



# Differential expression of glycoprotein IV on monocyte subsets following high-fat diet feeding and the impact of short-term low-dose aspirin treatment

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## ABSTRACT

**Objective:** To assess the levels of glycoprotein GPIV (CD36) expression on peripheral blood monocyte subsets, in a mouse model of glucose intolerance. Moreover, to determine the effect of; low-dose aspirin (LDA) alone, LDA combined with metformin, or clopidogrel alone, on the expression of CD36 on subsets of circulating monocytes.

**Method:** The study consisted of two experimental phases. In experiment one, the mice ( $n = 14$ ) were randomised to receive a low-fat diet (LFD) or a high-fat diet (HFD) for eight weeks. Whereas the secondary phase of the experiment, comprised of twenty-four HFD-fed mice treated with LDA alone (3 mg/kg), or in combination with metformin (150 mg/kg), or clopidogrel alone (10 mg/kg) for six weeks. The surface expression of CD36 on monocytes was measured using flow cytometry.

**Result:** The levels of CD36 expression on monocytes were upregulated in the HFD-fed compared to LFD-fed group ( $p < 0.05$ ). In addition, HFD group showed; no significant changes in body weight ( $p = 0.3848$ ), however, blood glucose ( $p = 0.0002$ ) and insulin ( $p = 0.0360$ ) levels were markedly increased following HFD-feeding. Interestingly, all treatments reduced the expression of CD36 on monocytes, decreased fasting blood glucose levels ( $p = 0.0024$ ) and increased circulating monocyte levels ( $p = 0.0217$ ) when compared to the untreated HFD group. Moreover, treatment with LDA alone increased basophils levels ( $p = 0.0272$ ), while when combined with metformin showed an improved effect in enhancing eosinophil levels ( $p = 0.0302$ ).

**Conclusion:** HFD-feeding increased the expression of CD36 on monocyte subsets. LDA as a monotherapy or combined with metformin was as effective as clopidogrel monotherapy, in downregulating the expression of CD36 on monocyte subsets. These treatments may be of relevance in preventing cardiovascular complications associated with impaired glucose tolerance.

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## 1. Introduction

Chronic inflammation and the persistent activation of peripheral monocytes and neutrophils are associated with the pathogenesis of type 2 diabetes mellitus (T2DM). Activated monocytes can further initiate atherosclerosis and increase the risk of complications in people living with T2DM [1–3]. For example, the

monocyte chemoattractant protein 1 (MCP-1) plays a pivotal role in the recruitment of monocytes in response to atherogenic stimuli such as hyperglycaemia and dyslipidaemia [4]. The efficacy of low-dose aspirin (LDA) in combination with clopidogrel in the secondary prevention of cardiovascular disease (CVD) has been reported [5,6]. In addition, the pleiotropic properties of metformin, a widely used first-therapy drug in patients with T2DM, include both anti-inflammatory and anti-thrombotic properties [7]. Metformin has also been shown to suppress the production of tumour necrosis factor-alpha (TNF)- $\alpha$  and reactive oxygen species (ROS), while also

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downregulating the expression of the membrane glycoprotein IV (CD36) [8]. Importantly, CD36 is a class B scavenger receptor that plays a crucial role in innate immune responses [9,10], including the modulation of lipid metabolism and inflammation [9,11].

The ligation of CD36 by oxidised low-density lipoprotein (oxLDL) initiates the formation of foam cells with the subsequent formation of an atherosclerotic plaque [12]. In such a case, CD36 plays an essential role in the development of atherosclerosis [1]. Notably, in hyperglycaemic states, activated monocytes can upregulate the expression of CD36 [9], which has a high affinity for oxLDL and apoptotic cell surfaces [13,14], as well as advanced glycation end products (AGEs) [15]. Furthermore, the levels of the soluble form of CD36 (sCD36) are increased in patients living with T2DM, and can also accelerate the risk of CVD in these patients [16,17]. Currently, the use of low-dose aspirin (LDA) in the primary prevention of cardiovascular events in patients living with T2DM remains controversial [18]. Interestingly, LDA has been shown to partially downregulate sCD36 levels, consistent with reduction of platelet activation in patients with T2DM [9]. Furthermore, a recently published preclinical study showed beneficial effects of LDA in suppressing vascular inflammation and maintaining atherosclerotic plaques stability in LDL receptor-deficient mice [19].

Although CD36 is highly expressed on monocytes and upregulated in atherosclerosis and T2DM, it remains unclear whether the levels of circulating monocyte subsets differentially express CD36 in a state of impaired glucose tolerance. To date, the differential expression of CD36 expression on circulating monocyte subsets in a state of impaired glucose metabolism remains unclear. The enumeration and quantification of monocyte subsets in a state of impaired glucose tolerance may allow for risk stratification of individuals at increased risk of atherosclerosis. In this study, short term high-fat diet (HFD) was used to induce glucose intolerance in C57BL/6 male mice, which is an important model to study complications linked with early development of T2DM, as demonstrated elsewhere [20,21]. Therefore, the current study reports on the expression of CD36 on monocyte subsets in HFD fed mice with glucose intolerance. Furthermore, we assessed whether treatment with; low-dose aspirin (LDA) alone; LDA in combination with metformin or clopidogrel alone could modulate the expression of CD36 on monocyte subsets in glucose tolerance.

