Evaluating immunosuppression in obesity and short-term oral contraceptive use: using an experimental model of diet-induced atherothrombosis.

By

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Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy - Physiology

In the School of Laboratory Medicine and Medical Science, College of Health Sciences at the University of KwaZulu Natal.

2023

Preface

This thesis fulfils the requirements for a Doctor of Philosophy degree in the College of Health Sciences.

Chapter 1 describes the problem statement, the aims, and the research questions covered in the thesis.

Chapter 2 covers the literature review and provides the rationale for the current project. The chapter comprises a protocol and systematic review with meta-analyses.

Three manuscripts submitted for peer review in accredited journals make up chapters 3, 4, and 5.

Chapter 6 is the synthesis and conclusion summarising the entire project.

This work has not been submitted in any form for any other degree or diploma at another institution.

The use of other people's work has been acknowledged accordingly in-text.

Oyesanmi A. Fabunmi [redacted] Date 6/10/2023

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As the candidate's supervisors we approve the submission of this thesis.

Declaration

I Oyesanmi A. Fabunmi declare that,

I. The research reported in this thesis, except where indicated, is my 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.

IV. This thesis does not contain other person's writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) their words have been re-written but general information.

Publications to international peer-reviewed journals

Published manuscript.

1. **Fabunmi OA.**, Dludla PV., Ngcobo S.R., Nkambule BB., (2023). Investigating the risks of cardiovascular disease among premenopausal women using oral contraceptive: a protocol for a systematic review and meta-analysis. ***BMJ Open 2023; 13: e071118.***

2. **Fabunmi OA.,** Dludla PV., Nkambule BB (2023). Investigating cardiovascular risk in premenopausal women on oral contraceptives: Systematic review with meta-analysis. ***Front. Cardiovasc. Med. 10:1127104. doi: 10.3389/fcvm.2023.1127104.***

3. **Fabunmi O.A.,** Dludla P. V., Nkambule B.B., (2024). High-dose oral contraceptives induce hyperinsulinemia without altering immune activation in diet-induced obesity which persists even following a dietary low-fat diet intervention. ***J. Reprod. Immunol. 104234. https://doi.org/10.1016/J.JRI.2024.104234.***

4. **Fabunmi OA.,** Dludla PV., Nkambule BB., (2023). High fat diet promotes coagulation and endothelial activation in Sprague Dawley rats: Short-term effects of combined oral contraceptives. ***Clínica e Investig En Arterioscler 2024;36:60–70. https://doi.org/https://doi.org/10.1016/j.arteri.2023.10.001.***

Under review

1. **Oyesanmi A. Fabunmi**, Phiwayinkosi V. Dludla, Bongani B. Nkambule. Short-term treatment with low-dose aspirin improved metabolic status and attenuated atherothrombotic risk factors in female rats exposed to combined oral contraceptives.

Conference presentations

1. **OA Fabunmi**, PV Dludla, BB Nkambule (2022). Systematic review and meta-analysis of association between oral contraceptives and cardiovascular disease (CVD) in premenopausal women. **Cochrane, South Africa National Symposium, South Africa**, 22-23 November 2022.

2. **OA Fabunmi**, PV Dludla, BB Nkambule. High-dose oral contraceptives induce hyperinsulinemia without altering immune activation in diet-induced obesity which persists even following a dietary low-fat diet intervention. **Annual College of Health Science Research Symposium, K-RITH Tower Building, Nelson R Mandela School of Medicine Campus, UKZN, South Africa,** 15-16 August 2023.

3. **OA Fabunmi**, PV Dludla, BB Nkambule. High-dose oral contraceptives induce hyperinsulinemia without altering immune activation in diet-induced obesity which persists even following a dietary low-fat diet intervention. **18th International Congress of Immunology, Cape Town International Convention Centre, Cape Town, South Africa,** 27 November - 2 December 2023.

4. **OA Fabunmi**, PV Dludla, BB Nkambule. High-fat diet promotes coagulation and endothelial activation in Sprague Dawley rats: Short-term effects of combined oral contraceptives. **Cross-Talk of Cells in the Heart: Novel Mechanisms of Disease and Arrhythmias, University of Liverpool, UK**. 11 – 12 September 2023.

5. **OA Fabunmi**, PV Dludla, BB Nkambule. Short-term treatment with low-dose aspirin improved metabolic status and attenuated atherothrombotic risk factors in female rats exposed to combined oral contraceptives. **Annual School Research Day, Senate Chambers, Westville Campus, UKZN, South Africa,** 15-16 August 2023.

Dedication

This thesis is dedicated to "**God" the "G.I"** and/or **"Oyesanmi A. Fabunmi".**

Acknowledgement

I thank "**God the G.I**" for the successful completion of this doctoral degree.

To my mother, "**Chief Mrs CM Fabunmi"**, **Mrs RF Olaleye *et al.,*** and every family member, both dead and alive, I appreciate you all.

To my supervisor, "**Prof BB Nkambule"**, and co-supervisor "**Prof PV Dludla"**, thank you for signing me on trial to be part of the research team despite my shortcomings, your tutelage, kind support, and guidance throughout this research project was full of insight at every time point. Nevertheless, I am always grateful, cheers!

I thank the entire **Immune Activation and Coagulation in Chronic Inflammation (IACCI)** research study group for their support and contribution to the success of this study. I am indeed privileged to have learned from you all. Nonetheless, **"keep raising tha "bar"**!

To all my colleagues, friends, and most especially my **"foes"**, I thank you all. God bless.

I thank the University of KwaZulu-Natal (UKZN) Biomedical Resource Unit (BRU) for their assistance in rat handling training, procedures, and housing facilities. Furthermore, I would like to extend my gratitude to the UKZN Department of Human Physiology, School of Laboratory Medicine and Medical Science (SLMMS), and College of Health Sciences (CHS) for their kind support at every time point. Cheers!

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Section 2.4. Systematic review and meta-analysis

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Abstract

Introduction

Obesity is a prominent feature of metabolic syndrome that can predispose an individual to an increased risk of developing type 2 diabetes (T2D) and cardiovascular disease (CVD). More so, oral contraceptives are associated with an increased risk of cardiovascular-related complications such as arterial and venous thrombosis in some women of reproductive age. There is a need to understand how the usage of combined oral contraceptives (COC) affects women with diverse metabolic complications. Thus, we aimed to evaluate CVD-related risk factors, especially those implicating atherothrombosis, in a preclinical model of high-fat diet (HFD) exposure to COC. We also assessed whether switching to a low-fat diet or pharmacologic intervention with low-dose aspirin (LDA) could improve the metabolic status or alleviate CVD risk using this preclinical model of HFD.

Methods

The study was divided into three phases to achieve its aims. The study's first phase was used to establish a preclinical model of impaired glucose tolerance and to test the efficacy of COC. Thus, female Sprague Dawley rats were randomly assigned to receive HFD and low-fat diet (LFD) for eight weeks before assessing basic metabolic parameters or CVD-related abnormalities. The study's second phase involved testing the detrimental effects of COC, where rats switched from HFD to LFD for an additional six weeks while receiving either a high (HCOC) or low dose (LCOC) of COC. The third phase of the study involved rats exposed to COC for six weeks before treatment with LDA for another four weeks. At the end of each experimental phase, measurements for basic metabolic status and CVD-related parameters were taken. These included the animal body weights, insulin levels, lipid profiles, fasting blood glucose, hematological indices, blood pressure and heart rate, as well as markers of immune activation such as interleukin (IL)-6, tumor necrosis factor-alpha (TNF)-

α, monocyte chemoattractant protein-1 (MCP-1), changes in the coagulation cascade, tissue factor (TF) and D-dimer, Von Willebrand factor (vWF) and nitric oxide.

Results

The results of the first phase indicated that exposure to HFD led to a marked weight gain, impaired glucose tolerance, and abnormal lipid profiles, including obscured triglyceride-glucose index when compared to rats in the LFD group ($p < 0.001$). Rats exposed to HFD also presented with increased markers of CVD risk, accompanied by a pro-inflammatory state, as displayed by increased levels of IL-6 and TNF-α compared to the LFD group ($p < 0.05$). Interestingly, dietary intervention and switching from an HFD to an LFD could improve metabolic status and potentially lower CVD-risk-related markers. However, this improvement was not seen in rats that received HCOC, as these animals persistently showed impaired metabolic state that was accompanied by alteration in the levels of immune activation, coagulation, and endothelial function. However, the third phase of the study showed that short-term LDA treatment for four weeks could improve the metabolic status and decrease the markers of immune activation (IL-6, TNF-α, and MCP-1) in animals that received HCOC. LDA also decreased the bleeding time and makers of hypercoagulation (TF and dimer), as well as improved endothelial function by increasing the availability of nitric oxide and decreasing levels of vWF in rats that were exposed to HCOC treatment ($p < 0.05$).

Conclusion

Our results indicated that exposure to HFD was consistent with impaired glucose tolerance and increased CVD risk in female rats. Exposure to HCOC was associated with an increased risk of atherothrombotic disorder in HFD animals despite dietary intervention that involved switching from an HFD to an LFD. Short-term LDA attenuates the risk of atherothrombotic disorder by improving the metabolic status and decreasing markers of immune activation, hypercoagulation, and endothelial dysfunction during exposure to COC treatment in animals.

Our result demonstrated that an increased risk of thrombotic events during COC treatment may potentially be associated with the dose and duration of treatment. While the use of LDA may be of potential therapeutic benefit against the risk of atherothrombotic disorder following toxic exposure to COC. However, further studies are needed to confirm the interaction mechanism between the several types of available COC, further revealing the potential therapeutic value of LDA.

Chapter 1: Introduction

1.1 Background

Obesity remains a major risk factor for chronic disease and metabolic complications, which have reached epidemic dimensions in the past decades [1,2]. Obesity is characterized by adipose tissue expansion [3]. More so currently, over 1.9 billion adults across the world aged 18 years and older are overweight, and of these, over 650 million adults are obese (11% of men and 15% of women) [4]. Although the United States of America accounts for the highest prevalence of obesity, with 40%; South Africa accounts for 28% of the global prevalence of obesity [4]. Alarmingly, South African women within 40-43 years of age have the highest prevalence of obesity in sub-Saharan Africa, at over 42% [4].

Oral contraceptive pills (OCPs) containing either estrogen and progestogen (COC), or progestogen-only pills (POPs) remain one of the most widely used modern methods of contraception among women of reproductive age and the prevalence of use cut across and also differs in all the geographical regions [5]. More so, the use of oral contraceptive pills and the risk of increased weight gain remain high among users [6]. Notably, combined oral contraceptives (COCs) are also associated with a 3- to 6-fold elevated risk of venous and arterial thrombosis [8] that account for the most common cause of mortality [9]. Cardiovascular disease (CVD) is a predominant phenomenon and is often associated with atherosclerotic damage and inflammation, while venous thrombosis, on the other hand, is generally thought of as a disorder in plasma coagulation [10].

Most of the CVD risks associated with the use of COC are attributed to the estrogen component of COC, although the progesterone/progestin component seems to antagonize some of these effects [11]. A previous study showed that estrogen can aggravate fat

accumulation in the cell or adipose tissue, while progestogen is associated with an increased appetite, which facilitates anabolism [6,12]. Thus, the effect of COC and the risk of obesity-related complications may contribute to the development of CVDs [13], and these effects may depend on the dosage and duration of COC treatment in susceptible individuals [14].

Over the years, antiplatelet therapy such as Acetylsalicylic acid (aspirin) has remained clinically relevant in the primary prevention of cardiovascular events in high-risk individuals [15]. Emerging evidence suggests a decreased risk of myocardial infarction and stroke in patients on aspirin [15]. More so, evidence from primary prevention trials performed by the Antithrombotic Trialists' (ATT) Collaboration also showed a 12% reduction in serious vascular events following the administration of aspirin [16]. In contrast, a meta-analysis by Berger et al. [17] showed no significant reductions in myocardial infarction (MI), stroke, ischemic stroke, or mortality following aspirin treatment. However, a null association between aspirin treatment and reduction in either cardiovascular death or cancer mortality in patients without prior cardiovascular events has also been reported [18].

5.2. Problem statement

Obesity is a prominent feature of metabolic syndrome [20], and it is associated with insulin resistance, dyslipidemia, a high glycemic index, elevated blood pressure, and pro-inflammatory and prothrombotic states [21,22]. Individuals with these metabolic abnormalities are usually predisposed to an increased risk of type 2 diabetes (T2D) and CVDs [23]. Systemic inflammation in obesity results in the production of pro-inflammatory cytokines, recruitment of M1 macrophages, as well as infiltration of the effector (CD4+) T cell into the adipose and vascular tissues [24,25], which can lead to endothelial dysfunction and hypercoagulable state [26,27]. Typical features of a prothrombotic state include increased platelet activation, elevated concentrations, and increased activities of plasma coagulation

factors such as thrombin. The increased release of these biomarkers is partly due to the excess release of tissue factor [28] and increased production of plasminogen activator inhibitor-1, which impairs fibrinolysis and leads to an increased risk of arterial and venous thrombotic events [27].

The impact of the association between COCs and metabolic perturbations [29] also contributes to the onset and progression of a prothrombotic state [30,31] and endothelial dysfunction [32,33] that leads to cardiovascular complications. However, the impact of COC on certain hemostatic parameters remains inconsistent [34,35]. While COC are known to modulate the susceptibility to autoimmune diseases, their profound effects on immune activation remain inconclusive [36]. Sex hormones have their receptors present in a wide range of tissues, including innate and adaptive immune cells [37] with estrogens acting to enhance cell proliferation and humoral immune responses [38] while progesterone/progestin acts as natural immune suppressors [39].

For instance, evidence from previous studies demonstrated the suppressive effect of COC on certain immune populations in the innate and adaptive immune systems [40]. Progestin can interfere with the nuclear factor of kappa B (NF-κB) by inhibiting gene transcription and suppressing the activation of macrophages and dendritic cells, decreasing inflammatory responses [41]. It is plausible that suppression of certain immune cells may contribute to the lack of resolution during thrombotic events. However, the interplay between the sex hormones and the immune system remains very complex, and there is relatively little data to elucidate their role in certain conditions like obesity. Moreover, a better understanding of the intricate immunomodulatory effects of hormonal therapy, especially progestins, may pave the path to developing clinically meaningful therapeutic interventions in various conditions and autoimmune diseases [42]. The awareness of current demand and unmet need for

contraceptives, existing trade-offs between unwanted pregnancies, and maternal and infant mortality must be considered and weighed against complications attributed to off-target effects of COC. Thus, the need to evaluate the potential effects of accumulative exposure to the available COCs and CVD risk in women of reproductive age may provide insight and guidance during consultation and when making contraceptive choices.

5.3. Aim of study

To investigate immune regulation and thrombotic risk in obese rats on oral contraceptives and treated with low-dose aspirin.

5.4. Objectives

1. To investigate the impact of COC on the activation of innate immune cells in rats on a high-fat diet.
2. To investigate the impact of low-dose aspirin (LDA) on the vascular and cellular components of coagulation in rats on a high-fat diet subjected to COC.

5.5. Research question

1. Does COC alter monocyte-macrophage function in rats fed high-fat diets?
2. Does LDA alter endothelial cell function and coagulation cascade in high-fat diet-fed rats subjected to COC?

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Prologue

The next chapter is a critical review of current evidence on obesity and the use of oral contraceptives (OC) and how the association with several complications are linked to cardiovascular disease (CVDs). We conducted a systematic review and meta-analysis to provide insight into the topic of interest. Our systematic review and meta-analysis protocol **(published)** outlines the rationale behind the subsequent review and thoroughly describes the planned methods to be followed in conducting them. This was essential for the subsequent systematic review and meta-analysis **(published),** which investigated the risk of CVDs in premenopausal women on OC. Our study highlighted the impact of OC on markers of endothelial dysfunction and other traditional cardiovascular variables in premenopausal women, and these impacts vary across different geographical locations.

Chapter 2: Literature Review

2.1. Overview of obesity

Obesity is a multifaceted disease that originates from complex pathologic conditions involving biological, psychosocial, socioeconomic, and environmental factors, as well as heterogeneity in the routes and mechanisms that lead to negative health effects [1,2]. There is an increase in the prevalence of obesity worldwide, and efforts to understand this massive increase over the past decades are challenging [3]. Although current data shows that the rate of increase in obesity in most high-income countries is declining, it is suggested that the rate of obesity will increase exponentially in low-income and middle-income countries shortly [4].

The hallmark of obesity involves the pathological expansion of the adipose tissue (AT) due to long-term metabolic surplus, which involves the differentiation of adipocytes [5]. Obesity leads to several complications in the body system, such as impaired immune responses to viral infection [6], and increased risk for type 2 diabetes (T2D) [7]. Recent evidence reported high levels of hospitalization in obese individuals during the COVID-19 pandemic, which also lends support to the impact of obesity on communicable diseases [8–10].

The interplay between several factors, such as elevated calorie intake, diet composition, lack of physical activity, and changes in the gut microbiome, contributes significantly to obesity [11,12]. Genetic susceptibility to obesity has been reported with over a hundred single genetic polymorphisms (SNP) [13–15]. These polymorphisms may only explain a modest change in body weight over time among individuals [16–19]. Alterations of certain genes allow adipocytes to respond to excess energy intake through the accumulation of lipids,

leading to sudden changes in size (hypertrophy) [20]. The increased changes in adipose tissue mass result in increased adiposity, causing variations in systemic physiology [21].

Adiponectin (also known as ACRP30), a hormone that reduces hepatic gluconeogenesis and lipid oxidation in muscle, is inversely linked with adiposity [21]. Adiponectin binds with the AdipoR1 receptor to inhibit muscle fatty acid oxidation via activating Adenosine Monophosphate-Activated Protein Kinase (AMPK) and also binds with the AdipoR2 receptor to induce other signal transduction proteins such as peroxisome proliferator-activated receptor alpha (PPARα), which in turn inactivates acetyl CoA carboxylase (ACC), glucose uptake, nitric oxide (NO) synthesis, lactate production in myocytes, and reduced liver production of molecules involved in gluconeogenesis [22–24].

In rodents, adiponectin was also reported to improve insulin secretion and sensitivity [16]. Furthermore, adiponectin has anti-inflammatory and anti-atherogenic properties in humans [25]. Other known hormones have been implicated in appetite control, which includes ghrelin, Cholecystokinin (CCK), Glucose-dependent insulinotropic polypeptide (GIP), Glucagon-like peptide 1 (GLP-1), oxyntomodulin, glicentin, peptide YY (PYY) and neurotensin [16]. Several studies have highlighted the role of oral contraceptives in the regulation of these hormones in certain conditions [26–29]. For instance, a prospective clinical trial showed an increased level of ghrelin in polycystic ovarian syndrome (PCOS) patients receiving oral contraceptives containing ethinyl estradiol and drospirenone in a 3 months [29]. In contrast, 3-month treatment with ethinyl estradiol/drospirenone did not alter the levels of Ghrelin, PYY, CCK, and satiety index (SI) in women with PCOS [27].

Despite significant progress in understanding the pathophysiology of obesity-related metabolic dysregulation and its implications [16,17], as well as potential therapeutic and prevention strategies [30,31], the devastating impact of obesity in association with an

increasing burden of debilitating disease, including cardiometabolic, digestive, respiratory, neurological, musculoskeletal, infectious and CVD related diseases, remains alarming [32]. Thus, prompt prioritization of preventive strategies and interventions is required to mitigate the risks and complications of obesity.

1.11. Role of immune cell activation in obesity-related complications

The inflammatory responses are essential for tissue repair and involve converging cellular signaling pathways in tissues and organs [33]. Conversely, a diet rich in fats can cause metabolic stress [34], which can, in turn, trigger chronic systemic inflammation and lead to obesity-related complications [5]. Interestingly, white adipose tissue (WAT) promotes the release of pro-inflammatory cytokines [20].

Different types of innate and adaptive immune cells accumulate in the AT during obesity [35]. The pro-inflammatory macrophages are the most dominant, constituting up to 40% of the AT cell population [36]. There is also consistent infiltration and activation of these pro-inflammatory macrophages and other immune cells, which result in the synthesis and secretion of pro-inflammatory cytokines and chemokines [37]. The inflammatory state of adipose tissue macrophages (ATM) can be characterized by traditionally activated “M1” macrophages being proinflammatory and alternatively activated “M2” macrophages being anti-inflammatory [38]. It remains unclear how diet-induced obesity induces the polarization of monocytes from classical to their different subtypes, but the MCP-1/CCR2 axis is suggested to be involved in this polarization [36].

Proinflammatory mediators such as LPS and IFN-γ stimulate the classically activated "M1" macrophages [35,36]. M1 macrophages produce more proinflammatory cytokines (TNF-, IL-6, IL-12) and reactive oxygen species such as nitric oxide via inducible nitric oxide synthase (iNOS) activation (Nos2) [36]. M2 macrophages, on the other hand, produce anti-

inflammatory cytokines like IL-10 [36]. Hence, the polarization of M2 macrophages is crucial in preventing inflammatory reactions and promoting tissue repair [39].

In M2 macrophages, arginase production is also elevated [35]. This enzyme inhibits iNOS function in several ways, such as competing for the arginine substrate needed for NO generation [40]. Previous experimental studies have shown that short-term HFD feeding induces both M1 and M2 macrophage polarization in adipose tissue [41–43]. Interestingly, acute HFD feeding is linked to increased M2 macrophage polarization in adipose tissue, which is mediated by natural killer T (NKT) cells [41]. The M2 macrophage promotes arginase 1 expression in adipose tissue via (IL)-4 [41].

1.12. The role of inflammation during atherogenesis and thrombotic event.

Current evidence shows that an altered lipid profile is associated with meta-inflammation in atherogenesis [44]. During dyslipidemia, lipids accumulate in the intimal layer, which results in fatty streak formation [45]. The accumulated lipids become oxidized and elicit a series of inflammatory responses that can impair the endothelium and inhibit the release of NO [46]. The impaired endothelium expresses certain adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1) and the vascular cellular adhesion molecule 1 (VCAM-1), which in turn drives monocyte adhesion and leukocyte diapedesis [44]. Thus, the expression of adhesion molecules and accumulation of monocytes in the endothelium contributes to endothelial dysfunction. Endothelial dysfunction is a systemic, reversible disorder that occurs during the earliest pathologic process of atherothrombosis [47].

While the interaction between oxidized low-density lipoprotein (OxLDL) and the expression of MCP-1 [48], as well as macrophage colony-stimulating factor (M-CSF) [49], contributes to the progression of atherogenesis, the presence of smooth muscle cells primarily from the media can aggravate the plaque and fibrous cap formation [50]. However, the specific role of

vascular smooth muscle cells in atherothrombotic events is unclear. Emerging evidence suggests that vascular smooth muscle cells contributed to the development of atheroma by producing proinflammatory mediators such as MCP-1 and VCAMs [51]. When the plaque becomes calcified due to the accumulation of hydroxyapatite mineral [52], matrix metalloproteinases accumulate in the lesion and predispose the plaque to rupture or ulceration, resulting in tissue factor (TF) exposure and thrombus formation [53]. Strong clinical evidence suggests there is upregulation of the TF pathway in obesity and MetS [54]. Thus, exploring the TF pathway may provide insight into the intersection between obesity, inflammation, and thrombosis.

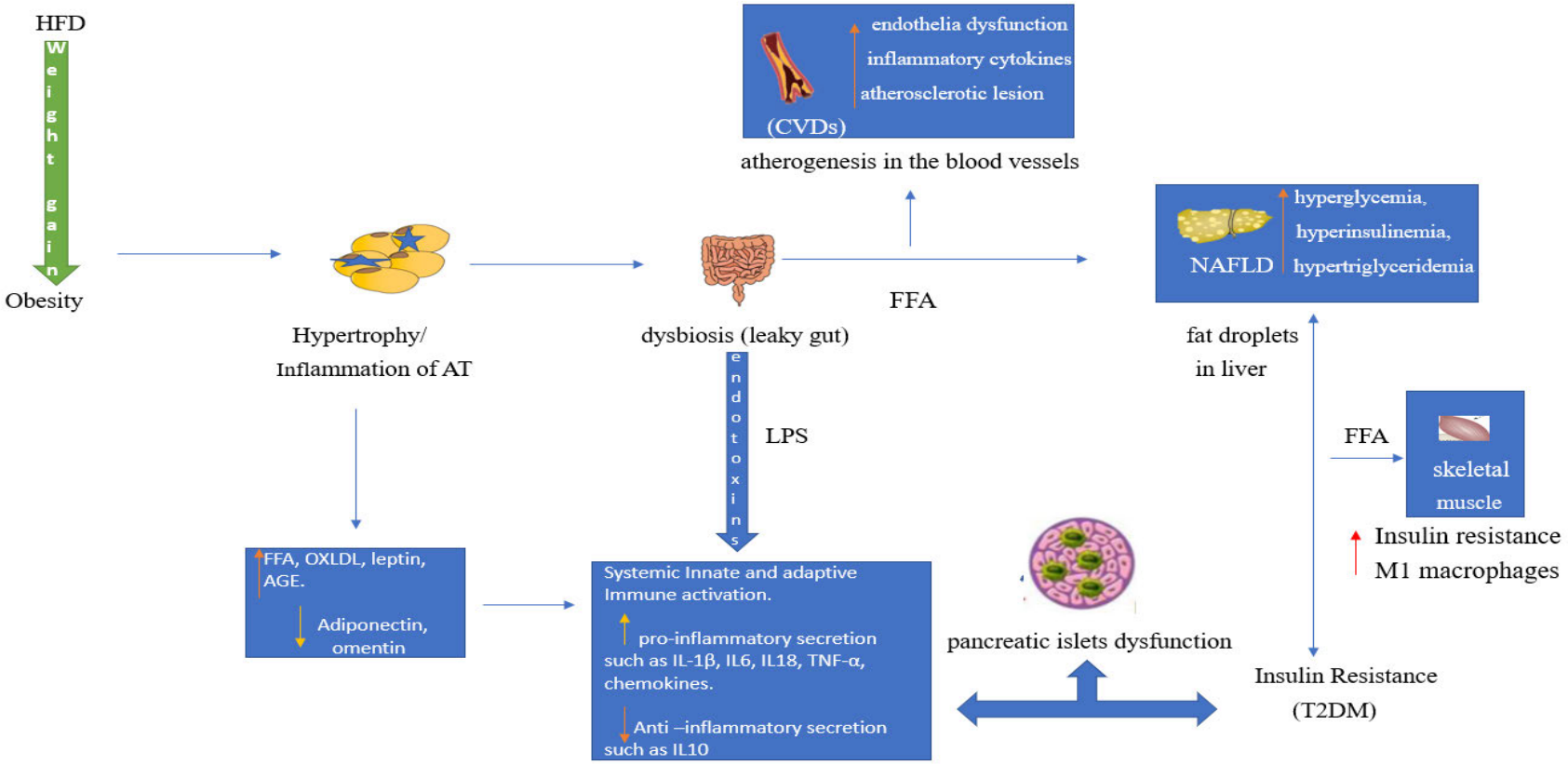

Figure 1: Overview of HFD (high-fat diet) induces chronic metabolic low-grade systemic inflammation and its complications.
Key: **T2DM** (type 2 diabetes mellitus) **NAFLD** (non-alcoholic fatty liver disease); **AGE** (advanced glycation end products); **OxLDL** (oxidized low-density lipoprotein); **CVDs** (cardiovascular diseases); **FFA** (free fatty acid); **AT** (adipose tissue); **LPS** (lipopolysaccharide); **IL** (interleukins); **TNF- α** (tumor necrosis factor-alpha).

2.2. Overview of COC usage for birth control.

The use of oral contraceptives remains one of the most relevant means of controlling birth among women of reproductive age [55] and the prevalence of use remains prominently high among premenopausal women [56]. Of note, oral contraceptive pills are majorly in three forms: combined estrogen-progesterone, progesterone-only, and continuous or extended-use pills [57]. COC can also be classified based on generation: the first-generation progestin includes norethindrone, lynestrenol, ethynodiol diacetate, and norethisterone, while the second-generation includes levonorgestrel, and norgestrel, and the third generation includes desogestrel, gestodene, and norgestodene [58].

Notably, the progesterone component in COC prevents pregnancy, while the estrogen component controls menstrual bleeding [57]. COC is also used in several other health conditions, especially menstrual-related disorders, fibroids, endometriosis-related pain [59,60], certain types of cancer such as ovarian and colon cancers and treatment of acne and hirsutism [57,61]. However, the risk of CVD, such as venous and arterial thrombosis, is 3 to-6-fold in women using COC [62].

2.2.1 Mechanism of action

Hormonal contraception mechanistically acts by inhibiting follicular development and prevents ovulation [63]. The progesterone component of COC provides negative feedback to the hypothalamus to suppress the pulsatile frequency of the gonadotropin-releasing hormone. This, in turn, leads to reduced secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland, which prevents ovulation[57,64].

2.2.2. Consideration of COC usage and adverse effect

When considering the choice of hormone contraceptives such as COC, certain risks such as arterial and venous thrombosis and other side effects are associated with the use of COC [63]. Major side effects accounted for are bleeding, nausea, headaches, abdominal cramping, breast tenderness, mood change, weight gain and increased vaginal discharge or decreased libido. The risks and side effects of COC may be influenced by the duration of use, type and dose of estrogen and the progestin component [65]. When COC is discontinued, certain side effects may subside [57].

Notably, venous thromboembolism (VTE) remains the most significant adverse event associated with the use of combined hormonal contraception and is mediated through the estrogen component [62,66]. Whereas ethinylestradiol and other estrogens, which are metabolized by the liver, can activate the coagulation system [67]. The risk of VTE events per 10,000 women years using COC is approximately 7 to 10 [68,69]. The estrogen component can impact the vascular wall and promote endothelial dysfunction as well as disrupting the coagulation system [70,71].

COC may also induce changes in lipid [72] and glucose metabolism [73]. Furthermore, previous studies also showed a decrease in insulin sensitivity during COC administration [73]. The use of hormonal contraceptives can also increase the risk of developing hypertension [74]. COC treatment in normotensive women increased their blood pressure [71]. More so, at high doses, the estrogen component of the COC can also increase the risk of ischemic stroke or myocardial infarction [67]. However, the long-term consequences of COC use require further longitudinal investigation [75].

2.2.3. The use of COC in obesity

The increased use of hormonal contraceptives and the high prevalence of obesity among women of reproductive age is a major public health concern due to increased thrombotic risks associated with obesity and the use of hormonal contraceptives [76–78]. There are uncertainties around the efficacy of hormonal contraceptives use in obesity, which is attributed to the generational types of OC, among other things [72]. While some studies have shown that obesity affects the efficacy and effectiveness of COC [79,80], other studies reported contradictory findings [81,82]. Although evidence suggests possible mechanisms by which obesity may affect the effectiveness and efficacy of COC [83]. The increased fat distribution in the adipose tissue of obese women is suggested to impact the availability of steroid hormones below therapeutic potential in the serum [83]. More so, an increased metabolic rate and hepatic clearance in obese women may also lead to a shortened half-life of COC in circulation [83].

