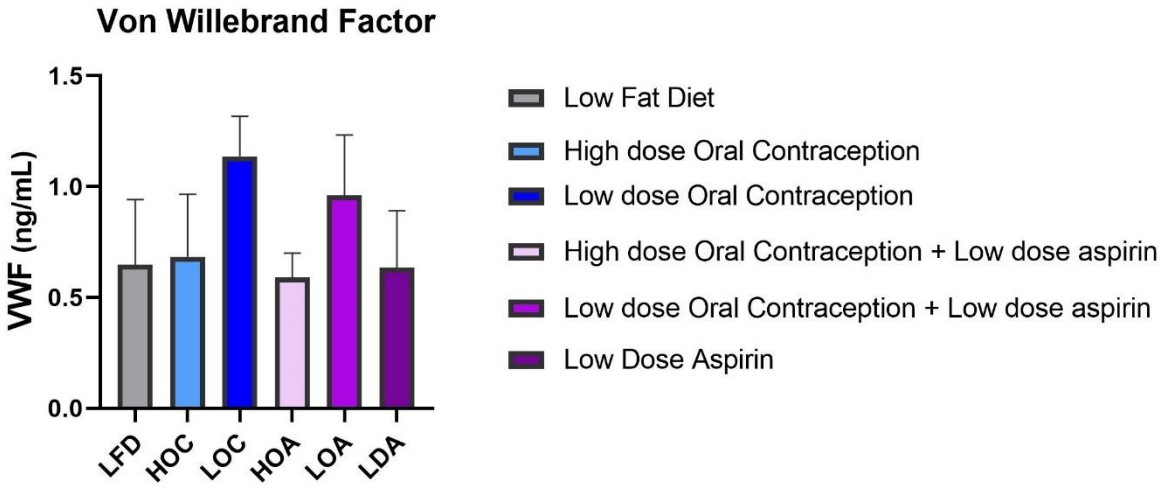


Figure 4: Shows changes in vWF levels following 8 weeks of HFD and LFD, 5 weeks of low and high dose OC, and 5 weeks of aspirin treatment. The data is presented as the mean, ± standard deviation

### 3.3.6 Changes in T helper cytokines following high and low doses of oral contraceptive usage in Sprague Dawley rats and 5 weeks of aspirin treatment

Alterations in T helper cytokines were assessed to determine the effect of LOC and HOC use. There were significant changes in IFN-γ levels across the groups, ($F_{(3.832, 34.49)}$ = 5.572, $p$ = 0.0016), TNF-α ($F_{(2.743, 24.69)}$ = 5.041, $p$ = 0.0086), IL-4 ($F_{(1.974, 17.76)}$ = 16.61, $p$ = <0.0001), IL-6 ($F_{(2.342, 21.08)}$ = 14.40, $p$ = <0.0001), IL-10 ($F_{(2.490, 22.41)}$ = 11.18, $p$ = 0.0002),Table 3. Briefly, post hoc analysis showed that HOC use significantly increased the levels of IFN-γ, TNF-α, IL-4, IL-6, and IL-10 when compared to the LFD with no treatment group (as shown in table 3). In contrast, the levels of IFN-γ, TNF-α, IL-4, IL-6, IL-10 were comparable between the LOC and the control group.

Interestingly, the post hoc analyses showed that the levels of IFN-γ, TNF-α, IL-4 and IL-6 were similar in HOC group compared to HOA.