## 2. Materials and methods

### 2.1. Animal care and feeding

In this study, six-week-old male C57BL/6 mice ( $n = 36$ ) were purchased from the Biomedical Research Unit (BRU) at the University of KwaZulu-Natal, South Africa. The UKZN Animal Research Ethics Committee (AREC) approved the animal experiments and study procedures, complying with existing UKZN animal handling standards which are in accordance to the principle and guidelines of Canadian Council on Animal Care (protocol number: AREC/086/016) and reported as per Animal Research Reporting *In Vivo* Experiments (ARRIVE) guidelines (Supplementary doc 1). The animals were housed in a temperature-regulated room ( $22 \pm 2^\circ\text{C}$ ) in a 12-h light and 12-h dark cycle (light was switched on from 06:00 p.m.-06:00 a.m.). After a 1-week acclimatisation period, the mice were assigned into two diet groups matched for macronutrient type and source. These mice were enclosed in clean cages ( $n = 6/\text{cage}$ ). In addition, they had unlimited access to water, food pellets and fresh air throughout the experiments.

#### 2.1.1. The diet composition of a high and low-fat diet

Low-fat diet (LFD; D12450J) contained 20% kcal protein, 70% kcal carbohydrate and 10 kcal% of fat. While the high-fat diet (HFD;

D12492J) contained 20% kcal protein, 20% kcal carbohydrates and 60 kcal% of fat.

### 2.2. Study design and experimental protocols

The experiments comprised of two phases (Fig. 1). The first phase of the experiment included mice ( $n = 14$ ) with six kept on a low-fat diet (LFD), and eight mice kept on HFD for eight weeks (Fig. 1A). The second phase of the experiment comprised of mice ( $n = 24$ ) kept on an HFD diet for eight weeks (Fig. 1B). In the second phase of the study, the animals were randomly assigned into four groups ( $n = 6/\text{group}$ ) comprised of an untreated HFD group and three treatment groups, following short-term six-week treatment.

**Experimental phase 1:** In the first phase of the experiment, we measured peripheral blood monocyte subtypes and basal CD36 expression on classical and pro-inflammatory monocytes following a short-term HFD-feeding. In this phase of the study, fourteen six-week-old male C57BL/6 mice were assigned into two experimental diet groups, the LFD ( $n = 6$ ) and HFD ( $n = 8$ ). The animals were kept on the respective diets for eight weeks (Fig. 1A). We then measured the haematological parameters using the Act 5 diff haemo-analyser (Beckman Coulter, Brea, CA, USA). Plasma insulin and glucose levels were measured at experimental week 8, while body weights were measured weekly. The oral glucose tolerance test (OGTT) was also performed at experimental week 8, following previously described methods [22]. All glucose measurements were performed using the OneTouch® Select® handheld glucometer (LifeScan Inc., Milpitas, CA, USA). While fasting serum insulin levels were determined following, using an enzyme-linked immunosorbent assay (ELISA) kit (Thermo Fisher, Massachusetts, U.S.A).

**Experimental phase 2:** To determine whether the anti-inflammatory and antithrombotic drugs, modulate the expression of CD36 on monocyte subsets in conditions of impaired glucose tolerance. A total of 24 HFD-fed mice were randomly assigned into four groups ( $n = 6/\text{group}$ ); (i) untreated HFD; (ii) LDA alone (3 mg/kg); (iii) LDA in combination with metformin (150 mg/kg); (iv) clopidogrel alone (10 mg/kg) (Fig. 1B). All drugs were administered daily, for six weeks via oral gavage. Blood was drawn after six weeks of treatment, and the analysis of glycoprotein IV (CD36) was performed using flow cytometry (Fig. 1 and S). The animals were then euthanised using halothane.

#### 2.2.1. Biochemical and haematological analysis

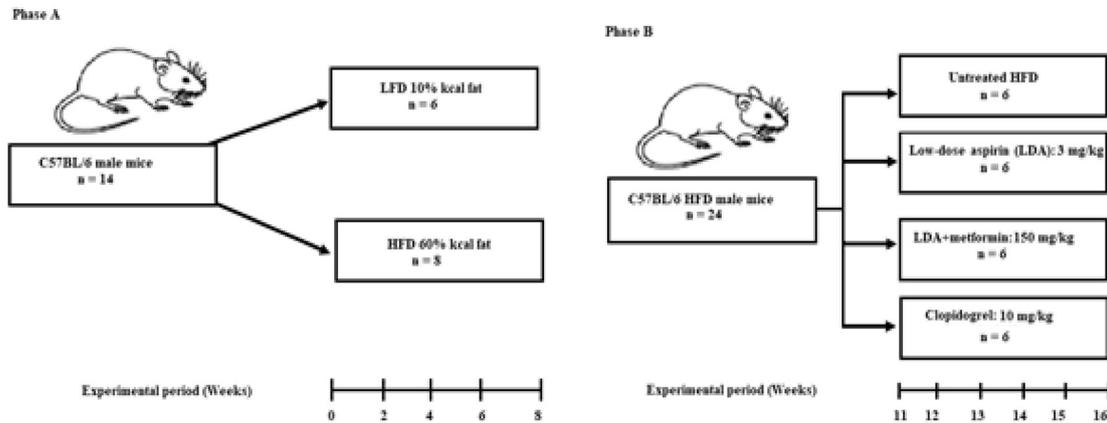
Blood (200  $\mu\text{l}$ ) was drawn from the lateral tail vein into serum separator (SST) and ethylenediaminetetraacetic acid (EDTA) microtainer tubes (BD Bioscience, USA), as previously reported [23]. Haematological parameters were measured using the Act 5 diff haemo-analyser (Beckman Coulter, Brea, California, United States).

