Investigating Immune Metabolism and Exhaustion in Type 2 Diabetes

By

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Preface

This thesis is in fulfilment of the requirements for Doctor of Philosophy degree in Human Physiology. Chapter 1 describes the problem statement, the aims and objectives covered in the thesis. Chapter 2 covers the literature review and provides the rationale of the current project. The chapter comprises of a total of six manuscripts, a protocol and five systematic reviews and meta-analyses. All of these manuscripts have already been published in accredited journals. Chapters 3, 4 and 5 includes the experimental work conducted using the mice model and are research articles 1, 2 and 3, respectively. Chapter 3 and 4 experimental papers have also been published in accredited journals and chapter 5 is currently under the review process. Chapters 6 is the synthesis and conclusion section that summarises the entire project and provides future directions of the work presented in this thesis.

This work has not been submitted in any form for any other degree or diploma at another institution. Use of other people's work has been acknowledged accordingly in-text.

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

Declaration

I……Tawanda Maurice Nyambuya……. 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 as general information.

Publications directly related to this thesis.

Published Manuscripts

1. **Nyambuya TM**, Dludla PV, Nkambule BB. T cell activation and cardiovascular risk in type 2 diabetes mellitus: a protocol for a systematic review and meta-analysis. *Syst Rev*. 2018 Oct 20;7(1):167. doi: 10.1186/s13643-018-0835-1. PMID: 30342529; PMCID: PMC6195734.

2. **Nyambuya TM**, Dludla PV, Mxinwa V, Nkambule BB. Obesity-induced inflammation and insulin resistance: A mini-review on T-cells. *Metabol Open*. 2019 Aug 10;3:100015. doi: 10.1016/j.metop.2019.100015. PMID: 32812921; PMCID: PMC7424835.

3. **Nyambuya TM**, Dludla PV, Mxinwa V, Nkambule BB. T-cell activation and cardiovascular risk in adults with type 2 diabetes mellitus: A systematic review and meta-analysis. Clin Immunol. 2020 Jan;210:108313. doi: 10.1016/j.clim.2019.108313. Epub 2019 Nov 22. PMID: 31765833.

4. **Nyambuya TM**, Dludla PV, Mxinwa V, Nkambule BB. Obesity-related asthma in children is characterized by T-helper 1 rather than T-helper 2 immune response: A meta-analysis. *Ann Allergy Asthma Immunol*. 2020 Oct;125(4):425-432.e4. doi: 10.1016/j.anai.2020.06.020. Epub 2020 Jun 16. PMID: 32561508.

5. **Nyambuya TM**, Dludla PV, Mxinwa V, Mokgalaboni K, Ngcobo SR, Tiano L, Nkambule BB. The impact of metformin and aspirin on T-cell mediated inflammation: A systematic review of in vitro and in vivo findings. *Life Sci*. 2020 Aug 15;255:117854. doi: 10.1016/j.lfs.2020.117854. Epub 2020 May 26. PMID: 32470453.

6. **Nyambuya TM**, Dludla PV, Mxinwa V, Nkambule BB. A Systematic Review and Meta-Analysis on the Regulation of Programmed Cell Death-1 on T-cells in Type 2 Diabetes. Medicine. 2021 doi: 10.1097/MD.0000000000025488.

7. **Nyambuya TM**, Dludla PV, Nkambule BB. Diet-Induced Obesity Promotes the Upregulation of Fas Expression on T-cells. *Biology*. 2021; 10(3):217. Doi: 10.3390/biology10030217

8. **Nyambuya TM**, Dludla PV, Nkambule BB. The aberrant expression of CD69 on peripheral T-helper cells in diet-induced inflammation is ameliorated by low-dose aspirin and metformin treatment. *Cell Immunol*. 2021;363:104313. doi:10.1016/j.cellimm.2021.104313

Submitted Manuscripts

1. **Nyambuya TM,** Dludla PV, Mxinwa V, Nkambule BB. The Modulatory Effects of Short-term Treatment with Metformin and Fluvastatin on Glucose and Lipid Metabolism cells **[To be Submitted]**

Dedication

In loving memory of my late father

Patrick Nyambuya
(06 June 1956 – 02 May 2003)

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I thank the Lord almighty for constant protection and guidance in pursuit of my goals.

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List of abbreviations

AMPK: adenosine-monophosphate-activated protein kinase

AT: adipose tissue

$CD4^+$: T-helper cells

$CD8^+$: Cytotoxic T-cells

CRP: C-reactive protein

CVD: cardiovascular disease

DIO: diet induced obese

IFN-γ: interferon gamma

IL: interleukin

IR: insulin resistance

JAK: Janus kinase

JAK/STAT3: Janus kinase/signal transducers and activators of transcription 3

LDA+Met: low-dose aspirin and metformin

mTOR: mechanistic target of rapamycin

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B-cells

PD-1: Programmed cell death-1

PD-L1/2: programmed death ligand ½

PI3K/AKT: phosphoinositide 3-kinase/protein kinase B

STAT: signal transducer and activator of transcription

T_H: T helper cell

T_{regs}: regulatory T-cells

T2D: type 2 diabetes

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Abstract

Introduction

In the era of rapid modernisation and urbanisation, the global incidence of non-communicable diseases such as type 2 diabetes (T2D) has significantly increased. This has been attributed to increased sedentary lifestyles and the adaptation of unhealthy diets, particularly in low-to-middle-income countries like South Africa. These changes promote the development of obesity, which is one of the major risk factors for T2D. Obesity, further plays a pivotal role in the pathoimmunological changes that are associated with outwards effects of poor glucose control and insulin resistance. Chronic inflammation and increased immune activation are a hallmark of T2D, and both these processes are partially mediated by T-cells. Interestingly, these pathological consequences are identified as early as in pre-diabetes before the onset of overt T2D. Upon activation, T-cells release cytokines that induce the activation of other immune cells and polarises T-cells towards the pro-inflammatory subset. This consequently leads to a pro-inflammatory milieu that alters T-cell function and predisposes individuals with pre-diabetes or patients with T2D to developing cardiovascular disease (CVD). Although few studies have implicated activated T-cells in mediating inflammation and altering myocardial function in poor glucose control, the underlying mechanisms and sequence of events remains scarce. Therefore, this study made use of a short-term high-fat diet (HFD)-induced mouse model of pre-diabetes to investigate inflammation and immune responses mediated by T-cells. Furthermore, it assessed and compared the modulatory effects of low-dose aspirin (LDA), metformin and fluvastatin (statin), well-acknowledged anti-inflammatory, anti-hyperglycaemic and cholesterol lowering drugs, respectively, on inflammation, T-cell activation, and cardiovascular risk.

Methods

This study involved the use of a diet-induced pre-diabetes and inflammation mouse model of glucose intolerance. Briefly, in phase one of the experiment, a total of 27 six-week-old male C57BL/6 mice were randomised into either a high-fat diet (HFD) (n=21) or low-fat diet (LFD) (n=6) groups for a total of 8 weeks. Phase two of the experiment subsequently initiated at week 9 whereby HFD-fed mice were randomised into a short-term treatment with either metformin, LDA or in combination with metformin (LDA+Met) or statin over an additional 6-week period (n=6/7group). Changes in body weights were monitored on a weekly basis. Glucose profiles, cholesterol levels, complete blood counts, T-cell associated cytokines, and the expression on T-cell markers were measured at the end of phase one (week 8) and phase two (week 14) of the experiments. The Kolmogorov-Smirnov test with Dallal-Wilkinson-Lillie was performed for normality testing. For parametric data, the mean differences between the LFD- and HFD-fed groups were assessed using unpaired student *t-test* and were reported as mean ± standard error. The Man Whitney U test was used for non-parametric data and reported as the median and interquartile range [IQR]. Comparisons across the diet and treatments groups were assessed using a Two-way analysis of variance (ANOVA). A posthoc Tukey's multiple comparisons test was performed if the F-value reached statistical significance ($p<0.05$). The Kruskal-Wallis test, followed by a Dunn's

posthoc test, was used for non-parametric data. A p-value of < 0.05 was considered statistically significant. The GraphPad Prism version 6 software (GraphPad Software Inc, CA, USA) was used for all statistical analysis.

Results

The HFD-fed group had significantly increased weight gain (29.17%) in comparison to the LFD-fed group (21.74%) after the 8-week period. Notably, HFD-feeding (HFF) was associated with impaired metabolic function marked by poor glucose control and a state of hypercholesterolemia. In particular, the HFD-fed group had increased fasting glucose ($p<0.0001$) and 2-hour postprandial area under curve ($p=0.0029$) when challenged with an oral glucose tolerance test in comparison to the LFD group. In addition, total cholesterol (Tc) ($p=0.0039$) and low-density lipoprotein (LDL)-c ($p=0.0447$) levels were higher in the HFD-group than LFD-group, whilst high-density lipoprotein (HDL)-c levels were comparable between the groups ($p=0.1749$). HFF was associated with enhanced levels of inflammation and generalised immune activation, marked by increased white cell count (WCC) ($p=0.008$) and elevated levels of interleukin (IL)-6 ($p<0.0001$), IL-2, tumour necrosis factor (TNF)-α ($p=0.0312$) and IL-17A ($p<0.0001$). Most importantly, HFF upregulated Fas (CD95) and downregulated CD69 ($p=0.0009$) expression on T-cells without altering the levels of programmed-cell death 1 (PD-1) ($p=0.6408$). The elevated levels of Fas were directly associated with body weight gain ($r=0.93$, $p=0.0333$). Short-term treatment with LDA+Met lowered insulin levels ($p=0.0475$) and fasting blood glucose ($p<0.0001$) when compared the untreated HFD-fed group. Although treatment with LDA monotherapy did not affect any cholesterol levels, metformin monotherapy and statin significantly lowered Tc and LDL-c when compared to the untreated HFD-fed group ($p<0.05$). Treatment with LDA+Met lowered WCC ($p=0.0095$), lymphocyte count ($p=0.0264$), IL-6 ($p=0.0002$), TNF-α ($p=0.0465$), IL-2 ($p=0.0001$) and IL-17A ($p<0.0001$), when compared to the untreated HFD-fed group. Lastly, LDA+Met ($p=0.0010$) but not LDA ($p=0.147$), upregulated the expression of CD69 on T-cells whilst both treatment groups had no impact on PD-1 levels. Treatment with fluvastatin had no effect on the levels of inflammation ($p>0.05$).