**Table 4.** Impact of oral contraceptive usage and aspirin treatment on T helper cytokines in Sprague Dawley rats.

| MFIs | LFD | HOC | LOC | HOA | LOA | LDA | *p* – value |
|---|---|---|---|---|---|---|---|
| IFN-γ | 285326±3272 | 291929±3599 | 286975±2428 | 289295±4117 | 291149±5074 | 288155±2518 | **0.0016** |
| TNF-α | 288194±4880 | 295223±3250 | 289152±3598 | 293102±4655 | 293866±4487 | 293323±2688 | **0.0086** |
| IL-10 | 277733±3188 | 286140±3737 | 279652±2129 | 281832±2439 | 282437±2721 | 282277±3061 | **0.0002** |
| IL-4 | 492916±3304 | 505588±2854 | 496263±3397 | 499041±4017 | 501838±3325 | 501848±4006 | **<0.0001** |
| IL-6 | 515350±4659 | 529108±3944 | 517760±3225 | 521808±3771 | 523254±4127 | 524292±5053 | **<0.0001** |

The results are reported as mean ± SD. Significance (P<0.05) shown in bold. MFI: Mean Fluorescence Intensity. ILC: Innate Lymphoid Cell. IL: Interleukin. Low Fat Diet. HOC: High Oral Contraceptive dosage. LOC: Low Oral Contraceptive dosage. HOA: High Oral Contraceptive dosage + Low Dose Aspirin. LOA: Low Oral Contraceptive dosage + Low Dose Aspirin. LDA: Low Dose Aspirin.

### 3.4. Discussion

The aim of this study was to investigate the effect of oral contraceptives on the development of thrombosis, and further investigate the effect of low-dose aspirin prophylaxis in animals on high and low-dose OC. Previous studies have shown that high and low dose OC treatment on these rats causes a significant increase on the risk of thrombosis development in comparison to a LFD (12,19). Upon assessing some of the metabolic parameters, we found that there were significant changes on insulin levels. Low OC dosage caused an increase in insulin levels compared to the LFD. This finding is similar to Godsland *et al*, where it was reported that increased insulin secretion was associated with oral contraceptive (Levonorgestrel) usage (20). This might be due to the decreased insulin sensitivity associated with OC usage, leading to an increased insulin production and high insulin levels in blood. Another factor might be due to raised glucose levels associated with OC usage thereby resulting in a higher demand for insulin in the body. We also discovered noticeable changes on oral glucose tolerance test which might have been affected by the alterations in insulin levels. Interestingly, in our study we found that aspirin usage managed to slightly reduce insulin levels.

We also monitored the changes in lipid profiles which included LDL, HDL, and total cholesterol (TC) levels. Although there were no significant changes on LDL levels, high and low-dose OC caused a slight increase on LDL levels which also remained elevated even after the use of LDA. Similarly, no significant changes were observed with HDL on both the OC and aspirin phases. These results indicate that OC usage might not have a severe impact on lipid profiles. In contrast to our findings, recent studies have concluded that OC usage might cause significant elevations to lipid profiles i.e HDL, LDL and

TC levels (21,22). This potentially reveals that the usage of OCs may disrupt normal lipid metabolism causing a greater risk to diseases such as cardiovascular disease and atherosclerosis. The HDL and LDL differences between our study and previous research might be due to differences in the diets used and subjects used, amongst other factors. Total cholesterol levels were significantly altered after OC and aspirin phases which may have been due to insulin resistance and/or dyslipidaemia (22). High and low dose OC caused elevation of total cholesterol levels which were much higher in the high OC dosage group (HOC). After aspirin administration, total cholesterol levels were reduced on the high-dosage OC group but remained the same on the low-dosage OC group (23). In the present study, a slight increase in platelet count was observed after the high dose OC use which may have been an indication of increased platelet aggregation (11,24). This may have been due to the associated thrombotic risk of OCs (12). Aspirin treatment caused a slight reduction of platelet count on the group that received high dosage of OCs (25). A slight increase was also observed on the mean platelet volume for both high and low OC dosages. Furthermore, aspirin reduced mean platelet volume in both OC groups (25).