#### 2.2.2. Monocytes isolation

For monocyte isolation, whole blood samples were incubated with microbeads and then magnetic cell sorting (MACS) buffer at  $4^\circ\text{C}$  for 15 min. MACS consisted of phosphate-buffered saline (PBS) (Gibco BRL, Frederick, MD, USA) with bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA) and 2 mM of EDTA (VWR international, Leuven, Belgium). MACS buffer was used at  $4^\circ\text{C}$  for all washing stages with centrifugation at 600 g for 5 min. Ice-cold LD and MS MACS columns were kept at  $4^\circ\text{C}$ , and air bubbles were prevented during incubation and magnetic separation. Isolation and characterisation of mouse monocytes were performed, as explained previously [24].

#### 2.2.3. Immunophenotyping of monocyte subsets

All data acquisition was carried out using the BD FACS CANTO II flow cytometer (Becton Dickson, NJ, USA). In order to control for



**Fig. 1. Study design.** The figure illustrates the two experimental phases. The first phase was used to establish the pathological state (A), where fourteen animals were randomly allocated into a high-fat diet (HFD) ( $n = 8$ ) or low-fat diet (LFD) ( $n = 6$ ). The mice were kept on the respective diets for eight weeks. The oral glucose tolerance test (OGTT) was then performed to determine glucose tolerance. In the second phase (B), twenty-four mice fed on HFD for eight weeks showing signs of impaired glucose tolerance and were then randomised into four groups ( $n = 6$ /group); (i) untreated HFD group; (ii) Low-dose aspirin group (LDA); (iii) Low-dose aspirin in combination with metformin (LDA + Met); and (iv) Clopidogrel group. The treatment was administered daily, through oral gavage for six weeks.

non-specific binding a fluorescence minus one (FMO) control was used to control for non-specific binding. Antibody titrations were also performed to determine the optimal antibody concentrations used. The antibody cocktail included CD11c-PE/Cy7 and CD36-PE (BD Biosciences, San Diego, CA) (Table 1S). CD11c-PE/Cy7 antibody was used to identify monocytes, and the CD36 antibody was used to measure the expression of glycoprotein IV (CD36) on monocyte subsets. Briefly, 50  $\mu$ L (50  $\mu$ l) of isolated monocytes sample were stained with titrated volumes (1:100) of the antibody cocktail. For each sample, at least a 1000 events were acquired at a medium flow rate. Monocyte subsets were identified by their distinctive forward and side scatter properties and further classified as classic (CD11c<sup>+</sup>) or pro-inflammatory (CD11c<sup>++</sup>) monocytes.

### 2.3. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 version 8.2.1 (441) Software, (GraphPad Software Inc, San Diego, CA, USA). For normality testing, Kolmogorov-Smirnov (KS)

normality test was performed. Differences between LFD and HFD groups in normal distribution was assessed using a two-tailed unpaired Student's *t*-test, while the Mann-Whitney test was used for nonparametric data. In instances of normally distributed data, a one-way ANOVA was used with Tukey's as a post hoc test while for nonparametric data, the Kruskal-Wallis test was used followed by a Dunn's post-hoc test. A *p*-value of  $< 0.05$  was considered statistically significant. The effect size was also calculated using Cohen's *D* method.

### 3. Results

Initially, we determined whether the HFD induced a state of impaired glucose tolerance following short-term 8-week HFD-feeding period. Blood glucose and insulin levels were determined after animals were kept on their respective diets for eight weeks. As anticipated, HFD-feeding resulted in impaired glucose tolerance with a higher postprandial blood glucose level (6.75 (6.45–7.05) when compared to the LFD-feed group [3.1 (3.0–3.85)],  $p = 0.0002$

**Table 1**

Baseline metabolic and haematological characteristics of low-fat diet (LFD) and high-fat diet (HFD)-fed mice.

Parameters	LFD ( $n = 6$ )	HFD ( $n = 8$ )	<i>p</i> -value
Weight (g)	24.93 $\pm$ 1.91	25.81 $\pm$ 1.98	0.3771
Insulin ( $\mu$ U/L)	4.63 $\pm$ 0.19	6.02 $\pm$ 1.43	<b>0.0465</b>
<b>2 HOUR POSTPRANDIAL GLUCOSE</b>			
Glucose (mmol/L*120 min)	3.1 (3.0–3.85)	6.75 (6.45–7.05)	<b>0.0002</b>
AUC (mmol/L* 120 min)	557.40 $\pm$ 201.80	1032.00 $\pm$ 194.10	<b>0.0151</b>
WBC ( $10^3/\mu$ L)	5.98 $\pm$ 1.50	8.08 $\pm$ 2.99	0.1155
Monocytes ( $10^3/\mu$ L)	0.08 $\pm$ 0.04	0.09 $\pm$ 0.06	0.8774
Neutrophils ( $10^3/\mu$ L)	0.42 $\pm$ 0.19	0.72 $\pm$ 0.29	0.0848
Lymphocytes ( $10^3/\mu$ L)	4.54 $\pm$ 0.76	7.16 $\pm$ 2.73	0.1094
Basophils ( $10^3/\mu$ L)	0.01 (0.01–0.01)	0.01 (0.00–0.01)	0.3215
Eosinophils ( $10^3/\mu$ L)	0.01 (0.00–0.01)	0.01 (0.01–0.01)	0.8852
RBC ( $10^9/\mu$ L)	6.56 $\pm$ 1.01	5.93 $\pm$ 1.41	0.3502
Hgb (g/dL)	25.43 $\pm$ 3.33	19.04 $\pm$ 4.13	0.0571
Hct (%)	28.68 $\pm$ 4.25	22.01 $\pm$ 5.56	0.0746
MCV (fl)	42.00 $\pm$ 0.89	43.13 $\pm$ 0.83	<b>0.0016</b>
MCH (pg)	48.00 $\pm$ 1.31	38.44 $\pm$ 10.60	0.1141
RDW (%)	10.53 $\pm$ 0.63	10.38 $\pm$ 0.42	0.6086
Platelets ( $10^3/\mu$ L)	671.0 (625.5–842.8)	739.0 (570.3–782.5)	1.0000

Significance ( $p < 0.05$ ) shown in boldface. Data reported as mean  $\pm$  SD or median (IQR).