The Progestin component of COC is associated with hyperphagia and an increased anabolic process, while the estrogen component aggravates the accumulation of fat in the cell or adipose tissue [84]. The metabolic effect of COC was demonstrated in a previous study that showed increased weight gain in individuals who used COC for more than 6 months when compared with non-users [85]. In contrast, previous longitudinal studies and randomized controlled trials showed that COC usage does not impact weight gain [86–88]. While obesity is a known risk factor for CVD [89], COC treatment may exacerbate cardiovascular-related complications in obese women [83]. A previous study showed an impaired metabolic status and a higher risk of VTE in obese women receiving COC treatment [78,90]. Thus, both obesity and COC can independently predispose an individual to higher CVD risk [91]. Taken together, the clinical significance of COC use in the condition of obesity remains inconclusive and needs further research [72,83].

2.2.4. Impact of COC on the endothelial system

The use of OC is associated with endothelial activation, which is linked to an increased risk of CVD in susceptible individuals [91–93]. The vasodilatory impact of estrogen on the vascular wall is associated with the release of nitric oxide (NO) due to the stimulation of endothelial nitric oxide synthase; however, certain progestins can antagonize this effect by promoting the release of vasoactive endothelin-1 (ET-1) leading to an unbalanced endothelial function [94,95].

Endothelin-1 is a potent vasoconstrictor and mitogenesis inducer that plays a role in migrating and activating smooth muscle cells in the vasculature during atherosclerosis. [96]. In a prospective cross-over study including healthy premenopausal women, the use of OC, 30 microg ethinylestradiol/150 microg levonorgestrel (EE/LNG) and 30 microg ethinylestradiol/75 microg gestodene (EE/GSD) for six months was shown to impact the endothelial cells by causing it to release endothelin-1 and NO even though there were no significant major changes in circulating NO and endothelin-1 plasma levels. [97]. In contrast, evidence from a randomized prospective study shows that the use of OC (30 microg ethinylestradiol/150 microg levonorgestrel [EE/LNG] and 30 microg ethinylestradiol/75 microg gestodene [EE/GSD]) does not significantly change NO [98].

A previous study by Friedman et al [99] reported an increased flow-mediated dilation (FMD) in the participants who used COC. In contrast, several other studies showed a significantly lowered FMD significant during COC treatment [70,100,101]. Previous studies also showed an increased mean CCA-IMT in the COC participants compared to non-users [70,101]. Evidence from a recent study also showed decreased asymmetrical dimethylarginine (ADMA) levels in Female cohorts receiving COC when compared with non-users. ADMA is an endogenous nitric oxide synthase inhibitor and an index of endothelial dysfunction [102].

More so, recent animal studies have also shown the effect of COC on the endothelial and coagulation system that regulates thrombotic events [103,104]. COC treatment induces a high release of C-reactive protein (CRP), uric acid (UA) and plasminogen activator inhibitor-1 (PAI-1), which can promote thrombotic injury and cause tissue damage due to oxidative stress [103,104]. However, the magnitude by which COC impacts the endothelial system remains inconclusive [105].

2.2.5. Impact of COC on immune regulation

The interplay between the sex hormones and the immune system appears to be very complex [106]. Evidence suggests that sex hormones have profound effects on the immune system [107] because they have their receptors (PRs) present in a wide range of tissues, including immune cells such as NK cells, macrophages, DCs, T cells [108]. However, the use of COC suppressed the activation of macrophages and DCs [109]. Progestins can also interfere with the transcription factor NF-kB by inhibiting gene transcription, which results in decreased inflammatory responses [109]. In fact, experimental and a recent cross-sectional study also showed a suppressive effect of COC on DC activation and function as well as inhibiting the formation of pathogen-specific T cell immunity and memory cell development [110,111].

In a previous study, Hall et al [112] showed that administration of exogenous progesterone (at concentrations that mimic the luteal phase) to progesterone-depleted adult female mice conferred protection from both lethal and sublethal influenza A virus (IAV) infection. The progesterone treatment altered the inflammatory environment of the lungs and promoted faster recovery by increasing TGF-β, IL-6, IL-22, numbers of regulatory Th17 cells expressing CD39, and cellular proliferation, reducing protein leakage into the airway, improving pulmonary function, and upregulating the epidermal growth factor amphiregulin (AREG) in the lungs, but had no effects on viral load [112].

Furthermore, evidence from a prospective nonrandomized controlled trial showed changes in immune regulation following low-dose OC treatment across the hormonal cycle [113]. There was a significantly higher level of CD3+ CD8+ cells throughout the pill cycle compared to controls [113]. Whereas OC caused lower levels of natural killer (NK) cells during the period, there was an increase in CD20+ and CD20+ CD5+ cells from days 1-8 of the pill cycle. Furthermore, the number of Cytotoxic lymphocytes, which are responsible for first-line immunological defense, and the number of B cells, which are involved in autoimmune illnesses, were both affected by OC use [167]. It is plausible that most of the noticeable immunomodulatory effects of oral contraceptives may be due to the dosage, progestin type, and duration of use. Nevertheless, more studies are needed to understand and elucidate the role of COC on immune regulation, which may provide insight into developing clinically meaningful therapeutic interventions in various conditions and autoimmune diseases [106].

2.2.6. Conclusion

Hence, depending on the condition, knowing the magnitude at which the various available COC formulations affect immune function in premenopausal women may help provide insight and guidance when making an informed decision about the available COC during consultation and follow-up. In addition, it will also guide the planning of future experimental studies, which will involve digging into the various mechanisms of how COC regulates immune responses.

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2.3. Systematic review and meta-analysis Protocol

Open access Protocol

BMJ Open

Investigating the risks of cardiovascular disease among premenopausal women using oral contraceptive: a protocol for a systematic review and meta-analysis

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To cite: Fabunmi OA, Dludla PV, Ngcobo SR, *et al*. Investigating the risks of cardiovascular disease among premenopausal women using oral contraceptive: a protocol for a systematic review and meta-analysis. *BMJ Open* 2023;**13**:e071118. doi:10.1136/bmjopen-2022-071118

► Prepublication history and additional supplemental material for this paper are available online. To view these files, please visit the journal online (http://dx.doi.org/10.1136/bmjopen-2022-071118).

Received 16 December 2022
Accepted 03 January 2023

Check for updates

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ABSTRACT

Introduction The use of oral contraceptives (OCs) is linked to an increased risk of cardiovascular diseases (CVDs) in women of reproductive age. CVD remain one of the top causes of death worldwide, with at least three-quarters of deaths occurring in low-income and middle-income nations. The impact of various types of combined oral contraceptive (COC) on several modifiable risk factors associated with CVDs in premenopausal women is inconsistent regardless of genetic mutations. The aim of this systematic review will be to provide a comprehensive synthesis of the available evidence on the impact of COC usage on modifiable risk factors associated with CVDs and assess ethnic and geographic disparities in the reported prevalence of CVD.

Methods and analysis This systematic review protocol was prepared in accordance with the Preferred Reporting Items for Systematic reviews and Meta-Analyses Protocols 2015 statement. An extensive search on the Embase, MEDLINE and Cochrane Library will be conducted from inception until. Two reviewers will independently screen for eligible studies using a predefined criterion. The risk of bias and quality of included studies will be assessed using the modified Downs and Black's checklist. Whereas the overall quality of included studies will be assessed using the Grading of Recommendations, Assessment, Development and Evaluation assessment tool.

Ethics and dissemination This is a review of existing studies and will not require ethical approval. The findings will be disseminated through peer-reviewed publication. The use of OC and the risk of CVDs including arterial and venous thrombosis remain a major concern among women of reproductive age. Thus, given the impact of COCs on the risk variables linked with CVDs, this review may provide an insight and assistance during COC use.

PROSPERO registration number CRD42020216169.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ This review will be a comprehensive synthesis of available evidence on the link between oral contraceptive (OC) usage and the risk of cardiovascular diseases (CVDs).
⇒ A comprehensive search strategy will be used to identify and retrieve articles relevant to our research question.
⇒ This will be the first systematic review and meta-analysis to assess the geographic disparities in the prevalence of CVD linked to OC usage.
⇒ The main limitation will be the heterogeneity of the studies available in terms of clinical, methodological, type of study and reported outcomes.

INTRODUCTION

Women are exposed to estrogen fluctuations throughout their life which can either endogenous or exogenous in nature.[1] The use of exogenous combined oral contraceptives (COCs) remains one of the most commonly used modern methods of birth control due to their high efficacy and safety profile in premenopausal women.[2] The prevalence of COC use in women of reproductive age is around 16% representing around 151 million women worldwide, and in developed countries this accounts for over 30%[3 4], despite certain risk identified to be associated with the usage.[2 5]

The use of oral contraceptives (OCs) is associated with an increased risk of cardiovascular events[6 7] such as coronary heart disease, arterial and venous thrombosis, ischaemic or haemorrhagic stroke, and myocardial infarction among others.[8] Although, these cardiovascular events appear to differ by geographic region, age and gender.[9 10] The risk for CVD increases when other modifiable cardiovascular factors, such as smoking, obesity, dyslipidaemia, diabetes mellitus, haemostatic disorders and hypertension, are considered.[11]

Notably, available data regarding the impact of COC on modifiable risk factors associated with cardiovascular diseases (CVDs) in women of reproductive age in different population settings are inconsistent.[12–14] For instance, findings from an epidemiological study reported changes in levels of systolic blood pressure and diastolic blood pressure

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following the administration of OCs.[13] In contrast, the study by Kharbanda *et al*, observing the cardiovascular effects of COCs in adolescents, reported no changes in blood pressure.[12]

Furthermore, findings from previous studies investigating the impact of COC on haemostatic parameters are also inconsistent.[5 15] Nevertheless, the impact of COC on the modifiable risk factors associated with CVDs is usually attributed to the direct influence of the oestrogen component, which is known to impact the vascular wall and stimulate endothelial dysfunction, as well as altering the coagulation system.[2 16] The impact of the oestrogen appears to be countered by the progesterone component, which may be dependent on the dose and duration of use.[2 17] Prior studies have also revealed the effect of COCs on metabolic parameters, such as changes in lipid profiles,[14 18] and insulin sensitivity, all of which play a role in the development of a prothrombotic condition.[19 20]

In spite of high efficacy and safety profile attributed to OCs, data available regarding the impact on the occurrence of atherosclerosis, thrombosis, vasomotion and arrhythmogenesis among others are conflicting and insufficient.[21] More so, evidence are currently limited as regard the role of geographical areas and ethnicity on the association between the use of OC and risk of CVDs,[22] although emerging evidence suggest that ethnic differences can influence CVD risk even at a young age, with pronounced differences in some modifiable risk factors.[9] The justification for exploring the effect of OCs on cardiovascular events is simply due to the fact that the prolong use of COC and risk of CVD in women of reproductive age still remain an important issue despite premenopausal women having low risk.[2 23] Thus, determining the impact of COC usage on certain modifiable risk factors associated with CVDs, will help to provide insight and guidance in making an informed decision at each contraceptive consultation (initial and follow-up) in different population settings. Therefore, the aim of this systematic review and meta-analysis will be to provide a comprehensive synthesis of the available evidence on the impact of OC usage on modifiable risk factors associated with the occurrence of CVDs and to further assess the role of ethnic and geographic disparities in the reported prevalence of CVD in women on OCs.

Objectives

The objectives of this study are as follows:

1. To determine the prevalence of CVDs in premenopausal women using OC.
2. To determine the effect of OC on vascular and cellular markers of coagulation in association with immune cell activation in premenopausal women on OC.

METHODS

The systemic review protocol was prepared according to the Preferred Reporting Items for Systemic review and Meta-Analysis Protocol.

Patient and public involvement

There will be no patient and public involvement in the design, interpretation or dissemination of the findings.

Participants

The participants of the systematic review will mainly include healthy adolescent and adult premenopausal women between the age of 18 and 45 years who are not obese and non-smokers.

Exposure

This systematic review will include studies that report on the use of second-generation, third-generation and fourth-generation type of COC.

Comparators

The comparator will include healthy adolescent and adult women who not OCs.

Inclusion and exclusion criteria

This systematic review and meta-analysis will include cross-sectional, cohort, case–control studies and randomised control trials. Studies reporting on the effect of COC usage as form of hormonal therapy or as a method of contraception on the risk factors associated with CVD in healthy premenopausal women who are non-obese, and non-smokers will be included. There will be no language restriction. Reviews, books, letters to editors and studies will be excluded.

Outcomes

The study outcomes will include the following.

Primary outcome

Prevalence or incidence of cardiovascular events.

Surrogate outcome

1. Vascular and cellular markers of coagulation and endothelia dysfunction (endothelin 1, D-dimer, NO, fibrinogen and proinflammatory cytokines (TNF-α, IL-6, C-reactive protein).
2. Traditional cardiovascular risk variables (BMI, systolic and diastolic blood pressure, lipid profile, glucose intolerance and hyperinsulinaemia).

Information sources

A search strategy will be developed using medical subject headings (MeSH) words for MEDLINE, EMBASE and Google Scholar database. The electronic database search will be augmented by searching the Cochrane Central Register of Clinical trials. Papers published in both commercial and in non-commercially operated databases (grey literature) will also be considered. In addition, the reference list of the selected studies will be scanned to identify relevant literature.

Search strategy

The search strategy will be developed using MeSH terms and keywords related to OCs, CVD and premenopausal (online supplemental file). The keywords and

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MeSH terms will include OC pills, birth control pills or OCs or contraceptives, premenopausal women, CVD, hypertension, or coronary heart disease.

Study selection

The screening of studies will be conducted by two independent authors (OAF and SRN) to avoid inconsistency in terms of eligibility of studies to enhance objectivity and prevent mistakes. At the initial stage, studies will be screened by the titles, abstracts, keywords and synonyms, then followed by the identification of the full-text articles. Should discrepancies arise (PD) will screen such studies, and consensus will be reached through discussion.

Data management

The Mendeley desktop reference manager (V.1.19.4) will be used to manage retrieved studies, archiving of relevant and excluded studies with reasons. Importantly, reference lists of included studies will be screened to confirm that no relevant studies are left out. Eligible will then be subjected to data collection, critical appraisal, risk and quality evaluation.

Data items and collection process

Relevant data items will be extracted using a structured form depending on the type of study design. To avoid errors during data entry from selected studies, two authors (OAF and SRN) will independently perform this process. Should discrepancies arise, the third and fourth authors (PVD) will be invited for arbitration. The author and year of publication, the country, population (sample size), study design, types, dosage and duration of contraceptive usage, plasma levels of cellular and vascular markers of coagulation, endothelia dysfunction and inflammation or immune activation, lipid profiles, glucose profiles, body mass index (BMI) and haemodynamic indices of hormonal contraceptive users and non-users will be extracted. In case of insufficient data, the main authors of studies will be contacted to obtain enough information.

Risk of bias in individual studies

The risk of bias and quality of included studies will be assessed using the modified Downs and Black's checklist.[24] Two reviewers (OAF and BBN) will make independent judgements based on the domains of the tool: reporting bias (10 items), external validity (3 items), internal validity (6 items) and selection bias (7 items). The scores will be rated as excellent (25–26), good (20–24), moderate (14–19), poor (11–13) and very poor (<10). In case of disagreements, PVD will be consulted to arbitrate.

Data synthesis

Review Manager (RevMan) V.5.3 (The Nordic Cochrane Centre, The Cochrane Collaboration, 2014) will be used for statistical analysis. Clinical and methodological heterogeneity assessment will be conducted first which will then be followed by an assessment of statistical heterogeneity. The χ^2 and I^2 statistic tests will be used to determine the level of heterogeneity across the included studies. An I^2 value of >25 will be considered as moderate or substantial heterogeneity.[25] If studies are similar with regard to (participants, intervention, comparisons and outcomes), and more so, information extracted from this sufficient number of studies included are homogeneous, we will conduct a fixed-effect meta-analysis using R statistical Software (The R foundation for statistical computing, Vienna, Austria). A random-effects model will be employed where studies show a substantial level of heterogeneity. Furthermore, a subgroup analysis base on the reported outcome and meta-regression will be conducted to assess the role of geographical areas and ethnicity in association with the use of COC and risk of CVD. The levels of inter-rater agreement will be assessed using Cohen's kappa[26] and the funnel plots will be used to assess publication bias.

Quality cumulative evidence

The quality of evidence for primary outcomes will also be evaluated using the grading of recommendations assessment, development and evaluation tool.[27] The findings will be summarised and presented in the summary of findings table.

Sensitivity analysis

The sources of statistical heterogeneity will be assessed by performing a sensitivity analysis and excluding studies with high risk of bias based on the risk of bias assessment.

Ethics and dissemination

This is a review of existing studies and will not require ethical approval. The findings will be disseminated through peer-reviewed publication. The use of OC and the risk of CVDs including arterial and venous thrombosis remain a significant concern among premenopausal women. Thus, given the impact of COCs on the risk variables associated with CVDs, this review may provide an insight and assistance during COC use.

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Contributors OAF and BBN conceptualised and designed the study. OAF drafted the protocol. SRN and PVD reviewed the drafted the protocol. All authors wrote and approved the final manuscript. BBN is the guarantor of the review.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting or dissemination plans of this research.

Patient consent for publication Not required.

Ethics approval Not applicable.

Provenance and peer review Not commissioned; peer reviewed for ethical and funding approval prior to submission.

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2.4. Systematic review and meta-analysis

frontiers | Frontiers in Cardiovascular Medicine

TYPE Systematic Review
PUBLISHED 25 April 2023
DOI 10.3389/fcvm.2023.1127104

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Hypertension, a section of the journal Frontiers in Cardiovascular Medicine

RECEIVED 19 December 2022
ACCEPTED 09 March 2023
PUBLISHED 25 April 2023

CITATION
Fabunmi OA, Dludla PV and Nkambule BB (2023) Investigating cardiovascular risk in premenopausal women on oral contraceptives: Systematic review with meta-analysis. Front. Cardiovasc. Med. 10:1127104. doi: 10.3389/fcvm.2023.1127104

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Investigating cardiovascular risk in premenopausal women on oral contraceptives: Systematic review with meta-analysis

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Background: The use of oral contraceptives (OCs) is associated with an increased risk of cardiovascular events such as arterial and venous thrombosis (VTE). Cardiovascular diseases (CVDs) are the leading cause of death worldwide, with low- and middle-income nations accounting for over three-quarter of CVD deaths. The aim of this systematic review is to provide a comprehensive synthesis of the available evidence on the link between OC use and CVD risk in premenopausal women and to further assess the role of geographic disparities in the reported prevalence of CVD risk in women on OCs.
Methods: A comprehensive search of databases such as MEDLINE, Academic Search Complete, Cumulative Index to Nursing and Allied Health Literature (CINAHL), and Health Source: Nursing/Academic Edition was conducted, right from the inception to the present, by using the EBSCOhost search engine. The Cochrane Central Register of Clinical trials (CENTRAL) was also searched to augment relevant sources of information. OpenGrey, which is a repository of information providing open access to bibliographical references, was searched and the reference list of the selected studies was also scanned. The potential risk of bias of the included studies was assessed using the modified Downs and Black checklist. Data analysis was performed using the Review Manager (RevMan) version 5.3.
Results: We included 25 studies that comprised 3,245 participants, of which 1,605 (49.5%) are OC users, while 1,640 (50.5%) are non-OC users. A total of 15 studies were included for meta-analysis, and the overall pooled estimates suggested a significant increase in the traditional cardiovascular risk variables [standardized mean difference (SMD) = 0.73, (0.46, 0.99) ($Z = 5.41$, $p < 0.001$)] and little to no difference in endothelial activation among OC users when compared with non-OC users [SMD = −0.11, (−0.81, 0.60) ($Z = 0.30$, $p = 0.76$)]. Europe [SMD = 0.03, (−0.21, 0.27), ($Z = 0.25$ $p = 0.88$)] had the least effect size, while North America had the highest effect size [SMD = 1.86, (−0.31, 4.04), ($Z = 1.68$ $p = 0.09$)] for CVD risk in OC users when compared with non-OC users.
Conclusion: The use of OCs suggests a significant increase in the prevalence of traditional cardiovascular risk variables with little to no difference in the risk of endothelial dysfunction when compared with non-OC users, and the magnitude of CVD risks varies across different geographical regions.
Registration and protocol: This systematic review was registered in the international prospective register of systematic reviews (PROSPERO) under the registration number: CRD42020216169.

KEYWORDS
oral contraceptives, combined oral contraceptives, ethinylestradiol, progestins, cardiovascular disease

Candidate's contribution: OAF (the candidate) was involved in the conceptualization of the study, data analysis and writing of the manuscript.

1. Introduction

Hormonal contraceptives, primarily oral contraceptive pills (OCPs), are one of the most commonly-prescribed modern methods of birth control for premenopausal women aged 15–49 (1) because of its high efficacy and safety profile (2–5). There are an estimated 151 million women using OCPs worldwide (6) and developed countries account for over 30% of such women (6, 7). Despite the known health benefits of OCPs that include preventing pregnancy and treating reproductive disorders among others (8–10), their physiological impact on women's health, combined with the risk of cardiovascular events (2, 11) such as arterial and venous thrombosis (ATE and VTE), ischemic and hemorrhagic stroke, and myocardial infarction (12–15) at various phases of life, remains a major concern (1). Nonetheless, a previous report showed that the incidence of cardiovascular events is rare in young female adults (1–2 per 10,000 per year) but the rate increases to ~1% per year in the elderly (16, 17), indicating age as a strong predisposing risk factor of cardiovascular disease (CVD) among women, especially in developed countries (18, 19).

Since the introduction of the first-generation combined oral contraceptives (COCs), efforts to reduce their adverse cardiovascular side effects has led to the development of subsequent second-generation and third-generation medications (levonorgestrel; LNG and desogestrel; DSG or norgestimate, respectively) with lower estrogen dose and a varying progestin component called "gonanes," including the recent fourth-generation medication (drospirenone; DRSP) (1). However, emerging evidence shows conflicting differences regarding the individual impact of COCs on several cardiovascular risk variables such as metabolic, hemodynamic, and hemostatic parameters (1, 10, 13, 20–23), and their impact is attributed to the dose of estrogen and progestin type (24, 25) and the duration of use (26).

Notably, evidence from previous studies showed an association between third-generation COCs (desogestrel; DSG and gestodene; GSD) and elevated risk of thrombosis when compared with the second-generation COC (levonorgestrel) (27–29). More so, the reported incidence of thrombotic events associated with third-generation COCs, when compared with second- and fourth-generation COCs, remains high at 6.6 per 10,000-woman (27). However, the incidence rates for ATE events is lower in women on DRSP-containing OCs compared to other COCs (30, 31). More so, the relative risk of ATE for COCs containing 30–35 μg ethinylestradiol and gestodene, desogestrel, cyproterone acetate, or DRSP was similar, and approximately 50%–80% higher than, the second-generation LNG (32, 33).

In contrast, a previous study showed that the use of COCs are not associated with the occurrence of acute myocardial infarction in young women because no excess risk was reported among users of desogestrel and gestodene when compared with LNG (14). In fact, the study further reported a high amelioration of CVD-risk among smokers using the third-generation COC when compared with the second-generation LNG (14), which contradicts with the finding of another multicenter, case-control study that reported a 3-fold increased risk of ischemic stroke among COC users (34, 35). However, the incidence and risk of ischemic stroke attributable to OC use in the study was reportedly low in women of reproductive age who are non-smokers with no hypertension (34, 35).

Furthermore, a recent study showed an increased number of adverse events relating to CVD in fourth-generation COC (DRSP) users when compared with second/third-generation COC users, and the number of reported events was the highest in the 20-year age group, followed by the 30-year age group, and finally in those over 40 years (36). Meanwhile, available data on the risk of cardiovascular events among different formulations of COC remain inconclusive and further research is needed to identify the causality between COCs and CVDs (36). Therefore, the aim of this systematic review and meta-analysis was to provide a comprehensive synthesis of the available evidence on the link between COC use and CVD risk in premenopausal women and to further assess the role of geographic disparities in the reported prevalence of CVD risk in women on COCs.

2. Methods

This systematic review and meta-analysis was prepared according to the preferred reporting items for systematic reviews and meta-analysis (PRISMA) guidelines (37) and the protocol was published (38). A comprehensive and systematic search of published studies was conducted to address the following research questions:

1. Do COCs impact cellular and vascular markers of endothelial activation?
2. What is the role of COCs in traditional cardiovascular risk variables?

2.1. Eligibility criteria

We included cross-sectional, cohort, and case control studies and randomized control trials. Studies reporting on the effect of OC use as a method of contraception on the risk of CVDs in healthy premenopausal women were also included. There were no language restrictions.

2.2. Exclusion criteria

Reviews, books, letters to editors including gray literature were excluded, the bibliographies that were searched for relevant citations.

2.3. Search strategy and information sources

The search strategy was developed using medical subheadings (MeSHs) and keywords related to oral contraceptives,

cardiovascular disease, and premenopausal women (Supplementary File S1). The keywords and MeSH terms used included oral contraceptive pills, premenopausal women, cardiovascular disease, or coronary heart disease. A comprehensive search of databases such as MEDLINE, Academic Search Complete, Cumulative Index to Nursing and Allied Health Literature (CINAHL), Health Source: Nursing/Academic Edition, APA PsycInfo, and MasterFILE Premier was conducted from inception to the present by using the EBSCOhost search engine. Furthermore, the Cochrane Central Register of Clinical trials (CENTRAL) was searched including OpenGrey (System Information on Grey Literature in Europe) (www.open.eu) to obtain relevant sources of information. In addition, the reference list of the selected studies was scanned, and forward citation tracking was done using Google scholar to identify the relevant literature. In instances of disagreements, a third reviewer (BBN) was consulted to conduct arbitration proceedings.

2.4. Study selection

The screening of studies was performed by two independent reviewers (OAF and PVD) to avoid inconsistencies with regard to the eligibility of the studies. The abstracts were screened, and the full texts of eligible studies were retrieved. In instances of discrepancies, BBN was consulted for arbitration.

2.5. Outcomes

The primary outcomes of this systematic review and meta-analysis were endothelial activation measured by nitric oxide (NO) and endothelin 1 (ET-1) level, flow-mediated dilation (FMD), and common carotid artery intima–media thickness (CCA-IMT). The secondary outcomes was cardiovascular risk evaluated by changes in blood pressure, lipid profile, and blood glucose levels.

2.6. Data items and collection process

A data extraction sheet was used to extract data items that included the name of the author, year of publication, country, population (sample size), study design, types of OC, dosage, and main findings of the study. Mendeley desktop reference manager software (version 1.19.4) was used to examine the retrieved citations and to remove study duplicates.

2.7. Quality assessment and risk of bias

The potential risk of bias of the included studies was assessed using the modified Downs and Black checklist (39). The tool assesses four domains, namely, reporting bias, external validity, internal validity, and selection bias. Each study was graded and scored as either "excellent" (24–28 points), "good" (19–23 points), "fair" (14–18 points), or "poor" (<14 points).

2.8. Certainty of evidence

The quality of evidence was evaluated using the grading of recommendations assessment, development, and evaluation (GRADE) tool (40). The findings are summarized and presented in the summary of findings table (Table 4).

2.9. Data synthesis and statistical analysis

Higgin's I^2 statistic was used to assess statistical heterogeneity. In instances of substantial heterogeneity ($I^2 > 50\%$), a random-effects model was used to generate pooled effect estimates (41). Outcomes with same-effect estimates were reported as the mean difference (MD), while different-effect estimates were reported as the standardized mean difference (SMD) and a 95% confidence interval (CI). To explore potential sources of statistical heterogeneity, we conducted a subgroup analysis on the basis of the study design. Data analysis was performed using the software Review Manager (RevMan) version 5.3. The levels of inter-rater agreement were assessed using Cohen's kappa (39), in which a score of values 0.01–0.20 indicate none to slight agreement, 0.21–0.40 fair, 0.41–0.60 moderate, 0.61–0.80 substantial, and 0.81–1.00 an almost perfect agreement (42). A p-value of ≤ 0.05 was considered statistically significant.

2.10. Sensitivity analysis and publication bias

Sensitivity analysis was performed to test the robustness of our reported effect estimates by following a stepwise removal of studies. We performed repeated meta-analysis by taking into account participants' characteristics and study design, and thereafter, sensitivity analysis was conducted on the basis of geographical location. Furthermore, the method of visual inspection of funnel plots was used to assess publication bias.

3. Results

A total of 165 studies were identified and retrieved using the search strategy and screened for eligibility. A total of 25 studies met the inclusion criteria, while total of 140 studies were excluded. Among the excluded studies, 17 were reviews, and 123 were not relevant to the topic of interest (Figure 1). In all, only 15 studies were shortlisted for quantitative and meta-analyses.

3.1. Characteristics of the included studies

The included studies were published between 1998 and 2019, and the study characteristics are given in Table 1. The included studies comprised 3,245 participants, of which 1,605 (49.5%) were on OCs, while 1,640 (50.5%) were non-OC users. Furthermore, 11 studies were cross-sectional studies (43–45, 47–50, 55, 56, 63, 64), seven were randomized control trials (51, 52,

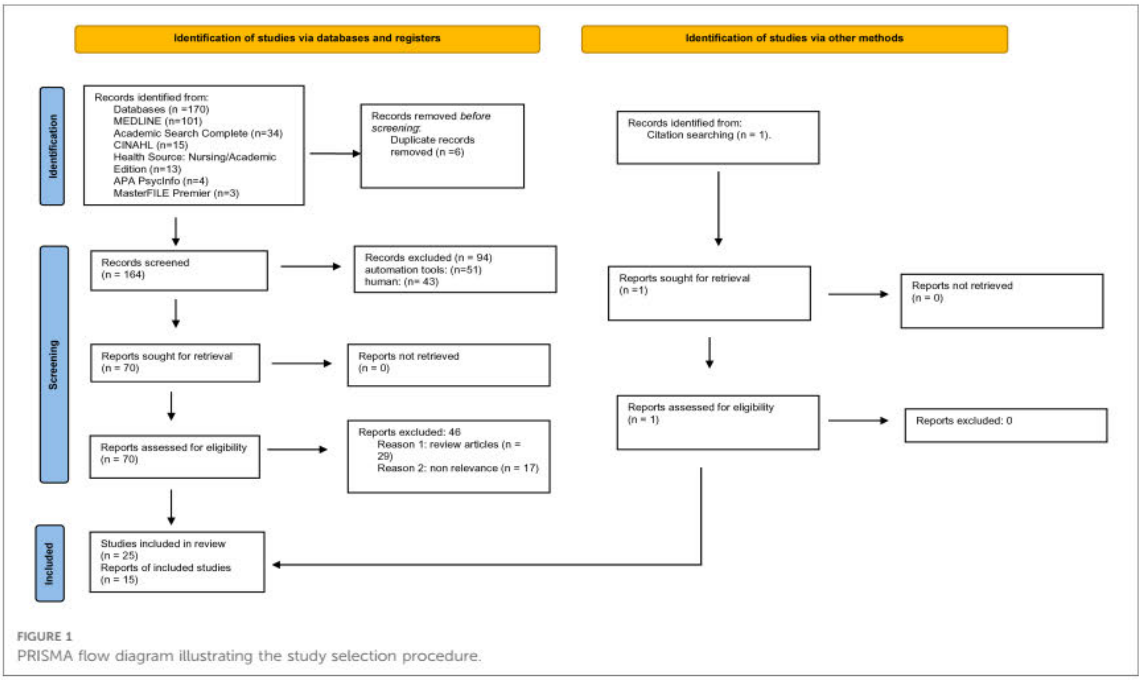

FIGURE 1
PRISMA flow diagram illustrating the study selection procedure.