In the present study we assessed some of the coagulation markers associated with a prothrombotic state. We found that OC usage caused an increase in D-dimer levels both under low and high dose OC usage, with a much greater significance on the LOC group when compared to the LFD (26). This is also in agreement with some of the previous studies that have linked OC usage with elevated D-dimer levels in a state of increased thrombotic risk (14,26). Additionally, we found that OC usage caused increased thrombin levels both under low and high dose OC usage and this is in line with what has been reported in other studies (12,27). This further links OC usage to an increased risk of thrombosis especially in high thrombosis risk patients such as those affected by obesity (12). Thrombin plays a key role in the coagulation cascade by converting fibrinogen into fibrin strands in the blood clotting process (28). In our study, the levels of fibrinogen were not altered following 5 weeks of high dose and low dose oral contraceptive. In contrast to our finding, a study by Bonnar found increased levels of fibrinogen associated with OC usage (12). The slight decrease in fibrinogen levels under OC dosage might have been due to other thrombotic conditions such as dysfibrinogenemia, which is another condition that is worth looking into for upcoming research (29). We also found a slight increase in the levels of vWF after 5 weeks of HOC and LOC use (13,30). Von Willebrand factor (vWF) is glycoprotein that is vital for primary haemostasis through platelet and subendothelial collagen adhesion, and it is also involved in the intrinsic coagulation cascade, through stabilisation of factor VIII (31). Furthermore, a slight decrease in vWF levels was also observed after treatment with aspirin (32).

Lastly, we found that proinflammatory T helper cytokines (IL-4, IL-6, IL-10, INF-γ and TNF-α) were altered by HOC use and that highlights the associations between thrombosis and inflammation. There is a link between CRP and IL-6 levels since CRP is usually produced from the liver in response to IL-6 (33). In our study, we found increased expression of IL-6 levels under OC usage. A study by Kangasniemi and colleagues found elevated levels of CRP levels under OC usage (34). This finding

also supports increased risk of inflammation under OC usage. Similarly, a study by Masama *et al* found a significant increase in CRP levels under hormonal contraception although no significant changes were caused by hormonal contraception on IL-6 and TNF-α levels in women aged 17-29 (35). We also found that OC usage caused an increase in IFN-γ and IL-10 levels in Sprague Dawley rats. A similar finding to our study was also noticed in work done by Fernández-Martínez *et al* where they found elevated levels of TNF-α and IL-10 associated with OC usage in female rats (36). Another study by Larsen and colleagues found no significant changes caused by OCs on IFN-γ, IL-10 and TNF-α levels under OC usage although it caused a slight increase in IL-6 levels compared non-OC users (37). Similarly to our study, Divani *et al* has also found an increased level of IL-6 associated with oral contraceptive usage (38). These differences may be attributed to the unclear mechanism of association between hormonal contraception and inflammation.

In actual fact, oral contraceptive usage has been associated with a state of inflammation according to previous research (37–39). However, the use of aspirin has been associated with a controlled state of inflammation as it acts as an anti-inflammatory drug and causes inhibition of the cyclooxygenase enzymes thereby preventing inflammation (40).

### 3.5. Conclusion

Oral contraceptive usage is highly associated with increased risk of thrombosis and inflammation especially in a state of obesity. High oral contraceptive dosage further increases the risk of thrombosis development compared to low dose oral contraceptive. Aspirin usage reduces the risk of thrombosis development, however, users must first consult with health professionals for safety precautions. Future studies should consider looking more on the effects of OC usage on immunity and the associated pathways that trigger the immune response to open more research on therapeutic approaches. More research needs to be conducted on human samples before final conclusions can be made.

### Authors' contributions

MM, VM, OF and BBN conceptualised and designed the study. All authors including drafted and approved the final manuscript.

### Competing interests

None declared.

### Ethics approval

The study was approved by the University of KwaZulu Natal's Animal Research Ethics Committee (AREC) with the ethics number: AREC/00003067/2021.