**Abbreviations:** LFD: low-fat diet; HFD: high-fat diet; AUC: area under the curve; WBC: white blood cell; RBC: red blood cell; Hgb: haemoglobin; Hct: haematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; RDW: red cell distribution width.

(Fig. 2B and C). Furthermore, the elevated levels of postprandial blood glucose levels were associated with a 1.9-fold increase in the area underneath the curve (AUC) compared to the LFD mice ( $p = 0.0068$ ) (Table 1). In a similar manner, insulin levels ( $\mu\text{U/L}$ ) were increased in HFD ( $6.02 \pm 1.43$ ),  $p = 0.0360$ ) compared to LFD-fed mice ( $4.63 \pm 0.19$ ) (Fig. 2B, Table 1). However, the animal weights across both diet groups were comparable ( $p = 0.3848$ ) following the short-term diet feeding of 8 weeks (Table 1). There were no adverse events observed in any of the experimental phases of this study.

### 3.1. Changes in haematological indices following short-term HFD compared to LFD

The haemoglobin concentration (g/dL) were comparable between the HFD group ( $19.04 \pm 4.13$ ) when compared to the LFD ( $25.43 \pm 3.33$ ),  $p = 0.0571$  (Table 1). The haematocrit levels were also comparable between the HFD ( $22.01 \pm 5.56$ ) compared to the LFD group ( $28.68 \pm 4.52$ ),  $p = 0.0746$ . While the mean corpuscular volume (MCV) was slightly increased in HFD group ( $43.13 \pm 0.83$ ) compared to LFD group ( $42.00 \pm 0.89$ ),  $p = 0.0016$  (Table 1). Lastly, the levels of circulating monocytes, basophils and eosinophils were comparable ( $p > 0.05$ ) between the two groups. Whereas, neutrophils and lymphocytes were increased in the HFD compared to the LFD Group,  $p < 0.05$  (Table 1).

### 3.2. Short-term treatment with LDA alone, or in combination with metformin improves glucose tolerance in HFD-fed mice

To assess the changes in the body weights, glucose and insulin levels following short-term treatment. A one-way analysis of variance (ANOVA) was performed. There were significant changes in the body weights following the short-term treatment with the various drugs ( $F_{(3, 20)} = 9.474$ ,  $p = 0.0004$ ). Tukey's post hoc analysis, showed a significant reduction in the body weights of mice treated with LDA monotherapy ( $24.80 \pm 1.19$ ), when compared to those on HFD only ( $25.81 \pm 1.19$ ), or when LDA was used in combination with metformin ( $28.27 \pm 1.42$ ),  $p < 0.05$  (Table 2). In addition, the treatments also altered the blood glucose levels ( $H = 18.38$ ,  $p = 0.0024$ ). The Dunn's post-hoc analysis showed a marked reduction in the blood glucose levels in; LDA + metformin [3.7 (2.60–4.15)], and clopidogrel alone [2.45 (2.38–3.78)], treated groups compared to the untreated HFD group [6.75 (6.45–7.05)],  $p = 0.0024$ . However, all treatments did not affect insulin levels ( $F_{(3, 21)} = 1.992$ ,  $p = 0.1460$ ) (Table 2, Fig. 3).

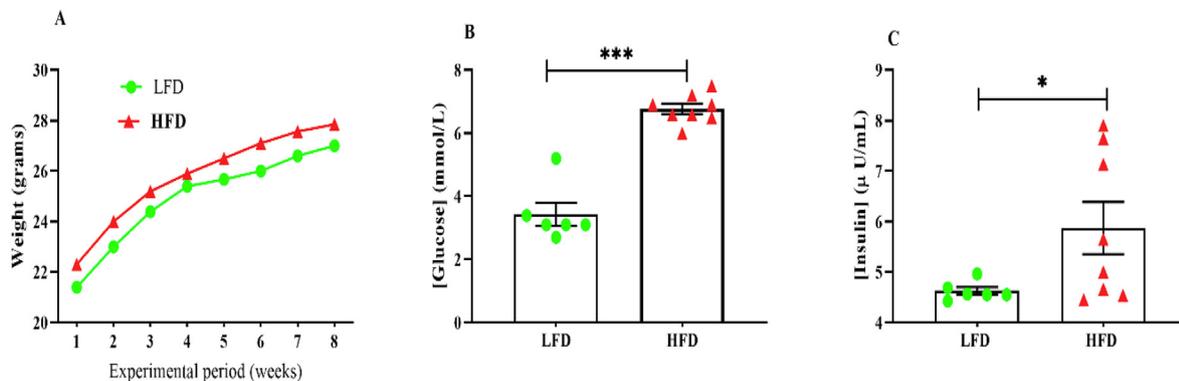
### 3.3. The effect of short-term treatment on haematological parameters in HFD-fed mice