59–61, 65, 66), three were cohort studies (54, 57, 62), two were clinical trials (53, 67), and one each was a prospective longitudinal study (46), and a case control study (58). In addition, the geographical distribution of the included studies comprised Europe ($n = 6$) (46, 51, 52, 58, 65, 66), North America ($n = 6$) (43–45, 47, 54, 57), South America ($n = 6$) (49, 53, 56, 59, 61, 67), Asia ($n = 4$) (50, 55, 62, 64), Africa ($n = 2$) (48, 63), and Australia ($n = 1$) (60).

3.2. Quality assessment and risk of bias of the included studies

The risk of bias was independently assessed by two reviewers (OAF and PVD) using the modified Downs and Black checklist (39). Overall, the included studies were rated as fair, with an average score of 18 out of a possible 26. Overall, the studies were scored as excellent for reporting the bias domain (with a score of nine out of a possible 10), poor for external validity (with a score of one out of a possible three), moderate for the internal validity domain (scoring three out of a possible seven), and moderate for selection bias (with a score three out of a possible six). The inter-rater reliability per domain was scored as $k = 0.86$ (CI = 0.8, 0.93) for reporting bias (perfect agreement), $k = 0.54$ (CI = 0.41, 0.68) for external validity (moderate agreement); $k = 0.68$ (CI = 0.53, 0.83) for internal validity (substantial agreement), and $k = 0.63$ (CI = 0.49, 0.77) for selection bias (substantial agreement) (**Supplementary additional file S1**, **Figure 2**).

3.3. The impact of OC use on reported markers of endothelial activation in premenopausal women

Overall, the results of our meta-analysis showed little to no difference in the pooled estimate for endothelial activation among participants on OCs when compared with non-users [SMD = −0.11, 95% CI (−0.81, 0.60), $Z = 0.30$, $p = 0.76$, low certainty evidence]. However, these results showed a substantial level of statistical heterogeneity ($I^2 = 94\%$, $p < 0.00001$) (**Figure 3**) and subgroup analyses based on study design, following which the reported measure of effect size of endothelial activation was estimated (**Figure 3** and **Table 2**).

3.3.1. NO level

The qualitative findings of our study, as reported in **Table 1**, showed that at the basal level, NO production and release was enhanced by OCs [second-generation (LNG) and third-generation gestodene and desogestrel (GSD, DSG) types], but upon stimulation with different dosages of acetylcholine, the plasma level of NO remained unchanged (58). Meanwhile, a study by Merki-Feld et al. showed that the use of second-generation (LNG) and third-generation (GSD) OC did not alter the plasma levels of nitric oxide (46). In contrast, the use of second-generation (LNG) OCs was associated with reduced plasma levels of NO when compared with the control group (55). However, the pooled estimate of our subgroup analysis suggests that OC use may result in little to no difference in the plasma level of NO when compared with non-OC users

TABLE 1 Characteristics of the included studies ($n = 25$).

Author	Year	Country	Main finding	Aim of study	Study design	Type of contraceptive	Dosage	Duration of use	Sample size	Age (years)
Friedman et al. (43)	2011	United States	OCPs significantly decreased serum iron and transferrin saturation and significantly increased FMD in the brachial artery.	To characterize the link between OCP use, iron stores, and cardiovascular risk in premenopausal women	Cross-sectional	COC	Estranes/gonanes and DRSP; 20–25 µg, >25 µg	>1 year	**23** (OC: 23; Non-user: NR)	OC (24.4 ± 2.3) Non-user (NR)
Blacknore et al. (44)	2011	Canada	OC use significantly lowered IGF-1 levels among younger women (18–21) when compared with non-users and older women (31–40).	Effect of past OC use and timing on circulating IGF-1	Cross-sectional	COC	NR	>1 year	**329** (OC: 137; Non-user:192)	OC (27.5 ± 2.1) Non-user (27.5 ± 2.1)
Meendering et al. (45)	2008	United States	MPA treatment antagonized estradiol administration by decreasing endothelium-dependent vasodilation and increasing resting plasma ET-1 in healthy young women.	To investigate whether medroxyprogesterone acetate (MPA) antagonizes the favorable effects of exogenous estradiol on vascular function and biomarkers of cardiovascular risk in young women.	Cross-sectional	Progestin only	5 mg MPA	10 days	**14** (OC: 14; Non-user: NR)	OC (23 ± 5.7) Non-user (NR)
Merki-Feld et al. (46)	2002	Switzerland	No significant changes in the plasma levels of nitric oxide, ET-1, blood pressure (BP), and BMI. However, there was a significant negative correlation between nitric oxide and endothelin-1, and nitric oxide and cholesterol, and a positive correlation between endothelin-1 and cholesterol.	To examine the influence of LNG and GSD on plasma levels of the vasodilator NO, the vasoconstrictor endothelin 1, and the plasma lipids, cholesterol and HDL	Prospective Longitudinal	COC	30 µg EE/150 µg LNG; 30 µg EE/75 µg GSD.	6 months	**12** (OC: 12; Non-user: NR)	OC (21.7 ± 4.3) Non-user (NR)
Odutayo et al. (47)	2015	Canada	Baseline levels of angiotensinogen, angiotensin II, aldosterone, and plasma renin activity were significantly higher in OCP subjects compared with normotensive control and contraceptive patch subjects, while the mean arterial pressure and BMI were non-significant.	Effects of the contraceptive patch and OCP on circulating renin–angiotensin–aldosterone system (RAAS) mediators and systemic blood pressure.	Cross-sectional study	COC, Transdermal patch	30 µg EE/150 µg LNG, 20 µg EE/150 µg norelgestromin	3 months	**25** (OC: 10; Non-user: 15)	OC (28 ± 1) Non-user (24 ± 1)
Asare et al. (48)	2014	Ghana	Diastolic blood pressure, TC, LDL, Castelli risk indices I (TC/HDL) and II (LDL/HDL), and BMI were significantly higher in the HC group than in the control group.	Effect of hormonal contraceptives on lipid profile and the risk indices for CVD in a Ghanaian community	Cross-sectional	COC, Progestin only	0.03 mg EE/0.15 mg LNG 0.035 mg norethindrone	>1 year	**43** (OC: 19; Non-user: 24)	OC (33.1 ± 6.3) Non-user (29.3 ± 8.1)
Lizarelli et al. (49)	2009	Brazil	The OC group had significantly lower FMD and HDL when compared with the control group, while the impact on CCA-IMT was not significantly different.	Both a combined oral contraceptive and depot medroxyprogesterone acetate impair endothelial function in young women.	Cross-sectional	COC, Progestin only	EE30 µg/LNG 150 µg DMPA (150 mg)	6 months	**75** (OC: 25; Non-user: 50)	OC (23.7 ± 3.2) Non-user (23.4 ± 3.6)
Heidarzadeh et al. (50)	2014	Iran	COC use significantly reduced FMD % in comparison with the control group, while the mean CCA-IMT was significantly but slightly higher when compared with the age-matched control group	The effect of low-dose combined oral contraceptive pills on brachial artery endothelial function and CCA-IMT thickness	Cross-sectional	COC	EE 30 µg/LNG150 µg	>1 year	**60**(OC: 30; Non-users: 30)	OC (33.3 ± 4.6) Non-users (33.9 ± 5)

(*continued*)

TABLE 1 Continued

Author	Year	Country	Main finding	Aim of study	Study design	Type of contraceptive	Dosage	Duration of use	Sample size	Age (years)
Yildizhan et al. (51)	2009	Turkey	OCs resulted in significant reductions in systolic and diastolic BP and LDL levels and a significant increase in TG and HDL levels, resulting in increasing the HDL/LDL ratio.	Effects of two combined oral contraceptives containing ethinyl estradiol 30 µg combined with either gestodene or drospirenone on hemostatic parameters, lipid profiles, and BP	RCT	COC	EE 0.03 mg/GSD 0.075 mg, EE 0.03 mg/DRSP 3 mg	1 year	**160** (OC: 160; Non-user: NR)	OC (27.5 ± 10.6) Non-user (NR)
Wiegratz et al. (52)	2004	Germany	OCs caused significant changes in the hemostatic parameters by increasing the levels of fibrinogen, prothrombin fragment 1 + 2, factor VII, protein C, plasminogen, Plasmin-Alpha-2-Antiplasmin (PAP) complexes, and D-dimer, while total and free protein S, t-PA, and PAI levels were significantly reduced.	Effect of four oral contraceptives on hemostatic parameters	RCT	COC	30 µg EE/2 mg DNG, 20 µg EE/2 mg DNG, 10 µg EE/2 mg EV/2 mg DNG, 20 µg EE/100 µg LNG	6 months	**100** (OC: 100; Non-user: NR)	OC (26.5 ± 12) Non-user (NR)
Giribela et al. (53)	2012	Brazil	The contraceptive formulation did not cause any significant changes in BP, heart rate (HR), cardiac output (CO), total peripheral resistance (TPR), and arterial endothelial function.	Effect of combined oral contraceptives containing drospirenone on endothelial function and hemodynamic parameters in healthy young women	Clinical trial	COC	20 µg EE/3 mg DRSP	6 months	**71** (OC:43; Non-user: 28)	OC (29.2 ± 6.8) Non-user (30.6 ± 6.8)
Kharbanda et al. (54)	2014	United States	COCs were not associated with clinically meaningful changes in weight or blood pressure.	Initiation of oral contraceptives and changes in blood pressure and BMI in healthy adolescents	Observational cohort	COC	30/35 µg EE	>1 year	**1,422** (OC: 510; Non-user: 912)	OC (16.4 ± 1) Non-user (16.4 ± 1)
Fallah et al. (55)	2012	Iran	OCs significantly elevated the levels of homocysteine (HCY) and reduced the levels of NO concentration in the plasma.	Influence of oral contraceptive pills on homocysteine and nitric oxide levels	Cross-sectional	COC	30 µg EE/150 µg LNG	>1 year	**100** (OC: 50; Non-user: 50)	OC (27.5 ± 10.6) Non-user (27.5 ± 10.6)
Dos Santos et al. (56)	2018	Brazil	There was a significant increase in the levels of TG, HDL-cholesterol, CRP, and systolic blood pressure values in COCs. There was also a significant increase in plasma-oxidized LDL values when compared with the control group.	Elevation of oxidized lipoprotein of low density in users of combined oral contraceptives	Cross-sectional	COC	150 µg LNG/30 µg EE	>1 year	**42** (OC: 21; Non-user: 21)	OC (23 ± 3.1) Non-user (23 ± 3.4)
Harvey et al. (57)	2015	United States	Blood pressure was significantly higher in OC users than in OC non-users. Muscle sympathetic nerve activity (MSNA) at rest, as well as CO and TPR, is similar between the two study groups.	Effect of oral contraceptive use on MSNA, and systemic hemodynamics in young women	Retrospective cohort study	COC	20–30 µg EE/3 mg DRSP, 150 µg LNG/30 µg EE	NR	**127** (OC: 53; Non-user: 74)	OC (25 ± 1) Non-user (25 ± 1)

(*continued*)

TABLE 1 Continued

Author	Year	Country	Main finding	Aim of study	Study design	Type of contraceptive	Dosage	Duration of use	Sample size	Age (years)
John et al. (58)	2000	Germany	At the basal level, NO production and release was enhanced by oral contraceptive use, while upon stimulation, NO bioavailability remained unchanged in the participants' group.	Effects of oral contraceptives on the vascular endothelium in premenopausal women	Case control	COC	0.035 mg EE/0.125 mg LNG; 0.03 mg EE/0.15 mg DG; 0.03 mg EE/0.075 mg GSD; 0.03 mg EE/0.05 mg LNG; 0.02 mg EE/0.15 mg DSG.	1 year	**16** (OC: 8; Non-user: 8)	OC (27 ± 2) Non-user (27 ± 2)
Nadai et al. (59)	2015	Brazil	There was no significant difference in BP associated with the use of combined oral contraceptives containing DRSP irrespective of the EE dose used	Effects of two contraceptives containing drospirenone on blood pressure in normotensive women: a randomized-controlled trial	RCT	COC	30 μg EE/3 mg DRSP, 20 μg EE/3 mg DRSP	6 months	**44** (OC: 44; Non-user: NR)	OC (24.7 ± 4.5) Non-user (NR)
Straznicky et al. (60)	1998	Australia	OCs significantly increased 24 h systolic and diastolic blood pressure levels, triglyceride levels, and insulin area under the curve in users when compared with non-users.	Effects of oral contraceptive use and dietary fat intake on blood pressure, cardiovascular reactivity, and glucose tolerance in normotensive women	RCT	COC	0.03 mgEE/0.15 mg LNG,0.05 mg EE/ 0.125 mg LNG,0.03 mg EE/0.05 mg LNG, 0.04 mg EE/0.075 mg LNG, 0.03 mg EE/0.125 mg LNG	>1 year	**32** (OC: 16; Non-user: 16)	OC (29.8 ± 7.8) Non-user (31.3 ± 7.7)
Franceschini et al. (61)	2013	Brazil	A COC containing LNG is associated with a more pronounced reduction in FMD and increased IMT of healthy women than a COC containing CMA and non-hormonal contraception.	Effects of combined oral contraceptives containing levonorgestrel or chlormadinone on the endothelium	RCT	COC	EE 30 μg/CMA 2 mg, EE 30 μg/LNG 150 μg	6 months	**64** (OC: 43; Non-user: 21)	OC (24.2 ± 6.1) Non-user (28.3 ± 3.7)
Zahra et al. (62)	2019	Iran	OCs significantly increase the plasma levels of homocysteine (HCY), TG, cholesterol (TC), and LDL-c among users when compared with non-users.	Effects of low-dose contraceptive pills on the risk factors of cardiovascular diseases among 15-35-year-old women	A retrospective cohort study	COC	30 μg EE/150 μg LNG.	>1 year	**100** (OC: 50; Non-user: 50)	OC (30.1 ± 3.7) Non-user (30.1 ± 4.1)
El-Haggar and Mostafa (63)	2015	Egypt	The uptake of combined oral contraceptive pills (COCPs) significantly lowered adiponectin concentration and significantly increased leptin and resisting levels and the atherogenic index when compared with other studied groups.	To evaluate the associated cardiovascular risk in Egyptian healthy consumers of different types of COCPs.	Cross-sectional study	COC	30 μg EE/150 μg LNG, 0.03 mg EE/0.075 mg GSD, 30 μg EE/3 mg DRSP	6 months	**120** (OC: 90; Non-user: 30)	OC (31.2 ± 2.7) Non-user (31.7 ± 1.8)
Fallah et al. (64)	2011	Iran	Low-dose COC uptake results in a significant decrease in serum adiponectin concentration and an increased atherogenic lipid profile by significantly increasing LDL levels.	Adiponectin, leptin, and lipid profile evaluation in oral contraceptive pill consumers	Cross-sectional study	COC	30 μg EE/150 μg LNG	>1 year	**100** (OC: 50; Nonuser: 50)	OC (31.7 ± 7.9) Nonuser (33.9 ± 6.3)
Winkler et al. (65)	2009	Germany	OC treatments significantly increased triglyceride and Apo AI levels and HDL levels, while LDL levels were reduced in OC users when compared with non-users.	The effects of two monophasic oral contraceptives containing 30 μμg of ethinyl estradiol and either 2 mg of chlormadinone acetate or 0.15 mg of desogestrel on lipids, hormones, and metabolic parameters	RCT	COC	EE 30 μg/CMA 2 mg, EE 30 μg/DSG 0.15 mg	6 months	**43** (OC: 43; Non-user: NR)	OC (27.2 ± 5) Non-user (NR)

(*continued*)

TABLE 1 Continued

Author	Year	Country	Main finding	Aim of study	Study design	Type of contraceptive	Dosage	Duration of use	Sample size	Age (years)
Piltonen et al. (66)	2012	Finland	The use of oral, transdermal, and vaginal combined contraceptives (CCs) decreases androgenicity, worsens insulin sensitivity, and increases the level of markers of chronic inflammation at the same rates.	Effect of alternative administration routes of CCs on androgen secretion, chronic inflammation, glucose tolerance, and lipid profile	RCT	COC, Patch, vaginal ring	EE 20 µg/DSG 0.15 mg, EE 20 µg/norelgestromin 150 µg, EE 15 µg/120 µg etonogestrel	2 months	**54** (OC: 18; Non-user: NR)	OC (23.5 ± 3.1) Non-user (NR)
Marcelo et al. (67)	2014	Brazil	There were no significant alterations in blood pressure, heart rate variability, and baroreflex sensitivity of healthy women during a 6-month period of use of a COC containing EE and DRSP.	To evaluate the effect of a contraceptive containing 20 µg of ethinyl estradiol and 3 mg of drospirenone on the heart rate variability, baroreflex sensitivity, and blood pressure of healthy women.	Prospective clinical trial	COC	20 µg EE/3 mg DRSP	6 months	**69** (OC: 36; Non-user: 33)	OC (28.8 ± 1.1) Non-user (30.3 ± 1)

NR, not reported; COC, combined oral contraceptives; EE, ethinylestradiol; DSG, desogestrel; DNG, dienogest; DRSP, drospirenone; CMA, chlormadinone acetate; LNG, levonorgestrel; MPA, medroxyprogesterone acetate; GSD, gestodene; IGF-1, insulin-like growth factor 1; RCT, randomized control trial.

(SMD = −0.73, 95% CI (−2.60, 1.14), $p = 0.44$ (low certainty evidence) with a substantial level of heterogeneity ($I^2 = 97\%$, $p < 0.00001$) (Figure 3).

3.3.2. Flow-mediated dilation

The qualitative findings of our study, as reported in Table 1, showed that the use of fourth-generation drospirenone (DRSP) OC significantly increased flow-mediated dilation (FMD) in the brachial artery of the participants (43), which contrasted with the findings of other studies (49, 50, 61), where the use of second-generation levonorgestrel (LNG) and fourth-generation chlormadinone acetate (CMA) OC by the participants significantly lowered FMD when compared with non-users. However, the results of our meta-analysis suggest little to no difference in the pooled estimate for FMD in the participants on OCs when compared with non-OC users [SMD = −0.22, 95% CI (−1.12, 0.68), $p = 0.63$ (low certainty evidence) with a substantial level of heterogeneity ($I^2 = 87\%$, $p = 0.0004$)] (Figure 3).

3.3.3. Common carotid artery intima–media thickness

The qualitative findings of our study, as reported in Table 1, showed that the mean CCA-IMT was significantly higher in participants who used second- and third-generation OCs (50, 61), which contrasted with the findings of a study by Lizarelli et al. that reported no significant difference between users of the second-generation levonorgestrel (LNG) and non-users (49). However, the results of our meta-analysis showed a significant increase in the pooled estimate for CCA-IMT in participants not on OCs when compared with OC users [SMD = 0.62, 95% CI (0.02, 1.21), $p = 0.04$, low certainty evidence], although a substantial level of statistical heterogeneity was observed in these studies ($I^2 = 71\%$, $p = 0.03$) (Figure 3). Thus, our evidence suggests that OC use may result in a significant reduction in CCA-IMT among users.

3.4. Prevalence of traditional cardiovascular risk variables among OC users when compared with non-users

The overall pooled estimates of our meta-analysis suggest an increased CVD risk among OC users when compared with non-users [SMD = 0.73, 95% CI (0.46, 0.99), $Z = 5.41$, $p < 0.001$] ($I^2 = 94\%$, $p < 0.001$, low certainty evidence). However, due to a substantial level of heterogeneity, a subgroup analysis of the reported effect estimates was conducted (Table 2).

3.4.1. Blood pressure measurements

3.4.1.1. Systolic blood pressure

The qualitative findings of our study, as reported in Table 1, showed that systolic blood pressure increased significantly among users of second- (levonorgestrel; LNG) and third- (gestodene; GSD) generation COCs (56, 57, 60, 62, 64), which contrasted with those of a study by Franceschini et al. that reported a significant reduction among users of second (LNG)-generation

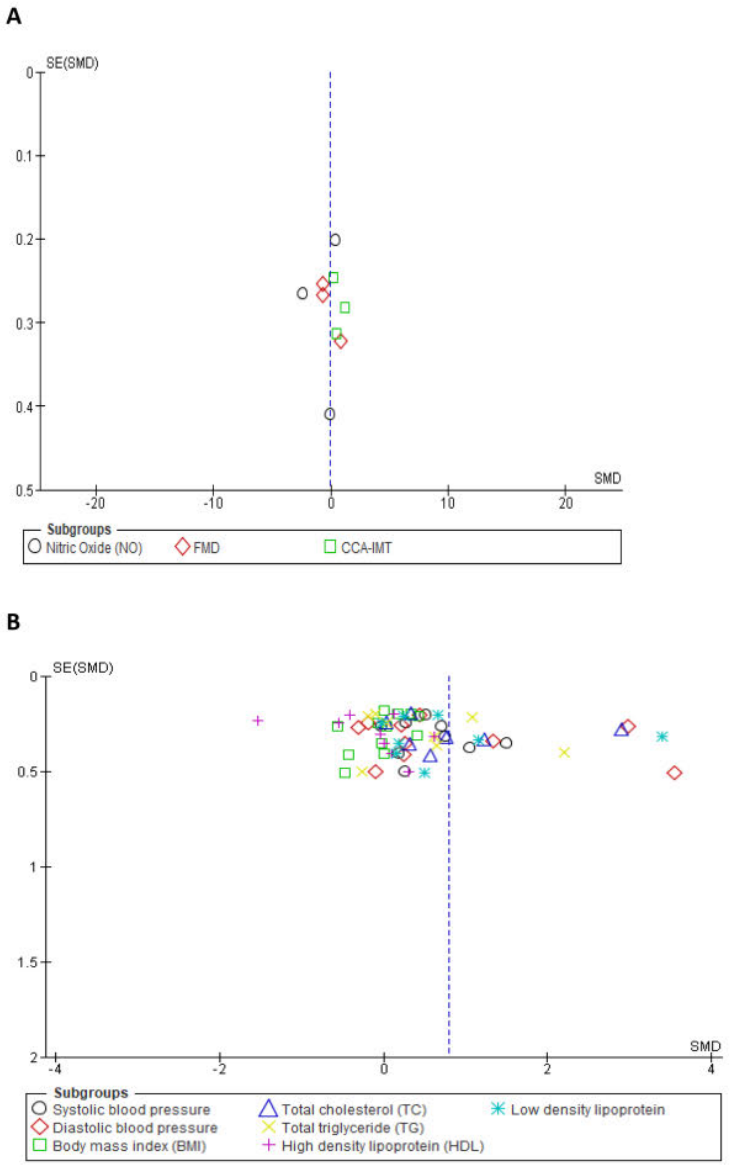

FIGURE 2
Quality assessment of the included studies.

COC when compared with non-users (61). However, several other studies reported a non-significant change in systolic blood pressure (SBP) among COC users despite the similarity in the duration of use (46, 48–50, 58, 67). Furthermore, the results of our subgroup analysis suggest a significant increase in the SBPof participants on OCs when compared with non-users [SMD = 1.96, 95% CI (0.94, 2.97), $p = 0.002$, low certainty evidence] and a substantial level of heterogeneity ($I^2 = 97\%$, $p < 0.001$) (Table 2).

3.4.1.2. *Diastolic blood pressure*

The qualitative findings of our study, as reported in Table 1, showed that diastolic blood pressure (DBP) increased

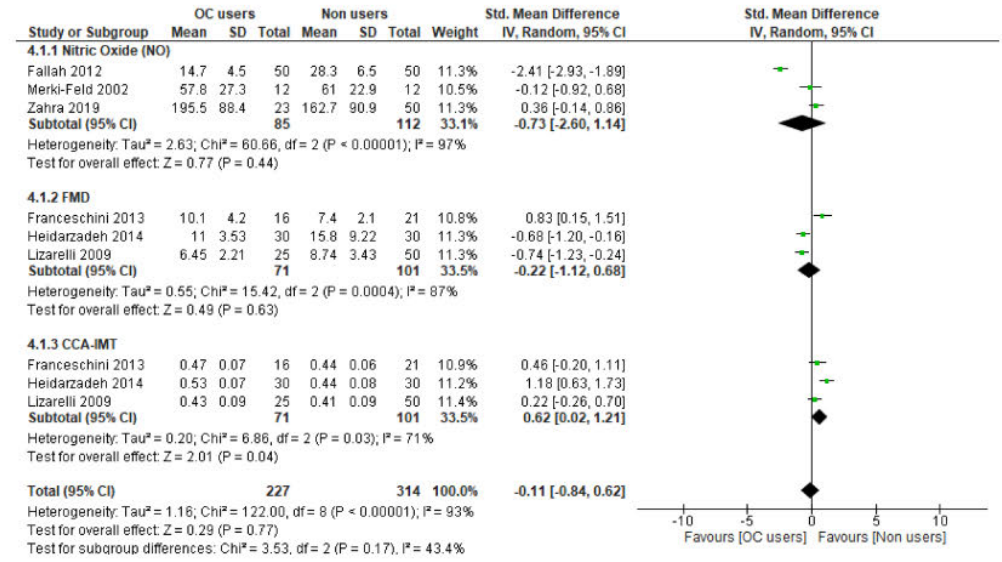

FIGURE 3
Forest plot of cellular and vascular markers of endothelial activation in premenopausal women on OCs vs. non-OC users.

significantly among users of second- (levonorgestrel; LNG) and third- (gestodene; GSD) generation COCs (48, 57, 64), which contrasted with those of a study by Franceschini et al. that reported a significant reduction among users of second- (LNG) and fourth- (CMA) generation COCs when compared with non-users (61). However, several other studies reported a non-significant change among COC users despite the similarity in the duration of use (46, 49, 56, 58, 60, 62, 67). In addition, evidence from our meta-analysis suggests a significant increase in DBP of participants on OCs when compared with non-users [SMD = 1.74, 95% CI (0.86, 3.03), $p = 0.001$, low certainty evidence], although there was a substantial level of heterogeneity ($I^2 = 97\%$, $p < 0.001$) (Table 2).

3.4.2. Body mass index

The qualitative findings of our study, as reported in Table 1, showed that the use of second- (levonorgestrel; LNG) and third- (gestodene; GSD, desogestrel; DSG) generation COCs does not significantly increase body mass index (BMI) (46, 47, 49, 50, 55–58, 60, 61–63, 67), which contrasted with those of a study by Asare et al. that reported a significant increase in BMI among users of the second- (LNG) generation COC despite the

TABLE 2 Traditional cardiovascular-risk variables of included participants.

Effect Measure	Number of Studies	Number of participants	Effect estimate				
			Model	SMD	95% CI	I^2, p-value	p-value
Blood pressure							
SBP	**12**, (46, 48, 62, 67, 49, 50, 55–58, 60, 61)	752	RE	1.96	0.94–2.97	97%, $p < 0.001$	3.78, $p = 0.002$
DBP	**12**, (46, 48, 62, 67, 49, 50, 55–58, 60, 61)	752	RE	1.74	0.71–2.78	97%, $p < 0.001$	3.3, $p = 0.001$
BMI	**14**, (46, 47, 61–63, 67, 48–50, 55–58, 60)	897	RE	0.22	−0.14–0.57	82%, $p < 0.001$	1.21, $p = 0.23$
Lipid metabolism							
Total cholesterol	**8**, (46, 48, 55, 56, 60, 62, 63)	536	RE	0.94	0.22–1.66	92%, $p < 0.001$	2.55, $p = 0.01$
HDL cholesterol	**9**, (46, 48, 49, 55, 56, 58, 60, 62, 63)	552	RE	−0.20	−0.64–0.25	82%, $p < 0.001$	0.85, $p = 0.39$
LDL cholesterol	**8**, (48, 55, 56, 58, 60, 62, 63)	509	RE	0.79	−0.04–1.59	92%, $p < 0.001$	1.93, $p = 0.05$
Triglycerides	**8**, (48, 55, 56, 58, 60, 62, 63)	528	RE	0.48	−0.02–0.99	85%, $p < 0.001$	1.87, $p = 0.06$
Glucose metabolism							
Fasting blood glucose	**3**, (55, 56, 60)	87	RE	0.07	−0.23–0.37	0%, $p = 0.59$	0.45, $p = 0.65$
Total effect estimate	**14**	4,320	–	0.74	0.47–1.01	94%, $p < 0.001$	5.41, $p < 0.001$

SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; RE, random effects, MD, mean difference; SMD, standard mean difference.

similarity in the duration of use (48). However, the pooled estimate of our subgroup analysis suggests that OC use may result in little to no difference in BMI when compared with non-users [SMD = 0.22, 95% CI (−0.14, 0.57), $p = 0.23$, low certainty evidence] and a substantial level of heterogeneity ($I^2 = 82\%$, $p < 0.001$) (Table 2).

3.4.3. Lipid profile

3.4.3.1. Total cholesterol

The qualitative findings of our study, as reported in Table 1, showed that second- (levonorgestrel; LNG), third- (gestodene; GSD), and fourth-generation (drospirenone; DRSP) COCs significantly increased the total cholesterol (TC) level among users when compared with non-users (48, 55, 62, 63). However, some studies reported no significant difference among users of second- and third-generation COCs when compared with non-users despite similarity in the duration of use (46, 49). Furthermore, evidence from our subgroup analysis suggests a significant increase in the total cholesterol level among OC users when compared with non-users [SMD = 0.94, 95% CI (0.22, 1.66), $p = 0.01$, low certainty evidence] and a substantial level of heterogeneity ($I^2 = 92\%$, $p < 0.001$) (Table 2).

3.4.3.2. High-density lipoprotein

The qualitative findings of our study, as reported in Table 1, showed a significant increase in the high-density lipoprotein (HDL) level among users of second- (levonorgestrel; LNG) and third-generation (gestodene; GSD) COCs when compared with non-users (46, 56). However, these findings contrasted with the results of other studies that reported a significant decrease in the HDL level among users of second- (LNG) generation COC when compared with non-users and among third- (GSD) and fourth- (drospirenone; DRSP) generation COC users (49, 63). Nonetheless, several other studies reported non-significant changes in the HDL level among COC users despite similarity in the duration of use (55, 58, 60, 62). Furthermore, our subgroup analysis suggests that OC use may result in little to no difference in HDL levels when compared with non-users [SMD = −0.20, 95% CI (−0.64, 0.25), $p = 0.39$, low certainty evidence] and a substantial level of heterogeneity ($I^2 = 82\%$, $p < 0.001$) (Table 2).