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**Prologue**

This is the last chapter, and it focuses on the overall findings of the project. This chapter summarizes all aspects reviewed in this paper forms part of the synthesis of this project. In this chapter we highlighted the associations of oral contraceptive usage and the risk of thrombosis. We also covered the pathogenesis of thrombosis and mentioned some of the markers we used to assess for thrombosis. The main findings in this chapter are increased pro-coagulant and inflammatory markers under oral contraceptive usage. According to our findings, low dose aspirin might be more effective in treating thrombosis. The risk of oral contraceptive usage is also found to be dose and type dependant according to previous research. Therefore, precaution must be taken when prescribing oral contraceptives with regards to BMI as an underlying obesity state might further increase thrombosis risk.

## CHAPTER 4: SYTHESIS

Oral contraceptives are one of the most common types of birth control method with an estimated usage of approximately 150 million women worldwide (1). They contain synthetic hormones oestrogen and progesterone which prevent ovulation and cause closure of the cervix to prevent sperm from entering (2). However, in the past years, they have been associated with an increased risk of thrombosis development due to their sensitivity to some of the blood coagulation factors thereby resulting abnormal blood clot formation (3). The risk is further increased up to 10-fold with women living with obesity (4). Thrombosis is one of the most common vascular diseases affecting approximately 1 per 1000 adults each year (5).

The oestrogen content found in oral contraceptives stimulates activation of some of the coagulation factors such as thrombin, fibrinogen and tissue factor which further facilitates the clotting process (3). Elevated d-dimer levels have also been associated with oral contraceptive usage which further supports increased risk of thrombosis development associated with OC usage (6). Abnormal activation of pro-coagulant factors by OC usage eventually results in suppression of anti-coagulant factors such as antithrombin (3,7). A pro-inflammatory state has also been linked to OC usage which is supported by an increased level of pro-inflammatory cytokines under OC usage (8).

In our experimental study, we found increased activation of pro-coagulant factors such as thrombin, Von Willebrand factor and increased d-dimer levels associated with oral contraceptive usage. High and low OC dosage both caused elevated thrombin levels when compared to LFD with the high OC dosage having slightly higher thrombin levels than low dose OC. As previously described, thrombin levels are elevated under OC usage (3). Thrombin facilitates the conversion of fibrinogen to fibrin in the coagulation cascade (9). An increase in d-dimer levels was also observed under high and low dose OC which further associates OC usage with risk of pro-thrombotic events (6). We also discovered a slight increase under OC usage with regards to vWF levels and platelet count further supporting the development of either thrombosis or inflammation. These findings support the pro-thrombotic events that have been associated with OC usage (3,4,6). We further found that the use of low dose aspirin, although not significant, but helps in lowering some of these pro-coagulation factors in a state of thrombosis. According to previous studies, the use of aspirin in treating pro-thrombotic disorders has been shown great improvement (10–12).

We also looked at some of the pro-inflammatory cytokine changes under OC usage and aspirin treatment to determine the effects of OCs in inflammation. We found that both high and low dose OC result in increased IFN-γ, TNF-α, IL-10, IL-4 and IL-6 levels when compared to the control with a slightly higher count under high OC dosage. These results reveal the pro-inflammatory state associated with OC usage and further link the development of thrombosis and inflammation under OC usage (8,13). In our study, we found that the use of low dose aspirin assists in reducing some of the elevated pro-inflammatory

cytokines under OC usage (10,11). The exact mechanism at which low dose aspirin works is by targeting the cyclooxygenase enzymes thereby causing their inhibition leading to a controlled pro-inflammatory state (10). OC usage was also found to have an effect on some of the metabolic parameters such as insulin and glucose levels. We found that OC usage had increased insulin and glucose levels which were also reduced almost to normal levels under low dose aspirin treatment. Increased insulin secretion associated with OC usage has also been reported according to previous research (14).

Therefore, our results suggest that low dose OC usage may be safer compared to high dose OC usage as high dose may result in an increased risk of thrombosis development due to its association with a much more hypercoagulation state. Our results further suggest that low dose aspirin usage might be beneficial in treating pro-thrombotic related diseases.