Conclusion

This study showed that T-cell dysfunction is congruent with a state of inflammation, hypercholesterolaemia and poor glucose control in the early stages of obesity. Notably, the altered T-cell function is partially mediated by the aberrant expression of Fas and CD69. The combinational treatment of LDA with metformin was more effective than the use of LDA only in improving glucose control, ameliorate inflammation, and moderate T-cell functions. These findings outline the pathological link between the development of inflammation, immune activation and altered lipid metabolism in a pre-diabetic state. More importantly, it highlights the cardiovascular risk properties of statins and enhanced anti-inflammatory efficacy of LDA when combined with metformin in poor glucose control. Therefore, alleviating inflammation and lowering glucose levels during the early development of T2D may be an effective strategy to attenuate T-cell remodeling in diet-driven metabolic disturbances.

CHAPTER 1: Introduction

1.1 Background

The global burden of non-communicable diseases such as type 2 diabetes (T2D) has drastically increased over the years, particularly in developing countries [1]. This is attributed to an increased sedentary lifestyle which is associated with the development of obesity, a major risk factor for T2D [2]. Emerging evidence has provided an association between T2D and low-grade inflammation as well as chronic immune activation [3–5]. Interestingly, the pre-diabetic state which precedes overt T2D is also congruent with persistent immune activation [6,7]. Immune dysfunction in patients with pre-diabetes or T2D is associated with increased incidence of consequences of ongoing immune activation, particularly thrombotic complications [6–8]. In fact, the involvement of T-cells in mediating abnormal immune responses in these patients has been described in recent studies [9,10]. Whereby, the polarisation of T helper (T_H) cells towards the pro-inflammatory subsets coupled with a reduction of anti-inflammatory T_H cells and regulatory T-cells (Tregs) exacerbates inflammation and insulin resistance [11]. Consequently, this dysregulation of immune homeostasis leads to the activation of various metabolic and inflammatory pathways which include, the aldose reductase pathway [12], the protein kinase C (PKC) pathway [13], phosphatidylinositol 3 kinase/ protein kinase B (PI3K/Akt), mitogen-activated protein kinase (MAPK), the nuclear factor-kappa B (NF-kB) and the Janus kinase signal transducer-activator of transcription (JAK-STAT) signalling pathways [13–16]. The activation of these pathways results in altered T-cell metabolism which promotes T-cell dysfunction and the pathogenesis of metabolic disorders. Notably, the continuous stimulation of T-cells in T2D induces the aberrant expression of T-cell activation markers which may lead to T-cell exhaustion [17], a progressive loss of effector function. These processes are usually mediated by increased expression of T-cell markers such as programmed cell death 1 (PD-1), Fas (CD95) and CD69 [18–21].

Chronic inflammation and T-cell dysfunction are hallmarks of T2D. Consequently, several pharmacological drugs that aim to alleviates inflammation and modulate T-cell responses are being investigated. One of these drugs is metformin, an anti-hyperglycaemic drug that downregulates pro-inflammatory signals mediated by STAT3 and the mechanistic target of rapamycin (mTOR) activity [22,23]. Although the anti-inflammatory properties of metformin have been described, there is no strong evidence of the drug offering effective cardio-protection in patients with T2D who are increased risk of cardiovascular disease [24]. This has led to an increased interest in using metformin in combination with well-established anti-inflammatory drugs with cardio-protective properties such as aspirin [25]. Although, both metformin and aspirin can ameliorate inflammation [22,23,26–28], their impact on mediating T-cell function is not well understood, and therefore remains elusive.

1.2 Problem statement

The prevalence of non-communicable diseases such as T2D has drastically increased in low-to-middle-income countries (LMICs) over the years [29]. This has led to reduced life-expectancy and increased strain on national healthcare budgets, particularly in the sub-Saharan Africa [5]. Despite over two-thirds of the cases being undiagnosed in this region, a staggering 15.9 million people were diagnosed with diabetes in 2017, and this number is expected to exponentially increase to a total of 41.6 million cases by 2045 [5]. Of the countries in sub-Saharan Africa, South Africa has the second largest number of people with diabetes, where the prevalence is currently estimated to be 12.8% [30]. Notably, over 90% of these cases are T2D and a major source of morbidity and mortality in South Africa.

The rapid modernisation and urbanisation in LMICs results in people eating unhealthy diets and living sedentary lifestyles [4]. These changes promote the development of obesity, one of the main risk factors for T2D and insulin resistance [31]. Notably, the excessive adipose tissue in obesity is associated with exacerbated release of pro-inflammatory cytokines and increased levels of immune activation. Whereby, there is polarisation of T_H cells towards the pro-inflammatory subsets that promotes the development and progression of insulin resistance, and the pathogenesis of thrombotic events [32–34]. Current evidence shows close association between increased T-cell activation and the development of cardiovascular disease (CVD) such as coronary atherosclerotic heart disease, carotid atherosclerosis and coronary artery disease in patients with T2D [34–37]. As a result of this, there is dire need to unravel the pathophysiological mechanisms and exact roles played by T-cells since CVD is the leading cause of death in patients with diabetes [38]. Therefore, deducing the role of T-cells in the development of CVD in patients with poor glucose control will be of great benefit in reducing cardiovascular risk in individuals with pre-diabetes or patients with T2D, as well as managing those with CVD.

It is well-acknowledged that T-cell dysfunction drives the pathogenesis of asthma, a chronic inflammatory condition that is characterised by aggravated T_H2 inflammation [39,40]. Notably, the poor pulmonary function symptoms in this condition are exacerbated by obesity. The association between obesity and asthma is strengthened by the presence of insulin resistance [41]. Consequently, patients with T2D are at increased risk of developing asthma since over two-thirds of patients are obese [20]. Obesity, however, polarises the immune response toward T_H1 rather than the classical T_H2 in obese-related asthma [39,40]. This altered pathophysiology of asthma in these patients negatively impacts the efficacy of therapeutic strategies [42], particularly those that targets T-cell modulation. Due to the involvement of T-cells in mediating the pathogenesis of various non-communicable diseases, there has been grown interest in exploring treatment strategies that modulates T-cell function and ameliorates inflammation.

The use of immune checkpoint inhibitors in oncology is one of the therapeutic strategies that has made great strides in modulating T-cell responses [43]. An outstanding example is the use of drugs that targets the programmed cell death 1/programmed death-ligand (PD-1/PD-L) signalling pathway, whose transduction is exacerbated due to chronic inflammation in cancer. Notably, the blockage of PD-1 signalling resuscitates T-cell function in patients with various forms of cancer [41,44,45]. In consideration of T2D being a well-acknowledged chronic inflammatory condition, it remains important to assess the expression of T-cell activation and regulatory markers as well as exploring the potential therapeutic benefits of targeting their signalling in poor glucose control. Therefore, this study used a diet-induced mouse model of pre-diabetes to investigate inflammation and immune responses mediated by T-cells. This mouse model is a suitable and well-acknowledged model to explore pathophysiological mechanisms of T2D [46–48].

1.3 Aim of the study

1. To investigate immune responses mediated by T-cells in an inflammatory pre-diabetic state.
2. To further assess and compare the modulatory effects of anti-inflammatory and anti-hyperglycaemic drugs on the function of these T-cell.

1.4 Study objectives

1. To optimise a flow cytometry-based assay to measure the expression of markers associated with T-cell activation and exhaustion in the pathogenesis of T2D using a diet-induced mouse model of pre-diabetes.
2. To assess T-cell responses in a chronic inflammatory state by measuring the levels of cytokines associated with T_H cell function using a (HFD)-induced mouse model of pre-diabetes.
3. To evaluate changes in metabolic profiles, inflammation status and the expression of T-cell function markers in diet-induced mouse model of pre-diabetes.
4. To further determine the modulatory effects of treatment with metformin, statin, LDA and its combination with metformin on these metabolic parameters and inflammatory profiles.
5. To determine if there are any associations between metabolic disorders, denoted by abnormal metabolic profiles, and the expression of the T-cell function markers in pre-diabetes.

1.5 Research questions

1. How does a pre-diabetic state during the development of T2D affects T-cell function?
2. Is there any link between T2D-associated metabolic complications and impaired immunological responses, particularly those that are mediated by T-cells?
3. What is the role of T-cell activation in the development of CVD-related complications in T2D?

4. Does metformin or LDA alter T-cell function during the pathogenesis of T2D? If so, how do these drugs modulate immune response mediated by T-cells in normal physiology and a chronic inflammatory state?
5. Are circulating T-cells exhausted in T2D?

1.6 Study approach

The overall objectives of this study were to investigate immune metabolism and exhaustion mediated by T-cells during the development of T2D, and to assess the modulatory effects of metformin and low-dose aspirin on T-cell function. This was achieved by conducting a series of systematic reviews and meta-analyses involving cohorts of patients with T2D, as well as performing various experimental studies using a diet-induced mouse model of pre-diabetes. In this model, the main purpose was to induce a chronic inflammatory state which is strongly associated with T2D. In addition, the diet-induced pre-diabetes model was used to assess the effects of these drugs on the levels of generalised immune activation and immune responses, particularly those modulated by T-cells.

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Prologue

The following chapter involves a synthesis of literature on T-cell function in diabetes and its related metabolic disorders. In order to critically review current literature on this topic of interest, we conducted a series 6 of systematic review and meta-analyses which all have been published in accredited journals.

Section 2.1 constitutes of the systematic review and meta-analysis protocol **(published)** which outlines the rationale behind the subsequent reviews and thoroughly describes the planned methods to be followed in conducting them.

Section 2.2 includes a systematic review on the role of T-cells in obesity-induced inflammation and insulin resistance **(published)**. Here, we outline the evolving concepts linking insulin resistance with impaired immunological responses mediated by T-cell in obesity. Moreover, we highlight important avenues to be explored as therapeutic strategies in ameliorating T-cell mediated inflammation and preventing the pathogenies of obesity linked cardiovascular disease.

Section 2.3 includes a systematic review and meta-analysis on the impact of T-cell activation in T2D and the stratification of cardiovascular risk in these patients **(published)**. In this manuscript, we report on increased T-cell activation and a skew towards the frequency of pro-inflammatory T_H subsets coupled with enhanced expression of T-cell negative co-stimulatory molecules. We also report on cardiovascular risk in patients with T2D. These findings highlight the possible benefits of modulating T-cell function as a strategy to reduce inflammation and cardiovascular risk in T2D.