3.4.3.3. Low-density lipoprotein

The qualitative findings of our study, as reported in Table 1, showed an increased level of low-density lipoprotein (LDL) among users of second- (LNG) generation COC when compared with non-users and among third- (GSD) and fourth- (DRSP) generation COC users (48, 55, 62, 63). This contrasted with the findings of other studies that reported no significant differences among users of second- (LNG) and third- (GSD, DSG) generation COCs when compared with non-users despite similarity in the duration of use (55, 56, 58, 60). Nevertheless, the pooled estimate of our subgroup analysis suggests a significant increase in LDL levels among OC users when compared with non-users [SMD = 0.79, 95% CI (−0.04, 1.59), $p = 0.05$, low certainty evidence] and a substantial level of heterogeneity ($I^2 = 92\%$, $p < 0.001$) (Table 2).

3.4.3.4. Triglyceride

The qualitative findings of our study, as reported in Table 1, showed an increased level of triglyceride (TG) among second-generation (LNG) users when compared with non-users (56, 62). While several studies reported no significant differences among users of second- (levonorgestrel; LNG) and third- (gestodene; GSD, desogestrel; DSG) generation COC users (48, 55, 58, 60), a study by El-Haggar and Mostafa showed a significant reduction in the levels of TG among users of second-generation COC (LNG) when compared with non-users and among third- (GSD) and fourth- (drospirenone; DRSP) generation COC users (63) despite similarity in the duration of use. In addition, the pooled estimate of our subgroup analysis suggests that OC use may result in little to no difference in triglyceride levels when compared with non-users [SMD = 0.48, 95% CI (−0.02, 0.99), $p = 0.06$, low certainty evidence] and a substantial level of heterogeneity ($I^2 = 85\%$, $p < 0.001$) (Table 2).

TABLE 3 Sensitivity analysis of outcomes based on geographical location.

Outcome	Geographical location	Number of studies	Omitted studies	SMD (95% CI)	I^2 (%), *p*-value	Overall effect: Z, *p*-value
Vascular markers of endothelial dysfunction	All	**6,** (1–6)	None	−0.11 (−0.81, 0.60)	94%, $p < 0.00001$	$Z = 0.30$, $p = 0.76$
	Europe	**1,** (1)	**5,** (3–7)	−0.12 (−0.92, 0.68)	N/A	$Z = 0.30$, $p = 0.76$
	Asia	**3,** (3, 5, 7)	**3,** (1, 4, 6)	−0.39 (−1.83, 1.06)	97%, $p < 0.00001$	$Z = 0.52$, $p = 0.60$
	South America	**2,** (4, 6)	**4,** (1–3, 5)	0.17 (−0.49, 0.82)	82%, $p = 0.0007$	$Z = 0.49$, $p = 0.62$
Traditional cardiovascular risk variables	All	**14,** (1–6, 8–15)	None	0.74 (0.47, 1.01)	94%, $p < 0.00001$	$Z = 5.34$, $p < 0.00001$
	Europe	**2,** (1, 10)	**12,** (2, 3, 14, 15, 4–6, 8, 9, 11–13)	0.03 (−0.21, 0.27)	0%, $p = 0.88$	$Z = 0.25$, $p = 0.88$
	North America	**2,** (9, 14)	**12,** (1, 2, 13, 15, 3–6, 8, 10–12)	1.86 (−0.31, 4.04)	98%, $p < 0.00001$	$Z = 1.68$, $p = 0.09$
	South America	**4,** (4, 6, 11, 13)	**10,** (1–3, 5, 8–10, 12, 14, 15)	0.24 (0.01, 0.47)	72%, $p < 0.00001$	$Z = 2.03$, $p = 0.04$
	Africa	**2,** (8, 12)	**12,** (1, 2, 14, 15, 3–6, 9–11, 13)	1.44 (0.51, 2.38)	97%, $p < 0.00001$	$Z = 3.02$, $p = 0.003$
	Asia	**3,** (1, 2, 12–15, 3–6, 8–11)	**11,** (1, 2, 12–15, 3–6, 8–11)	1.30 (0.69, 1.91)	96%, $p < 0.00001$	$Z = 4.19$, $p < 0.001$
	Australia	**1,** (15)	**13,** (1, 2, 12–14, 3–6, 8–11)	0.33 (0.05, 0.61)	7%, $p = 0.38$	$Z = 2.34$, $p = 0.02$

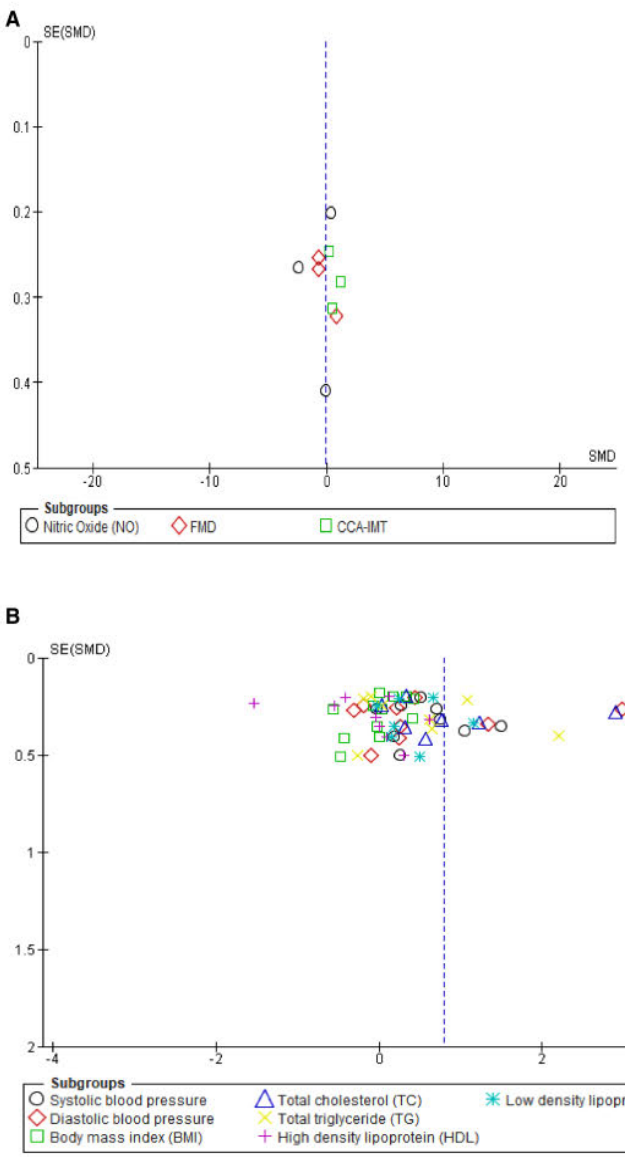

FIGURE 4
Funnel plot of vascular markers and cardiovascular risk factors showing a perfect symmetry. Hence, there was no publication bias in these studies. (A) Vascular markers, (B) Traditional cardiovascular risk variables.

3.5. Glucose metabolism

3.5.1. Fasting blood glucose

The qualitative findings of our study, as reported in Table 1, showed no significant change in fasting blood glucose (FBG) among users of second- (levonorgestrel; LNG) generation COC when compared with non-users (55, 56, 60). Moreover, the pooled estimate of our subgroup analysis also suggests that OC use may result in little to no difference in FBG levels [SMD = 0.07, 95% CI (−0.23, 0.37), $p = 0.45$, low certainty evidence] when compared with non-users ($I^2 = 0\%$, $p = 0.59$) and a low level of heterogeneity (Table 2).

TABLE 4 Summary of findings: use of oral contraceptives in premenopausal women compared with non-users.

Oral contraceptive treatment compared with non-users (controls)						
Patient or population: [premenopausal women]						
Intervention: [oral contraceptive]						
Comparison: [non-user]						
Outcomes	Anticipated absolute effects* (95% CI)		Relative effect (95% CI)	No. of participants (studies)	Certainty of the evidence (GRADE)	Comments
	Risk with [comparison]	Risk with [intervention]	–	197 (3 observational studies)	⨁⨁◯◯ Low[a,b]	
Cellular marker of endothelial activation—NO	–	SMD **0.73 lower** (2.6 lower to 1.14 higher)				
Vascular marker of endothelial activation—FMD	–	SMD **0.22 lower** (1.12 lower to 0.68 higher)	–	172 (2 observational studies, 1 RCT)	⨁⨁◯◯ Low[a,b]	
Vascular marker of endothelial activation—CCA-IMT	–	SMD **0.62 higher** (0.02 higher to 1.21 higher)	–	172 (2 observational studies, 1 RCT)	⨁⨁◯◯ Low[a,b]	
Traditional cardiovascular risk variables	–	SMD **0.74 higher** (0.47 higher to 1.01 higher)	–	4,320 (12 observational studies, 2 RCTs)	⨁⨁◯◯ Low[a,b]	

CI, confidence interval; SMD, standardized mean difference.
GRADE Working Group grades of evidence High certainty: We are very confident that the true effect lies close to that of the estimate of the effect.
Moderate certainty: We are moderately confident in the effect estimate: the true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.
Low certainty: Our confidence in the effect estimate is limited: the true effect may be substantially different from the estimate of the effect.
Very low certainty: We have very little confidence in the effect estimate: the true effect is likely to be substantially different from the estimate of the effect.
*The risk in the intervention group (and its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI).

3.6. Sensitivity analyses and publication bias

We assessed the robustness of our results and further explored the sources of heterogeneity in the reported outcomes by performing sensitivity and subgroup analyses. The meta-analysis was repeated by a stepwise omission of studies based on the geographical location of each reported outcome. The sensitivity analysis of the traditional cardiovascular risk variables showed that studies conducted in Europe [SMD = 0.03, 95% CI (−0.21, 0.27), (I^2 = 0%, p = 0.88)] and Australia [SMD = 0.33, 95% CI (0.05, 0.61), (I^2 = 7%, p = 0.38)] had low levels of heterogeneity when compared with other studies conducted in Africa, Asia, and North and South America; however, the effect size was quite small in South America when compared with that in Africa, Asia, and North America (**Supplementary additional file S1** and **Table 3**). This suggested geographical location to be a potential source of statistical heterogeneity in the included studies. However, an assessment of the funnel plot suggests evidence of publication bias (**Supplementary additional file S1** and **Figure 4B**).

4. Discussion

The aim of this systematic review was to provide a comprehensive synthesis of the available evidence on the link between OC use and CVD risk in premenopausal women. Cumulative evidence summarized in this review highlights the impact of OC use on endothelial function and some traditional cardiovascular risk variables. The results of our study show that the use of progestin-only type of OC is associated with increased levels of plasma endothelin 1 (ET-1) in healthy young women (45). In contrast, the use of second-generation (levonorgestrel; LNG) and third-generation (gestodene; GSD) COCs does not significantly impact the plasma levels of ET-1 and NO (46). It is noteworthy that the imbalance in quotient between NO and ET-1 can impact the vascular tone. Meanwhile, a study by John et al. showed that the use of second-generation (LNG) OC significantly impacted the production and release of NO at the basal level and the levels of NO remained unchanged despite stimulating its release with acetylcholine and sodium nitroprusside (58). However, change in several hemodynamic, mechanical, and chemical factors, including blood pressure, vascular resistance, angiotensin II, as well as transforming growth factor-β, among others, can influence the activation and functions of endothelial cells leading to multiple inflammatory responses involving the innate and adaptive immune cells across the body system.

Furthermore, our study findings showed that fourth-generation (drospireneone; DRSP) OC significantly increased FMD (43). In contrast, the findings of other studies (49, 50, 61) involving the use of second-generation levonorgestrel (LNG) and another type of fourth-generation CMA OC showed lowered FMD. However, the reported pooled estimate of our meta-analysis showed no significant change in FMD in participants who used the second-(LNG) and third-generation (GSD, DSG) OCs (49, 50, 61).

More so, our study findings showed a significantly increased mean Common Carotid Artery Intima–Media thickness (CCA-IMT) in those who used second-generation (LNG) OC when compared with non-users and among fourth-generation (CMA) OC users (50, 61). However, the pooled estimate of our meta-analysis showed a significant decrease in CCA-IMT of participants on OCs when compared with non-users (49, 50, 61). In clinical settings, both FMD and IMT are strong predictors of

endothelial dysfunction where FMD reflects early and predominant functional changes in the vascular wall, and IMT serves as a marker of more advanced structural changes (68). Nonetheless, understanding these changes may provide an insight into the power and effectiveness of the deep nerve stimulation to regulate systemic blood pressure (69).

Of note, endogenous estrogen is known to guard against vascular damage and atherosclerosis via the estrogen receptor (Eps), especially ERα and Erβ (70). However, the demonstrated changes in endothelial activation markers can be attributed to the type of progestin where a COC containing LNG was shown to result in 3–7.5-fold greater reduction in mean FMD among users when compared with non-users (61) and among users of fourth-generation (CMA) OC, which is derived from 17-hydroxyprogesterone, with high affinity for the progesterone receptor (PR) and moderate antiandrogenic activity (61). Furthermore, high androgenic properties associated with second-generation LNG progestin can antagonize the vasodilatory effects of estrogens and impact endothelial function (71, 72).

Furthermore, evidence emerging from our summary of findings showed that the OC use significantly increased systolic and diastolic blood pressure levels (60, 73, 74). Chronic use of COCs can induce increases in arterial pressure, primarily by activating the renin–angiotensin system (61) and via oxidative stress (75). However, some studies reported contradictory findings where the use of OCs did not significantly impact the blood pressure of the participants irrespective of the estrogen component (59, 67). Of note, endogenous female sex hormones are known to play a role in maintaining body fluid homeostasis (76) during the menstrual cycle. However, emerging evidence suggests that exogenous sex hormones may alter body fluid homeostasis in women of reproductive age (77, 78), which may depend on progestin type (76). While the progestin component may increase plasma volume through the combined mechanisms of increased osmolarity in the vascular space as well as overall expansion of ECF, the estrogen component may increase the plasma volume by reducing the operating point for osmoregulation of arginine vasopressin (AVP) and thirst, leading to a greater fluid retention in the vascular space (76).

AVP is a key hormone synthesized in the paraventricular and supraoptic nuclei of the hypothalamus (79, 80) they are released together with copeptin from the axonal terminals of the magnocellular neurons located in the posterior lobe of the pituitary gland (79). They are involved in the regulation of other body functions besides the control of the body's osmotic balance, respiratory and blood pressure regulation, sodium homeostasis, kidney functioning (80), fear conditioning, and love making (81–83). It is noteworthy that the synthetic progestins, apart from acting at the PR, can also influence the activity of other steroid receptors to induce androgenic, glucocorticoid, antiandrogenic, and antimineralocorticoid effects (84, 85).

Furthermore, findings from our data synthesis showed that the use of OCs is associated with dyslipidemia. Due to imbalance in the lipid profile, dyslipidemia may result in cardiovascular complications (86). The results showed that second- (LNG), third- (GSD), and fourth- generation (DRSP) COCs significantly increased the TC levels of OC users when compared with non-OC users (48, 55, 62, 63). In contrast, the findings from other studies showed that second- and third-generation COCs do not impact the TC level (46, 49). Furthermore, our study results showed that second- (LNG) generation COC increased the levels of LDL in users when compared with non-users, as also third- (GSD) and fourth- (DRSP) generation COCs (48, 55, 62, 63). This contrasted with the findings of other studies that showed that second- (LNG) and third- (GSD, DSG) generation COCs do not impact the LDL levels (55, 56, 58, 60). However, the pooled estimate of our meta-analysis showed that OC significantly increased the levels of TC and LDL in OC users when compared with non-users (62–67).

In addition, the results showed that second- (LNG) and third-generation (GSD) increased the HDL levels (46, 56). However, these findings contrasted with the results of other studies where second- (LNG) generation COC decreased the HDL levels when compared with third- (GSD) and fourth- (DRSP) generation COCs (49, 63). Nonetheless, the findings of several other studies showed that COCs do not impact the HDL levels (55, 58, 60, 62). More so, our study results showed that second- (LNG) generation COC increased the levels of TG (56, 62). On the other hand, second-generation COC (LNG) reduced the levels of TG when compared with the third- (GSD) and fourth- (DRSP) generation COCs (63). However, several other studies showed that COCs do not impact the TG levels (48, 55, 58, 60). Furthermore, the pooled estimate of our subgroup analysis showed an insignificant increase in the levels of TG and HDL among OC users.

Moreover, the results showed that COCs do not impact BMI (46, 47, 49, 50, 55–58, 60, 61–63, 67), although a study by Asare et al. showed that second- (LNG) generation COC increased BMI (48). However, the pooled estimate of our subgroup analysis showed that OCs do not impact BMI as well as FBG levels. Of note, emerging evidence showed the existence of regional disparities in cardiovascular disease incidence and mortality (87, 88). Moreover, three-quarter of the world's CVD deaths occur in low- and middle-income countries (89). Despite limited data on known risk factors to explain these regional variations in CVD among women of reproductive age, the results of our meta-analysis showed a high prevalence of traditional cardiovascular risk variables among OC users from North America when compared with Europe and other regions, which had the lowest prevalence.

There are several limitations in the evidence presented in this systematic review. These include substantial levels of statistical heterogeneity among included studies and unavailability of data on some prespecified effect measures. Therefore, caution should be exercised in interpreting and extrapolating these findings in different populations of various geographical locations.

5. Conclusion

The evidence presented in this review highlights the impact of second-generation (LNG) OC use on FMD, CCA-IMT, and NO

levels in premenopausal women. In conclusion, evidence from our findings suggests that second-generation OC may result in little to no difference in endothelial activation. Although, among the variables assessed, our evidence suggests that the use of LNG may result in a significant reduction in CCA-IMT among users. Furthermore, our evidence suggests that the use of LNG may significantly increase other traditional cardiovascular risk variables. However, more independently conducted studies are needed to determine the long-term impact of individually available COCs on CVD risk.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

OAF and BBN conceptualized and designed the study, and OAF drafted the protocol. PVD helped draft the protocol. All authors wrote and approved the final manuscript. BBN is the guarantor of the review. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Prologue

Obesity-induced inflammatory response is linked to several major adverse cardiovascular events, such as atherosclerosis, myocardial infarction, coronary heart disease. Oral contraceptives containing either estrogen or progestogen (COC) are associated with weight gain, and alteration of the metabolic and immunological pathways. In this chapter (Experimental 1), we focused on the impact of COC on immune activation in diet-induced obesity (DIO), we also assessed whether the dietary intervention of switching from a high-fat diet (HFD) to a low-fat diet (LFD) attenuates undesired immunological responses.

Chapter 3: Experimental article 1

Abstract

Journal of Reproductive Immunology 163 (2024) 104234

Contents lists available at ScienceDirect

Journal of Reproductive Immunology

journal homepage: www.elsevier.com/locate/jri

High-dose oral contraceptives induce hyperinsulinemia without altering immune activation in diet-induced obesity which persists even following a dietary low-fat diet intervention

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ARTICLE INFO

Keywords:
Combined oral contraceptive
Diet switching
High-fat diet
Low-fat diet
Immune activation
Sprague Dawley rats

ABSTRACT

Combined oral contraceptives (COCs) are known to cause weight gain and alter metabolic and immunological pathways. However, modifications in arterial or venous thrombotic risk profiles of women of reproductive ages on COC remain unclear. The study aimed at assessing the impact of COC on immune activation in diet-induced obesity. We further established whether the dietary intervention of switching from a high-fat diet (HFD) to a low-fat diet (LFD) attenuates immunological responses. Twenty (n=20) five-week-old female Sprague Dawley rats were randomly divided into two diet groups of HFD (n=15) and LFD (n=5) and were monitored for eight weeks. After eight weeks, animals in the HFD group switched diets to LFD and were randomly assigned to receive high-dose COC (HCOC) or low-dose COC (LCOC) for six weeks. Animals on HFD significantly gained weight and had a higher lee index when compared to the LFD group ($p < 0.05$). Moreover, the triglyceride-glucose index, insulin, and other metabolic parameters also increased in the HFD group compared to the LFD group ($p < 0.001$). Consistently, the levels of interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α), were elevated in the HFD group when compared to the LFD group ($p < 0.05$). Upon switching from a high-fat to a low-fat diet, insulin levels persistently increased in animals receiving HCOC treatment compared to the LFD and HFD/LFD groups ($p < 0.05$). Thus, in a rat model of HFD-feeding, short-term HCOC treatment induces long-term metabolic dysregulation, which persists despite dietary intervention. However, further studies are recommended to confirm these findings.

1. Introduction

Obesity has become a global pandemic worldwide over the years (The Lancet Gastroenterology and Hepatology, 2021) with more than 1.9 billion adults being overweight and 650 million were obese (WHO, 2018). Of note, obesity is associated with metabolic syndrome (Marques et al., 2016a) prothrombotic state (Grundy et al., 2004) and increased risk of major adverse cardiovascular events (Khan et al., 2018). In obesity, low-grade inflammation may occur as result of a compromised gut diversity and increased monocyte activation in the adipose tissue through several molecular mechanisms (Jialal et al., 2012). This process induces the polarization of the resident macrophage (M1) cells towards a pro-inflammatory phenotype (Esser et al., 2013). The polarization of macrophages towards M1, is followed by the infiltration of neutrophils influx into the adipose tissue. Neutrophil infiltration is a characteristic features of obesity-induced inflammation that contributes to the development of metabolic syndrome (Artemniak-Wojtowicz et al., 2020). The latter describes a cluster of metabolic complications that increases the risk of developing type 2 diabetes and atherosclerotic cardiovascular disease (CVDs) (Grundy, 2016). Hence, there is need to understand the

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https://doi.org/10.1016/j.jri.2024.104234
Received 1 July 2023; Received in revised form 24 February 2024; Accepted 7 March 2024
Available online 8 March 2024
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Candidate's contribution: OAF (the candidate) was involved in the conceptualization of the study, animal handling, running laboratory assays, data analysis and writing of the manuscript.

1. Introduction

Obesity has become a global pandemic worldwide over the years [1] with more than 1.9 billion adults being overweight and 650 million were obese [2]. Of note, obesity is associated with metabolic syndrome [3] prothrombotic state [4] and increased risk of major adverse cardiovascular events [5]. In obesity, low-grade inflammation may occur as result of a compromised gut diversity and increased monocyte activation in the adipose tissue through several molecular mechanisms [6]. This process induces the polarization of the resident macrophage (M1) cells towards a pro-inflammatory phenotype [7]. The polarization of macrophages towards M1, is followed by the infiltration of neutrophils influx into the adipose tissue. Neutrophil infiltration is a characteristic features of obesity-induced inflammation that contributes to the development of metabolic syndrome [8]. The latter describes a cluster of metabolic complications that increases the risk of developing type 2 diabetes and atherosclerotic cardiovascular disease (CVDs) [9]. Hence, there is need to understand the pathophysiological mechanisms involved in the development of obesity-associated complications, including chronic inflammation and impaired immune response. This is essential to comprehend and establish the effectiveness of currently used interventions.

The use of combined oral contraceptives (COCs) has been associated with an increased risk of arterial and venous thrombosis in women of reproductive age [10]. While the risk of these cardiovascular events are rare in young women, the magnitude of the risk and the effect of different hormonal contents of COC preparations remain unclear [11]. However, evidence has shown that the use of COC may potentially exacerbate the risk of major adverse cardiovascular events in conditions of obesity [12,13]. This may be linked with impaired metabolic and exacerbated inflammatory responses which may occur subsequent to the development of major adverse cardiovascular events (MACEs) [14]. Despite the risk for CVDs associated with the use of COC in conditions of impaired metabolism [15], there is

still controversy regarding the efficacy of contraceptives in obese individuals in terms of the duration of use, dosage, and generational-type of COC [16].

Of note, estrogen and progesterone are known to play a role in modulating the development and function of both innate and adaptive immune system [17–19]. The modulatory role of these hormones during immune activation may be influenced by the dose and duration of administration [18,20–22] and their action may also counteract each other [19,23]. A previous study on infected ovariectomized female C57BL/6 mice reported on an increased neutrophil count and production of neutrophil chemoattractants proteins following recruitment following estrogen treatment [17]. Estrogen and progesterone also modulate macrophage functions [19,24] as well as T cell functions [22,25,26]. For instance, in clinical studies, exogenous estrogen enhanced the production of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-alpha (TNF-α) [27], while in a murine model, high concentrations of estrogen suppresses the production of these cytokines by monocyte or macrophage cells [28]. More so, in lipopolysaccharide (LPS)-stimulated dendritic cells in female rats, progestin treatment suppressed the production of TNF-α and IL-1 β in a dose dependent manner [29]. In contrast, a study by Bouman and colleagues showed that neither exogenous estrogen nor progesterone enhances the production of cytokine in human LPS-stimulated monocytes [30]. Taken together, the magnitude by which exogenous estrogen and progesterone modulates the function of both innate and adaptive immune cell remain elusive.

The aim of this study is to determine the impact of COC treatment within rat model of diet-induced obesity (DIO). We further assessed whether switching from a high-fat diet (HFD) to low-fat diet (LFD) restores the levels of immune activation as measured using acute phase reactants such as IL-6 and TNF-α and monocyte chemoattractant protein (MCP)-1.

2. Materials and methods

2.1. Animal handling

Twenty (n=20) female Sprague Dawley rats that are five weeks old and weighing between 150-200g were purchased and housed at the Biomedical Research Unit (BRU) at the of University KwaZulu-Natal. The animals were acclimatized for 2 weeks with an unrestricted access to food and water. They were maintained under standard environmental conditions of temperature (22±2°C), humidity (55±5%) and controlled 12-h light cycle (6:00–18:00) and dark cycle (18:00–6:00). While the animals were regularly monitored, and the cages were cleaned daily. Ethical clearance for this study was granted by the UKZN animal research ethics committee (AREC/00003067/2021).

2.2. Experimental design and treatment of animals

The study was conducted in two phases, which are illustrated in Figure 1. The first experiment phase used a model of DIO, where basic metabolic parameters, lipid profiles, and acute phase reactant proteins were assessed after animals were fed HFD (n=15) for 8 weeks. Control animals were kept on LFD (n=5), for both experimental phase (Figure 1, Experiment A). The LFD composition was 10 Kcal% derived from fat (19 g% protein, 67 g% carbohydrates, 4 g% fat, Research Diets #D12450), while the HFD group was 60 Kcal% derived from fat (26 g% protein, 26 g% carbohydrates, 35 g% fat, Research Diets #D12492) [31]. A Lee index greater than 310 was used to define obesity in the animals, as previously described [32].

$$\text{Lee index} = \frac{\sqrt[3]{body\ weight\ (g)}}{Nose - to - Anus\ Length\ (cm)}\ x\ 1000$$

In the second experimental phase, the HFD diet was switched to a LFD as previously described [33] in order to assess the implication of COC treatment on the metabolic changes and makers of immune activation following a dietary intervention. COC treatment was prepared accordingly as previously described [34]. The animals that were initially placed on a HFD (n=15) were further randomized to receive a LFD + low dose COC (LCOC; n=5) or LFD + high dose COC (HCOC; n=5), or LFD + placebo (distilled water; n=5) (Figure 1, Experiment B) [34]. The LCOC treated group received (via oral gavage) a combination of 4.5 µg of levonorgestrel / 0.9µg of ethinylestradiol while the HCOC treated group was administered a combination of 9µg levonorgestrel /1.8µg ethinylestradiol (Aspen Pharmacare, South Africa) which was adjusted for animal body weight. The treatment period with COC was for 6 weeks as previously decsribed [35], and the entire experimental phase was 14 weeks (Figure 1). Both at week 8 and week 14 experimental phase blood samples were drawn from the lateral tail vein into EDTA microtainer and vacutainer citrate tubes (BD Bioscience, USA) for analysis.

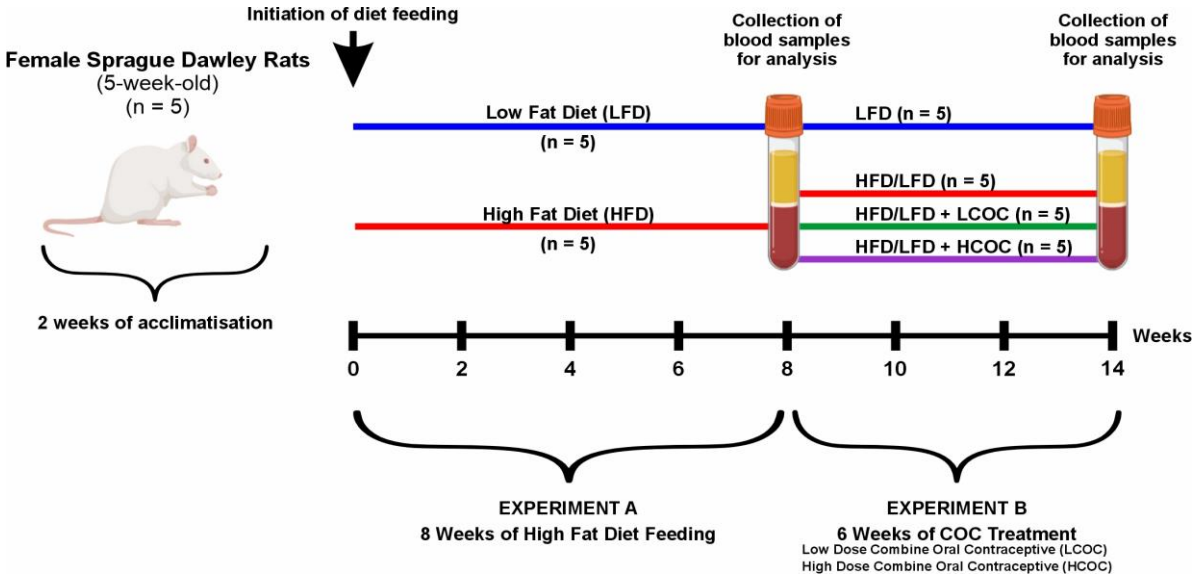

Figure 1: Experimental design. 20 five-week-old female Sprague Dawley rats were used in this study. The 5-week-old rats were acclimated for 2 weeks, and therefore, they were 7-week-old rats at the start of the treatment. In experiment A rats were randomly allocated into two diet groups low-fat diet (LFD) and high-fat diet (HFD), (n=5) for a period of eight weeks. In experiment B, this was after the initial 8-week period, in animals receiving HFD diet was switched to LFD. These animals were randomised to receive high dose combined oral contraceptive (HCOC) and low dose combine oral contraceptive (LCOC) for six weeks, to give total experimental period to be 14 weeks. The animal weights and other anthropometrics were monitored weekly, while metabolic changes were determined at both week eight and week fourteen.

2.3. Anthropometric assessment

The body weight of the animals was measured on a weekly basis. To determine the Naso–Anal length (NAL) and the abdominal circumference, each rat was placed in the ventral position. Naso–Anal length (NAL) of rats was measured by a non-extensible thread and readings taken using a ruler with an accuracy of 0.1 cm as described [36]. The abdominal circumference (AC) was assessed on the largest zone of the rat abdomen in front of the hind legs using a non-extensible thread. The readings of the abdominal circumferences were taken as previously described [36].

2.4. Oral glucose tolerance test (OGTT) and insulin resistance (IR)

At week 8 of the high-fat diet feeding and week 6 of COC treatment, the rats were fasted for 12-h overnight and given a glucose challenge to test for glucose tolerance. All glucose measurements were determined using the OneTouch® Select® handheld glucometer (LifeScan Inc., Milpitas, CA, USA) as previously described [37]. The IR was determined using the homeostasis model assessment for insulin resistance (HOMA-IR) and triglyceride-glucose index (TyG) [38].