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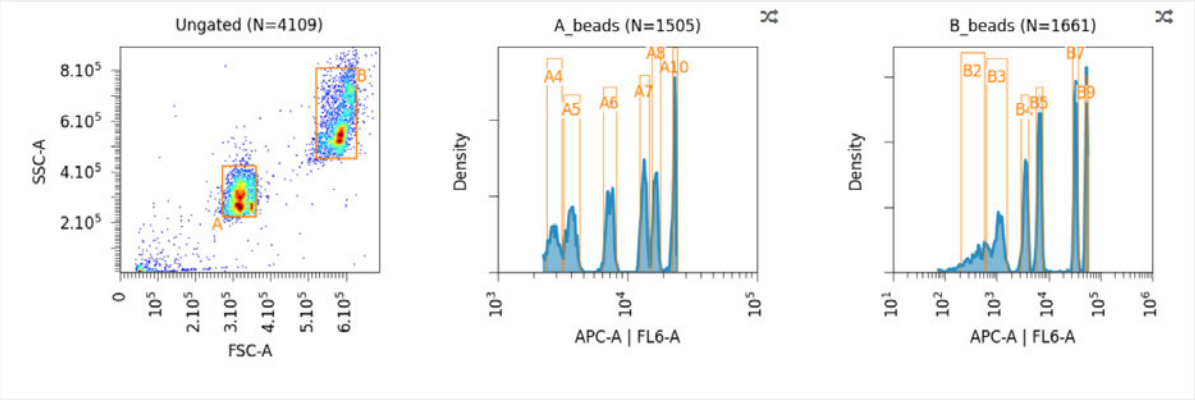
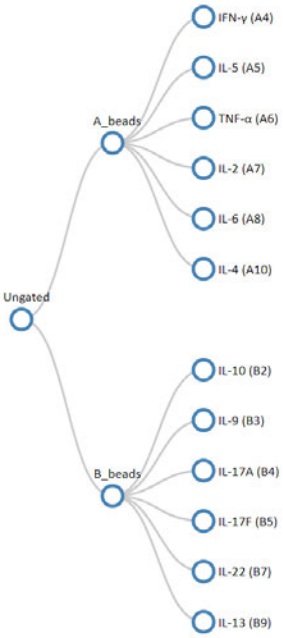
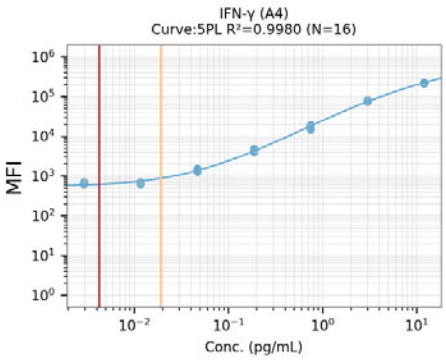
## APPENDICES

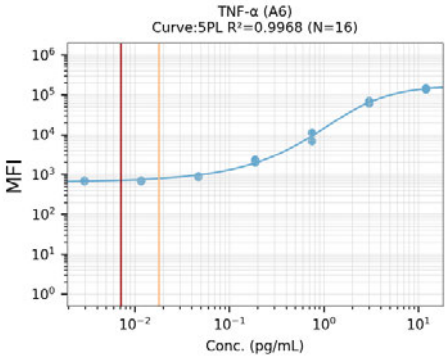
## APENDIX:A Gating strategy for cytokine beads A and B with density plots and histogram