Section 2.4 involves a systematic review and meta-analysis on T-cell exhaustion in T2D **(published)**. Here, we report on increased expression of PD-1, a negative co-stimulatory molecule known to promote T-cell exhaustion. In this study, we concluded that immune dysfunction in T2D is at least in part due to T-cell exhaustion mediated by an upregulation of PD-1 expression. We highlight how the use of immune checkpoint inhibitors may be an effective therapeutic strategy in restoring T-cell effector function and at the same time how it poses a risk of exacerbating inflammation in patients with T2D.

Section 2.5 encompasses a systematic and meta-analysis on the impact of obesity on the pathogenesis and progression of asthma, a chronic respiratory disease whose incidence is increased in T2D **(published)**. In this manuscript, we report on how obesity alters the pathophysiology of asthma by polarising the immune response towards T_H1 rather than the classical T_H2. We further describe the implications of such changes on the efficacy of therapy, particularly those that modulates T-cell responses.

Section 2.6: After establishing the involvement of T-cells in the pathogenesis and progression of T2D and its associated complications, we then assessed the effect of aspirin or its combination with metformin on T-cell function **(published)**. We report on the anti-inflammatory effects of both drugs on T-cell mediated responses and further highlighted an overlap in their mechanism of action.

CHAPTER 2: Literature Review
CHAPTER 2.1: Systematic Review and Meta-analysis Protocol

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Systematic Reviews

PROTOCOL Open Access

T cell activation and cardiovascular risk in type 2 diabetes mellitus: a protocol for a systematic review and meta-analysis

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Abstract

Introduction: The burden of non-communicable diseases such as type 2 diabetes mellitus (T2DM) and cardiovascular diseases (CVDs) has drastically increased in developing countries over the years. Although recent evidence points to chronic immune activation to be a significant aspect in the pathogenesis and development of T2DM and CVDs, the exact role of T cells is not fully understood. Therefore, we aim to investigate T cell function and cardiovascular risk in T2DM. In addition, the therapeutic effect of blood glucose-lowering drugs to reverse hyperglycaemia induced T cell dysfunction and myocardial infarction will be reviewed.

Methods: This will be a systematic review and meta-analysis of published studies assessing T cell activation and cardiovascular risk in adults with T2DM. The search strategy will include medical subject headings (MeSH) words for PubMed/MEDLINE database. The search terms will also be adapted to grey literature, Embase and Cochrane Central Register of Controlled Trials electronic databases. Studies will be independently screened by two reviewers using predefined criteria. Relevant eligible full texts will be screened, and data will be extracted. Data extraction will be performed using a pre-piloted structured form. To assess the quality and strengths of evidence across selected studies, the Grading of Recommendations Assessment Development and Evaluation approach will be used. The Cochran's Q statistic and the I^2 statistics will be used to analyse statistical heterogeneity between studies. If included studies show substantial level of statistical heterogeneity, a random-effects meta-analysis will be performed using R statistical software.

Discussions: This review will not require ethical approval, and the findings will be disseminated through peer reviewed publication and conferences. Although other previous studies have reported deregulated T cell function in hyperglycaemia, the underlying mechanisms remain controversial. However, evidence suggests that T cells may be a key component in the development of T2DM and CVDs as its complication. Furthermore, they are a potential diagnostic and therapeutic target in the management of the disease.

Systematic review registration: PROSPERO CRD42018099745

Keywords: Cardiovascular diseases, Inflammation, T cell activation and exhaustion, Type 2 diabetes mellitus

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Background

In an era of rapid urbanisation and modernisation, the burden of non-communicable diseases has drastically increased worldwide, especially in developing countries [1, 2]. Of particular interest is type 2 diabetes mellitus (T2DM), a low-grade chronic inflammatory condition that is characterised by hyperglycaemia (high blood glucose level), insulin resistance and chronic activation of T cells [3–5]. Individuals living with T2DM have elevated levels of pro-inflammatory cytokines that may lead to immune dysfunction and increased risk of cardiovascular diseases (CVDs) [6–9]. Notably, the risk of morbidity and mortality due to CVDs is over fourfold higher in individuals with T2DM, compared to normoglycaemics [10].

The bidirectional relationship between T2DM and inflammation has been well described and involve the role of inflammation in causing both insulin resistance (IR) and hyperglycemia, which in turn further exacerbate inflammation [11–14]. For instance, chronic hyperglycaemia triggers activation of several metabolic and inflammatory pathways which include the aldose reduction pathway [15], advanced glycated end products (AGE) pathway [16], reactive oxygen intermediate pathway [17] and protein kinase C (PKC) pathway [11]. Furthermore, the AGE pathway modulates the nuclear factor-kappa B (NF-kB), phosphatidylinositol 3 kinase/protein kinase B (PI3K/Akt) and mitogen-activated protein kinase (MAPK) pathways resulting in further amplification of pro-inflammatory signals [11, 12]. This chronic exposure to proinflammatory mediators leads to the activation of cytokine signalling proteins which competes with insulin for binding sites and ultimately blocks the insulin signalling receptor resulting in the development of IR and hyperglycaemia [18].

Obesity-induced inflammation and insulin resistance play an important role in the pathogenesis of T2DM. The increased release of interleukin-6 (IL-6) and tumour necrosis factor alpha (TNFα) in individuals with T2DM enhances IR by inhibiting the activity of lipoprotein lipase which is responsible for the hydrolysis of triglycerides into free fatty acids [19, 20]. This reduces the uptake of glucose uptake by adipocytes. Furthermore, in obese T2DM individuals, the adipose tissue becomes hypertrophic and this triggers the production of proinflammatory cytokines and chemokines which attract immune cells [13]. This process causes the infiltration of innate immune cells such as pro-inflammatory macrophages (M1) into adipose tissue, moreover the switching of resident anti-inflammatory macrophages (M2) to M1 subtype [13]. These changes then lead to the initiation of an adaptive immune response. Infiltration of $CD4^+$ T cells into the adipose tissue and their subsequent activation by adipocytes expressing major histocompatibility complex (MHC) class II has been implicated in the early stages of IR in obesity [21]. In addition, during the development of obesity, there is infiltration of B cells and their subsequent production of pathogenic antibodies in adipose tissue which leads to the activation of M1 macrophages and T cells and ultimately the development of IR [22].

It is well documented that chronic hyperglycaemia dysregulates T cell function [23, 24]; however, the underlying mechanisms remain controversial. In fact, contradictory findings of both elevated [13, 25–27] and decreased [28] levels of T cell activation have been reported in hyperglycaemic individuals. Furthermore, previous studies highlight the role of hyperglycaemia in activating pro-inflammatory T helper (Th) subsets [14, 25, 29]. Decreased expression of interleukin 2 receptor (CD25) on activated T cells has been reported in individuals with T2DM [30]. This may be indicative of a loss of the natural regulatory mechanism mediated by T cells in T2DM which further exacerbates T cell activation and inflammation. In contrast, others suggest that hyperglycaemia inhibits T cell activation by disrupting calcium transduction signalling [28]. Therefore, evidence on T cell function in metabolic diseases remains inconclusive.

The involvement and role of T cells in myocardial function and dysfunction has been well described. For instance, lymphocyte-deficient (RAG1 KO) mice revealed significantly smaller infarct sizes compared to the wild-type mice [31]. However, reconstitution of RAG1 KO mice by adoptive transfer of $CD4^+$ T cells reversed this protection and showed an increase in the infarct sizes, therefore suggesting that $CD4^+$ T cells promote myocardial ischaemia-reperfusion injury. A study on patients with acute coronary syndromes (ACS) reported a significant reduction in the number of regulatory T cells (Tregs) compared to the group of individuals with normal coronary arteries [32]. Furthermore, the study reported compromised function activity of Tregs in ACS compared to the control group. These findings implicate T cell activation and inability to suppress T cell function in the development of ACS.

Current T2DM drugs have been proven to be highly effective in the management of hyperglycaemia albeit offering limited cardio-protection [33, 34]. One of these drugs is metformin, a first-line oral anti-diabetic drug which lowers blood glucose levels through direct suppression of hepatic glucose production and the activation of adenosine-monophosphate-activated protein kinase (AMPK). Interestingly, AMPK regulates cellular energy homeostasis and T cell differentiation [35, 36]. However, the exact impact of metformin on T cell function and cytokine release is

not fully understood. A study by Zarrouk et al. reported on a decreased expression of CD25 and activation inducer molecule (CD69) in antigen-activated T cells exposed to metformin when compared to the control group [35]. Furthermore, the study reported failure of metformin-treated T cells to express transferrin receptors and inability to increase glucose uptake [35], thus suggesting alterations in T cell function during metformin treatment, subsequent to the aggravation of a pro-inflammatory response.

This systematic review will for the first time assess available literature on the effect of hyperglycaemia on T cell function, including activation and exhaustion. Furthermore, it will assess the role of T cells in inducing myocardial dysfunction and the therapeutic intervention of glucose-lowering drugs to reverse these effects.

Research question

What is the role of T cell activation in the development of cardiovascular diseases in T2DM? Furthermore, what is the effect of anti-hyperglycaemic drugs on T cell function?

Objectives

1. To investigate T cell function in T2DM
2. To evaluate Th_1 and Th_2 T cell function in treated individuals with T2DM and their association with increased risk of CVDs.
3. To assess whether metformin is effective in reversing hyperglycaemia-induced T cell activation and protect against myocardial injury.

Methods

Protocol and registration

The systematic review protocol has been prepared according to the Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols (PRISMA-P) 2015 guidelines [37].

The protocol has been submitted on PROSPERO for registration. A detailed checklist for this review protocol is provided as PRISMA-P checklist (see Additional file 1 attachment).

Eligibility criteria

This study will include both observational and interventional studies inclusive of cross-sectional and case-control studies with a clearly defined control population. In addition, randomised controlled trials (RCTs) and retrospective and prospective cohort studies with defined time points highlighting data points before and after intervention will be included. Animal studies, case studies and case reports will be excluded from the review. Furthermore, we will also include studies that report the exclusion of participants using steatogenic medications or drugs that interfere with the immune system. Studies that include pregnant women and patients with a known history of T cell malignancy will be excluded.

Participants

Studies on T cell function in adults (> 18 years) with both T2DM and CVDs will be included.

Interventions

We will consider studies that have clearly defined the anti-hyperglycaemic drugs used.