2.5. Biochemical analysis

Plasma insulin levels, IL-6, TNF-α and MCP-1 were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Elabscience Biotechnology Co., Ltd., Houston, TX, USA). High density lipoprotein was determined using high density lipoprotein quantitation kit (Sigma–Aldrich St. Louis, MO, USA) and triglyceride (TG) levels was determined using triglyceride assay kit (Elabscience Biotechnology Co., Ltd., Houston, TX, USA). The plasma levels of very low-density lipoprotein (VLDL) were determined using the Friedewald formula [39]. All assays were performed according to the recommended manufacturer's protocol.

2.6. Statistical analysis

Normality testing was performed using the Kolmogorov-Smirnov test with Dallal-Wilkinson-Lillie. The mean differences between the LFD and HFD-fed groups were determined using unpaired student t-test for parametric data and reported as means ± standard error of mean (SEM). While the Man Whitney U test was used for non-parametric data and reported as median and reported as median and interquartile range (IQR). Correlations were performed between Lee index and acute phase reactants, as well as TyG and acute phase reactants using Pearson coefficients. In the experimental groups, one-way (treatment factor) analysis of

variance (ANOVA) was used for the comparison of the mean values of measured variables. A post hoc Tukey's multiple comparisons test was performed if the F-value reached statistical significance ($p < 0.05$). All the statistical analysis were performed using the GraphPad Prism version 8.0 software (GraphPad Software Inc, CA, USA).

3. Results

3.1. Baseline anthropometric characteristics of rats following 8 weeks of HFD feeding.

To induce obesity the animals were kept on a HFD for a duration of eight weeks. The HFD-fed group showed a significant increase in weight (g) (289 ± 4.1) when compared with the LFD group (270 ± 5.2) ($p < 0.021$) (Figure 2A). The HFD group was classified as obese based on the Lee index (337 ± 5.9) when compared to the LFD group with a lower Lee index (307.7 ± 6.3) ($p < 0.001$). HFD group also showed an increased abdominal circumference (cm) (15.6 ± 0.2) when compared to the LFD group respectively (13.1 ± 6.3) ($p < 0.001$) (Table 1).

Table 1. Characteristics of animals after 8 weeks of low- fat diet (LFD) vs high-fat diet (HFD) feeding (n=5/group)

Parameters	**LFD group**	**HFD group**	**p-value**
Anthropometric measurements			
Initial body weight (g)	189.2 ± 21.1	188.4 ± 15.6	0.947
Body weight at Week 8 (g)	270 ± 5.2	289 ± 4.1	**0.021**
weight gain (%)	43.6 ± 9.5	53.8 ± 11.3	0.180
Lee index	307.7 ± 6.3	337 ± 5.9	**<0.001**
Abdominal circumference (cm)	13.1 ± 6.3	15.6 ± 0.2	**<0.001**
Metabolic profiles			
High density lipoprotein (µg/µL)	1.77 (1.47 - 2.07)	2.04 (1.42 - 2.65)	0.691
Triglyceride (mg/dL)	78.7 ± 6.3	108.6 ± 8.7	**0.024**
Very low-density lipoprotein (mg/dL)	15.7 ± 1.3	21.7 ± 1.7	**0.024**
Fasting glycemia (mmol/L)	3.8 ± 0.6	5 ± 0.5	**0.006**
Acute phase reactant proteins			
IL-6 (pg/mL)	52.8 ± 16.4	107.8 ± 10.4	**0.022**
TNF- α (pg/mL)	49.8 ± 6.9	101.1 ± 14.5	**0.013**
MCP-1 (ng/mL)	1.08 ± 0.03	1.21 ± 0.07	0.115

Results expressed as mean ± SEM and median interquartile range. Significance ($p < 0.05$) shown in boldface. ***IL-6****: interleukin 6;* ***TNF-α****: tumor necrosis factor-alpha;* ***MCP-1****: monocyte chemoattractant protein-1.*

3.2. Metabolic changes and lipid profiles of HFD-fed rats compared to LFD group after 8 weeks.

The HFD group had significantly elevated levels of fasting blood glucose (mmol/L) following eight-weeks of HFD-feeding (5 ± 0.5) when compared with LFD group (3.8 ± 0.6) (p = 0.006). Furthermore, HDL was comparable between the groups (p = 0.691), however, TG levels (mg/dL) was significantly higher in HFD group (108.6 ± 8.7) when compared to LFD group (78.7 ± 6.3) (p = 0.024) (Table 1). The VLDL levels (mg/dL) was also

significantly higher in HFD group (21.7 ± 1.7) when compared to the LFD group (15.7 ± 1.3) (p = 0.024) (Table 1). The HFD-fed group showed a significant elevated 2-h post load glycemia (OGTT) (mmol/L) including an increased postprandial area under the curve (AUC) (mmol/L x 120mins) (941 ± 100) when compared with LFD-fed group (714 ± 59), (p = 0.002) (Figure 2B and C). In addition, HFD-fed group showed a significant increase in fasting plasma insulin (µU/L) (27.1 ± 3) when compared with LFD group (17.7 ± 3.3), (p < 0.001), HOMA-IR was also significantly elevated in HFD group (6.1 ± 1) in comparison with LFD group (2.9 ± 0.5) (p < 0.001), while TyG was significantly higher in HFD group (4.6 ± 0.1) when compared with LFD-fed group (4.2 ± 0.07), (p < 0.001) (Figure 1C-E).

3.3. Inflammatory status of HFD-fed rats compared to LFD group after 8 weeks.

In terms of inflammatory response, the plasma levels of the IL-6 (pg/mL) were significantly higher in HFD-fed group (107.8 ± 10.4) when compared with LFD group (52.8 ± 16.4), (p = 0.022). HFD group also showed an elevated TNF-α (pg/mL) level (101.1 ± 14.5) when compared with LFD group (49.8 ± 6.9), (p = 0.013). Lastly, plasma level of MCP-1 was comparable between the groups (p > 0.05) after the eight weeks of HFD-feeding (Table 1).

3.4. Correlation between the inflammatory acute phase reactants, lee index and TyG index of HFD group after 8 weeks of high fat diet.

We assessed the interdependent association between inflammatory acute phase reactants, lee index and TyG index in obese condition. Briefly, there was a significant association between the Lee index and IL-6 (r = 0.95, p < 0.014) (Figure 3A), TNF- α (r = 0.96, p = 0.001) (Figure 3B), and MCP-1 (r = 0.92, p < 0.025) (Figure 3C) in the HFD group. There was also a significant association between TyG index and IL-6 (r = 0.90, p < 0.018) (Figure 3D), TNF-α levels (r = 0.93, p < 0.022) (Figure 3E). However, there were no significant associations between the TyG index and MCP-1 levels (Pearson r = 0.53, p = 0.344) (Figure 3F).

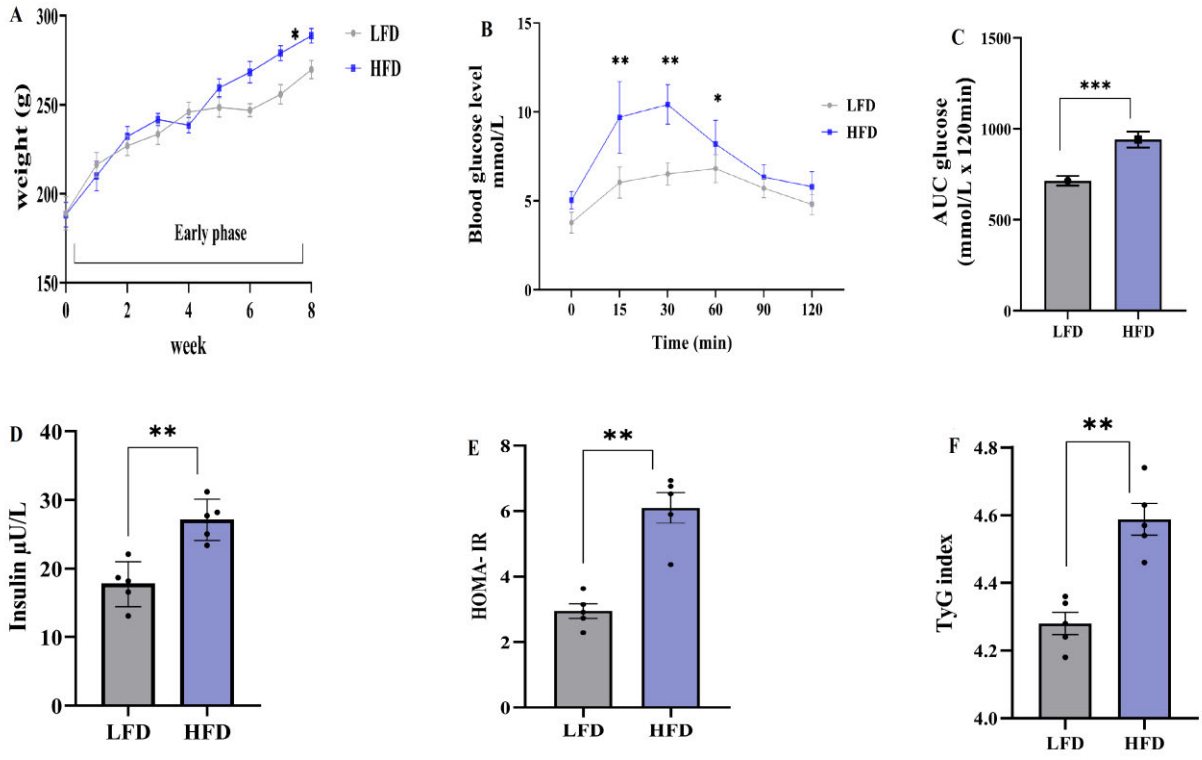

Figure 2. *Effect of high fat diet feeding* ***(A)*** *weight gain;* ***(B)*** *2-hour postprandial glucose test* ***(C)*** *area under the curve (AUC);* ***(D)*** *fasting insulin;* ***(E)*** *HOMA-IR; and* ***(F)*** *triglyceride-glucose index (TyG). All results are presented as mean ± SEM. *p < 0.05, **p < 0.001, ***p < 0.0001.*

Key: LFD: *low-fat diet;* ***HFD****: high-fat diet.*

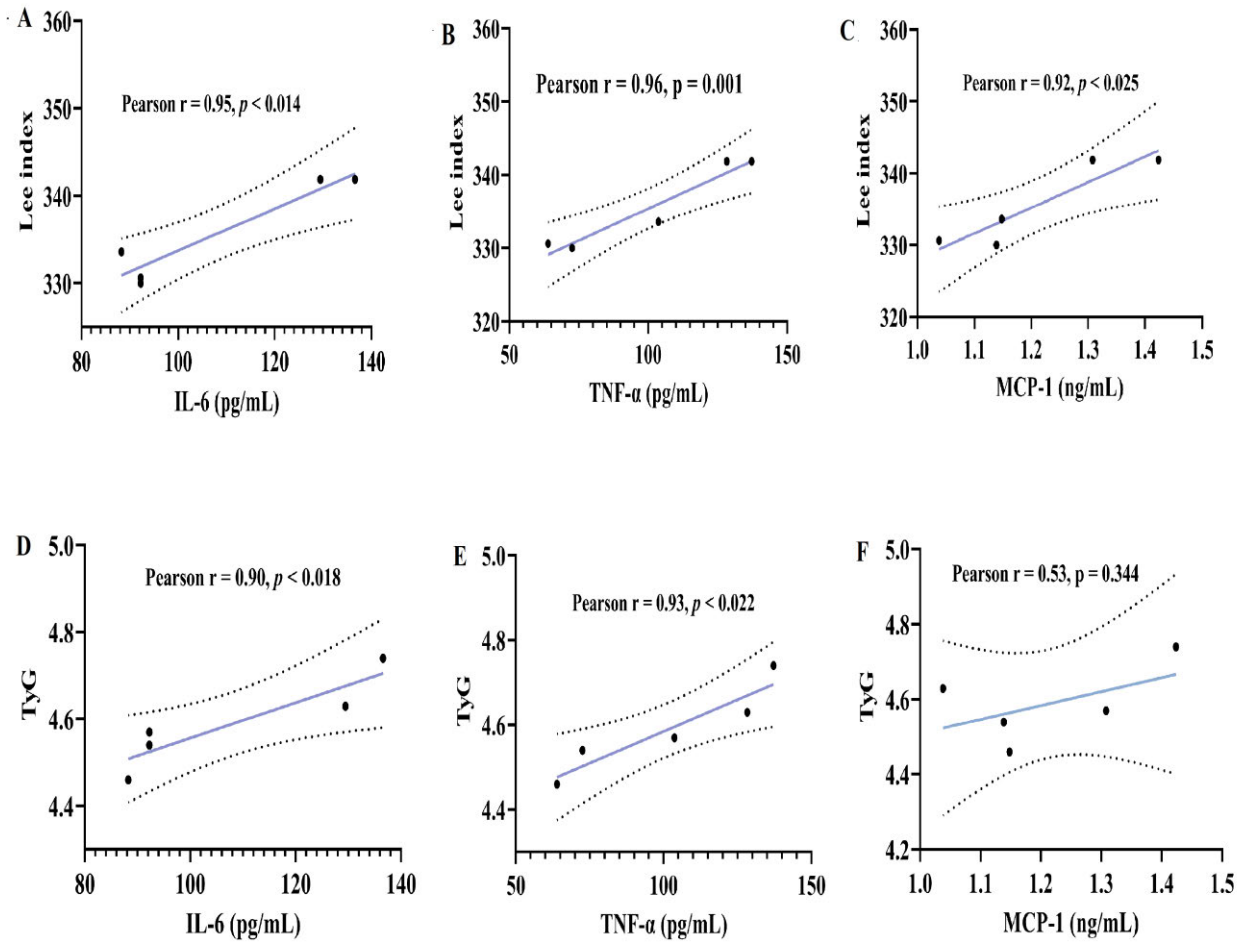

Figure 3: *Correlation between the inflammatory acute phase reactants, lee index* ***(A-C)*** *and TyG* ***(D-F)*** *of HFD group after 8 weeks of high fat diet. Correlations are presented as Pearson r* 95% confidence interval (p<0.05).

Key*:* ***IL-6*** *(Interleukin- 6)* ***TNF-alpha*** *(Tumour necrosis factor-alpha)* ***MCP-1*** *(monocyte chemoattractant protein-1.*

3.5. The metabolic status of HFD-fed rats following the switch to LFD and short-term treatment with different dosage of COC.

The weight and the abdominal circumference were comparable across the experimental groups after six weeks of COC treatment ($p > 0.05$) (Table 2). The level of fasting blood glucose following six weeks of COC treatment were comparable across the experimental groups ($p > 0.05$) (Table 2). Meanwhile, 2-h post load glycemia (OGTT) ($F_{(3, 16)} = 3.881$; $p = 0.03$) (Figure 4A) including the postprandial area under the curve (AUC) (Table 2) ($F_{(3, 16)} = 2.784$; $p = 0.020$) varied across the group following six weeks of COC treatment. In the post-hoc analysis, HFD/LFD + HCOC group showed a significant elevated 2-h post load glycemia (OGTT) (Figure 4A) including increased postprandial area under the curve (AUC) when compared with LFD-fed group ($p < 0.05$) (Table 2).

In addition, there was significant changes in fasting plasma insulin ($F_{(3, 16)} = 5.577$; $p = 0.008$) across the experimental groups following short-term COC treatment (figure 4B). The post-hoc analysis showed an elevated fasting plasma insulin in the HFD/LFD + HCOC group when compared with both HFD and LFD groups, respectively ($p < 0.05$) (figure 4B). In terms of insulin resistance, HOMA-IR ($F_{(3, 16)} = 4.009$; $p = 0.03$) and TyG levels ($F_{(3, 16)} = 3.284$ $p = 0.02$) varied across the experimental groups. The post-hoc analysis showed an elevated levels of both HOMA-IR and TyG in HFD/LFD + HCOC group when compared with LFD-fed group ($p < 0.05$) (Figure 4C-D).

3.6. Lipid profiles of HFD-fed rats following the switch to LFD and short-term treatment with different dosage of COC.

We also determined the alterations in the lipid profiles which are linked to an increased CVD risk. Briefly, the HDL ($F_{(3, 16)} = 3.9$; $p = 0.03$), TG ($F_{(3, 16)} = 5.1$; $p = 0.006$) and VLDL ($F_{(3, 16)} = 5.8$; $p = 0.007$) levels all varied across the experimental groups (Table 2). The post-

hoc analysis showed a significant lower level of HDL in the HFD/LFD + HCOC group when compared with LFD group ($p < 0.05$) (Table 2). The post-hoc analysis showed a significant higher level of TG levels in HFD/LFD + HCOC group when compared with LFD group ($p = 0.004$) as well as increased VLDL levels in HFD/LFD + HCOC group when compared with LFD group ($p = 0.005$) (Table 2).

3.7. Inflammatory status of HFD-fed rats following the switch to LFD and short-term treatment with different dosage of COC.

The release of acute phase reactant proteins is an indicator of inflammatory responses. Briefly, there were significant changes in the plasma levels of both IL-6 ($F_{(3, 16)} = 4.189$; $p = 0.02$) and TNF- α ($F_{(3, 16)} = 5.848$; P=0.007) across the experimental groups following six weeks COC treatment. In the post-hoc analysis, HFD/LFD + HCOC showed a significant increase in IL-6 ($p < 0.05$) and TNF- α levels ($p < 0.001$) when compared with LFD group (Figure 5A-B). Meanwhile, plasma level of MCP-1 was comparable in all the experimental groups ($p > 0.05$).

Table 2. Characteristics of animals following 6-week short-term treatment with different dosage of combined oral contraceptive (COC) (n=5/group)

Parameters	**LFD**	**Untreated HFD**	**HFD + high dose COC**	**HFD + low dose COC**	**p-value**
Anthropometric measurements					
Body weight	287.2 ± 9.9	299.8 ± 17	281.8 ± 26.2	295.2 ± 11.3	0.387
Abdominal circumference	14.7 ± 0.2	15.8 ± 0.2	15.2 ± 0.39	15.1 ± 0.37	0.120
Metabolic profile					
High density lipoprotein (mg/dL)	3.25 ± 1.3	2.19 ± 0.6	1.65 ± 0.35 **a**	2.03 ± 0.46	**0.030**
Triglyceride (mg/dL)	76.1 ± 14.5	88.3 ± 8.9	112.4 ± 16.6 **a**	96.50 ± 14.4	**0.006**
Very low-density lipoprotein (mg/dL)	15.2 ± 2.9	17.7 ± 1.8	22.5 ± 3.3 **a**	19.3 ± 2.9	**0.007**
Fasting glycemia (mmol/L)	3.52 ± 0.52	4 ± 0.37	3.58 ± 0.49	3.94 ± 0.59	0.342
AUC glucose (mmol/L x 120min)	622.8 ± 26.1	654.9 ± 23.1	741 ± 42 **a**	698.6 ± 29.4	0.020

*Data presented as mean ± SEM. Significance (**a** $p < 0.05$) compared with LFD shown in boldface.* ***Key: AUC*** *(area under the curve).*

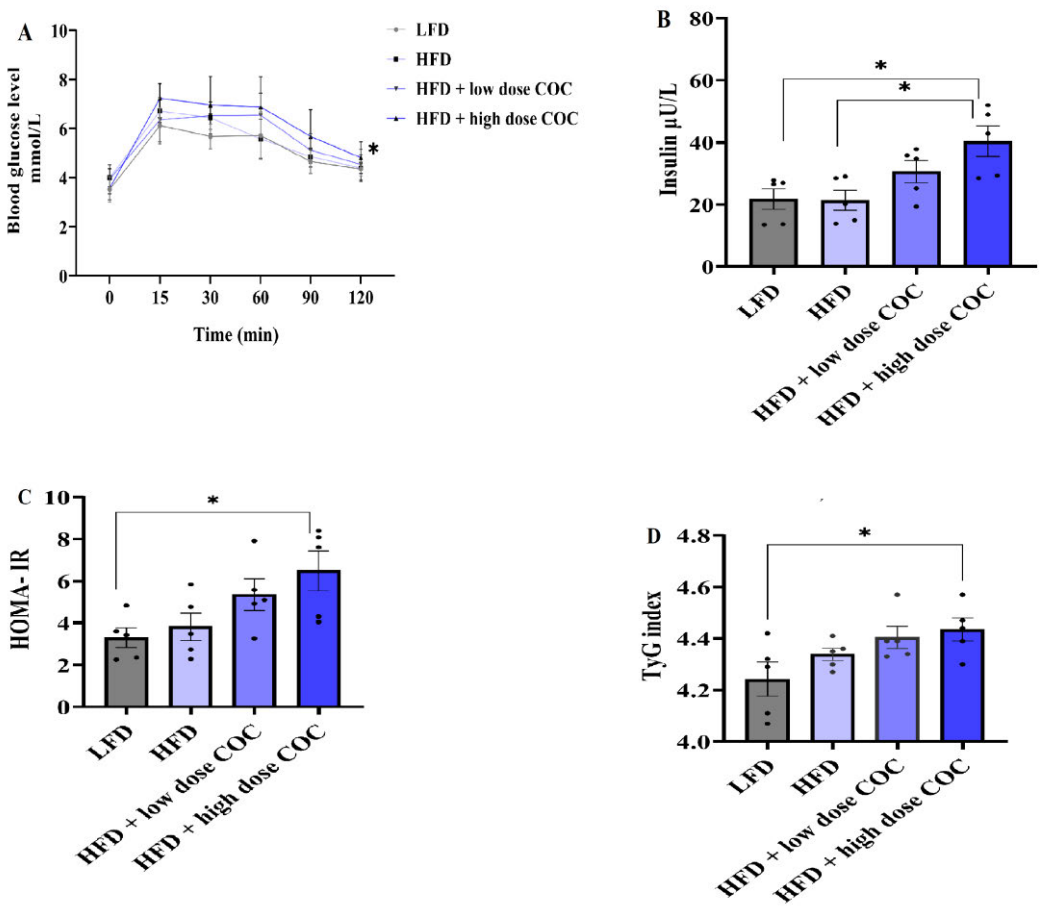

Figure 4. *Impact of 6week COC treatment in DIO rats on* ***(A)*** *oral glucose tolerance test (OGTT)* ***(B)*** *fasting insulin* ***(C)*** *HOMA-IR and* ***(D)*** *triglyceride-glucose index (TyG). All results are presented as mean ± SEM. *p < 0.05 compared with CON and HFD.*

Key: ***LFD:*** *Low fat die;* ***HFD:*** *high fat diet;* ***COC:*** *combined oral contraceptive.*

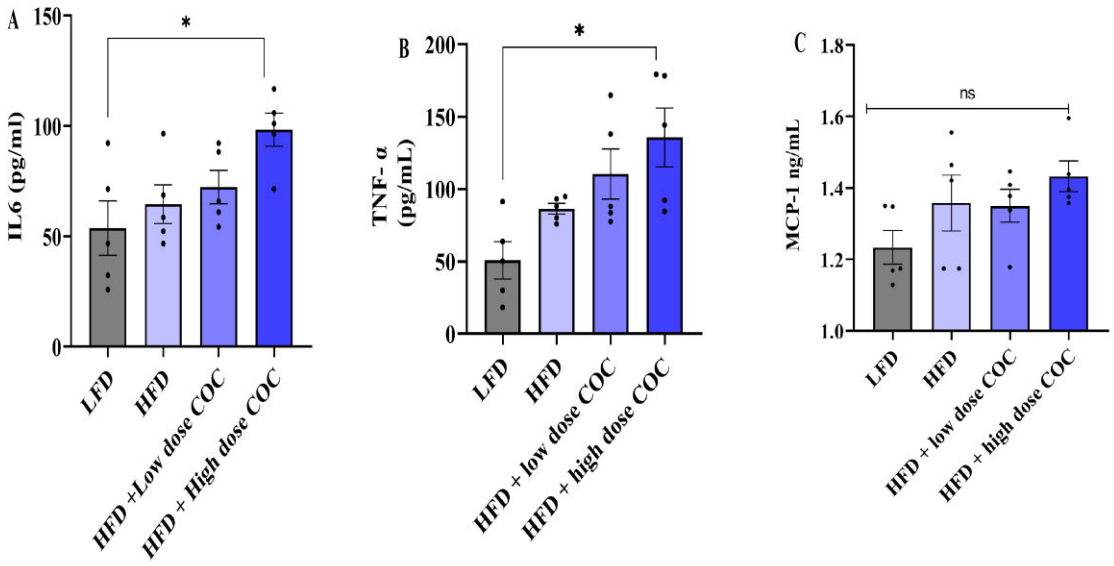

Figure 5A-C. *Effect of COC on acute phase reactants:* ***(A)*** *IL-6* ***(B)*** *TNF- α* ***(C)*** *MCP-1. All results are presented as mean ± SEM. *p < 0.05, **p < 0.001.*

Key: LFD: *Low fat die;* ***HFD:*** *high fat diet;* ***COC:*** *combined oral contraceptive;* ***IL-6:*** *interleukin 6;* ***TNF-α:*** *tumour necrosis factor alpha;* ***MCP-1:*** *monocyte chemoattractant protein-1;* ***ns****: not significant.*

4. Discussion

The aim of this study was to determine the impact of COC treatment on female Sprague Dawley rats using a DIO model. Moreover, we explored whether a dietary intervention of switching from a HFD to LFD restored the levels of immune activation such as IL-6, TNF-α and MCP-1 following 6-weeks of COC use. In our study, HFD-fed rats demonstrated a comparable change in weight gain within four weeks of HFD-feeding when compared with LFD group. Interestingly, this was similar to previous study by Taraschecnko et al that also reported a comparable change in weight of female Sprague Dawley rats during the course of 34 days [40]. Notably, the developmental stage of obesity in Sprague Dawley rats upon exposure to HFD is associated with variable responses depending on the sex, age, and duration of feeding [41,42]. At eight week, the HFD group showed a mildly obese phenotype with an increased weight gain, Lee index and abdominal circumference when compared with the LFD group which is congruent to previous findings [43,44]. This was a major indication these rats have developed the metabolic syndrome, as observed with significantly enhanced levels of triglycerides, VLDL, and fasting glycemia.

Briefly, HFD-fed group demonstrated a hyperglycemic state during 2-hour OGTT which is associated with insulin resistance and/or glucose intolerance when compared with LFD group. This results are similar to findings of other previous studies in HFD-fed Sprague Dawley rats [43,45,46]. Under physiological conditions, insulin promotes lipogenesis and inhibits lipolysis which leads to elevated levels of glucose and lipids in circulation thus resulting in impaired insulin secretion [47]. Interestingly, our result showed an elevation of TG and VLDL levels in HFD-fed rats when compared to LFD-fed rats which is congruent with previously published findings [48,49]. Whereas there were no significant changes in the

levels of HDL-c in between the two diet groups. Perhaps highlighting that short term HFD-feeding could have an influence on circulating lipid profile particularly HDL-c levels.

Furthermore, evidence from our study also showed increased plasma levels of IL-6 and TNF-α without concomitant changes in the levels of MCP-1 in HFD group when compared with the LFD group. The outcome of our study showed an association between the degree obesity as measured by Lee index and alterations with acute phase proteins like IL-6, TNF-α and MCP-1. Similarly, we observed an association between TyG, IL-6 and TNF-α levels in the HFD group. Our findings support the interdependent relationship between insulin insensitivity and high levels of pro-inflammatory cytokines seen in the progression of metabolic syndrome [50,51]. In a previous study, IL-6 levels were also associated with a decreased expression of the glucose transporter-4 and insulin receptor substrate-1 which are linked to increased insulin resistance [52]. Taken together, these findings may suggest that dyslipidaemia maybe associated with inflammation in individuals on COC. These findings further support the monitoring of lipid profiles in individuals on COC which may be essential in the CVD-risk stratification of individuals with obesity [53]. Thus, it may be reassuring to see that the metabolic effects of long-term COC use are normalized upon stopping regardless of the generational-type and formulation of the available oral contraceptives [54].

In order to assess the role of diet modification in obesity, the animal diet were switched from a HFD to LFD followed by COC treatment. Briefly, the weight of all the experimental groups were comparable after 6 weeks of COC treatment. However, a previous study showed significant reduction in body weight in lean animals treated with COC for the duration of 10 weeks when compared to controls [55]. The observed changes were associated to the hypophagic effect of the oestrogen component leading to a reduced events of excessive calorie consumption [55].

Furthermore, the HFD/LFD group receiving HCOC remained at a hyperglycemic state with decreased insulin sensitivity when compared with HFD/LFD and LFD groups respectively. Our findings are similar with previous animal and human studies where COC treatment resulted in the development of IR and reduced insulin sensitivity [56,57]. The androgenicity of the progestin component may have contributed to the impaired glucose uptake and decreased insulin sensitivity [58,59]. Furthermore, there was no significant change in lipid profile following short-term COC treatment in the obese rats despite switching diet. However, the result of our study showed increased TG and VLDL levels in the HFD/LFD group treated with HCOC, while HDL-c was significantly decreased when compared with LFD group. In line with our study, Piltonen and colleagues also reported on an elevated levels of TG in premenopausal women on COC whereas they reported an increased HDL level which is in contrast to our finding [57].

In our study, the plasma levels of both IL-6 and TNF-α in HFD-fed rats that received HCOC treatment remained significantly higher when compared with LFD-fed rats. However, HFD-fed rats did not show any significant change in the plasma levels of IL-6 and TNF-α after short-term COC treatment despite switching from HFD to LFD, although there was a slight increase in the magnitude of these biomarkers when compared with HFD/LFD group. In addition, the plasma levels of MCP-1 were comparable in all the experimental groups. The outcome following short-term COC treatment in HFD-fed rats contradicts previous findings where COC promote the release of proinflammatory mediators [60,61]. In fact, a study by Campesi et al showed a significant increase in the release of TNF-alpha in women treated with COC [61]. Similarly in a previous study involving both premenopausal and postmenopausal women showed COC did not alter levels of IL-6 and TNF-α [62–64].

Notably, ectopic lipid accumulation due to hyperlipidaemia is associated with the expression of proinflammatory mediators and the recruitment of M1 macrophages [65], thus aggravating chronic systemic low-grade type 1 inflammation [66] and impairs insulin signalling [67]. Previous study also showed a correlation between IL-6 expression and increased expression of TNF-α, MCP-1, Interferon gamma-induced protein 10 (IP-10) and macrophage infiltration into adipose tissue [68,69]. Furthermore, the presence of IL-6 and TNF-α in vascular smooth cells during the development and rupture of atherosclerotic plague also orchestrates a pro-coagulant state [70,71]. Whereas, MCP-1 mediates the attraction of monocytes-leukocytes interaction via diapedesis through the endothelium into the intima where they magnify the innate inflammatory response and promote adaptive immune regulation by T cells [70] which all aggravate the pathogenesis of atherosclerosis [68]. It is worth nothing that most studies on CVD risk in COC users have mostly reported on women of reproductive age, among whom the absolute risk for CVD events are generally low [72]. Thus, the need to evaluate the potential effects of accumulative exposure of the available COCs at reproductive age on the subsequent CVD risk at older age may provide insight and guidance during consultation and when making contraceptive choices [54].