The changes in the haematological indices following treatment with the anti-inflammatory and anti-thrombotic drugs were assessed. Treatment had a no effect on levels of circulating white blood cells (WBC) ( $F_{(3, 22)} = 0.2557$ ,  $p = 0.8564$ ) and red blood cells (RBC) ( $F_{(3, 22)} = 1.417$ ,  $p = 0.2644$ ). Whereas all the treatment altered the haemoglobin ( $F_{(3, 18)} = 7.803$ ,  $p = 0.0015$ ); haematocrit ( $F_{(3, 22)} = 3.230$ ,  $p = 0.0420$ ) and mean corpuscular volume (MCV) ( $F_{(3, 22)} = 6.905$ ,  $p = 0.0019$ ), while other RBC indices remained unchanged ( $p > 0.05$ ) (Table 2). Notably, the haemoglobin levels were increased following treatment with LDA alone ( $28.97 \pm 4.97$ ,  $p = 0.0090$ ), or when combined with metformin ( $30.57 \pm 3.90$ ,  $p = 0.0025$ ), in comparison to the untreated HFD group ( $19.04 \pm 4.13$ ) (Table 2). Moreover, a significant decrease in MCV was observed in both LDA alone ( $41.50 \pm 0.55$ ) and clopidogrel ( $41.00 \pm 1.41$ ) treatments when compared to the untreated HFD group ( $43.13 \pm 0.83$ ),  $p < 0.05$  (Table 2). LDA + Met significantly increased the haematocrit levels when compared to the untreated HFD group ( $p = 0.041$ ), (Table 2).

There were significant interactions between various treatments and levels of circulating monocytes ( $F_{(3, 22)} = 3.937$ ,  $p = 0.0217$ ). While no interactions were observed between the treatment and the levels of circulating lymphocytes ( $F_{(3, 22)} = 0.2769$ ,  $p = 0.8414$ ) (Table 2). Unexpectedly, monocytes significantly increased in mice treated with LDA alone ( $0.26 \pm 0.11$ ) and in LDA + Met ( $0.26 \pm 0.10$ ), when compared to the untreated HFD group ( $0.09 \pm 0.06$ ) (Table 2). Treatment also altered the levels of circulating basophils and eosinophils. Notably, treatment with LDA alone was effective at increasing the levels of circulating basophils when compared to the untreated HFD group ( $p = 0.0179$ ). Whereas LDA + Met increased the levels of circulating eosinophils when compared to clopidogrel treated mice ( $p = 0.0177$ ) (Table 2).

### 3.4. Pro-inflammatory monocytes ( $M_1$ ) in HFD-fed mice express elevated levels of CD36

Upregulation in the expression of CD36 is associated with an increased risk of thrombosis. However, the cellular origins of CD36 in metabolic disease remain unclear. We measured the levels of CD36 circulating classical ( $M_\Phi$ ) and pro-inflammatory ( $M_1$ ) monocytes following short-term HFD feeding. The levels of circulating monocytes subsets were elevated in HFD compared to LFD ( $p < 0.05$ ). As expected, the levels of CD36 expression on  $M_\Phi$  and  $M_1$  monocytes were also elevated in the HFD group when compared to



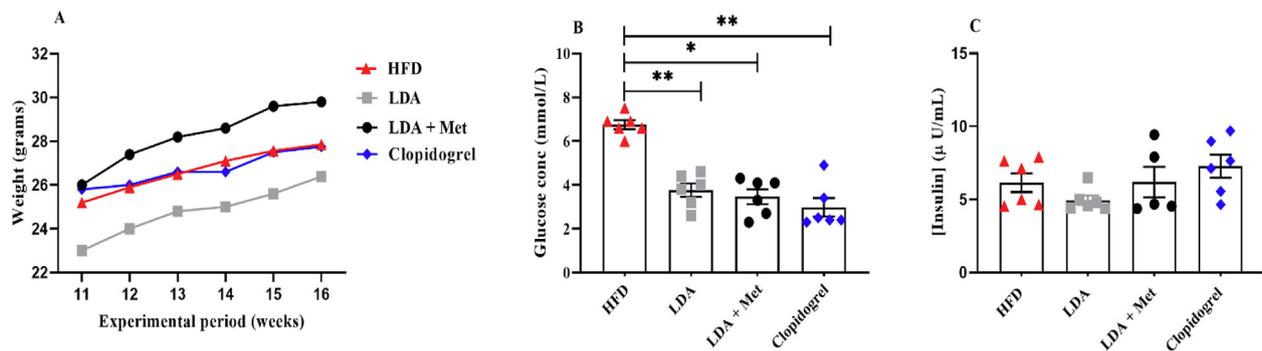
**Fig. 2. Metabolic changes following eight weeks of high-fat diet (HFD) and low-fat diet (LFD) feeding.** Figure A-Illustrates the changes in body weight from experimental week 1–8. Figure B and C demonstrates the postprandial glucose and insulin levels following eight weeks of HFD-feeding compared to LFD. Data are reported as mean  $\pm$  SEM, \*  $p < 0.05$ , \*\*\*  $p < 0.0001$  vs. LFD group.

**Table 2**

Haematological, and metabolic changes following the short-term treatment with low-dose aspirin (LDA); LDA + Met or clopidogrel.