Comparators

The primary comparisons will include:

1. Individuals with T2DM vs the normoglycaemic group (control)
2. Individuals with T2DM on treatment vs control group
3. Individuals with T2DM on treatment vs individuals with T2DM not on treatment group

Outcomes

Primary outcomes will include:

1. T-cell activation reported as mean percentage expression or mean florescence intensity of HLADR, CD38, CD69 and CD95 or $Th_{1/2}$ cytokine secretion.
2. T cell exhaustion reported as mean percentage expression or mean florescence intensity of PD-1.
3. Cardiovascular events associated with T2DM.
   a. Coronary artery events: fatal myocardial infarction, non-fatal myocardial infarction, unstable angina and stable angina.
   b. Cerebrovascular events: fatal stroke, non-fatal stroke (ischaemic or haemorrhagic), transient ischaemic attack and vascular events.

Secondary outcomes will include:

1. For T2DM: insulin resistance, impaired glucose tolerance and increased glycated haemoglobin (HbA1c).

2. For cardiovascular risk (total cholesterol, high-density lipoprotein cholesterol level, systolic blood pressure, dyslipidaemia and smoking).
3. For T cell activation: increased biomarker levels of inflammation (CRP), leucocytosis and high erythrocyte sedimentation rate (ESR).

Search strategy

The systematic search will be conducted without any language restrictions. However, for none-English articles, only those google translatable will be searched. The search strategy will consist of the following major keywords and their respective synonyms: type 2 diabetes mellitus, hyperglycaemia, inflammation, CVDs, T cell activation and exhaustion. For each keyword, multiple synonyms will be searched in the title or abstract. In addition, the reference lists of selected studies will be scanned to identify relevant literature. A search strategy will be developed using medical subject headings (MeSH) words and their respective synonyms on MEDLINE (see Additional file 2). The search strategy will also be adapted to grey literature, Embase and Cochrane Central Register of Controlled Trials electronic databases and will be peer-reviewed by a librarian specialist.

Study selection

A standard data extraction sheet will be used to extract data from the screened and selected studies. The appraisal worksheet will enable the extraction of the following information: aims and objectives of the study, study population, country where the study was conducted, funding source, participant demographics, year published, study type, treatment drugs used, methods and techniques used to assess T cell activation and statistical methods used and limitations of the study. The study selection process will be carried out independently by two reviewers (TMN and BBN) to eliminate any discrepancies and inconsistencies regarding reviewers' inclusion and exclusion of studies. In case of disagreements, PVD will be consulted for arbitration. The appraisal of studies will be documented using Microsoft Excel, and the V.1.18 Mendeley reference manager (Elsevier, Amsterdam, Netherlands) will be used to identify duplicates.

Data collection process

To ensure effective data collection from the selected studies, a pre-piloted structured form will be used to collect data items (listed below). The titles, abstracts and full texts yielded by the search against the inclusion criteria will be used to collect relevant data. To minimise data entry errors, selected studies will be carefully and independently assessed by two different authors (TMN and PVD) to extract relevant data. The other author (BBN) will be consulted for arbitration in case of any disagreements.

Data items

The data items that will be extracted include the name of the authors, year of publication, cohort sample size and duration of follow-up. In addition, participant characteristics such as average age, gender ratio, glucose metabolic profile (blood glucose levels, glycated haemoglobin and fasting insulin) levels of inflammatory biomarkers (C-reactive protein and cytokines), levels of T cell activation and exhaustion markers and their treatment status will also be extracted. In addition, details related to the assays used to measure the levels of T cell function (activation and/or exhaustion) as well as the techniques used will be extracted. Since CVD is a broad category, we will extract the type of cardiovascular event reported from each respective study. Furthermore, the CVDs will be categorised into micro- and macrovascular diseases. The surrogate outcomes for T2DM will include insulin resistance and impaired glucose tolerance that may be reported based on varying outcome measures. In cases where there are no reported data amputation techniques on priority outcomes and when the effect size cannot be calculated, the authors will be contacted for additional information.

Data simplification

As a data simplification measure, studies that mention that participants were on diabetic treatment will be grouped as the treatment group irrespective of the drugs used and those with diabetes and not on treatment as the non-treatment group. Furthermore, the levels of T cell activation and exhaustion will be reported as a continuous variable and will be compared by calculating the standardised mean difference (SMD).

Risk of bias in individual studies

The Cochrane risk of bias tool will be used to assess risk of bias in included randomised controlled trials [38]. The Joanna Briggs Institute (JBI) Critical Appraisal tools with specific checklists for non-randomised experimental studies will be used for other types of studies [39]. A judgement on the possible risk of bias of extracted information will be made based on each of the six domains. The judgement will be made independently by two reviewers (PVD and BBN) based on the criteria defined for judging the risk of

bias. In instances where these two reviewers disagree, TMN will be consulted for arbitration. Furthermore, funnel plot analysis will be used to assess publication bias and the Harbord and Peters test will be used to test the funnel plot asymmetry.

Data synthesis

The Cochran's Q statistic [40] and the I^2 statistics will be used to analyse statistical heterogeneity between studies [38]. An I^2 value of > 25 will be considered as moderate or substantial heterogeneity [41]. If an efficient number of studies included are homogeneous in terms of extracted information, we will conduct a meta-analysis using R statistical Software (The R foundation for statistical computing, Vienna, Austria). The random-effects model will be used should there be significant levels of unexplained statistical heterogeneity [42]. In order to explore the sources of heterogeneity within the included studies, a subgroup analysis and meta-regression compare the study estimates from different study-level characteristics, which will include, age, gender, treatment drugs, reported measure of T cell activation (CD38, CD69, HLA-DR,CD95) and exhaustion (PD-1). Furthermore, data from clinical trials and observational studies will be analysed and used separately.

Cumulative evidence

To assess the quality and strengths of evidence across selected studies, two independent reviewers (PVD and BBN) will review the studies using the Grading of Recommendations Assessment Development and Evaluation (GRADE) approach [43]. The approach will be implemented by the downgrading of studies based on several factors such as study limitations, indirectness of results and publication or reporting bias. The scores will only be upgraded in exceptional cases where individual judgments (inconsistency, indirectness, imprecision and publication bias) are of low risk. Ratings for each outcome will be categorised as high, moderate or low. This will then be followed by the rating of the overall quality. The findings will be summarised and presented in the summary of findings table.

Discussion

Although other previous studies have reported immune dysfunction in a diabetic state, the involvement of adaptive immune response, particularly T cells, still remains limited and controversial. However, evidence suggests that T cells may be a key component in the development of T2DM and CVDs as its complication. Furthermore, they are a potential diagnostic and therapeutic target in the management of the disease. Therefore, the findings of this review will indicate novel avenues to explore at a molecular level in finding solutions in the management and treatment of diabetics. This in turn will help reduce the burden of diabetes and its complications on national health budgets.

Additional files

Additional file 1: PRISMA-P-checklist. (DOC 81 kb)
Additional file 2: Search strategy. (DOCX 19 kb)

Abbreviations
AGE: Advanced glycated end product; CD : Cluster of differentiation;
CVDs: Cardiovascular diseases; DM: Diabetes mellitus; IR: Insulin resistance; T2DM: Type 2 diabetes mellitus; Th: T helper cells

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Authors' contributions
TMN, PVD and BBN conceptualised, designed and drafted the protocol of the study. All authors wrote and approved the final manuscript. TMN is the guarantor of the review.

Ethical approval and consent to participate
Not applicable

Consent for publication
Not applicable. No individual person's data has been included in this manuscript.

Competing interests
The authors declare that they have no competing interests

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CHAPTER 2.2: Systematic Review 1

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Obesity-induced inflammation and insulin resistance: A mini-review on T-cells

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abstract

Excessive lipid accumulation in an obese state is linked with activation and release of detrimental cytokines and chemokines that promote metabolic dysregulation. In fact, emerging experimental evidence shows that abnormal modulation of T-cells in an obese state correlates with the development and progression of insulin resistance. Importantly, the evolving concept linking insulin resistance with impaired immunological mechanisms such as T-cell responses provides new prospects for understanding the role of inflammation in moderating metabolic complications.

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1. Introduction

Obesity is an independent risk factor of metabolic complications such as insulin resistance (IR) and inflammation during the pathogenesis of type 2 diabetes mellitus (T2D) [1]. Increased adipose tissue (AT) mass is a hallmark of chronic low-grade inflammation that is characterised by progressive infiltration of T-cells [2,3]. T-cells play an important role in orchestrating the adaptive immune response and are the second largest cell population in AT followed by macrophages [4]. Briefly, findings have shown that CD4+ and CD8+ T-cells can infiltrate both visceral and subcutaneous AT, with pro-inflammatory T helper (Th)-1, Th17, and CD8+ T-cells, concomitant to the development of IR in healthy overweight or obese human subjects [5]. Hence, T-cells are considered to play an important role in obesity-induced inflammation and IR.

Although previous studies have described T-cell involvement in AT inflammation [6,7], the exact mechanisms and sequence of events in obesity-induced inflammation and the development of IR is unknown. Moreover, conflicting reports on T-cell activation and function in obese AT have been reported. For instance, contrary to their well-known co-stimulatory effects, B7, CD28 and CD40L molecules have in fact been shown to maintain immune homeostasis by regulating the development of IR and ameliorating AT inflammation in diet-induced obese (DIO) mice [8-10]. On the other hand, recent findings show that OX40, a secondary costimulatory molecule could exacerbate AT inflammation and IR by promoting T-cell activation in a DIO mouse model [11]. This is in agreement with other studies showing an increased AT infiltration of both pro-inflammatory and anti-inflammatory T-cell subsets in an obese state [12,13]. Surprisingly, contradictory data presented by others have described decreased anti-inflammatory T-cells, particularly the regulatory T-cells (Treg) subset in various experimental models of obesity, including human studies [3,14,15]. Therefore, it remains essential to establish the precise involvement of T-cells in AT inflammation and IR in obesity and T2D. To explore such consequence, the current study synthesised and critically assessed available literature reporting on the role of T-cells in modulating AT inflammation and IR in obesity and T2D.

2. Methods

This mini-review was prepared in accordance with the Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols (PRISMA-P) 2015 guidelines [16]. Moreover, it forms part of a big project assessing published studies on T-cell function in T2D which was registered with the international prospective register of a systematic review (PROSPERO), registration number: CRD42018099745 and has been published [17].