The limitations of this study include the lack of digging and characterisation of the diverse lineage of tissue-resident cells especially macrophages in order to determine the direct cellular sources of the acute phase reactants [73]. Thus, further studies are required to understand the long-term effects and mechanism by which exogenous sex hormones may activate and socially engineer the functions of different tissue-resident cells. This would provide insight on the role of these acute phase proteins pertaining to various obesity related complications such as atherothrombotic-CVDs and host of other autoimmune and infectious diseases.

5. Conclusion

HFD-feeding promotes metabolic dysregulation and aggravates immune activation via the release of pro-inflammatory cytokines in rats. Short-term COC treatment induced metabolic dysregulation via hyperinsulinemia without aggravating immune activation in HFD-fed rats despite the switch from a high-fat to low-fat diet. However, additional studies are required to confirm these findings, especially long-term effects of this treatment on immune activation in conditions of obesity.

Acknowledgements

The authors are grateful to the Biomedical Resource Unit, University of Kwazulu-Natal, UKZN, for the technical assistance provided.

Funding

None

Disclosure statement

The authors declare no conflicts of interest.

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Prologue

Oral contraceptives (OCs) and obesity are independent risk factors of atherothrombotic disorder. The use of OC-containing both estrogen and progestin can exacerbate the progression of atherosclerotic lesions by promoting endothelial dysfunction and a procoagulant state in the presence of several other risk factors. In this chapter (Experimental 2), we focused on the impact of COC on selected markers of hemostatic changes and endothelial function in diet-induced obese (DIO) Sprague Dawley rats.

Chapter 4: Experimental article 2

Abstract

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ARTERI-690; No. of Pages 11
ARTICLE IN PRESS
Clinica e Investigacion en Arteriosclerosis xxx (xxxx) xxx–xxx
Sociedad Española de Arteriosclerosis
CLÍNICA E INVESTIGACIÓN EN ARTERIOSCLEROSIS
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ORIGINAL ARTICLE

High-fat diet promotes coagulation and endothelial activation in Sprague Dawley rats: Short-term effects of combined oral contraceptives

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Received 14 August 2023; accepted 9 October 2023

KEYWORDS
Combined oral contraceptive;
Cardiovascular disease;
High fat diet;
Obesity;
Coagulation;
Endothelial dysfunction

Abstract
Background: Combined oral contraceptives (COCs), use in individuals are associated with increased risk of thrombotic events. This highlights the significance of assessing the impact of COC on promoting coagulation and endothelial activation in high-fat diet (HFD)-fed Sprague Dawley rats.
Methods: Twenty (20) five-weeks-old female Sprague Dawley rats weighing between 150 and 200 g were subjected to both LFD and HFD-feeding for 8-weeks to determine its influence on basic metabolic status, hemostatic profile, hemodynamic parameters (blood pressure and heart rate), as well as selected biomarkers of coagulation (tissue factor and D-dimer) and endothelial activation (Von Willebrand factor and nitric oxide). Thereafter HFD-fed animals were treated with receive high dose combined oral contraceptive (HCOC) and low dose combine oral contraceptive (LCOC) for 6 weeks.
Results: Our results showed that beyond weight gain, HFD-feeding was associated with hyperglycemia, increased mean arterial pressure, and reduced nitric oxide levels when compared with LFD group ($p < 0.05$). Interestingly, treatment with high dose of COC for 6-weeks did not significantly alter atherothrombotic markers ($p > 0.05$). However, this study is not without limitation as regulation of these markers remains to be confirmed within the cardiac tissues or endothelial cells of these animals.

Abbreviations: CVDs, cardiovascular diseases; COCs, combined oral contraceptives; TF, tissue factor; NO, nitric oxide; vWF, Von Willebrand factor; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; MACEs, major adverse cardiovascular events.

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https://doi.org/10.1016/j.arteri.2023.10.001
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Please cite this article as: O.A. Fabunmi, P.V. Dludla and B.B. Nkambule, High-fat diet promotes coagulation and endothelial activation in Sprague Dawley rats: Short-term effects of combined oral contraceptives, Clinica e Investigacion en Arteriosclerosis, https://doi.org/10.1016/j.arteri.2023.10.001

Conclusion: HFD-feeding orchestrate the concomitant release of pro-coagulants and endothelial activation markers in rats leading to haemostatic imbalance and endothelial dysfunction. Short-term treatment with COC shows no detrimental effects in these HFD-fed rats. Although in terms of clinical relevance, our findings depict the notion that the risk of CVD in association with COC may depend on the dosage and duration of use among other factors especially in certain conditions. However, additional studies are required to confirm these findings, especially long-term effects of this treatment within the cardiac tissues or endothelial cells of these animals in certain conditions relating to postmenopausal state.
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Candidate's contribution: OAF (the candidate) was involved in the conceptualization of the study, animal handling, running laboratory assays, data analysis and writing of the manuscript.

1. Introduction

Cardiovascular disease (CVD) account for at least a third of the mortality occurring in low and middle-income countries (LMIC) [1,2]. Despite the advancements in the therapeutics and management of patients with chronic disease [3], CVD still account for a substantial number of deaths in people living with metabolic complications [4]. Obesity remains a predominant risk factor for thrombosis which is characterised by increased plasma levels or activity of coagulation factors and fibrinolytic proteins which contribute to major adverse cardiovascular events (MACEs) [5].

Of note, the pathological enlargement of the adipose tissue in obesity elicit the release of pro-inflammatory molecules that induce damage to the endothelium [6,7]. This further results in the altered release of nitric oxide (NO) and negatively affect other vasodilatory metabolites leading to endothelial dysfunction [8,9] and arterial stiffening [10,11]. Furthermore, the endothelium may be transformed from an antithrombotic to prothrombotic state in obesity which may orchestrate haemostatic imbalances [12–14]. During a prothrombotic state, tissue factor (TF), von Willebrand factor (vWF) and P-selectin promote the adhesion of platelet on the endothelium [15–19].

The use of oral contraceptives especially the combined oral contraceptives (COCs) is a commonly modern method for birth control [20,21]. However, the use of COC is also associated with endothelial and haemostatic dysregulation which increases the risk of CVD [22,23]. Some of the established mechanism by which COC impact the vasculature include the activation of aldosterone-mineralocorticoid receptor axis among others [20,24]. However, findings are inconsistent and others indicate no correlation exist between the use of COC and changes in major makers of endothelial function like NO and endothelin-1 in premenopausal

women [25]. In contrast, experimental study suggest that COC treatment may alter endothelial function and haemostasis and ultimately promote inflammation [26].

Nonetheless, there is a need to continuously assess the impact of hormonal contraceptives for their potential effects in causing vascular dysfunction in women at risk of developing CVD [27]. Beyond providing insight into the pathophysiology of vascular disease in those with metabolic anomalies, this would enhance our understanding on the potential influence of oral contraceptives in driving CVD-risk among susceptible individuals [28,29]. In the current study, we first assess the impact of high fat diet (HFD) on dysregulating biomarkers of coagulation and endothelial activation in Sprague Dawley rats. We further investigated the influence of COC treatment in these HFD-fed rats.

2. Materials and methods

2.1. Animal handlings

Twenty (n=20) five-week-old female Sprague Dawley rats weighing between 150-200g were used for this study. The animals were purchased and housed at the Biomedical Research Unit (BRU) at the of University KwaZulu-Natal (UKZN; South Africa). Animal handling followed guidelines and principles, as published by the Committee for the Care and Use of Laboratory Animals [30]. Briefly, the animals acclimatized for 2 weeks before commencement of the study, with an unrestricted access to food and water throughout the project. This was done within an environment- temperature (22±2°C), humidity (55±5%), controlled 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.

2.2. Study design

The study contained two major experiment phases. The first experiment group comprised of 10 female animals (n=5/group) that were kept on a HFD (containing 20% protein and 60% fat; #D12492) for eight weeks and a control group which was randomized to receive LFD (containing 20% protein and 10% fat; #D12450) (Research Diets, Inc.; New Jersey, United States). The diet composition and duration of HFD-feeding follows previously published literature [31]. The food and water intake were measured weekly via metabolic cages (Techniplats, Labotec, South Africa). Moreover, body weights were monitored weekly while the hemodynamic and haemostatic changes were determined at the last week of the experiment to minimise stress.

The second experimental phase contained 10 rats (n=5/group) that were kept on a HFD for 8 weeks before receiving levonorgestrel-containing oral contraceptives (COC) for 6 weeks [32], at low dose (4.5µg of levonorgestrel and 0.9µg ethinylestradiol) or high dose (9µg levonorgestrel and1.8µg ethinylestradiol) (Aspen Pharmacare, South Africa) [33]. Additional rats (n=10), exposed HFD (n=5/group) or LFD (n=5/group) alone, served as controls for diet-induced obesity and experimental control, respectively. Treatments were prepared in distil water as previously decsribed [34]. All the treatments were administered daily via oral gavage while the animals were regularly monitored for adverse reaction, and the cages were cleaned daily. After 6 weeks of COC treatment blood samples were drawn from the lateral tail vein into ethylenediaminetetraacetic acid (EDTA) microtainer and vacutainer citrate tubes (BD Bioscience, United States) for further analysis.

2.3. Measurement of hemodynamic variables

Hemodynamic parameters, including {systolic, diastolic, and mean arterial pressure (MAP)} were determined via the tail-cuff method using the non-invasive digital blood pressure (BP)

system (BIOPAC System, NTBP250, California, United States). Briefly, the machine has a built-in pump that automatically inflates and deflates the rat tail cuff to provide a linear drop in pressure. The equipment was calibrated each day before measurements and the animals were placed in a restrainer with a cuff attached to a heated tail to allow effective adaptation and habituation. During the day of measurement, the animals were kept warm at ± 37 ^{0}C in an enclosed chamber (IITC Model 303sc Animal Test Chamber, IITC Life Sciences, Woodlands Hills, California, United States) for 15 min before blood pressure recording to make the pulsations of the tail artery detectable. All measurements were conducted before mid-day to avoid diurnal variation. The mean arterial pressure was calculated using the formula as described previously [34].

$$\text{MAP} = \frac{[(2 \times \text{diastolic}) + \text{systolic}]}{3}$$

2.4. Hemostatic assessment

The bleeding time in the animals was determined non-invasively via the tail cut method while and this was recorded as previously demonstrated [35]. The tail of the rat was warmed for 1 min in water at 40°C and then dried before a small cut was made in the middle of the lower portion of the tail with a scalpel. Bleeding time started when the first drop touched the circular filter paper. It was checked at 30 secs intervals until bleeding stopped. The assessment was conducted before mid-day to avoid diurnal variation.

2.5. Biochemical analysis

Plasma levels of TF, D-dimer, NO and vWF were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Elabscience Biotechnology Co., Ltd., Houston, Texas, USA). All assays were performed according to the recommended protocol.

2.6. Statistical analysis

All experimental data were expressed as means ± standard error of mean (SEM). Normality testing was performed using the Kolmogorov-Smirnov test with Dallal-Wilkinson-Lillie. The mean differences between the LFD and HFD-fed groups were determined using unpaired student *t*-test for parametric data and reported as means ± standard error of mean (SEM). Correlations were performed between MAP, coagulation and endothelia activation markers using Pearson coefficients. Statistical significance for measured variables was determined by one-way (treatment factor) analysis of variance (ANOVA) for the comparison of the mean values of variables among the experimental groups. A post hoc Tukey's multiple comparisons test was performed if the F-value reached statistical significance ($p < 0.05$). All the statistical analysis were performed using the GraphPad Prism version 8.0 software (GraphPad Software Inc, CA, USA).

3. Results

3.1. Metabolic status of rats fed a high fat diet (HFD) compared to low fat diet (LFD) for 8 weeks.

There was significant decrease in food intake (g/kg/day) in HFD-fed group (37.2 ± 1.3) when compared with LFD-fed group (51.6 ± 2.3) ($p < 0.001$) (Table 1). Whereas water intake (ml/kg/day) was significantly increased in HFD-fed group (119 ± 4.6) when compared with LFD-fed group (88.1 ± 3.8) ($p < 0.001$) (Table 1). Furthermore, HFD-fed rats (289 ± 4.1) showed significant weight gain (g) in comparison to LFD-fed rats (270 ± 5.2) ($p = 0.002$) (Table 1). More so, fasting glycemia (mmol/L) was significantly increased in HFD-fed groups (5 ± 0.5) when compared with LFD-fed group (3.8 ± 0.6) ($p \leq 0.01$) (Table 1).

Table 1. Characteristic features of rats exposed to high fat diet (HFD)-feeding in comparison to the low-fat diet (LFD) group.

Parameters	**LFD group**	**HFD group**	**p-value**
Metabolic status			
Food intake (g/kg/day)	51.6 ± 2.3	37.2 ± 1.3	**<0.0001**
Water intake (ml/kg/day)	88.1 ± 3.8	119 ± 4.6	**<0.0001**
Body weight (g)	270 ± 5.2	289 ± 4.1	**0.02**
Fasting glycemia (mmol/L)	3.8 ± 0.6	5 ± 0.5	**0.006**
Hemodynamics			
SBP (mmHg)	129 ± 5.7	137 ± 7.2	0.082
DBP (mmHg)	90.8 ± 2.2	92.4 ± 0.9	0.166
MAP (mmHg)	103.6 ± 1.1	107.2 ± 2.9	**0.03**
HR (bpm)	333 ± 14.2	380 ± 20.7	0.174
Hemostatic profile			
Bleeding time (sec)	204 ± 39.1	156 ± 32.9	0.069
TF (pg/mL)	53.84 ± 6.7	76.6 ± 9.9	0.09
D-dimer (ng/mL)	175 ± 17.1	200 ± 23	0.403

Results expressed as mean ± standard error. Significance between groups ($p < 0.05$) shown in boldface. ***Key: SBP****: systolic blood pressure;* ***DBP****: diastolic blood pressure;* ***MAP****: mean arterial pressure;* ***HR****: heart rate;* ***TF****: tissue factor.*

3.2. Hemodynamic profile of LFD and HFD-fed rats for 8 weeks.

The systolic and diastolic blood pressure were comparable in both diet groups ($p > 0.05$); however, the MAP (mmHg) was significantly higher in HFD-fed group (107.2 ± 2.9) when compared to the LFD-fed group (103.6 ± 1.1) ($p \leq 0.03$). In addition, the heart rate of both diet groups was comparable ($p > 0.05$) (Table 1).

3.3. Haemostatic profile and endothelia markers of LFD and HFD-fed rats for 8 weeks.

The bleeding time which was used to determine the haemostatic changes between both diet groups was comparable ($p > 0.05$) (Table 1). Furthermore, plasma levels of tissue factor and D-dimer to determine changes in the clotting cascade were also comparable between diet groups ($p > 0.05$) (Table 1). In terms of endothelia activation, the plasma levels of Von Willebrand factor were comparable between diet groups (Figure 1A). Whereas the plasma levels of NO were significantly lower in HFD-fed group (8.1 ± 0.5) when compared to LFD-fed group (10 ± 1.3) ($p < 0.05$) (Figure 1B).

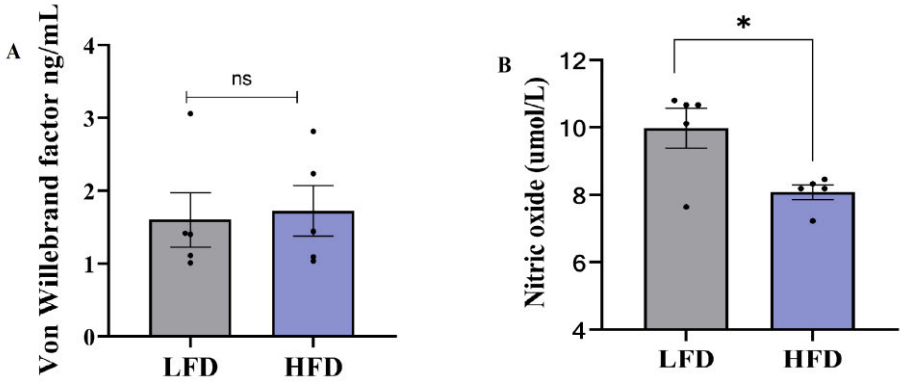

Figure 1: *Effects of low-fat diet (LFD) and high fat diet (HFD) feeding on endothelial activation including (A) Von Willebrand factor (B) nitric oxide levels. All results are presented as mean ± SEM with the significance represented by *p < 0.05.*
Key: LFD: *low-fat diet;* ***HFD:*** *high-fat diet;* ***ns:*** *(not significant).*

3.4. Associations between MAP and markers of coagulation cascade and endothelial activation in rats fed HFD for 8 weeks.

Next, we determined the relationship that exist between HFD-feeding and the presence of pathological features of atherothrombosis in rats. This was done by assessing if there was any association between MAP and the dysregulation of makers of coagulation and endothelial activation (Figure 2). The result showed that MAP was associated with TF (r = 0.97, $p < 0.007$) and D-dimer (r = 0.87, $p < 0.02$) (Figure 2A and B). MAP was also associated with Von Willebrand factor (r = 0.88, $p < 0.01$) and NO (r = -0.86, $p < 0.02$) (Figure 2C and D).

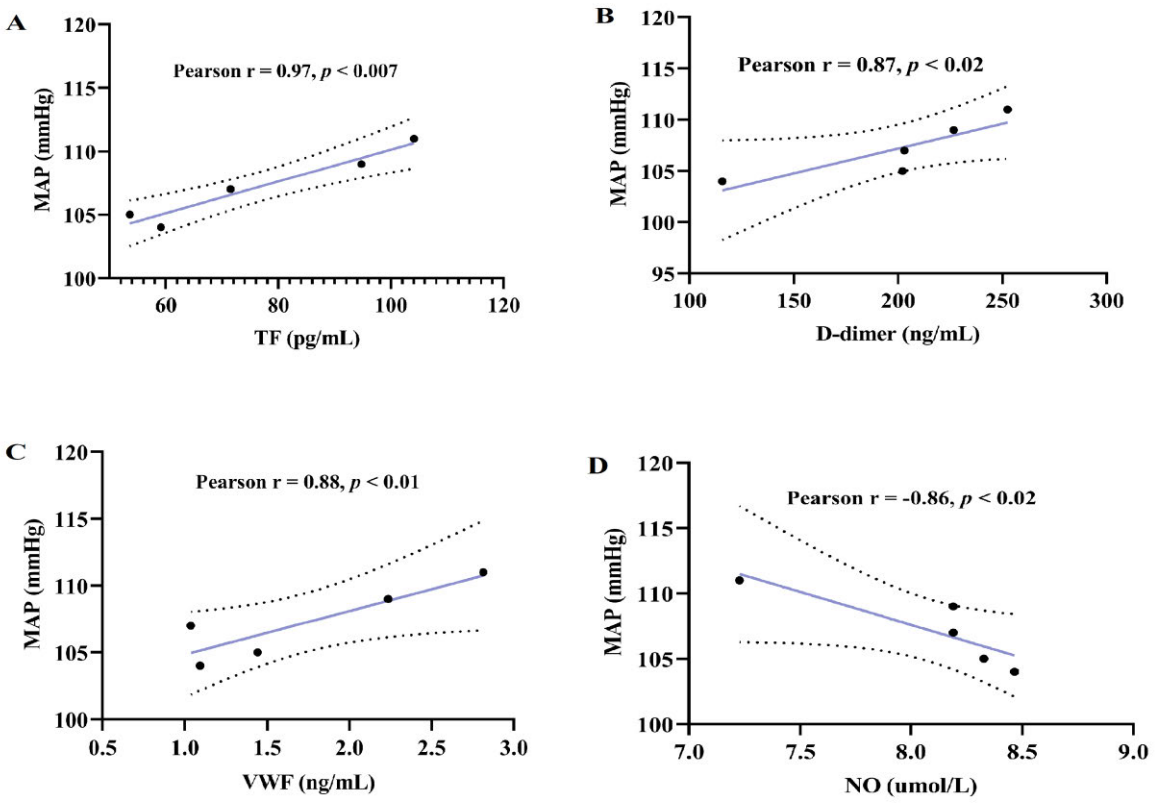

Figure 2: *Association between Mean arterial pressure (MAP) and biomarkers of coagulation cascade like tissue factor (TF) and D-dimer* ***(A-B)*** *as well as biomarkers of endothelia activation such as Von Willebrand factor (VWF) and nitric oxide (NO)* ***(C-D)*** *after 8 weeks high fat diet (HFD)-feeding in rats. Correlations are presented as Pearson r 95% confidence interval (p<0.05).*

3.5. Metabolic status of COC treated HFD-fed rats.

The body weights were comparable across experimental groups ($p > 0.05$) (Table 2). However, food intake ($F_{(3, 16)} = 3.674$; $p = 0.03$) varied across the experimental groups. The post-hoc analysis showed a significant decreased food intake in HFD fed rats that received the high dose COC when compared with LFD-fed rats ($p \leq 0.05$) (Table 2). Meanwhile, water intake was comparable among the groups despite COC treatment group displaying non-significant increase in this parameter ($p > 0.05$) (Table 2). More so, fasting glycemia was comparable across experimental groups ($p > 0.05$) (Table 2).

3.6. Effect of COC on hemodynamic parameters and haemostatic profile in HFD-fed rats.

The systolic blood pressure ($F_{(3, 16)} = 4.390$; $p = 0.02$) differed across the experimental groups. HFD-fed rats receiving high dose COC showed a significant increased systolic blood pressure when compared with the LFD group ($p \leq 0.05$) (Table 2). While the mean arterial pressure ($F_{(3, 16)} = 3.4$; $p = 0.03$) also varied across the experimental group, HFD-fed rats receiving high dose COC showed a significant increased mean arterial pressure when compared with the LFD group ($p \leq 0.05$) (Table 2). Meanwhile, the diastolic blood pressure and heart rate were comparable across all the experimental groups following 6 weeks of COC treatment ($p > 0.05$) (Table 2). Notably, the bleeding time assessment ($F_{(3, 16)} = 4.390$; $p = 0.02$) also differed across the experimental groups. HFD-fed rats receiving high dose COC showed significant increased bleeding time when compared with the LFD and HFD-fed rats ($p < 0.05$) (Table 2).

3.7. Effect COC on biomarkers of coagulation and endothelia activation haemostatic profile in HFD-fed rats.

To determine changes in the coagulation cascade, the plasma levels of tissue factor and D-dimer were determined following 6 weeks of COC treatment. Our data showed a varied plasma levels of tissue factor ($F_{(3, 16)} = 3.862$; $p = 0.03$) and D-dimer ($F_{(3, 16)} = 5.84$; $p = 0.007$) across the experimental groups respectively. Both tissue factor and d-dimer levels were significantly increased in HFD-fed rats receiving high dose COC when compared with LFD-fed rats ($p < 0.05$; $p < 0.001$) (Figure 3A and B). Furthermore, the plasma levels of Von Willebrand factor and NO were measured to determine changes in endothelia activation. Briefly, Willebrand factor ($F_{(3, 16)} = 3.73$; $p = 0.03$) and NO ($F_{(3, 16)} = 5.03$; $p = 0.01$) also differed across the experimental groups respectively. HFD-fed rats receiving high dose COC demonstrated a significant increase in plasma levels of Von Willebrand factor when compared with LFD-fed rats ($p < 0.05$) (Figure 4A). Whereas plasma levels of NO is significantly lower in the HFD-fed rats receiving high dose COC when compared with LFD-fed rats ($p < 0.001$) (Figure 4B).

Table 2. Characteristics of animals following 6-week short-term treatment with combined oral contraceptive (COC) (n=5/group)

Parameters	**LFD**	**Untreated HFD**	**HFD + low dose COC**	**HFD + high dose COC**	**p-value**
Metabolic status					
Food intake (g/kg/day)	67.6 ± 2.3	61.6 ± 3.6	62.1 ± 2.4	60 ± 5.9 [a]	**0.0347**
Water intake (ml/kg/day)	102.5 ± 4.6	107.6 ± 6.5	110 ± 4.4	116.5 ± 12.6	0.08
Body weight (g)	287.2 ± 9.9	299.8 ± 17	281.8 ± 26.2	295.2 ± 11.3	0.388
Fasting glycemia (mmol/L)	3.52 ± 0.52	4 ± 0.37	3.58 ± 0.49	3.94 ± 0.59	0.3424
Hemodynamics					
SBP (mmHg)	125.2 ± 5.4	135.8 ± 8	137.0 ± 7	140.4 ± 7.4 [a]	**0.0196**
DBP (mmHg)	88.2 ± 4.7	91.4 ± 4.8	91.8 ± 0.84	92.6 ± 6.15	0.5827
MAP (mmHg)	100.4 ± 4.3	106.4 ± 3.1	106.8 ± 2.6	108.4 ± 5.23 [a]	**0.025**
HR (bpm)	343.2 ± 39.5	361.6 ± 27.1	357 ± 17	385.6 ± 27.8	0.1775
Hemostatic profile					
Bleeding time (sec)	186 ± 33	168 ± 50.2	120 ± 37	96 ± 25.1[ab]	**0.005**

Data presented as mean ± SEM. ***SBP:*** *systolic blood pressure;* ***DBP:*** *diastolic blood pressure;* ***MAP:*** *mean arterial pressure;* ***HR:*** *heart rate. Significance ([a]P< 0.05 vs LFD; [b]P< 0.05 vs HFD) shown in boldface.*

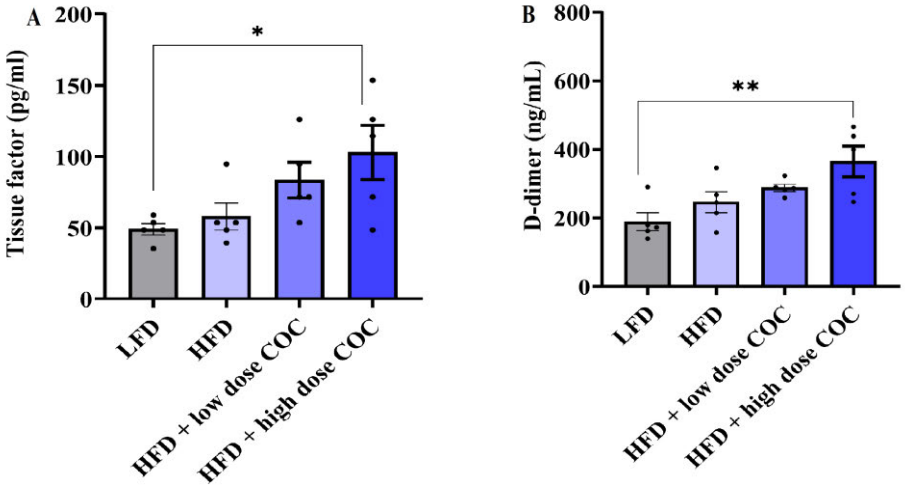

Figure 3. *Effect of combined oral contraceptive treatment on biomarkers of coagulation cascade like tissue factor (A) and D-dimer (B). All results are presented as mean ± SEM. Significance indicated between LFD and HFD + High dose COC. *p < 0.05; **p <* 0.001.
Key: LFD: *Low fat diet,* ***HFD:*** *high fat diet,* ***COC:*** *combined oral contraceptive.*

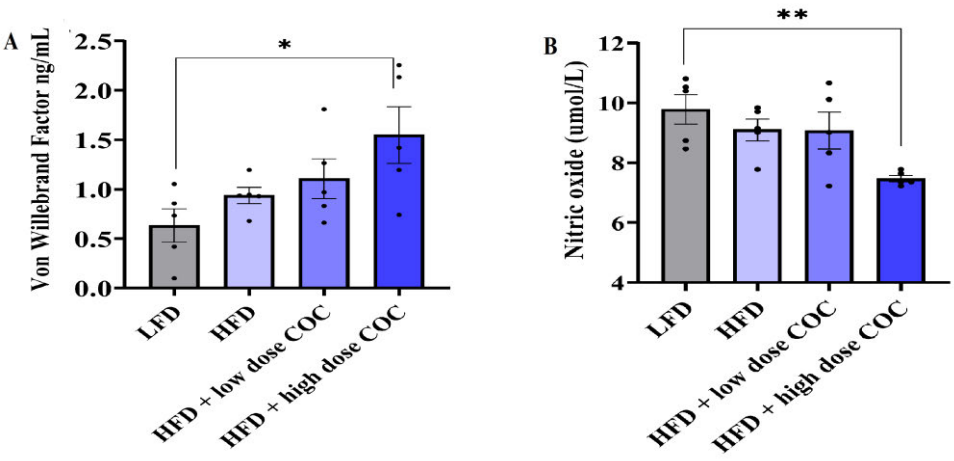

Figure 4. *Effect of combined oral contraceptive treatment on biomarkers of endothelia activation like Von Willebrand factor (A) and nitric oxide (B). All results are presented as mean ± SEM. Significance indicated between LFD and HFD + High dose COC. *p < 0.05; **p <* 0.001.
Key: LFD: *Low fat diet,* ***HFD:*** *high fat diet,* ***COC:*** *combined oral contraceptive.*

4. Discussion

In this present study we assessed the impact of HFD-feeding on selected biomarkers of coagulation and endothelial activation in HFD-fed rats. Firstly, HFD-feeding for 8-weeks promoted weight gain which was accompanied by alterations in food and water intake when compared with LFD-fed rats (Table 1). Interestingly, such phenotypic changes were similar to previous studies in experimental animals on HFD showing characteristic feature of metabolic syndrome [37–39]. In fact, our previous study has shown that 8-week HFD-feeding led to the development of metabolic syndrome and increased levels of biomarkers of atherothrombotic disorders and CVD [40,41].

In our study, the systolic and diastolic blood pressure including the heart rate were not affected by 8-weeek-treatment with HFD. It is noteworthy that the causal role of dietary fat on hemodynamic changes in animal models of obesity is subject to variation due to the strain of animal [42]. However, evidence from our study corroborates with previous studies where HFD over 10 weeks did not alter the hemodynamic profiles of the animals [43,44]. Nonetheless, the MAP was significantly higher in HFD-fed rats when compared with LFD-fed rats. This agreed with our results showing that MAP was associated with TF, D-dimer, and NO in these HFD-fed rats.

Furthermore, our results indicated that exposure of rats to HFD for 8-weeks in addition to COC treatment for a 6-week period did not affect the weight gain and fasting glycemia in these the animals, except for food intake, which was significantly reduced in response to a high dose of COC (Table 2). Our results indicated that COC treatment (especially the high dose) may have a significant role in influencing food intake, sex hormones, and eating behaviour in female rats, as previously reported in similar settings [45–47]. However, the short-term treatment period of 6-weeks may not be sufficient to induce marked changes in

HFD-fed rats. Nonetheless, it remained important to determine how COC treatment affected other markers related to the development of CVD.