Parameters	HFD	LDA	LDA + Met	Clopidogrel	p-value
Weight (g)	25.81 ± 1.98	24.80 ± 1.19 <sup>a</sup>	28.27 ± 1.42 <sup>b</sup>	26.71 ± 0.78 <sup>c</sup>	<b>0.0004</b>
Glucose (mg/dL)	6.75 (6.45–7.05)	3.90 (3.05–4.45) <sup>a</sup>	3.7 (2.6–4.15) <sup>d</sup>	2.45 (2.38–3.78) <sup>e</sup>	<b>0.0024</b>
Insulin (mmol/L)	6.02 ± 1.43	4.95 ± 0.79	6.19 ± 2.33	7.28 ± 1.94	0.1460
WBC (10 <sup>3</sup> /μL)	8.08 ± 2.99	8.43 ± 0.42	7.87 ± 1.70	7.17 ± 3.76	0.8564
Monocytes (10 <sup>3</sup> /μL)	0.09 ± 0.06	0.26 ± 0.11 <sup>a</sup>	0.26 ± 0.10 <sup>d</sup>	0.23 ± 0.17	<b>0.0217</b>
Neutrophils (10 <sup>3</sup> /μL)	0.63 (0.98–0.51)	0.69 (0.79–0.60)	0.77 (1.3–0.59)	0.50 (0.68–0.40)	0.1959
Lymphocytes (10 <sup>3</sup> /μL)	7.16 ± 2.73	7.45 ± 0.29	6.64 ± 1.34	6.35 ± 3.45	0.8414
Basophils (10 <sup>3</sup> /μL)	0.01 (0.01–0.0)	0.02 (0.02–0.02) <sup>a</sup>	0.01 (0.02–0.01)	0.01 (0.02–0.01)	<b>0.0272</b>
Eosinophils (10 <sup>3</sup> /μL)	0.01 (0.01–0.00)	0.01 (0.02–0.00)	0.02 (0.09–0.01) <sup>f</sup>	0.00 (0.0–0.00)	<b>0.0302</b>
RBC (10 <sup>6</sup> /μL)	6.55 ± 1.77	6.99 ± 1.13	7.21 ± 0.89	6.23 ± 1.71	0.2644
Hgb (g/dL)	19.04 ± 4.13	28.97 ± 4.97 <sup>a</sup>	30.57 ± 3.90 <sup>d</sup>	21.73 ± 8.00	<b>0.0015</b>
Hct (%)	22.01 ± 5.56	28.97 ± 4.97	30.57 ± 3.90 <sup>d</sup>	25.55 ± 7.35	<b>0.0420</b>
MCV (fl)	43.13 ± 0.83	41.50 ± 0.55 <sup>a</sup>	42.50 ± 0.82	41.00 ± 1.41 <sup>e</sup>	<b>0.0019</b>
MCH (pg)	38.44 ± 10.60	44.75 ± 1.49	31.80 ± 17.25	42.87 ± 1.60	0.5504
RDW (%)	10.38 ± 0.42	10.72 ± 0.74	10.95 ± 1.14	11.20 ± 1.35	0.4278
Platelets (10 <sup>3</sup> /μL)	739 (782.5–570.3)	839 (966.5–670.5)	820.5 (940.8–756.3)	756 (874–552.3)	0.4337

Significant values are shown in boldface (p &gt; 0.05).

Abbreviations: **WBC**: white blood cell; **RBC**: red blood cell; **Hgb**: haemoglobin; **MCV**: mean corpuscular volume; **MCH**: mean cell haemoglobin; **RDW**: red blood cell distribution width.<sup>a</sup> HFD vs LDA.<sup>b</sup> LDA vs LDA + Metformin.<sup>c</sup> LDA vs clopidogrel.<sup>d</sup> HFD vs LDA + Metformin.<sup>e</sup> HFD vs clopidogrel.<sup>f</sup> Clopidogrel vs LDA + metformin.**Fig. 3.** Changes in metabolic parameters following six-week treatment with low-dose aspirin (LDA) alone; LDA in combination with metformin (LDA + Met) or clopidogrel alone in high-fat diet (HFD)-fed mice. Figure A, B, and C show the body weights, blood glucose and insulin levels in untreated HFD-fed mice versus treated group following a 6-week short term treatment. Data are reported as mean ± SEM, (n = 6/group), \* p < 0.05, \*\* p < 0.005.

the LFD-fed group (p &lt; 0.001) (Table 3).

Furthermore, we investigated the effects of treatment on the mean differences of monocyte subsets ( $M_{\phi}$  and  $M_1$ ). Interestingly, in comparison to the untreated HFD group (52.60 [95% CI: 35.57–69.62], p = 0.0005); treatment with; LDA alone (43.70 [95% CI: 23.87–63.53], p = 0.0024); LDA + Met (44.89 [95% CI: 26.86–62.91], p = 0.0014) or clopidogrel alone (51.42 [95% CI:

33.18–69.65], p = 0.0008) significantly reduced the mean difference of circulating monocyte subsets in the LDA only group (43.70 [95% CI: 23.87–63.53], p = 0.0024); or when combined with (Table 4).

### 3.4.1. The effect of treatment on the expression of CD36 on monocytes subsets of HFD-fed mice

To determine which of the treatments induced a more significant effect on the levels of circulating monocytes expressing CD36. An effect size estimation was performed using Cohen's d method [25]. There was a larger reduction in the levels of  $M_1$  monocytes expressing CD36 following short-term treatment with LDA alone (Cohen's d ( $d_s$ ) = 0.71) and clopidogrel monotherapy ( $d_s$  = 0.66), compared to the dual therapy of LDA and metformin ( $d_s$  = 0.3) which showed smaller reduction in circulating  $M_1$  monocytes expressing CD36 (Table 5).