2.1. Search strategy

A comprehensive search was conducted on the Cochrane Library, Embase and PubMed electronic databases from inception up to 28 March 2019 by two investigators (TMN and PVD). Unpublished and ongoing studies as well as review articles were screened for primary findings. In cases of disagreements, the third reviewer (BBN) was consulted for arbitration. The search strategy was adapted to each database using keywords and medical subjects heading (MeSH) terms such as "T-cells", "adipose tissue", "obesity", "insulin resistance", "type 2 diabetes mellitus" and their respective synonyms and associated words or phrases. No language restrictions were applied to the search strategy.

2.2. Study selection

This review included both animal and human studies reporting on the role or effect of T-cells in obesity-induced AT inflammation and IR. However, reviews, editorials, books, and letters were excluded. Two investigators (TMN and PVD) independently reviewed all relevant articles and identified eligible studies. Any disagreements were resolved by consulting BBN.

2.3. Data extraction

The main outcome of this study was to determine the role of T-cells in obesity-induced inflammation and IR. Briefly, the extracted data items included; names of the authors, year of publication, experimental model used, interventions used and main findings of each study. The Mendeley reference manager version 1.19.4-dev2 software (Elsevier, Amsterdam, Netherlands) was used to manage extracted information including identifying and removing study duplicates.

2.4. Quality assessment

Two investigators (TMN and VM) with the assistance of a third reviewer (PVD), assessed the quality of individual studies included in this review by following Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines [18]. The modified Downs and Black checklist [19] was used to assess quality of included human studies.

3. Results

3.1. Characteristic features of included studies

An overall number of 125 studies were identified and screened for eligibility and a total of 29 articles met the inclusion criteria. All included studies were published between 2008 and 2017. A total of 31 articles were excluded because they were review articles and 59 were irrelevant. Few studies (n ¼ 6) were excluded due to study design, that is, the T-cells assessed in these studies were not AT resident but from peripheral circulation [20,21] (Fig. 1). Of the included studies, 24 were animal studies, 11 were human studies and 6 reported on both animals and humans (Tables 1 and 2). All human studies were observational studies.

3.2. Quality assessment and risk of bias

All included articles were published in peer-reviewed journals. For the animal studies, the ARRIVE guidelines were used to assess the quality of the included studies since it provides a precise method for scoring in vivo models. The median score and range of the 24 included studies was 16 (13-18) out of a possible score of 20, thus all studies met the minimum requirements for publication. Overall, all studies scored high in the introduction domain with a median of 4 (3-4) out of a possible score of 4 (overall agreement 92.97%, kappa = 0.96). Furthermore, the studies also scored high in the method and discussion domains with a median of 7 (5-9) out of the possible score of 9 (overall agreement 76.39%, kappa = 0.58) and 3 (2e3) out of a possible score of 3 (overall agreement 94.44%, kappa = 0.89), respectively. However, the studies scored low in the results section due to the study design, for example no baselines results and adverse events reported, resulting in a median of 2 (0-2) out of the possible score of 4 (overall agreement 69.79%, kappa = 0.40) (Table 1S).

For human studies, the Blacks and downs checklist was used to appraise the included studies and they all scored poorly (<13 points). The median score range of the 11 included studies was 10 (8-13). Overall, the included studies had a lower risk of reporting bias with a median of 5 (4-7) out of the possible score of 10 (overall agreement 90.91%, kappa = 0.82). In addition, the studies also had a relatively low risk of internal validity bias with a median of 3 (3-4) out of the possible score of 7 (overall agreement 88.31%, kappa = 0.95). However, all studies performed poorly on the external validity and selection bias domains (except one study) with a median of 0 (0-2) out of the possible score of 3 (overall agreement 87.88%, kappa = 0.76) and 1 (1-3) out of the possible score of 6 (overall agreement 81.82%, kappa = 0.64), respectively (Table 2S).

3.3. Overview of included animal studies on the role of T-cells

The search retrieved 24 studies that reported on the role of T-cells in AT inflammation and IR in various experimental models of obesity, published between 2008 and 2017. The sections below briefly discuss the different types of T-cells and their role in modulating obesity associated complications.

3.3.1. Infiltration of Th1, CD4+ and CD8+ T-cells in AT of obese animals promotes inflammation and IR

The overall evidence presented in this review clearly shows that DIO mice are among the well-recognized animal models used to investigate the role of T-cells in obesity (Table 1). Generally, these animals are fed a high caloric diet, usually rich in fat, which results in the development of obesity, mimicking that which is observed in humans. After just five weeks of high fat diet-feeding, Kintscher and colleagues were the first to show that infiltration of proinflammatory T-cells in the AT may occur before macrophages as a primary event in AT inflammation, concomitant to the development of obesity-induced IR [22]. These findings were further supported by subsequent studies that reported on an increased infiltration of interferon gamma (IFN-g) producing Th1, CD8+ T-cells in AT of DIO mice when compared to controls [6,12,23-25]. Thus, suggesting that T-cells in the AT are likely to play a major role in mediating inflammation and IR in DIO mice, including humans. In RAG-null (which are mice lacking CD3 or T-cell receptor) and CD8-null mouse models, it was further demonstrated that high fat diet feeding exacerbated AT inflammation [12,23]. However, the transfer of CD4+ and CD8+ T-cells in these respective models alleviated IR and aggravated AT inflammation, respectively.

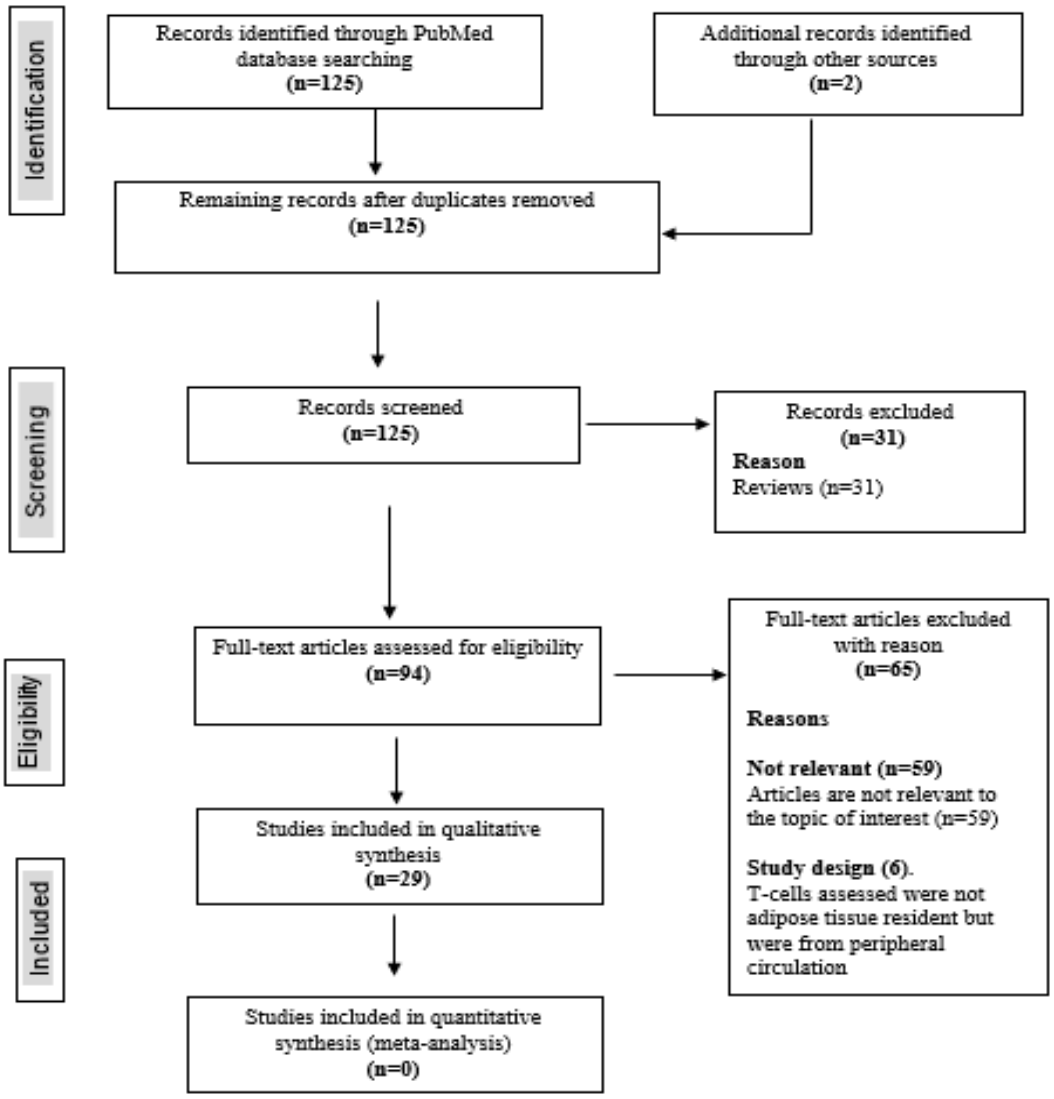

Fig. 1. Flow chart of study selection procedures.

In addition to increased CD4+ and CD8+ AT infiltration in DIO mice, the T-cells were reported to release increased proinflammatory IFN-g cytokine which significantly contributed to AT inflammation [7,26-28]. Remarkably, the removal of T-cells from DIO mice improved IR in early stages of obesity [7]. Like IFN-g, the signal transducer and activator of transcription 3 (Stat3) is known to be central in modulating cytokine-dependent inflammation and immunity within an obese state [29]. Indeed results summarised in this review showed that Stat3 transcriptional factor levels were elevated in both AT and AT-resident T-cells, this consequence promoted the release of IFN-g in DIO mice [26].

This study further demonstrated that Stat3-null mice showed improved IR and reduced AT inflammation. However, it is clear that other important components such as T-cell receptors remain important in regulating an inflammatory response within diverse specific disease conditions. For example, a sub-analysis of mice lacking T-cell a-chain (CD11a-null) showed markedly reduced accumulation and activation of T-cells in AT [28]. On the other hand, increased infiltration of interleukin (IL)-17 producing T-cells in AT was reported in DIO mice [30]. However, contrary to its well-established pro-inflammatory effects, IL-17 in fact regulated IR and reduced obesity, as well as AT inflammation in these mice. Nonetheless, a sub-analysis of IL-17-null DIO revealed increased obesity and IR [30], suggesting the diverse regulatory effects of Il-17 cytokine on AT inflammation in an obese state.