Notably, treatment of HFD-rats with a high-dose of COC significantly increased the systolic blood and MAP when compared to LFD group. However, such effects occurred without any changes in the hemodynamic profiles, including the diastolic blood pressure and heart rate. While the outcome of our study corroborates previous findings [48–50], it also suggested that a dose-dependent relationship between high COC and hemodynamic changes exist. In principle, endogenous female sex hormones are known to be cardioprotective [51] which maybe abrogated during hormonal contraceptive treatment thereby predisposing susceptible individuals to higher risk of MACEs in the presence of several comorbidities such as obesity, smoking and physical inactivity [27].

Importantly, beyond assessing the basic metabolic parameters, it remains essential to understand how different doses of COC affects some selected biomarkers of endothelial function in these HFD-rats. Obesity-related complications are multifactorial, and it encompasses alterations to blood flow, hypercoagulability, and endothelial dysfunction which all contribute to the early pathological process of atherothrombotic-CVD [52]. Although eight weeks of HFD-feeding did not alter levels of TF and D-dimer in our study. In contrast, other studies reported changes in the coagulation cascade in HFD-fed mouse model [53,54]. For instance, female C57Bl/6J mice demonstrated a prothrombotic phenotype after fourteen weeks of HFD-feeding [54]. Cleuren et al. also showed early-onset in procoagulant shift in HFD fed C57BL/6J male mice which persisted during sixteen weeks of HFD-feeding [53]. The physiological difference in the sex and specie of the animals in association with the handling of metabolic stress may contribute to the observed differential change in the coagulation cascade [55,56]. However, the plasma levels of TF and D-dimer were

significantly higher in HFD-fed rats receiving high COC when compared to LFD-fed rats in our study which contrast other previous finding where short-term ethinylestradiol treatment counteracts the prothrombotic phenotype in HFD-fed mice [54]. However, the orchestrated haemostatic imbalance observed in our study is similar with clinical findings by others where the release of TF and dimer was relatively higher in COC users when compared with women who did not use COCs [57–59]. The outcome of study suggest a dose-dependent relationship between COC and haemostatic changes in association with progestin type [60,61].

TF plays an important role in the initiation of the extrinsic coagulation process which is complemented by the intrinsic pathway that ensures thrombin generation and clot production [62]. D-dimer antigen originates as a product of the fibrinolytic system that regulates the removal of fibrin during clot formation [63]. In principle, the fibrinolytic system physiologically counteracts the hypercoagulable condition and maintains normal circulation during haemostasis [64]. However, haemostatic imbalance can cause excessive fibrin deposition inside the vascular channels and obstruct blood flow which may lead to MACEs such as arterial and venous thrombosis, ischemia, and myocardial infarction [64].

In terms of endothelial changes, the result of our study showed a significant increase in plasma levels of NO in HFD-fed rats when compared to LFD-fed rats after 8 weeks of HFD feeding. Meanwhile the plasma levels of vWF were comparable between both diet groups. This correspond with previous findings where NO level was reduced in HFD-fed rats [65–68]. Although recent evidence showed the critical role of vWF in the thrombo-inflammatory complex during obesity [69] where they mediate platelet aggregation and adhesion and promote leukocyte extravasation and several inflammatory responses [15]. Nonetheless, our result contrast other previous studies that reported elevated levels of vWF in HFD-fed experimental animals [15,69].

Furthermore, HFD-fed rats treated with high dose COC for 6 weeks demonstrated increased levels of VWF while NO levels were significantly reduced when compared with the LFD. However, no significant change was observed when compared with HFD only group. The observed endothelia changes orchestrated by COC treatment may have resulted from vasoactive effect of the progestin component which is known to antagonize the vasodilatory effect of oestrogen in healthy subjects [70,71]. Our result also corroborates previous findings that showed reduced NO bioavailability during COC administration in non-obese animals [49]. The limitations of this study include lack of determining vWF activity which is mainly secreted into circulation by endothelia cells. While vWF alone don't offer a complete functional mechanism, vWF:Ristocetin ratio is important in associating vWF levels with CVD risk [72]. Furthermore, TF activity and sources of NO were not determined. Thus, further studies are required to determine how the long-term COC treatment influences the activity of vWF and TF activity.

5. Conclusion

HFD-feeding aggravates the risk of atherothrombosis by orchestrating the concomitant release of pro-coagulants and endothelial activation markers leading to haemostatic imbalance and endothelia dysfunction. Short-term treatment with COC shows no detrimental effects. However, additional studies are required to confirm these findings, especially long-term effects of this treatment on CVD-related markers in conditions of obesity.

Declaration of Interest

The authors declare no conflict of interest.

Funding

None

Acknowledgements

The authors are grateful to the Biomedical Resource Unit, University of KwaZulu-Natal, UKZN for technical assistance.

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Prologue

Atherothrombotic disorder is an unpredictable progressive disease that can occur due to atherosclerotic lesions. Inflammation plays a key role both in the initiation and progression of atherosclerosis leading to multiple consequences and complications. The use of oral contraceptive containing estrogen and progesterone can exacerbate several complications that are associated with atherosclerotic lesions. On the other hand, compelling evidence suggest that the anti-inflammatory impact of low dose aspirin (LDA) may help prevent the initiation and progression of atherosclerotic lesions thereby reducing the risk of major adverse cardiovascular events (MACEs). In this chapter, we focused on the impact of LDA on the risk of atherothrombotic disorder in combined oral contraceptive (COC) treated rats.

Chapter 5: Experimental article 3

Short-term treatment with low-dose aspirin improved metabolic status and attenuated atherothrombotic risk factors in female rats exposed to combined oral contraceptives.

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Candidate's contribution: OAF (the candidate) was involved in the conceptualization of the study, animal handling, running laboratory assays, data analysis and writing of the manuscript.

(Will be submitted to the Journal of Fertility and Sterility).

Abstract

Background: Atherothrombosis is a chronic and progressive disease responsible for a significant number of deaths globally, and it has become essential to understand how this consequence contributes to enhanced cardiovascular disease in women on combined oral contraceptives (COC).

Objectives: To assess the impact of COC administration on metabolic status and the risk of atherothrombotic disorder in female rats beyond evaluating the therapeutic effects of short-term treatment with low-dose aspirin (LDA).

Methods: Thirty (n=30) five-week-old female Sprague Dawley rats were given low-dose COC (LCOC) or a high-dose COC (HCOC) for six weeks before treatment with LDA for another four weeks. These rats were compared to those that were only given LCOC or HCOC without treatment with LDA. Whereas rats that received distilled water (as a vehicle) and LDA only served as controls. The body weights and metabolic status were taken weekly. Whereas parameters related to glucose regulation, lipid profiles, inflammatory cytokines, hematological indices, coagulation, and endothelial dysfunction were recorded at the terminal end of the experiment.

Results: Rats exposed to HCOC presented with abnormal metabolic status and lipid profiles, as seen with impaired glucose tolerance, which was accompanied by significantly higher levels of insulin, triglycerides, and very low-density lipoprotein when compared to the controls. The HCOC treatment was also consistent with enhanced platelet count and elevated markers of inflammation and endothelial dysfunction, including interleukin 6; tumour necrosis factor-alpha; monocyte chemoattractant protein-1, as well as tissue factor, D-dimer, Von Willebrand factor, and low nitric oxide. Notably, systolic blood pressure and mean arterial pressure were also significantly higher in these animals, suggesting an increased cardiovascular disease risk in response to HCOC. Interestingly, short-term LDA could reduce cardiovascular risk by improving the metabolic status, alleviating inflammation, and lowering markers of endothelial dysfunction in rats that were exposed to HCOC.

Conclusion: A High dose of COC is associated with increased cardiovascular disease risk in female rats, while short-term LDA could alleviate this pathological effect by improving metabolic status, blocking inflammation, and improving endothelial function. However, additional studies are required to confirm the mechanism of interaction between LDA and COC.

Keywords: combined oral contraceptive; cardiovascular disease; low dose aspirin; inflammation; coagulation; endothelial dysfunction.

1. Introduction

Non-communicable disease (NCD) remains a leading common cause of death worldwide [1] especially in lower and middle-income countries (LMIC) [2]. This includes cardiovascular diseases (CVDs), especially those that involve arterial and venous events such as coronary artery diseases and stroke that are projected to exponentially exceed communicable, maternal, perinatal, and nutritional diseases as the most common causes of death by 2030 [3]. Poor lifestyle choices, such as the accumulation of diets rich in high fat in combination with physical inactivity, are likely to drive the development of metabolic diseases, including CVDs [4]. The pathogenesis and the progression of atherothrombosis are associated with endothelial dysfunction and chronic inflammatory responses [5,6]. Hence, there is a need to understand the contributing factors, including currently used medications, to the development of atherothrombosis associated with endothelial dysfunction to curb CVD-related deaths.

There is an increased risk of venous and arterial events during the use of oral contraceptive pills containing either estrogen or progestogen [7], and the prevalence of combined oral contraceptive (COC) use in women of reproductive age is projected to exponentially increase over the years across different geographical regions [8]. The prolonged use of COC may lead to metabolic dysregulation and alter the inflammatory milieu, which causes the release of certain cytokines and chemokines such as interleukin (IL)-6, tumor necrosis factor-alpha (TNF-α), and monocyte chemoattractant protein (MCP-1) which promotes endothelial dysfunction and asymptomatic atherosclerotic changes [9,10]. In the process of atherosclerotic development, inflammatory cells are internalized within the intimal layer of the arterial wall, leading to the proliferation and migration of vascular smooth muscle cells to the lesion site [11,12]. The vascular smooth muscle cells may undergo phenotypic switching, stabilizing plaque formation, leading to a thrombotic disorder [11,12]. This also explains a

strong interest in understanding how the therapeutic effects of certain drugs, including aspirin, may help prevent and manage CVD-related complications, especially through alleviating inflammatory responses [13,14]. The therapeutic use of aspirin in the secondary prevention of major adverse cardiovascular events has been described [15–17]. However, the role of aspirin in the primary prevention of cardiovascular events remains unclear. Current guidelines regarding the recommendation of aspirin in the management of CVDs are quite controversial [18,19], and these controversies centre around the dose regimen, risk of excessive bleeding, and interplay between gender and treatment response, among others [20–22]. Notably, some of the beneficial effect of aspirin in the management of cardiovascular events is associated with the permanent inactivation of cyclooxygenase activity and amelioration of inflammation [23,24], a therapeutic effect that is linked with inhibition of platelet aggregation and improved CVD-related outcomes [24,25]. Since COC is associated with a high risk of venous and arterial events [26–29], this study aims is to determine if short-term low-dose aspirin (LDA) administration will modulate the levels of selected biochemical molecules linked to the atherothrombotic disorder in experimental animals on different doses of COC treatment.

2. Materials and methods

2.1. Animal handling

Thirty (n=30) five-week-old female Sprague Dawley rats weighing between 150-200g were purchased and housed at the Biomedical Research Unit (BRU) at the University of KwaZulu-Natal (UKZN). The animals were allowed to acclimatize for 2 weeks with unrestricted access to food and water. They were maintained under standard environmental conditions of temperature (22±2°C), humidity (55±5%) and controlled 12-h light cycle (6:00–18:00) and dark cycle (18:00–6:00). Animal handling followed guidelines and principles, as published

by the Committee for the Care and Use of Laboratory Animals [33]. Ethical clearance for this study was granted by the UKZN animal research ethics committee (AREC/00003067/2021).

2.2. Experimental design and treatment of animals

The animals were randomly allocated into six groups (n = 5/group), respectively. The first two groups included experimental controls that received distilled water (vehicle), as well as the control for treatment drug (LDA: 20mg/kg). Another two groups consisted of the low dose COC (LCOC) and the high dose COC (HCOC), which was for six weeks. The last two intervention groups included those on LCOC and HCOC who also received LDA (at 20 mg/kg) for the additional four weeks to make the total experimental phase ten weeks. In terms of treatment composition, LCOC was made of a combination of 4.5 µg of levonorgestrel / 0.9µg of ethinylestradiol, while HCOC was a combination of 9µg levonorgestrel /1.8µg ethinylestradiol and was adjusted for animal body weight. The drugs were prepared accordingly, as previously described [31,32]. All the treatments were received daily (via oral gavage), while the animals were regularly monitored by a trained veterinarian and the cages were cleaned daily, to ensure a clean environment. The body weight of the animals was measured weekly using a digital weighing balance. The food and water intake were measured weekly via metabolic cages (Techniplats, Labotec, South Africa).

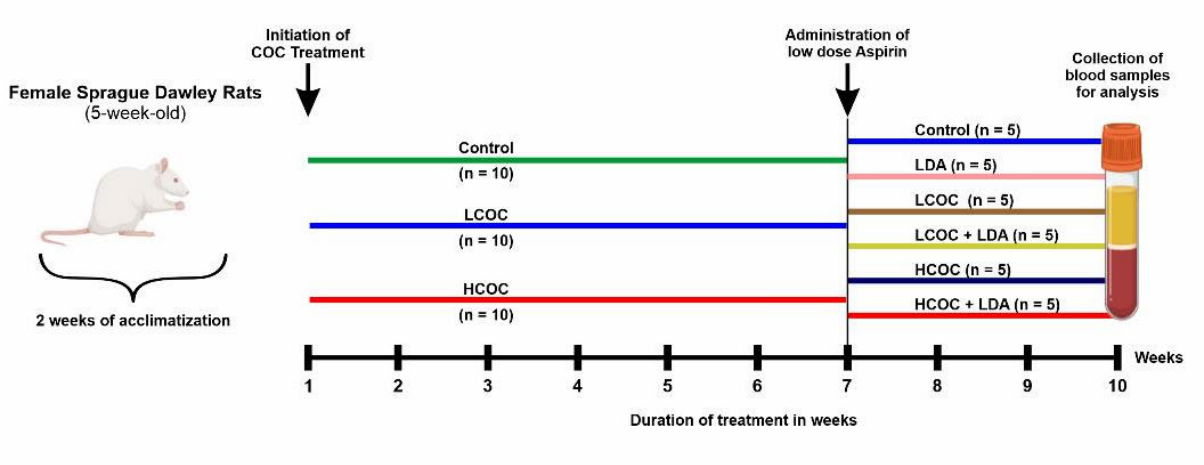

Figure 1: Experimental design. 30 five-week-old female Sprague Dawley rats were used in this study. Firstly, rats were randomly assigned to receive treatment as control, LCOC, and HCOC for six weeks. Thereafter, the rats were randomized to receive LDA treatment for another four weeks and were compared to those that were only given LCOC or HCOC without treatment with LDA to give the total experimental period to be ten weeks. Whereas rats that received distilled water (as a vehicle) and LDA only served as controls. The animal weights and metabolic status were monitored weekly.

2.3. Biochemical analysis, including insulins, inflammatory markers and lipid profiles

Plasma levels of insulin, IL-6, TNF-α, MCP-1, tissue factor (TF), D-dimer, nitric oxide, and von Willebrand factor (VWF) were determined using an enzyme-linked immunosorbent assay kit (Elabscience Biotechnology Co., Ltd., Houston, USA). High-density lipoprotein (HDL) was determined using a high-density lipoprotein Quantitation kit (Sigma–Aldrich, St. Louis, Missouri, USA), while triglycerides level was determined using the triglyceride assay kit (Elabscience Biotechnology Co., Ltd., Houston, USA), according to the manufacturer's protocol. The plasma levels of very low-density lipoprotein (VLDL) were determined using the formula described by Friedewald et al [36].

2.4. Oral glucose tolerance test (OGTT) and insulin resistance (IR)

Briefly, the OGTT was performed following a 12-hour overnight fast. Blood samples were collected using the tail prick method before glucose load, and blood glucose level was measured by a OneTouch® Select® handheld glucometer (LifeScan Inc., Milpitas, California, USA). Subsequently, glucose (2 g/kg bw) was administered orally (po), and blood glucose levels were determined after 15, 30, 60, 90, and 120 min as previously described [34,35]. The IR was determined using the homeostasis model assessment for insulin resistance (HOMA-IR) and triglyceride-glucose index (TyG) [36,37].

2.5. Measurement of hemodynamic variables

Hemodynamic parameters, including systolic and diastolic pressure as well as the mean arterial pressure (MAP) and heart rate were determined via the tail-cuff method using the non-invasive digital blood pressure monitoring system (BIOPAC System, NTBP250, California, USA). The animals were placed in a restrainer with a cuff attached to a heated tail to allow effective adaptation and habituation. During the day of measurement, the animals were kept warm at ± 37 ^{0}C in an enclosed chamber (IITC Model 303sc Animal Test Chamber, IITC Life Sciences, Woodlands Hills, California, USA) for 15 min to make the pulsations of the tail artery detectable before blood pressure recording. All measurements were conducted at a consistent time before noon to avoid diurnal variation. The MAP was calculated using the formula as described previously [38].

2.6. Determination of hematological profiles and hemostatic assessment

The hematological parameters were measured using the Beckman Coulter AcT5 Diff (Beckman Coulter, Miami, USA). The bleeding time was used determine hemostatic changes in the animals which was performed via the tail prick method. We recorded the bleeding time as previously described [39]. The tail of the animals was warmed using a water bath and the temperature was maintained between 37.5°C - 40°C. A small incision was made in the middle of the lower portion of the tail with a scalpel. Bleeding time was recorded when the first drop of blood was collected. Bleeding time was monitored at 30 secs intervals until bleeding stopped.

2.7. Statistical analysis

All experimental data were expressed as means ± standard error of the mean (SEM). Normality testing was performed using the Kolmogorov-Smirnov test with Dallal-Wilkinson-Lillie. In the treatment groups, one-way analysis of variance (ANOVA) was used for the comparison of the mean values of measured variables. A post hoc Tukey's multiple comparisons test was performed if the F-value reached statistical significance ($p < 0.05$). The GraphPad Prism version 8.0 software (GraphPad Software Inc, California, USA) was used for all statistical analysis.

3. Results

3.1. LDA improved the metabolic status of COC-treated rats.

The body weight as well as the visceral adiposity were comparable across the experimental groups ($p > 0.05$) (Table 1). The food intake varied across the experimental groups respectively ($F_{(5, 24)} = 5.6$; $p = 0.002$) (Table 1). The post-hoc analysis showed significantly increased food intake in groups receiving HCOC only when compared with the experimental control, LDA only and LCOC only ($p < 0.05$). The combination of LDA and COC treatment did not affect the food intake when compared with LCOC or HCOC only (Table 1). The water intake also varied across the experimental groups respectively (Table 1) ($F_{(5, 24)} = 5.6$; $p < 0.001$). The post-hoc analysis demonstrated significant increased water intake in the LCOC and HCOC only groups when compared with the experimental control and LDA only groups ($p < 0.05$). The combination of LCOC+LDA and HCOC+LDA treatment showed no significant effect on water intake when compared with LCOC and HCOC-only groups (Table 1).

3.2. LDA treatment improved the glucose metabolism of rats receiving COC treatment.

The fasting glycemia was comparable across the experimental groups ($p > 0.05$) (Table 1). The 2hr oral glucose tolerance test (OGTT) ($F_{(5, 24)} = 5.5$; $p = 0.002$) (Figure 2A) and the postprandial area under the curve (AUC) ($F_{(5, 24)} = 5.1$; $p = 0.003$) (Table 1) also varied across experimental groups. In the post-hoc analysis, HCOC showed a significant elevated 2-h post load glycemia (as measured by OGTT) when compared with the experimental control and LDA only group ($p < 0.05$), the combination of HCOC+LDA treatment improved OGTT when compared HCOC only ($p < 0.05$) (Figure 2A). The post-hoc analysis also showed a significant increase in postprandial AUC in the HCOC group when compared with the

experimental control and LDA-only groups ($p < 0.001$) (Table 1), the combination of HCOC+LDA treatment led to a significant decrease in the postprandial AUC when compared with HCOC only ($p < 0.05$) (Table 1). Furthermore, there were significant changes in fasting plasma insulin level ($F_{(5, 24)} = 5.4$; $p = 0.002$), HOMA-IR ($F_{(5, 24)} = 4.9$; $p = 0.003$) and TyG index ($F_{(5, 24)} = 4.9$; $p = 0.003$) across the experimental groups. In the post-hoc analysis, the HCOC group showed a significant increase in the fasting plasma insulin and HOMA-IR when compared with the experimental control ($p < 0.001$) and LDA-only group respectively ($p < 0.05$) (Figure 2B-C). HCOC group also showed a significantly increased TyG index level when compared with the experimental control group ($p < 0.05$) (Figure 2D). The combination of LCOC+LDA and HCOC+LDA treatment did not significantly impact fasting insulin, HOMA-IR and TyG index when compared with the LCOC and HCOC-only group.

3.3. LDA treatment improved the lipid profile of COC-treated rats.

The lipid profiles, including the HDL ($F_{(5, 24)} = 4.5$; $p = 0.005$), triglycerides ($F_{(5, 24)} = 4.7$; $p = 0.004$) and VLDL ($F_{(5, 24)} = 4.7$; $p = 0.004$) all varied across the experimental groups (Table 1). Briefly, the post-hoc analysis showed a significant decrease in HDL levels in the HCOC group when compared with the experimental control and LDA-only group ($p < 0.05$) (Table 1). However, LCOC+LDA and HCOC+LDA treatment did not significantly impact HDL levels when compared with the LCOC and HCOC-only group (Table 1). The post-hoc analysis also showed significantly higher levels of triglycerides and VLDL in HCOC group when compared with the experimental control, LDA only ($p < 0.05$) (Table 1). HCOC+LDA treatment significantly reduced the levels of triglycerides and VLDL when compared HCOC only group ($p < 0.05$) (Table 1). LCOC+LDA treatment showed no significant impact on the levels of triglycerides and VLDL when compared to LCOC only group (Table1).

Table 1. Anthropometric measurements and metabolic characteristics of rats on combined oral contraceptive (COC) following short-term low-dose aspirin (LDA) treatment (n=5/group)

Parameters	Control	LDA	Low COC	High COC	Low COC + LDA	High COC + LDA	p-value
Anthropometric							
Initial body weight (g)	189.2 ± 21.1	188.4 ± 15.6	183.4 ± 10.9	189.4 ± 15.7	181.2 ± 19.7	187 ± 11.8	0.952
Final body weight (g)	290.2 ± 10.5	307.8 ± 14.5	291.2 ± 22.4	310.8 ± 16.8	288.4 ± 13.9	288.8 ± 21.5	0.152
Weight gain (%)	54.6 ± 12.9	64.2 ± 13.2	58.8 ± 4.97	64.6 ± 12.5	60.4 ± 18.4	54.6 ± 13.1	0.724
Visceral adipose tissue (%)	1.3 ± 0.56	1.8 ± 0.95	1.1 ± 0.34	1.7 ± 0.46	1.5 ± 0.4	1.7 ± 0.7	0.470
Metabolic profile							
Food intake (g/kg/day)	66.9 ± 2.5	64.8 ± 3.1	68.1 ± 5.3	76.8 ± 4.4 **a b c e**	67 ± 4.98	71.2 ± 3.6	**0.002**
Water intake (ml/kg/day)	112.4 ± 3.6	106.1 ± 4.7	127.8 ± 6.7 **a b**	131.3 ± 8.5 **a b**	123.6 ± 5.7 **b**	123.6 ± 9.7 **b**	**<0.001**
Fasting glycemia (mmol/L)	3.52 ± 0.8	4.1 ± 0.7	4.2 ± 0.9	4.14 ± 0.3	4.1 ± 0.44	3.78 ± 0.43	0.530
AUC glucose (mmol/L x 120min)	588.3 ± 33.7	579.8 ± 34.8	653.7 ± 30.9	770.1 ± 36.6 **a b f**	681.2 ± 32.8	594.2 ± 26.3	**0.003**
Lipid Profiles							
High-density lipoprotein (mg/dL)	3.2 ± 0.9	3.3 ± 0.4	2 ± 0.7	1.8 ± 0.3 **ab**	2.9 ± 0.9	2.8 ± 0.6	**0.005**
Very low-density lipoprotein (mg/dL)	13.7 ± 1.8	14.5 ± 2.7	18.6 ± 2.4	20.2 ± 4.4 **a b f**	15.2 ± 1.9	14.3 ± 2.3	**0.004**
Triglyceride (mg/dL)	68.6 ± 9	72.6 ± 13.5	92.9 ± 11.8	100.8 ± 22 **a b f**	75.9 ± 9.4	71.4 ± 11.5	**0.004**

Results are expressed as mean ± SEM. Significance between groups ($^{a}p < 0.05$ vs control; $^{b}p < 0.05$ vs LDA; $^{c}p < 0.05$ vs LCOC; $^{e}p < 0.05$ vs LCOC+LDA; $^{f}p < 0.05$ vs HCOC+LDA) shown in boldface.
*Key: **AUC:** area under the curve.*

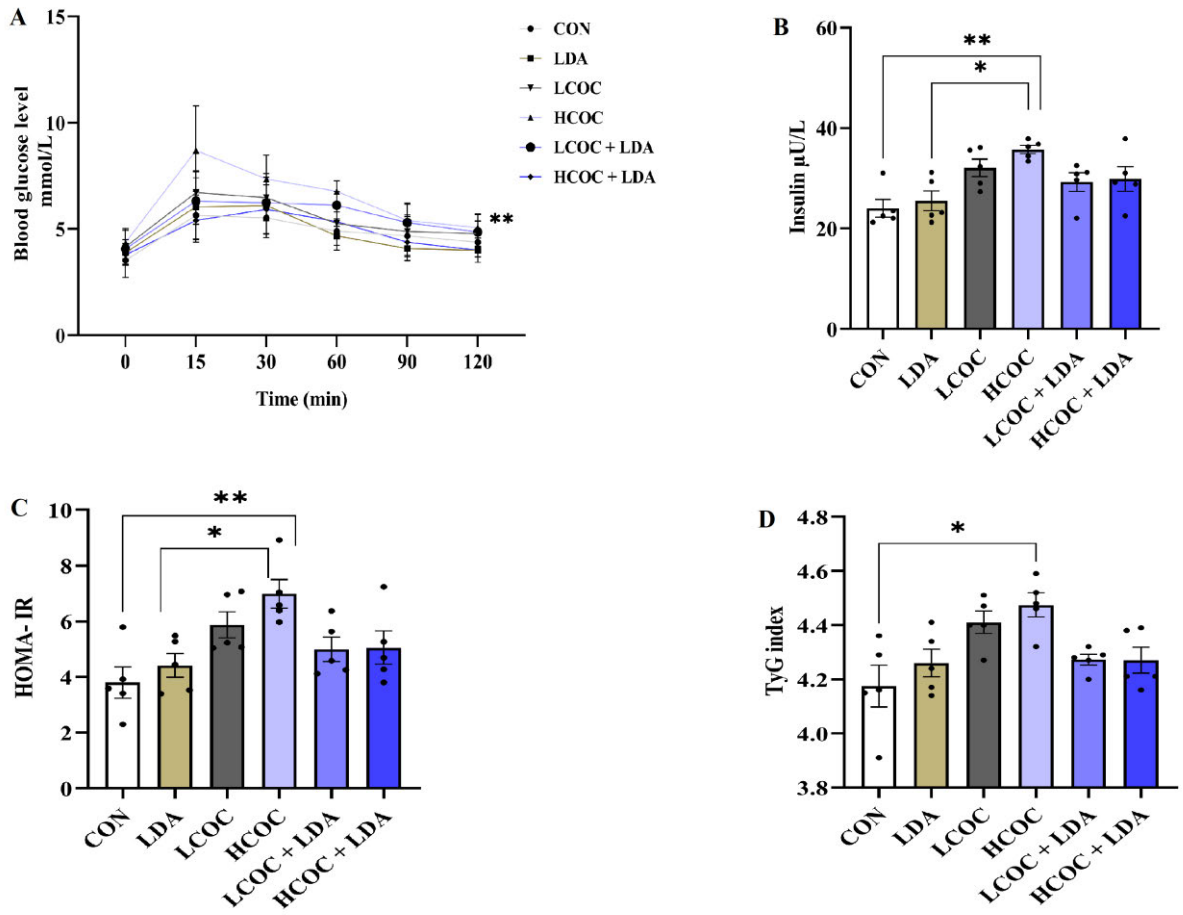

Figure 2: *Effect of low dose aspirin (LDA) and combined oral contraceptive (COC) treatment on* ***(A)*** *2-hour postprandial glucose test;* ***(B)*** *fasting insulin;* ***(C)*** *HOMA-IR; and* ***(D)*** *TyG in rats. All results are presented as mean ± SEM. (**p < 0.001 vs control; *p < 0.05 vs control, LDA).*
Key: ***CON:*** *control,* ***LDA****: low dose aspirin,* ***LCOC****: low dose combined oral contraceptive,* ***HCOC****: high dose combined oral contraceptive.*

3.4. Impact of LDA on the hematological indices of rats treated with COC.

The white blood count, red blood count, hemoglobin, hematocrit, lymphocyte, neutrophil, and monocyte counts were comparable across the experimental groups (Table 2). Platelet count varied across the experimental groups ($F_{(5,\ 24)} = 1.9$; $p = 0.022$). In the post-hoc analysis, platelet count was significantly higher in both HCOC and LCOC groups when compared with the experimental control group ($p < 0.05$), LCOC+LDA and HCOC+LDA treatment did not significantly impact the platelet count levels when compared with LCOC and HCOC only group (Table 2).

3.5. LDA improved hemodynamic changes in rats receiving COC treatment.

The systolic blood pressure ($F_{(5, 24)} = 3$; $p = 0.03$) varied across the experimental groups. The post-hoc analysis showed a significant higher systolic blood pressure in the HCOC group when compared with the experimental control group ($p < 0.05$). The MAP ($F_{(5, 24)} = 3.3$; $p = 0.02$) also varied across the experimental groups. In the post-hoc analysis, MAP was significantly higher in the HCOC group when compared with the experimental control and HCOC+LDA groups respectively ($p < 0.05$) (Table 2). However, LCOC+LDA and HCOC+LDA treatment did not significantly impact the systolic blood pressure when compared with the LCOC and HCOC-only group (Table 2). The diastolic blood pressure and heart rate were comparable across the experimental groups (Table 2).