## 4. Discussion

In this study, we evaluated the expression of glycoprotein IV (CD36) on monocyte subsets in a mouse model of high-fat diet

**Table 3**

Monocyte subsets and the expression of CD36 in the high-fat diet (HFD)-fed compared to low-fat diet (LFD)-fed mice.

Parameter	LFD (n = 6)	HFD (n = 8)	p-value
<b>Monocyte subsets</b>			
$M_{\phi}$	72.29 ± 26.06	79.70 ± 12.95	<b>0.0005</b>
$M_1$	6.75 ± 5.54	10.25 ± 8.50	<b>0.0001</b>
$M_{\phi}/M_1$	15.78 ± 8.35	20.33 ± 18.96	0.5525
<b>Monocyte-CD36 expression</b>			
$M_{\phi}$	39.72 ± 14.50	47.44 ± 15.15	<b>0.0002</b>
$M_1$	97.79 ± 2.5	99.56 ± 0.75	<b>&lt; 0.0001</b>
$M_{\phi}/M_1$	0.39 ± 0.14	0.49 ± 0.16	0.3053

Significant values are shown in boldface.

 $M_{\phi}$  - classical monocyte subset;  $M_1$ -pro-inflammatory monocyte subset.

**Table 4**  
The effect of treatment on the expression of CD36 on monocyte subsets in the HFD-fed mice.

Groups	M <sub>Φ</sub>	M <sub>1</sub>	Mean difference (95% CI)	p-value
HFD	43.29 ± 14.08	95.89 ± 3.66	52.60 (35.57–69.62)	0.0005
LDA	54.39 ± 19.80	98.09 ± 2.11	43.70 (23.87–63.53)	<b>0.0024</b>
LDA + Met	52.13 ± 18.35	97.02 ± 2.62	44.89 (26.86–62.91)	<b>0.0014</b>
Clopidogrel	46.59 ± 16.52	98.01 ± 1.63	51.42 (33.18–69.65)	<b>0.0008</b>

Significant values are shown in boldface ( $p < 0.05$ ). **HFD**, high-fat-diet; **LDA**, low-dose aspirin; **Met**, metformin; M<sub>Φ</sub>-classical monocyte subset; M<sub>1</sub>-pro-inflammatory monocyte subset.

(HFD)-induced impaired glucose tolerance. Furthermore, we assessed whether LDA alone; LDA + Met or clopidogrel alone, could modulate the expression of CD36 on monocyte subsets. Our results showed elevated pro-inflammatory monocytes (M<sub>1</sub>) in a mouse model of glucose intolerance and these levels of pro-inflammatory (M<sub>1</sub>) monocytes were associated with a high expression of glycoprotein IV (CD36). Moreover, all treatments were able to reduce the levels of CD36 expression on M<sub>1</sub> monocytes when compared to classical monocytes (M<sub>Φ</sub>) in HFD-fed mice. In this study, it was also evident that the short-term exposure to HFD could induce glucose intolerance and hyperinsulinemia, which were independent of increased body weight. This has been described in previous studies, where three major phases of HFD have been characterised, which include an early, intermediate and late phase [26,27]. Notably, the early phase occurs within three days of HFD and lasting until 12 weeks of HFD. The early phase is characterised by insulin insensitivity and a fold increase in the glucose tolerance area underneath the curve (AUC) without any marked changes in body weights [26]. Our findings point to the changes that occur in insulin sensitivity and impaired glucose metabolism during the early phase of HFD-feeding.

Interestingly, LDA as a monotherapy or in combination with metformin improved glycaemic index and this was independent of insulin. As, indicated by persistently elevated insulin levels even during treatment with the anti-inflammatory (LDA alone, LDA + Met) and antithrombotic drugs (clopidogrel) monotherapy. In addition, short-term LDA treatment led to a reduction in animal body weights, whereas a gradual increase in body weights of animals on LDA in combination with metformin and clopidogrel alone was observed. Consistently, although short-term LDA treatment is known to lower blood glucose levels [28], it provides no long-term therapeutic benefit in the prevention of clinical T2DM [29]. The administration of antithrombotic and anti-inflammatory drugs in high-fat diet C57BL/6 model, has been shown to ameliorate states of obesity and diabetes [30–32]. Moreover, impaired glucose tolerance was associated with elevated neutrophil and lymphocyte counts. This may further support the well-established association

between insulin insensitivity and increased risk of thrombosis. As elevated neutrophils [33,34] and lymphocytes have been associated with chronic inflammation and thrombosis [33]. The increased levels of circulating neutrophils and lymphocytes persisted even during treatment with LDA alone; LDA + Met or clopidogrel alone. Notably, the monocyte, basophil, and eosinophil levels were also increased following treatment with the various anti-inflammatory and antithrombotic drugs in HFD-fed mice. Although the RBC was normal, changes in the haemoglobin, haematocrit and mean corpuscular volume were observed. Interleukin-6, an anti-erythropoietic agent, alters the sensitivity of erythropoietin progenitors resulting in the destruction of immature red blood cells and a decreased concentration of haemoglobin [35–37].