3.3.2. The levels of Th2 and Tregs are reduced in AT of obese animals

It is well-established that T-cell anti-inflammatory subsets (Th2 and Tregs) decrease whilst the pro-inflammatory subsets increase as obesity progresses. Moreover, an imbalance between the modulation of Th1/Th17 and Th2/and Tregs has been associated with an exacerbated inflammatory response and the development of IR (Fig. 2). Evidence presented in this review shows that in addition to increased expression of major histocompatibility complex (MHC) class II, frequency of pro-inflammatory Th subsets and cytokines in AT were inversely proportional to the levels of Th2 subset and IL-13 cytokine in DIO mice [12,25]. Interestingly, MHC-null mice developed less AT inflammation and IR when compared to wild types, despite developing similar obesity associated abnormalities [25].

Winer and associates were the first to report on decreased Tregs in AT of DIO mice, which was linked with the progression of obesity linked complications [12]. These findings have also been supported by subsequent mice studies presented by others [3,14,15,25,31,32]. Briefly, it has been shown that stimulation of Tregs with IL-2 improves AT inflammation and IR mediated by increased IL-10 [14]. Furthermore, administration of visceral AT antigens could effectively increase the number of Tregs resulting in the inhibition of weight gain and IR in mice on high fat diet [15]. Moreover, this study showed that the number of Tregs inversely correlated with macrophages in the AT. Alternatively, the expression of proinflammatory IL-21 and its receptor's (IL-21R) mRNA were upregulated in the AT of DIO mice [31]. Interestingly in DIO IL-22-null mice, amelioration of AT inflammation and reversal of IR was linked to elevated number of Tregs. The overall findings are consistent that an obese state, illustrating that high fat feeding is responsible for reduced AT-resident ST2+ (IL-33R) Tregs promote AT inflammation and IR, as demonstrated in DIO or ST2-null mice [32,33]. Thus, suggesting that some interventions, as seen with administration of IL-33 in DIO mice [32,33], can be further developed to induce the release of Th2 cytokines leading directly or indirectly to increased number of $ST2^{+}$ Tregs.

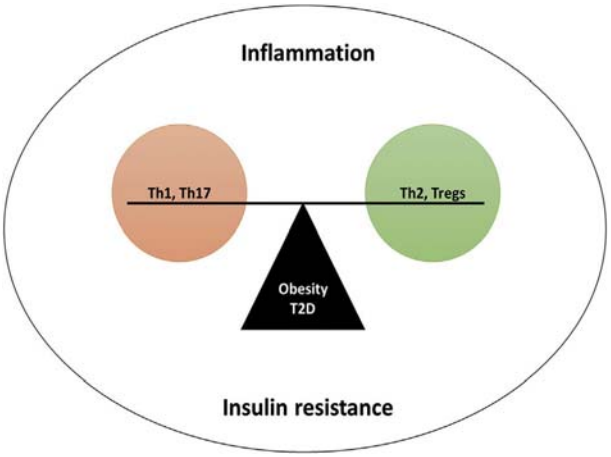

Fig. 2. Effective modulation of Th1/Th17 and Th2/and Tregs remains important in the regulation and amelioration of insulin resistance.

3.3.3. Double-edged sword effect of T-cell co-stimulatory molecules in obese animals

For the successful activation of T-cells, both T-cell receptor and co-stimulatory molecule signals are required. Thus in the absence of a co-stimulatory signals, a hypo-responsive state of T-cells termed anergy is induced despite active TCR signalling and IL-2 expression [34,35]. Therefore, co-stimulatory signals are essential for T-cell activation and function. Inhibition of CD40 signalling pathway by administration of anti-CD40L in DIO mice reduced accumulation of pro-inflammatory macrophages (M1) and AT inflammation and but did not improve IR [36]. However contrary to this, CD40-null mice showed exacerbated IR and AT inflammation mediated by increased accumulation of CD8+ T-cells and M1 macrophages [9,10,37]. Conversely, the activation of CD40 signalling improved IR and suppressed AT inflammation and the repopulation of RAG-null mice with CD40-null T-cells triggered AT inflammation and IR [37]. In agreement with findings by Montes and colleagues, DIO CD28-null mice showed a decrease of both proinflammatory T-cells and Tregs in AT without changing macrophages number [38]. However contrary to this, B7-null mice exhibited enhanced AT inflammation and IR in both DIO and lean mice [8]. In addition, it was shown that adoptive transfer of Tregs into B7-null mice could reverse AT inflammation and IR. Moreover, the same study reported on the decreased expression of B7 expression in an obese state [8]. The inhibition of another costimulatory molecule, CTLA-4, in DIO mice could reduce the number of T-cells in AT but not the levels of pro-inflammatory cytokines [36,38].

Table 1
An overview on included animal studies (n = 24).

Author and year	Experimental model	Intervention used	Role of T-cells/Findings
Kintscher et al., 2008 [22]	Male C57BL/6 J mice	None	Infiltration of pro-inflammatory T-cells in visceral adipose tissue (AT) preceded that of macrophages. Furthermore, the T-cells were identified in the initiation of AT inflammation and the development of IR.
Winer et al., 2009 [12]	RAG-null and diet induced obese (DIO) C57BL/6 J mice	$CD4^+$ T cell transfer	Increased infiltration of pathogenic interferon gamma (IFN-γ) secreting Th1 cells, Th2 and Tregs was identified in an obese state. Moreover, RAG-null mice showed exacerbated obesity and insulin resistance (IR). However, the transfer of $CD4^+$ T-cells into RAG-null DIO mice reversed weight gain and IR.
Rocha et al., 2009 [6]	Male DIO C57BL/6 mice	None	Visceral AT of DIO C57BL/6 mice had higher numbers of both $CD4^+$ and $CD8^+$ T-cells than lean controls. In *vitro* T-cells from obese AT released IFN-γ than in controls
Nishimura et al., 2009 [23]	Male DIO C57BL/6 J and CD8null mice	$CD8^+$ T cell transfer	There was increased infiltration of $CD8^+$ T-cells that preceded the accumulation of macrophages in AT of DIO mice. However, genetic depletion of $CD8^+$ T-cells lowered macrophage infiltration and reversed IR. Conversely, the adoptive transfer of $CD8^+$ T-cells to CD8-null mice aggravated AT inflammation
Feuerer et al., 2009 [14]	DIO C57BL/6 mice	Anti-IL-2	AT resident Tregs were decreased in obese mice and had no suppressive activity but a normal proliferative response in obesity.
Zúñiga et al., 2010 [30]	Male DIO C57BL/6 J and IL17-null mice	None	Stimulation of Tregs by exogenous anti-interleukin (IL)-2 ameliorated obesity-induced inflammation and IR mediated by increased levels of IL-10 Increased infiltration of IL-17 producing T-cells in obese AT inhibited adipogenesis, moderated infiltration of immune cells in AT and regulated glucose metabolism. Moreover, IL-17 deficient mice developed severe obesity and display altered glucose metabolism compared to the wild type.
Yang et al., 2010 [7]	Male DIO C57BL/6 mice were	None	AT T-cells from DIO mice released increased levels of pro-inflammatory cytokines such as IFN-γ upon T-cell receptor (TCR) ligation. Moreover, compared to splenic T-cells, AT T-cells exhibited markedly restricted TCR diversity. Interestingly, removal of T-cells in epidydimal fat enhanced insulin sensitivity in early stage of obesity
Strissel et al., 2010 [24]	Male DIO C57BL/6 mice	None	Enhanced priming for IFN-γ production suggested the contribution of $CD4^+$ and/or $CD8^+$ T-cells to cell-mediated immune responses promoting AT inflammation and IR in obesity. T-cell enrichment and IFN-γ gene induction occurred subsequent to AT macrophage recruitment and the development of IR
Miller et al., 2010 [33]	Genetically obese diabetic (*ob/ob*) and ST2-null mice	Recombinant IL-33	Treatment of AT cultures in vitro with IL-33 induced production of Th2 cytokines, and reduced expression of adipogenic and metabolic genes. Moreover, administration of recombinant IL-33 to *ob/ob* mice led to reduced adiposity and fasting glucose as well as improved glucose and insulin tolerance. HFD fed mice lacking endogenous ST2 (a receptor for IL-33) had increased body weight, impaired insulin secretion and glucose regulation compared to WT controls on HFD
Deiuliis et al., 2011 [3]	Male Foxp3-GFP "knockin" mice	None	DIO resulted in increased $CD4^+$and $CD8^+$ T-cells, with a significantly decreased Treg in visceral AT. Moreover, the number of Tregs inversely correlated with macrophages in the AT.
Priceman et al., 2013 [26]	DIO Stat3-null C57BL/6 mice	None	Regulation of AT T cell subsets by transcriptional factor, signal transducer and activator of transcription 3 (Stat3) is crucial for DIO and IR. The activity of Stat3 is elevated in both obese visceral AT and its resident T-cells. Stat3 in T-cells of DIO mice promoted the release of IFN-γ and blunts Tregs in visceral AT. Moreover, mice Stat3 null T-cells showed reduced DIO and improved IR and glucose tolerance, and suppressed visceral AT inflammation.
Morris et al., 2013 [27]	Male DIO C57BL/6 J mice	None	High fat diet (HFD)-induced obesity promoted conventional $CD4^+$ T-cell proliferation in mice visceral (AT). Dietary obesity was shown to activate the proliferation of IFN-γ producing $CD4^+$ T cells in adipose tissue
Montes et al., 2013 [36]	Male DIO C57BL/6 mice	AntiCTLA-4 Ig and AntiCD40L	$CD4^+$, $CD8^+$ and Tregs were increased in AT of DIO compared to lean controls. However, the administration of co-stimulatory inhibitors in DIO mice reduced inflammation but did not improve glucose tolerance
Jiang et al., 2013 [28]	Male DIO CD11-null C57BL/6 J mice	None	$CD8^+$ T-cells in AT of obese mice showed activated phenotypes with increased proliferation and IFN-γ expression. CD11a-null DIO mice displayed markedly reduced T-cell accumulation and activation in AT. Furthermore, $CD8^+$ T-cells from wild type mice, but not from CD11adeficient mice, infiltrated into AT of recipient obese wild type mice
Deng et al., 2013 [25]	Male DIO Major histocompatibility complex class II (MHC II)-null C57BL/6 mice	None	Expression MHC II in adipocyte was increased in obesity, which was parallel to increased pro-inflammatory and reduced anti-inflammatory AT T-cells. This exacerbated AT macrophage accumulation and M1 polarisation. Alternatively, MHC II-null mice developed less AT inflammation and IR than wild type mice, despite developing similar adiposity.
Zhong et al., 2014 [8]	Male B7-null DIO mice C57BL/6	Adoptive transfer of Tregs	Reduced B7 expressions in obesity directly impaired Treg proliferation and function in obese mice and led to exacerbated AT inflammation and IR. B7-null mice had enhanced AT inflammation and IR in both obese and lean mice. However, adoptive transfer of Tregs reversed IR and AT inflammation in B7 KO mice.
Yi et al., 2014 [9]	DIO CD40-null C57BL/6 mice	None	CD40 deficiency mice exhibited exacerbated AT inflammation and IR with $CD8^+$ T-cells being the major contributor. Contrary to its costimulatory effects, CD40 in fact regulated the development of IR DIO mice by ameliorating AT inflammation.
Wolf et al., 2014 [37]	Male DIO CD40-null and Rag1-null C57BL/6 mice	Anti-CD40 antibody Adoptive transfer of CD40-null T-cells	CD40 deficient mice exhibited increased weight gain, accumulation of inflammatory cells, impaired insulin secretion and enhanced pro-inflammatory gene expression in AT. Conversely, therapeutic activation of CD40 signalling blocked further weight gain, lowered glucose levels, improved insulin sensitivity and suppressed AT inflammation. Furthermore, repopulation of Rag1-null mice with CD40-null T-cells provoked AT inflammation and IR.