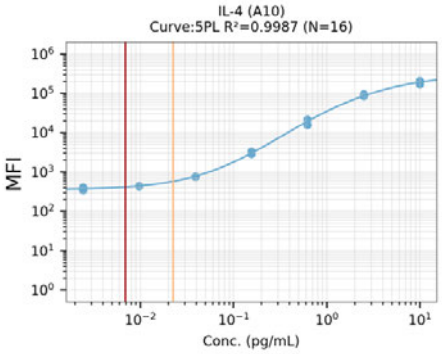


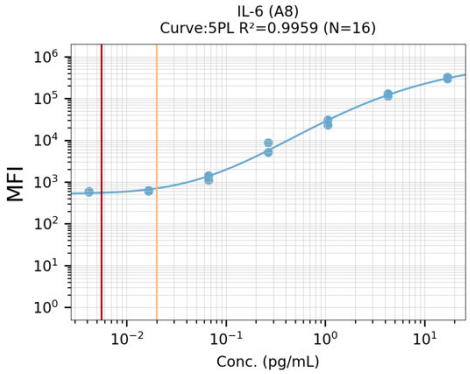



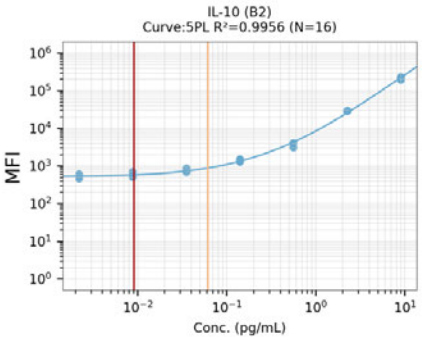

| Name | Count |
|---|---|
| Ungated | 4109 |
| A_beads | 1505 |
| B_beads | 1661 |
| IFN-γ (A4) | 190 |
| IL-5 (A5) | 255 |
| TNF-α (A6) | 259 |
| IL-2 (A7) | 251 |
| IL-6 (A8) | 217 |
| IL-4 (A10) | 202 |
| IL-10 (B2) | 155 |
| IL-9 (B3) | 295 |
| IL-17A (B4) | 250 |
| IL-17F (B5) | 336 |
| IL-22 (B7) | 272 |
| IL-13 (B9) | 221 |


IFN-γ (A4)
Curve:5PL R²=0.9980 (N=16)
MFI
Conc. (pg/mL)


TNF-α (A6)
Curve:5PL R²=0.9968 (N=16)
MFI
Conc. (pg/mL)


IL-4 (A10)
Curve:5PL R²=0.9987 (N=16)
MFI
Conc. (pg/mL)


IL-6 (A8)
Curve:5PL R²=0.9959 (N=16)
MFI
Conc. (pg/mL)


IL-10 (B2)
Curve:5PL R²=0.9956 (N=16)
MFI
Conc. (pg/mL)

## APENDIX B: Ethical Approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

18 January 2022

**Mr Oyesanmi Fabunmi (218087913)**
**School of Laboratory Medicine & Medical Sciences**
**Westville Campus**

Dear Mr Fabunmi,

**Protocol reference number:** AREC/00003067/2021
**Project title:** Evaluating immunosuppression in obesity and short-term oral contraceptive use: using an experimental model of diet-induced atherothrombosis.

**Full Approval – Research Application**

With regard to your revised application received on 28 October 2021, the Animal Research Ethics Committee has accepted the documents submitted and **FULL APPROVAL** for the protocol has been granted.

Please note: The researcher must monitor the weight gain of the animals, and reduce the number of rats per cage to 3 if they gain weight rapidly.

**Please note: There must be adherence to national and institutional COVID-19 regulations and guidelines at all times.** Researchers will be personally responsible and liable for non-adherence to national regulations. If in doubt, please contact the Research Ethics Chair and/or the University Dean of Research for advice.

**Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.**

**Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.**

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

**The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 17 January 2023.**

**Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.**

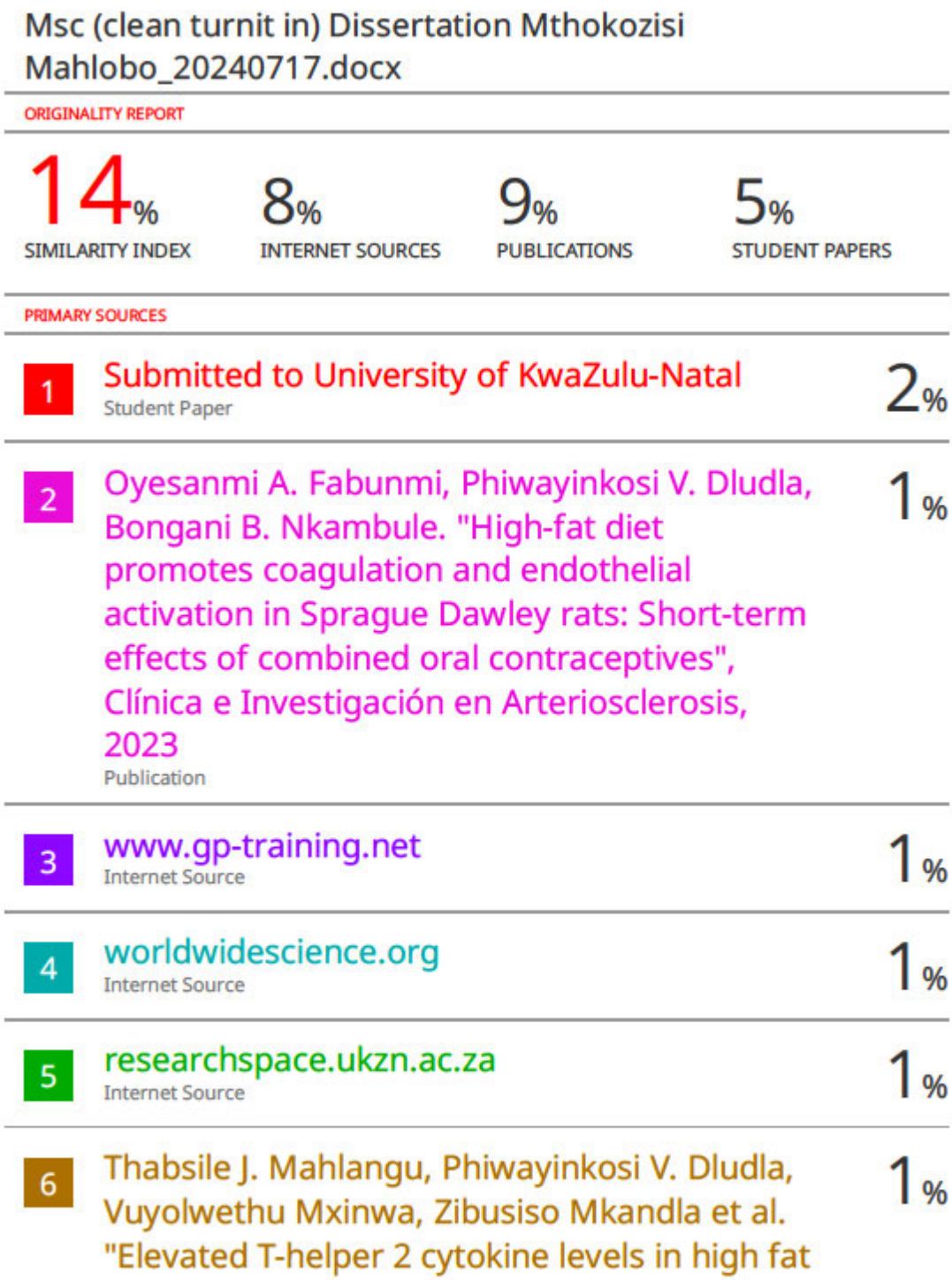
I take this opportunity of wishing you everything of the best with your study.

Yours faithfully [redacted]

**Dr Sanil D Singh, BVSc, MS, PhD**
**Chair: Animal Research Ethics Committee**
**/kr**
**cc Supervisor:** Prof Bongani Nkambule
**cc BRU Manager:** Dr Jaca

**Animal Research Ethics Committee (AREC)**
**Ms Karen Reinertsen (Administrator)**
**Westville Campus, Govan Mbeki Building**
**Postal Address:** Private Bag X54001, Durban 4000
**Telephone:** +27 (0) 31 260 8850 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
**Website:** http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

**APPENDIX C: Turnit in report**



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