Furthermore, due to their importance in regulating immune response [38], it was essential to explore the involvement and regulation of peripheral blood M<sub>Φ</sub> and M<sub>1</sub> monocytes in conditions of impaired glucose tolerance. In the first phase of the study, we showed increased levels of peripheral blood M<sub>Φ</sub> and M<sub>1</sub> monocytes following short-term HFD. Elevated levels of M<sub>1</sub> monocytes have been reported in patients living with T2DM [39]. Concomitantly, increased levels of circulating monocytes were associated with increased CD36 expression following HFD feeding. It is hypothesised that CD36 is upregulated during the early phases of atherosclerosis; this involves the differentiation of monocytes into macrophages which subsequently engulf oxLDL and promote the formation of foam cells [40,41]. Notably, the levels of sCD36 are associated with an increased risk of insulin resistance and an increased risk of T2DM [16]. Contradictory findings regarding the role of CD36 in T2DM exist, with a clinical study reporting on similar levels of sCD36 in patients with diabetes and healthy controls [42]. The cellular origins of sCD36 in T2DM remain unclear [43]. However, erythrocyte-derived micro-particles have been described as a significant contributor to the levels of sCD36 in patients with T2DM [43].

In the second phase of our study, the short-term treatment with either cyclooxygenase-1 (COX-1) dependent (LDA) or independent (clopidogrel) antithrombotic drugs reduced the expression of CD36 on circulating M<sub>1</sub> and M<sub>Φ</sub> monocytes. Interestingly, it has been previously demonstrated that LDA can promote CD36 expression on macrophage via peroxisome proliferator-activated-receptor (PPAR)  $\gamma$  independent pathways [44]. Moreover, it is known that aspirin can be metabolised into its active form, salicylic acid, and in the process, block the adhesion of monocytes to low-density-lipoprotein activated endothelial cells through mechanisms involving the inhibition of nuclear factor kappa-light-chain-enhancer of activated  $\beta$  cells (NF- $\kappa$ B) activity [45]. In T2DM, advanced glycation end products are also known to activate the NF- $\kappa$ B pathway, which promotes the polarisation of macrophages into the M<sub>1</sub> phenotype [46]. Importantly, CD36 orchestrates the patrolling of M<sub>1</sub> monocytes on the endothelium during the initial

**Table 5**  
Mean difference in the expression of CD36 on M<sub>Φ</sub> and M<sub>1</sub> subsets post treatment in high fat diet-fed mice.

Groups	M <sub>Φ</sub> Mean Difference [95%CI]	M <sub>1</sub> Mean difference [95%CI]	p-value
LDA	11.10 (-8.34–30.54)	2.20 (-1.18–5.58)	<b>&lt; 0.05</b>
LDA + Met	8.84 (-9.67–27.35)	1.13 (-2.47–4.73)	<b>&lt; 0.05</b>
Clopidogrel	3.30 (-14.07–20.67)	2.12 (-1.09–5.33)	<b>&lt; 0.01</b>

Significant values are shown in boldface ( $p < 0.05$ ). **HFD**, high-fat-diet; **LDA**, low-dose aspirin; **Met**, metformin; M<sub>Φ</sub> - classical monocyte subset; M<sub>1</sub>-pro-inflammatory monocyte subset.

stages of atherogenesis [47]. In addition, HFD feeding enhances the ratio of pro-inflammatory monocytes involved in CD36-dependent surveillance of peripheral blood vessels [47]. Elevated CD36 levels are associated with macrophage trapping, impaired insulin signalling, and thrombosis [48]. Altogether, our findings may suggest that an increased polarisation of monocytes accompanies the initial phases of impaired glucose tolerance into M<sub>1</sub> monocytes that express elevated levels of CD36. Although this did not establish mechanistic insights into the propensity of M<sub>1</sub> derived microparticles expressing CD36, our findings may suggest that M<sub>1</sub> monocytes may be a source of sCD36 in patients with insulin resistance.

A limitation of the current study includes the lack of lipid profiling and lipid-binding activity of monocyte subsets. However, previous studies have associated the upregulation of CD36 with increased oxLDL uptake and clearance of apoptotic cells [47,49]. In conclusion, the current study reports on differential expression of CD36 on classical and pro-inflammatory monocytes in a state of impaired glucose tolerance. Moreover, pro-inflammatory monocytes, express elevated levels of CD36, which may suggest that basal CD36 expression on classical and pro-inflammatory monocytes may enhance the formation of foam cells. Hence, the enumeration and quantification of monocyte subsets in prediabetes may allow for risk stratification of individuals at increased risk of atherosclerosis. In this study, LDA and clopidogrel monotherapies showed a more significant reduction in the levels of CD36 expression on circulating M<sub>1</sub>, as indicated by an effect size of > 0.6. While LDA in combination with metformin showed a smaller effect in reducing the levels of CD36 expression on circulating pro-inflammatory monocytes. The discordance between the efficacy of LDA compared to LDA combined with metformin could be due to the reported effect of metformin of increasing free fatty acid (FFA) uptake and reduced mitochondria oxidation [50] and thus modulating the expression of CD36, which is also an FFA transporter.

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### Authors contribution

**Kabelo Mokgalaboni:** Data curation, writing- Original draft preparation, investigation. **Phiywayinkosi V. Dlodla:** Conceptualization, Writing-reviewing and Editing, Supervision. **Zibusiso Mkandla:** Data curation, validation, Writing-Reviewing and Editing. **Tinashe Mutize:** Data Curation, validation, Writing-Reviewing and editing. **Tawanda Maurice Nyambuya:** Data curation, validation, Writing-Reviewing and Editing. **Vuyolwethu Mxinwa:** Validation, Writing-Reviewing and Editing, investigation. **Bongani B. Nkambule:** Conceptualization, Methodology, Supervision, Writing-reviewing and Editing, Supervision

### Declaration of competing interest

The authors have no conflict of interests regarding the work presented in this manuscript.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.metop.2020.100047>.

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