Table 1 (continued)

Author and year	Experimental model	Intervention used	Role of T-cells/Findings
Fabrizi et al., 2014 [31]	IL-21-null DIO C57BL/6 mice	None	IL-21 and IL-21R mRNA expression was upregulated in DIO and wild type mice in parallel to macrophage and inflammatory markers. Furthermore, DIO IL-21-null mice, showed reduced AT inflammation and improved IR due to increased infiltration of Tregs in AT.
Chatzigeorgiou et al., 2014 [10]	Male DIO CD40-null C57BL/6 mice	None	DIO CD40-null mice displayed worsened AT inflammation and IR when compared to wild-type mice. The worsened IR was associated with excessive AT inflammation mediated by increased accumulation of $CD8^+$ T-cells and M1 macrophages. However, CD40L mice ameliorated IR and AT inflammation.
Poggi et al., 2015 [38]	Male DIO CD28-null C57Bl/6 mice	Anti-CTLA4	CD28 deficiency decreased pathogenic T-cells and Treg content within AT without changing macrophages number. CTLA4-Ig injections reduced the number T-cells in AT but not inflammatory cytokines levels
Han et al., 2015 [32]	DIO C57BL/6 FOXP3 mice	IL-33 injections	DIO mice exhibited reduced AT-resident $ST2^+$ Tregs thereby promoting inflammation and IR. However, this effect was completely reversed by treatment with IL-33. Furthermore, IL-33 administration also increased the proportion of ST2 expressing Tregs in the AT by 3-fold in DIO mice.
Liu et al., 2017 [11]	Male DIO C57BL/6, OX40-KO and B6.Rag2/ Il2rg double knock mice	None	Increased expression of OX40 (CD134) on $CD4^+$ T cells, infiltration and expression of pro-inflammatory cells and genes respectively, was observed in the AT of DIO mice. Furthermore, DIO OX40-null mice exhibited significantly reduced weight gain and lower fasting glucose levels than the OX40 knocked in mice.
Chen et al., 2017 [15]	Male C57BL/6 J	VAT antigens	Oral treatment of visceral AT mixture antigens effectively inhibited weight gain, and improved IR in HFD mice by increasing the numbers of $CD4^+Foxp3^+$ Tregs that were depleted in obesity

Table 2
An overview of incl uded human studies (n = 11).

Author and year	Experimental model	Intervention	Role of T-cells
Kintscher et al., 2008 [22]	Individuals with T2D	None	Adipose tissue (AT) T-cell infiltration correlated with increased waist circumference in patients with type 2 diabetes mellitus (T2D).
Zeyda et al., 2011 [39]	Overweight and obese humans	None	Th1 and $CD8^+$ T-cells were significantly upregulated in obese AT and correlated with AT inflammation. Surprisingly, Th2 and Tregs were also increased in visceral AT of individuals with obesity compared to lean counterparts
Deiuliis et al., 2011 [3]	Obese humans	None	Humans with obesity showed increased $CD4^+$and $CD8^+$ T-cells with a decreased Tregs in visceral AT.
Yang et al., 2010 [7]	Obese humans	None	There was increased infiltration of $CD4^+$ and $CD8^+$ T-cells in visceral AT of obese individuals compared to lean
Fabbrini et al., 2013 [68]	Obese humans with metabolically abnormal IR	None	The number of AT resident $CD4^+$T-cells that produce interleukin (IL)-22 and IL-17 were 3e10 fold higher in obesity compared to lean subjects.
Deng et al., 2013 [25]	Obese women	None	Obesity enhanced major histocompatibility complex class II (MHC II) expression in adipocytes. Briefly, adipocytes activated AT resident $CD4^+$ T-cells via MHC class II and leptin to induce AT inflammation
Zhong et al., 2014 [8]	Obese humans	None	Reduced B7 expression in obesity impaired regulatory T-cells (Treg) proliferation and function and led to exacerbated AT inflammation and IR
McLaughlin et al., 2014 [5]	Overweight and obese humans	None	$CD4^+$ and $CD8^+$ T-cells infiltrated AT with pro-inflammatory T-helper (Th)1, Th17 and $CD8^+$ T-cells being significantly more frequent. Levels of Th2 in AT were inversely associated with systemic IR.
Fabrizi et al., 2014 [31]	Obese humans	None	IL-21 and IL-21R messenger RNA expression was upregulated in stromal vascular fraction from human obese subjects in parallel to macrophage and inflammatory markers.
Dalmas et al., 2014 [40]	Obese individuals with and without T2D	None	There was increased infiltration of IL-17 and IL-22-producing $CD4^+$ T-cells in individuals with T2D. Moreover, $CD4^+$ T-cell derived IL-22 amplified IL-1b driven inflammation in visceral AT and this was correlated with deterioration of glucose homeostasis.
Travers et al., 2015 [13]	Overweight and obese humans	None	Expression of $CD4^+$ T-cells, macrophages and FOXP3 RNA transcripts were elevated in obesity. Furthermore, AT $CD4^+$ and $CD8^+$ T-cells expressed increased expression of CD69 and CD25 which was associated with increasing degree of obesity. In addition, increased T-cell activation correlated with increased expression and secretion of both pro and anti-inflammatory cytokines in AT.

NB: All studies were observational studies.

On the other hand, the expression of another T-cell costimulatory marker, OX40, was reported to be increased on CD4+ T-cells in the AT of DIO mice [11]. Conversely, a sub-analysis of OX40-null mice showed significantly less weight gain and improved IR compared to the OX40 knocked in mice.

3.4. The impaired modulation of T-cells in obese human subjects promotes inflammation and IR

The search retrieved eleven human studies that reported on the role of T-cells in AT inflammation and IR, published between 2008 and 2015. The specific focus here was to establish whether the modulatory effect of T-cells on obesity associated complications compares to that observed in animal models.

Like the evidence observed in DIO mice (Table 1), increased infiltration of T-cells in obese AT of human subjects was consistent with exacerbated inflammation and it correlated with increased waist circumference [22]. An overwhelming number of studies presented in Table 2 reported on increased infiltration of Th1, CD4+ and CD8+ T-cells in AT of individuals with obesity when compared to lean counterparts [3,5,7,13,39]. Here, AT infiltrating T-cells were triggered in individuals with obesity, and this was demonstrated by elevation of activation markers such as CD69 and CD25, which are known to indicate immune activation and indirectly the degree of obesity in this case [13]. This was consistent with enhancement of pro-inflammatory cytokines like IL-17, IL-21 and IL-22 [31,40]. Elevated CD4+ T-cells in AT of individuals with obesity was also attributed to enhanced the expression of MHC class II [25], which strongly highlighted the consistent modulatory effects of T-cells in obesity induced inflammation.

Furthermore, obese individuals have been shown to present with reduced expression of B7 co-stimulatory molecule, which directly impairs both the proliferation and function of Tregs in AT [8]. In accordance with this, individuals with obesity display reduced levels of Tregs in AT, inversely correlating with IR [3,5]. However, contrary to this, increased AT infiltration by Th2 and Tregs was in fact reported in individuals with obesity [13,39].

4. Discussion

Obesity and its associated complications is persistently linked with impaired immune response and an aggravated inflammatory response [41]. However, the pathological mechanisms involved in these processes are not clearly understood. Therefore, this review aimed to synthesise and critically assess available literature on the role of T-cell function in AT inflammation in obesity or T2D. Most of the included studies showed a strong correlation between increased infiltration of Th1, CD4+ and CD8+ T-cells with an exacerbated pro-inflammatory state, leading to the development of IR. Experimental models of obesity and T2D persistently showed an enhanced infiltration of IFN-g secreting Th1 cells concomitant to reduced levels of Tregs [3,6,12,40]. Evidence presented in this study clearly demonstrated that nutrition plays a major role in the development of metabolic complications, since it was apparent that high fat feeding promoted spontaneous development of obesity that was accompanied by impaired T-cell function in both animals and human subjects [5,12,13,23]. Although a detailed molecular signature that better describes the complex relationship between diet and metabolic dysregulation is not completely understood, AT function within an obese state remains a major focus of ongoing studies [42,43].

Nevertheless, as an endocrine organ, the AT can greatly modulate an inflammatory response by promoting secretion of cytokines and chemokines such as IL-6, IL-8, and MCP-1 that are implicated in promoting ectopic lipid accumulation [41]. In fact, accumulative data summarised in this review showed a strong association between an abnormal inflammatory response and impaired glucose homeostasis that is characterised by an IR state [5,8,9,22]. Anyhow, a vicious circle has been acknowledged between IR and ectopic lipid accumulation, together increasing the risk for the development of metabolic inflammation [1,44,45]. The current study shows that adaptive immunity, especially regulation of T-cells is central in the development of metabolic inflammation and IR [46]. For instance, one study showed that the regulation of AT T-cell subsets by Stat3 is crucial in the pathogenesis of IR [26]. The activity of Stat3 appears to be elevated in both obese visceral AT and its resident T-cells. Evidence presented in Table 1 indicates that activation of Stat3 promotes the release of IFN-g and hinders that of Tregs in visceral AT of obese mice. Similarly, Stat3 null mice showed improved glucose tolerance and suppressed visceral AT inflammation. These findings suggest that besides vast involvement in other physiological processes [47], the STAT pathway plays a major role in modulating inflammatory response in obesity.