Table 2: Impact of low-dose aspirin (LDA) and combined oral contraceptive (COC) treatment on hematological and hemodynamic parameters in rats (n=5/group)

Parameters	Control	LDA	Low COC	High COC	Low COC + LDA	High COC + LDA	p-value
Hemostatic profile							
Bleeding time (sec)	150 ± 21.2	156 ± 49.3	102 ± 34.2	78 ± 16.4 **a b**	126 ± 25.1	120 ± 21.2	**0.004**
Hematologic indices							
WBC (10^3/uL)	2.96 ± 1.22	1.82 ± 0.35	2.7 ± 1.4	2.96 ± 0.92	2.5 ± 0.56	2.82 ± 0.42	0.36
RBC (10^6/uL)	4.3 ± 0.69	4.6 ± 0.87	4.92 ± 0.22	4.6 ± 0.25	4.84 ± 0.79	4.78 ± 0.18	0.59
HGB (g/dL)	6.92 ± 1.2	6.8 ± 1.1	7.6 ± 0.51	6.5 ± 0.74	7.7 ± 1.1	7.36 ± 0.54	0.22
HCT (%)	23.8 ± 3.7	24.6 ± 3.7	27 ± 0.93	25.3 ± 1.5	27.1 ± 3.4	26.4 ± 1.7	0.33
PLT (10^3/uL)	400 ± 37.4	415.2 ± 56.9	460.2 ± 23**a**	467.4 ± 60.1**a**	431.2 ± 36.5	449.6 ± 31.3	**0.02**
LYMP (%)	88.4 ± 4.3	87.1 ± 2.8	89 ± 2.5	90.3 ± 2.5	87.7 ± 2.6	90.4 ± 1.5	0.38
NE (%)	9.5 ± 3.7	11.5 ± 2.72	9.1 ± 1.67	8.2 ± 1.9	11 ± 2.62	8.1 ± 1.62	0.19
MO (%)	1.34 ± 0.49	1.14 ± 0.35	1.98 ± 1.3	1.28 ± 1.1	1.1 ± 0.19	1.38 ± 0.33	0.49
Hemodynamic indices							
SBP (mmHg)	124.8 ± 5.5	128.8 ± 7.3	134 ± 7.8	142.2 ± 6.5 **a**	131 ± 10.3	130.6 ± 7.4	**0.02**
DBP (mmHg)	91.4 ± 5.5	91 ± 2.7	91.4 ± 1.5	95.4 ± 2.2	90.4 ± 3.4	88.8 ± 4	0.12
MAP (mmHg)	102.2 ± 3.6	103.6 ± 3.4	105.6 ± 3	111 ± 2.6 **a e**	104 ± 5.2	102.8 ± 5.1	**0.02**
HR (bpm)	328 ± 17.6	345.4 ± 14.5	361.8 ± 32.42	363.2 ± 28.2	349 ± 32.6	336.8 ± 14.8	0.21

Results are expressed as mean ± standard error. Significance between groups ($^a p < 0.05$ vs control; $^e p < 0.05$ vs LCOC+LDA) shown in boldface.
***Key: WBC:** white blood count; **RBC**: red blood count; **HGB:** hemoglobin; **HCT** hematocrit; **PLT:** platelet; **LYMP**: lymphocyte; **NE:** neutrophil; **MO:** monocyte; **SBP:** systolic blood pressure; **DBP:** diastolic blood pressure; **MAP**: mean arterial pressure; **HR:** heart rate.*

3.6. LDA improved the haemostatic profile of COC-treated rats.

The bleeding time varied across the experimental groups ($F_{(5, 24)} = 4.8$; $p = 0.004$). In the post-hoc analysis, the HCOC group demonstrated a significant reduction in bleeding time when compared with the experimental control and LDA-only groups ($p < 0.05$), LCOC+LDA and HCOC+LDA treatment did not significantly impact the bleeding time when compared with LCOC and HCOC only group (Table 2). Furthermore, plasma levels of TF ($F_{(5, 24)} = 5.1$; $p = 0.002$) and D-dimer ($F_{(5, 24)} = 5.1$; $p = 0.003$) also varied across the experimental groups. In the post-hoc analysis, TF levels were significantly higher in the HCOC group when compared with the experimental control, LDA-only groups ($p < 0.05$) (Figure 3A). Meanwhile, the combination of HCOC+LDA treatment significantly reduced TF levels when compared with the HCOC-only group ($p < 0.05$) (Figure 3A). More so, plasma levels of D-dimer were significantly higher in the HCOC group when compared with experimental control and LDA-only groups ($p < 0.05$) (Figure 3B). The combination of HCOC+LDA treatment significantly reduced D-dimer levels when compared with the HCOC-only group ($p < 0.05$) (Figure 3B). However, LCOC+LDA did not significantly impact the levels of TF and D-dimers when compared with LCOC-only group (Figure 3A-B).

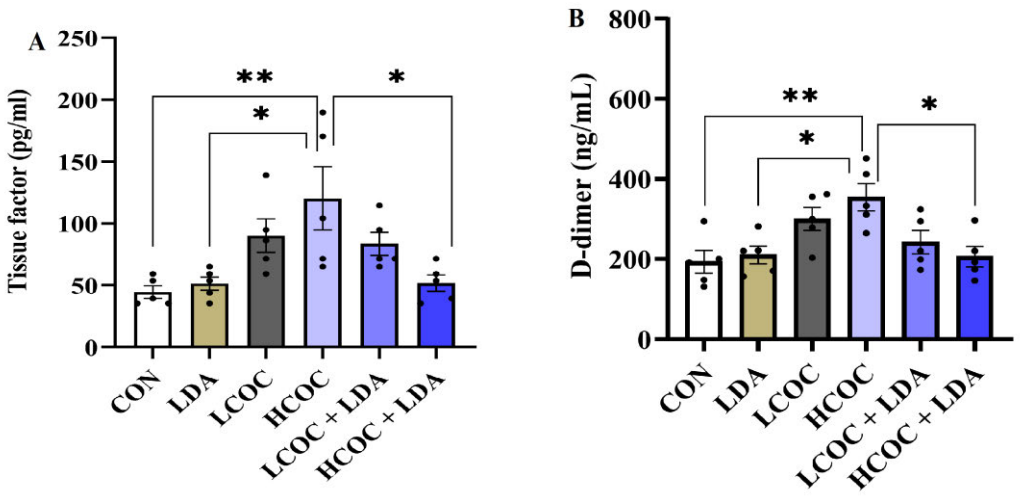

Figure 3: *Effect of low-dose aspirin (LDA) and combined oral contraceptive (COC) treatment on* ***(A)*** *tissue factor* ***(B)*** *D-dimer All results are presented as mean ± SEM. (**p < 0.001 vs control; *p < 0.05 vs control, LDA, HCOC+LDA).*
Key: CON: *control,* ***LDA:*** *low dose aspirin,* ***LCOC:*** *low dose combined oral contraceptive,* ***HCOC:*** *high dose combined oral contraceptive.*

3.7. LDA ameliorate the inflammatory changes in COC-treated rats.

The plasma levels of IL-6 ($F_{(5, 24)} = 5.5$; $p = 0.002$), TNF-α ($F_{(5, 24)} = 5$; $p = 0.003$) and MCP-1 ($F_{(5, 24)} = 5.1$; $p = 0.003$) all varied across the experimental groups. The post-hoc analysis showed a significantly increased level of IL-6, TNF- α, and MCP-1 in the HCOC group when compared with the experimental control ($p < 0.01$) and LDA-only groups ($p < 0.05$) (Figure 4A-C). Meanwhile, the combination of HCOC+LDA treatment significantly reduced IL-6, TNF- α, and MCP-1 levels when compared with the HCOC-only group ($p < 0.05$) (Figure 4A-C). However, LCOC+LDA did not significantly impact the levels of IL-6, TNF- α, MCP-1 when compared with LCOC-only group (Figure 4A-C).

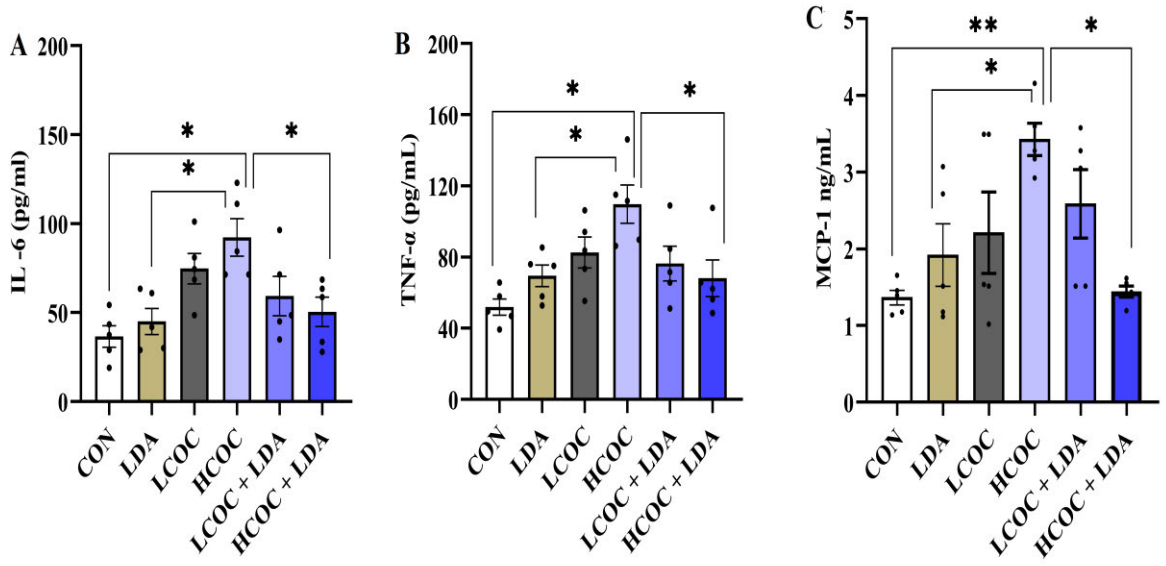

Figure 4: *Effect of low-dose aspirin (LDA) and combined oral contraceptive (COC) treatment on* ***(A)*** *IL-6* ***(B)*** *TNF-α* ***(C)*** *MCP-1 in rats. All results are presented as mean ± SEM. (**p < 0.001 vs control; *p < 0.05 vs control, LDA, HCOC+LDA).*
Key: IL-6: *interleukin 6;* ***TNF-α****: tumour necrosis factor-alpha;* ***MCP-1:*** *monocyte chemoattractant protein-1;* ***CON:*** *control,* ***LDA****: low dose aspirin,* ***LCOC:*** *low dose combined oral contraceptive,* ***HCOC:*** *high dose combined oral contraceptive.*

3.8. LDA improved the endothelia function in rats receiving COC treatment.

The plasma vWF levels ($F_{(5, 24)} = 4.2$; $p = 0.007$) and nitric oxide ($F_{(5, 24)} = 4.3$; $p = 0.006$) also varied across the experimental groups. In the post-hoc analysis, the plasma levels of vWF were significantly higher in the HCOC group when compared with the experimental control, LDA only ($p < 0.05$) (Figure 5A). However, HCOC+LDA treatment significantly reduced vWF levels when compared with the HCOC group respectively ($p < 0.05$) (Figure 5A). More so, the plasma level of nitric oxide was significantly reduced in the HCOC group when compared with the experimental control ($p < 0.05$) (Figure 5B). Meanwhile, HCOC+LDA treatment significantly increased the level of NO when compared to the HCOC group ($p < 0.05$) (Figure 5B). LCOC+LDA did not significantly impact the levels of vWF and nitric oxide when compared with LCOC only group (Figure 5A-B).

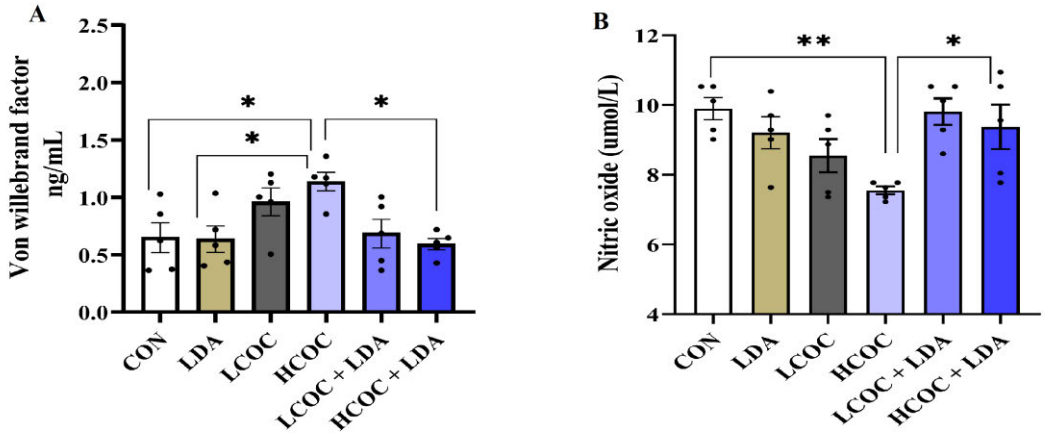

Figure 5: *Effect of low dose aspirin (LDA) and combined oral contraceptive (COC) treatment on* ***(A)*** *Von Willebrand factor;* ***(B)*** *nitric oxide in rats. All results are presented as mean ± SEM. (**p < 0.001 vs control; *p < 0.05 vs control, LDA, HCOC+LDA).*

Key: CON: *control,* ***LDA:*** *low dose aspirin,* ***LCOC:*** *low dose combined oral contraceptive,* ***HCOC:*** *high dose combined oral contraceptive.*

4. Discussion

In this study, we aimed to evaluate the impact of prolonged exposure to COC treatment on the risk factors associated with atherothrombotic disorder and whether short-term LDA treatment will alter these risk factors. Firstly, our study showed significant metabolic dysregulation during prolonged COC treatment in rats, which is known to provoke endothelial dysfunction and a prothrombotic state, thereby aggravating the risk of CVDs [42–44]. Briefly, HCOC treatment in our study led to impaired glucose tolerance, which was accompanied by poor insulin sensitivity, dyslipidemia, and hyperinsulinemia. The outcome of our study corroborates with previous findings in human and animal studies [39,45–47] where the most adverse effect of OC on metabolic changes was associated with the dose of estrogen and androgenicity of the progestin component [48,49]. However, study by Olatunji et al. [47] reported a contradictory finding on the impact of OC on HDL levels in fructose-fed rats where HCOC increased HDL levels, which may be due to the type of COC and duration of treatment, respectively. Nonetheless, the observed metabolic changes also reflect the increased food intake induced by HCOC despite reduced water intake and normal weight gain in our study.

Interestingly, short-term LDA treatment improved the metabolic dysregulation induced by prolonged exposure to HCOC in rats. The observed beneficial impact of LDA treatment on metabolic changes also corroborates previous studies' findings in high-fat-fed rats and patients with T2D [35,50–52]. A study by Abdelsadik and Amin [50] showed a reduction in hypertriglyceridemia, increased insulin sensitivity and glucose uptake in high-fat-fed male rats treated with LDA. A study by Yuan et al. [53] also highlighted the role of aspirin as an insulin-sensitizing agent in reversing hyperglycemia, hyperinsulinemia, and dyslipidemia in high-fat-fed mice. However, LDA treatment did not alter insulin sensitivity in COC-treated

female rats in our study, and this differential response may be attributed to the sex difference and dose regimen, among other factors [23–25].

Furthermore, our study also showed evidence of endothelial dysfunction during prolonged COC exposure in rats, which may have also contributed to the observed change in hemodynamic status, which is known to aggravate the risk of hypertension and other cardiovascular-related events among susceptible individuals [54–56]. Briefly, prolonged HCOC treatment in rats led to an increased plasma level of vWF and reduced nitric oxide bioavailability, which corroborates with other previous findings in rats and human [57–60]. The impact of altered endothelial function by prolonged HCOC exposure may have contributed to our study's observed increase in systolic blood pressure and MAP. For instance, a study by Olatunji et al. [59] showed that the exposure of female rats to COC for six weeks also led to an increased risk of hypertension with concomitant endothelial dysfunction. However, short-term LDA treatment led to a decrease in the MAP of the HCOC-treated rats, which also corroborates with previous findings that showed a reduction in MAP of hypertensive patients that received LDA for six month [61].

The vasoactive effect of estrogen at a high dose can alter the arterial wall's structural integrity by modifying the endothelium's elasticity and permeability, which subsequently switches endothelial cells to a prothrombotic state [62,63]. This can impact the vascular tone and promote vascular smooth muscle cell proliferation, leading to the apparent release of certain endothelial activation markers and undesired inflammatory responses [64–66]. Nonetheless, our study showed improved endothelial function of HCOC-treated rats receiving LDA therapy by reducing vWF plasma levels and promoting nitric oxide bioavailability in the circulation. Animal models of atherosclerosis also suggest that an extremely low or absent vWF level exerts a protective effect on the development and distribution of atherosclerotic

lesions [67]. The outcome of our study also corroborates with previous findings by Homonci and colleagues that showed reduced levels of vWF in individuals on aspirin [68] as well as improved nitric oxide production in patients with metabolic syndrome and coronary artery disease who received various doses of aspirin [69,70]. The beneficial impact of LDA in our evidence further supports the role in the primary prevention of CVD-related events in clinical settings [71].

Moreover, our study also assessed changes in the hematological and hemostatic profile of rats exposed to prolonged COC treatment. Briefly, most of the hematological variables, including white blood count, red blood count, hemoglobin, hematocrit, lymphocyte, neutrophil, and monocyte counts, were comparable across the experimental groups. However, platelet count was significantly higher in both HCOC and LCOC-treated rats. Furthermore, HCOC reduced bleeding time and altered the coagulation cascade by promoting increased TF and D-dimer levels, thereby predisposing the rats to a hypercoagulable state. The outcome of our study is also similar to previous findings that showed increased plasma levels for procoagulants among individuals exposed to COC treatment [72,73]. In a randomized controlled trial by Johnson et al. [73], OC treatment adversely affects vascular risk markers such as D-dimers. A study by Van Vliet and colleagues also showed a decreased level of tissue factor pathway inhibitor in women treated with different OC formulations, and some of the observed differential changes in the level of tissue factor pathway inhibitor (TFPI) were attributed majorly to the progestin component across OC formulations [72,74]. However, a study by Divani et al. [75] did not find any significant change in the levels of TF among individuals on COC despite reduced TFPI levels. TFPI is a glycoprotein produced by endothelial cells which is known to inhibit factor VII/tissue factor (FVIIa/TF) in the coagulation cascade [72].

The release of TF is crucial in the initial stage of the extrinsic coagulation pathway during tissue injury, where they are exposed to several blood components, such as circulating monocytes and also platelets, which facilitate hypercoagulability [76,77]. Elevated levels of D-dimers are a direct consequence of an impaired fibrinolytic pathway, which contributes to the thrombo-inflammatory cycle [78–80]. In our study, LDA treatment significantly reduced the TF and D-dimer levels in HCOC-treated rats, which is similar to previous findings in humans and mice where aspirin treatment reduced TF expression [81]. In contrast, other studies in humans showed no significant change in D-dimer levels during aspirin treatment, and this was linked to a high hypercoagulable state in the cohorts [78,82,83]. The impact of LDA in reducing D-dimer levels in HCOC-treated rats stems from the acetylation of fibrinogen, which impairs fibrin polymerization, thereby enhancing clot lyses and inhibiting the thrombo-inflammatory cycle [78,79,84–86].

Furthermore, our study also showed evidence of immune activation during prolonged COC treatment in rats. Previous evidence suggests a modulatory role of estrogen and progesterone in immune regulation [87–89]. Depending on the concentration, estrogen may enhance the production of pro-inflammatory cytokines (such as IL-1, IL-6, and TNF-α) from monocyte-macrophage cells [90,91]. Briefly, evidence from our study showed HCOC treatment promotes pro-inflammatory response via the release of acute phase reactants such as IL-6, TNF-α and MCP-1, which is similar to previous experimental and human studies [13,92]. A study by Campesi et al. [92] showed a higher release of TNF-α in the macrophages of individuals on COC, which was attributed to the androgenic properties of the progestin component. In contrast, a study by Divani et al. [12] reported that the levels of IL-6 and TNF-α among individuals on COC remain unaffected, which may probably be due to the small sample size population and the age of the participants. A study by Bouman et al. [93] also showed that COC did not influence the cytokine production capacity of LPS-stimulated

monocytes in postmenopausal women. Low androgenic COC may have influenced this outcome. However, a study by Calippe et al. [13] showed that chronic exposure of ovariectomized mice to estrogen treatment increased the levels of pro-inflammatory cytokines (IL-1beta, IL-6, and TNF-α).

Elevated levels of IL-6 and TNF-α are associated with an increased level of MCP-1, and their social interaction is associated with the engineering behind monocyte chemotaxis to the areas of inflammation such as endothelium where they differentiate into resident macrophages [12]. In response to atherogenic stimuli such as hyperglycemia and dyslipidemia, resident macrophages recruit more monocytes that cause vascular smooth muscle proliferation and endothelial dysfunction, which are known to increase the progression of atherogenesis and thrombotic risk [94,95]. In our study, LDA treatment reduced IL-6, TNF-α and MCP-1 plasma levels in HCOC-treated rats. The beneficial impact of LDA in reducing levels of inflammatory markers in COC-treated rats in our study also corroborates with previous studies that showed a reduction in the plasma levels of inflammatory markers in human and experimental animals treated with aspirin [35,50,96]. For instance, randomized controlled trials of aspirin therapy were shown to reduce levels of IL-6 and TNF-α among high-risk patients with cardiovascular disease [96,97]. Similarly, experimental studies also showed aspirin therapy reduced IL-6 and TNF-α in streptozotocin-induced type 2 diabetic rats and high-fat C57BL/6 mice, and the suggested mechanism is associated with inhibiting Nuclear factor kappa-β (NF-κB) activation [35,98]. The limitation of this study is that we did not determine the mechanism by which the interaction between low-dose aspirin and combined oral contraceptives regulates the release of these selected biomarkers. Exploring the interaction mechanism may further provide insight into their therapeutic potential in managing the risk of cardiovascular-related events among susceptible individuals.

5. Conclusion

Effective management of each component of the pathophysiological changes involved in atherothrombotic disorder is required to reduce the overall risk of CVD. Hence, Short-term low-dose aspirin (LDA) demonstrated a promising beneficial impact against the risk of atherothrombotic disorder by attenuating markers of metabolic dysregulation, pro-inflammation, hypercoagulation and endothelial dysfunction in female rats exposed to prolonged high-dose COC.

Acknowledgments

The authors are grateful to the Biomedical Resource Unit, University of Kwazulu-Natal, UKZN, for the technical assistance provided.

Funding

None

Disclosure statement

The authors declare no conflicts of interest.

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Chapter 6: General discussion and conclusion

Oral contraceptives remain one of the most commonly prescribed methods of birth control amongst women of reproductive age due to their safety profile [1]. The rising prevalence of obesity over the past decade [2], including its associated risk factors such as cardiovascular-related complications [3–6] has provided a need to understand how oral contraceptives affect such individuals. Increasing research is directed at establishing the potential link between prolonged use of COC and the high risk of thrombotic events in women of reproductive age [7,8]. Of note, certain individuals living with obesity may be at risk of experiencing thrombotic events [9–12] and a clear understanding is required as to whether the use of COC can aggravate the risks of thrombotic events [13,14]. With that being said, specific factors and conditions must be considered when administering COC to patients or individuals in need [14–16]. Of note, depending on the ongoing and future hormonal contraceptive choices being made by an individual, anticoagulants may be beneficial in some cases to protect against future thrombosis and its associated risk factors [17–19]. However, several guidelines on the use of some of the available anticoagulants, like LDA, are inherently inconsistent, especially when the dose, duration of use and risk of excessive bleeding are considered [20–23].

To dig and understand the above-mentioned inconsistencies, we simultaneously followed an evidence-based approach of developing a protocol to review systemically and quantitively analyzed available information on the impact of COC use and the risk of cardiovascular diseases among premenopausal women [24]. Surprisingly, the primary finding of this systematic information analysis supported a strong association between COC and the prevalence of traditional cardiovascular risk factors such as blood pressure, lipid profile, and body mass index with little to no difference in the risk of endothelial dysfunction [25].

However, the certainty of our evidence was quite low, and there was a lack of reported effects of various types of COC on measures of immune activation, coagulation, and endothelial function in these human studies. Be it as it may, we conducted several experimental studies to establish a link between COC use and the risk of cardiovascular disease in an impaired metabolic state. This involved exposing rats to different concentrations of COC for a predetermined period before assessment of metabolic status, immune activation, endothelial dysfunction, and hypercoagulability in a diet-induced obesity (DIO) experimental model. We also determined whether a dietary intervention (switching from a high-fat diet to a low-fat diet) or a pharmacological intervention (LDA) could alleviate the cardiovascular disease (CVD) risk by lowering thrombotic factors within this rat experimental model.

6.1. Implication of short-term COC use on metabolic status in an experimental model of a high-fat diet.

Obesity is associated with metabolic disorders and low-grade inflammation due to compromised adipose storage and gut diversity [21,26–29]. In obesity, monocyte activation usually favors the resident macrophage (M1) cell polarization, causing the release of pro-inflammatory cytokines and undesired immune activation [30]. The use of COC can also promote weight gain and alter immunological pathways, which may exacerbate the risk of arterial and venous thrombosis in susceptible women of reproductive age [31–34]. Despite conflicting data on the efficacy of COC use in obese individuals, lifestyle modification such as dietary intervention may be required to subdue several cardiovascular risk factors in high-risk individuals. In this study, we reported on obesity following a high-fat diet (HFD) for eight weeks, which was associated with impaired glucose and lipid metabolism in rats. This intervention could potentially lead to a marked increase in CVD-related factors such as high blood pressure and other complications such as weight gain and impaired metabolic status.

Nonetheless, our study showed evidence of elevated acute inflammatory responses without concomitant change in the vicious mechanism involving monocyte-driven tissue infiltration following HFD feeding in rats. The fact that there wasn't a significant association between the triglyceride-glucose index and monocyte chemoattractant protein-1 also aligned with the non-concomitant change in the vicious mechanism that is behind monocyte trafficking. However, upon dietary modification (switching to a low-fat diet) followed by short-term high-dose COC treatment for six weeks, metabolic dysregulation via hyperinsulinemia was persistent without changes in immune activation in HFD-fed rats. Possibly indicating that prolonged use or high doses of COC could be detrimental in those with impaired metabolic status. This has also been investigated in individuals, and the use of COC was associated with widespread metabolic and inflammatory dysregulations [35]. However, persistent use was not associated with cumulative dysregulations over time, and the metabolic perturbations were reversed upon discontinuation [35]. Thus, our study brings a unique perspective in understanding the potential detrimental effects of the prolonged use of oral contraceptives in conditions of impaired glucose tolerance despite diet modification. Additional studies, especially in clinical settings, are required to confirm this narrative, which will be vital to inform on the current policy by the World Health Organization on medical eligibility criteria and the national contraception clinical guidelines for contraceptive use among women in need of family planning depending on the clinical condition [15,16].

6.2. Impact of short-term COC use on thrombotic profile, including hypercoagulability and endothelial dysfunction in an experimental model of a high-fat diet.

The use of COCs in women living with obesity can lead to several complications that encompass alterations to blood flow, hypercoagulability, and endothelial dysfunction, which

exacerbates the risk of arterial and venous thrombotic events [36,37]. In the current WHO and national contraception clinical guidelines, it is important to check blood pressure before the initiation of COCs among women in need of family planning. In our study, the systolic and diastolic blood pressure, including the heart rate, were not affected by eight weeks of HFD as well as the markers of coagulation and endothelial activation, such as tissue factor and von Willebrand factor, except for nitric oxide that, was significantly reduced in our experimental model. However, the mean arterial pressure was significantly higher in obesity and was associated with hypercoagulability (tissue factor, D-dimer) and endothelial dysfunction markers like NO. Surprisingly, short-term treatment with COC showed no effects on the hemodynamic, hemostatic, and endothelial activation markers in HFD-fed rats. Our result also corroborates the findings of other previous studies in humans that reported no major changes in the hemodynamic and endothelial activation makers among COC users [38,39]. Thus, our study brings a unique perspective in understanding the potential effects of the prolonged use of oral contraceptives on multifactorial complications that can exacerbate the risk of arterial and venous thrombotic events in susceptible women of reproductive age. In lieu of these, regular blood pressure monitoring in clinical settings may be required where feasible to help make a better-informed decision for women needing family planning when considering the choice of available COC in association with the duration of use [15,16].

6.3. Modulatory role of LDA following long-term COC treatment and the risk of atherothrombotic disorder.

Recent guidelines on the use of aspirin for the primary prevention of cardiovascular events recommend the use of low-dose aspirin among susceptible individuals at high risk of CVD while considering the anticipated risk of excessive bleeding [40–44]. In our study, short-term four-week LDA treatment improved impaired glucose metabolism and reduced

hypertriglyceridemia during prolonged exposure to high COC treatment. LDA treatment also decreased MAP in rats treated with high doses of COC. LDA treatment also improved the coagulation cascade's prothrombotic state and endothelial function by decreasing vWF levels and enhancing nitric oxide bioavailability in high-dose COC-treated rats. The fact that women living obese are at higher risk of CVD, high-dose COC is not advisable for long-term use as monotherapy when viewed from the perspective of effectiveness and benefit. The outcome of our study also aligns with the findings of other previous studies that reported an increased risk of cardiovascular events after prolonged COC exposure among susceptible individuals [45–47]. Thus, our study brings a unique dimension to understanding the potential benefits of aspirin in the management of cardiovascular events that are associated with the long-term use of oral contraceptives in susceptible women of reproductive age.

6.4. Conclusion and future perspective

This current study demonstrated metabolic impairment and immune activation characterized by increased lee index and abdominal circumference, poor glucose uptake, altered lipid metabolism and increased plasma levels of IL-6 and TNF-α without concomitant change in the levels of MCP-1 in the DIO model. Moreover, there was an association between the Lee index and the acute phase proteins (IL-6, TNF-α and MCP-1) and between triglyceride-glucose index (TyG), IL-6 and TNF-α. These outcomes further support the interdependent relationship between metabolic impairment and immune activation in obesity. Meanwhile, when dietary intervention was introduced, followed by short-term high-dose COC treatment, metabolic impairment via hyperinsulinemia persisted without concomitant immune activation in HFD-fed rats, which also provided insight into the detrimental impact of long-term COC usage and poor glucose control among susceptible individuals. Furthermore, HFD-feeding in rats was also associated with an increased mean arterial pressure and reduced nitric oxide

levels. Interestingly, short-term high-dose COC treatment of these HFD-fed rats did not significantly affect atherothrombotic markers. Interestingly, these findings mirrored those found in our systematic review, where COC treatment in premenopausal women resulted in little to no difference in markers of endothelial dysfunction such as nitric oxide [9]. Notably, the outcome of our study is limited in terms of generalizability because the study was conducted on rats, which may not accurately reflect the effects of HFD-feeding and COC treatment in humans. Another notable limitation of our study is the lack of a measured parameter baseline. Thus, further exploration of the impact of other types of COC and HFD feeding is needed to confirm these findings in different conditions and provide insight and recommendations during prescription in a well-defined population study.

The findings of our study also showed the therapeutic benefit of LDA therapy in potentially reducing some of the markers associated with the development of atherothrombotic disorder during long-term COC treatment. LDA treatment improved metabolic impairment, prothrombotic state, and endothelial function in high-dose COC-treated animals. The outcome of our study depicts the notion that the impact of COC treatment depends on the type of progestin, dosage, and duration of use. Meanwhile, the introduction of LDA therapy in our study provides a unique understanding of the impact and the potential benefit regarding its recommendation for primary prevention of cardiovascular diseases (CVDs) among susceptible individuals. One of the limitations of our study is the lack of tissue-resident cell characterization, which may play a role in the observed effects. This limits the understanding of the mechanisms underlying the results. However, future studies are needed to confirm these findings and understand the mechanism of interaction between aspirin therapy and other different types of COC regimens.

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Appendix

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

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