Evidence synthesised in this review also highlights the impact co-stimulatory molecules could have in

modulating inflammatory responses within an obese state by inducing T-cell activation [10]. In fact, overwhelming evidence presented here suggests that their signalling pathways may in actual fact have a protective role in obesity and in the pathogenesis of T2D [9,37]. This evidence suggests the potential exploration of co-stimulatory molecules in understanding the role of T-cells in regulating pro-inflammatory responses and most importantly to determine ways to alleviate obesity-induced metabolic complications. In the context of obesity, CD40L is of particular interest since it has been shown that its administration could alleviate AT inflammation and IR in an obese state [36]. However, further studies are needed to confirm this aspect.

Furthermore, it is well-established that metabolites produced in the AT or other metabolic tissues may play an important role in immune response regulation [48e51. In fact, it is now well-established that AT is an active secretory organ that releases metabolites which have the ability to modulate body weight, insulin sensitivity and inflammation [48]. In the context of the latter, AT releases both pro- and anti-inflammatory adipokines which when imbalanced, contribute to the pathogenesis of obesity-linked complications [49]. One of the most studied AT derived adipokines is leptin, a pro-inflammatory metabolite that is significantly increased in obesity and has the ability to initiate and propagate a pro-inflammatory response [49-51]. Briefly, the binding of leptin to its specific receptor (Lep-R) expressed on T-cells is associated with activation of the Janus tyrosine kinase (JAK) pathway, which may results in the phosphorylation and activation of Stat3 [52,53]. Activation of Stat3 is positively correlated with elevated levels of detrimental cytokines such as IL-6 in obese individuals [53]. Interestingly, like leptin, IL-6 has the ability to activate the JAKStat3 signalling pathway [29]. Therefore, consistent with data summarised in this review [26], enhanced leptin secretion as a result of excess AT storage in an obese state may significantly contribute to the activation of the JAK-Stat3 signalling pathway in T-cells, thus contribute to aggravation of obesity-associated proinflammation.

On the other hand, AT is also known to secrete adipokines that oppose the actions of leptin and inhibit the pro-inflammatory stimuli. One of these adipokines is adiponectin, an anti-inflammatory metabolite that has been shown to increase insulin sensitivity and block lipid oxidation by activating the energy sensing, AMP-activated protein kinase (AMPK) [54,55]. Notably, adiponectin levels are significantly decreased in conditions of obesity, including individuals with T2D [56,57]. Concomitant to this, systematic and vector infusion of adiponectin in DIO mice has been shown to significantly inhibit the secretion and actions of IL-6 and TNF-a [58,59]. The latter has the ability to further activate and proliferate T-cells [60]. In addition, adiponectin inhibited cytotoxic activities of natural killer cells, the secretion of TNF-a and IFN-g as well as the signalling of pro-inflammatory nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kB) through the activation of AMPK [61,62]. Moreover, adiponectin can also prevent atherogenesis by inhibiting the expression of the chemokine receptor 3 (CXCR3) on activated macrophages and thus reduce the infiltration of T-cells into the atheroma [63]. These findings are consistent with its effect in blocking the differentiation of Th1 and Th17 cells in rodents [64]. Interestingly, the inhibitory effect of adiponectin on T-cell differentiation has been attributed to its ability to block the CD40-dependent co-stimulatory signalling [64]. Although studies included in the review did not particularly describe their role in T-cell regulation, in the context of obesity, AT derived metabolites such as leptin and adiponectin are skewed towards the pro-inflammatory subset, which could induce and worsen the activation of pro-inflammatory T-cells.

In summary, and to our knowledge, this is the first systematic review to comprehensively describe the role of T-cells in obesity, linking an exacerbated inflammatory state and IR. In addition, this review highlights the potential protective effects that could be established by effective regulation of T-cells, leading to the amelioration of obesity associated complications such as T2D. Therefore, this study paves the way for future studies to explore novel avenues in developing new drugs that alleviate AT inflammation and IR linked with an obese state. Also of note, are the limitations of the current review. Firstly, the included number of studies is low especially human studies. Furthermore, all human studies were observational studies whose evidence is of low quality. Lastly, due to unavailability of human participants' characteristics, we were unable to correlate any biochemical and immune markers with degree of AT inflammation and IR. However, further studies are required to address this aspect.

5. Concluding remarks

Lifestyle modification, including over nutrition coupled with physical inactivity significantly contribute to the development of metabolic complications, including obesity and T2D. Diverse molecular pathways and biological interactions have been explored to understand the impact of these complications to human health. In fact, much attention has been focused on the role of inflammation and immune response in the development of metabolic abnormalities. Data summarised in this review demonstrates that increased infiltration of Th1, $CD4^+$ and $CD8^+$ T-cells in an obese state coupled with decreased levels of Th2 and Tregs greatly impacts human health by exacerbating inflammation and IR. Furthermore, despite the double-edged sword effect of T-cell costimulatory molecules, therapeutic interventions targeting CD40L signalling may have the potential to alleviate inflammation and IR linked with obesity. Further studies assessing therapeutic interventions aimed at modulating these pathways in metabolic disease are needed.

Authorship

TMN, PVD and BBN conceptualised, designed and drafted the review. All authors, including VM wrote and approved the final manuscript.

Conflict of interest disclosure

Authors don't have any competing interests to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.metop.2019.100015.

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Review Article

T-cell activation and cardiovascular risk in adults with type 2 diabetes mellitus: A systematic review and meta-analysis

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ABSTRACT

Background: Chronic immune activation has been described in the development of cardiovascular diseases (CVDs) and in the pathogenesis of type 2 diabetes mellitus (T2D). However, the precise functional role of T-cells remains controversial. We therefore, assessed T-cell activation and cardiovascular risk in T2D.

Methods: The protocol was registered with PROSPERO [CRD42018099745]. We searched electronic databases and grey literature for eligible studies. The risk of bias and quality of evidence were assessed, and the random effects model was used in the meta-analysis.

Findings: Fifteen studies met the inclusion criteria. We report on increased T-cell activation in T2D and nondiabetics with CVD. Comorbidity of T2D and CVD (T2D + CVD) exacerbated T-cell activation. In addition, T2D + CVD comorbidity was associated with an increased CVD risk profile.

Conclusion: This meta-analysis suggests increased T-cell activation in T2D and nondiabetics with CVD. Moreover, an increased cardiovascular risk in patients with T2D which is exacerbated in T2D and CVD comorbidity.

Abbreviations: CVDs, cardiovascular diseases; Th, T helper cells; T2D, type 2 diabetes mellitus * Corresponding author.

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1. Background

The burden of non-communicable diseases (NCDs) has drastically increased in both developing and developed countries [1]. This has led to a significant reduction in life expectancy and an increased strain on national healthcare budgets worldwide [2]. Globally, NCDs are the leading cause of death and account for up to 70% of all-cause mortality [1]. The global prevalence of type 2 diabetes mellitus (T2D), which is one of the major contributing factors to NCDs has significantly increased in the past three decades [3]. This has been attributed to sedentary lifestyle, rapid urbanisation and modernisation [3,4]. T2D is a low-grade chronic inflammatory condition that is characterised by hyperglycaemia, insulin resistance and chronic T-cell activation [5,6]. These consequences are consistent with immune activation that may lead to immune dysfunction and increased risk of cardiovascular diseases (CVDs) [7,8]. The latter is known to be the leading cause of death in individuals with diabetes [9], hence the need to unravel pathophysiological mechanisms such as the role of T-cell activation in a hyperglycaemic state to better understand and prevent NCDs.

The role of activated T-cells in mediating inflammation and altering myocardial function has been previously described. Whereby, activated $CD4^+$ T-cells were shown to promote myocardial ischaemia-reperfusion injury in mice [10]. Increased levels of pro-inflammatory T-helper (Th) subsets have been implicated in the development of coronary atherosclerotic heart disease (CHD) [11,12], carotid atherosclerosis (CA) [13] and coronary artery disease (CAD) [14] in individuals with T2D. Moreover, a significant reduction in the number of regulatory T-cells (Tregs) and Treg/Th1 ratios have been described in individuals with T2D and CHD [11].

Currently, it is hypothesised that chronic hyperglycaemia dysregulates T-cell function. However, the underlying mechanisms remain controversial, with contradictory findings of both elevated [15] and decreased [16] levels of T-cell activation reported in individuals with obesity and T2D. Others have demonstrated reduced frequency of Tregs in T2D and thus loss of the natural regulatory mechanisms mediated by T-cells [11,17]. This suggests that contradictory findings regarding Tcell function in T2D exist, and it remains unclear whether they are dysfunctional or highly activated in a disease state. Although numerous studies reported on T-cell function in T2D [6,15,16], to date, available evidence has not been systematically reviewed to better inform on both T-cell activation and cardiovascular risk in T2D. Therefore, this systematic review was conducted to assess available literature on the impact of T-cell activation in T2D and whether their activation state has any association with the risk of developing CVD. Furthermore, we assessed whether the degree of T-cell activation is unique to individuals with both T2D and CVDs or independently associated with those without T2D but presenting with CVDs.

2. Methods

This systematic review was prepared in accordance with the Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA) guidelines [18]. A detailed checklist for this systematic review and meta-analysis is provided as PRISMA checklist (Supplementary file 1). The systematic review protocol was registered with the international prospective register of a systematic review (PROSPERO), registration number: CRD42018099745 and has been published [19].

2.1 Search strategy

A comprehensive search was conducted on the Cochrane Library, Embase and PubMed electronic databases from inception up to 20 October 2019 as previously described [19]. Briefly, two independent reviewers (TMN and VM) searched for relevant articles and a third reviewer (BBN) was consulted in cases of disagreements. Two search strategies were independently applied to identify relevant studies. The primary search strategy was on T-cell activation in individuals with T2D and CVDs (concept 1). Whilst the secondary search strategy was used to retrieve studies reporting on T-cell activation in nondiabetics with CVDs (concept 2). The search strategies were adapted to each database using keywords and medical subjects heading (MeSH) terms such as “Type 2 diabetes mellitus”, “hyperglycaemia”, “inflammation”, “CVDs”, “T-cell activation and exhaustion” and their respective synonyms and associated words/phrases. No language restrictions were applied. The study selection process was independently carried out by two reviewers (TMN and BBN). In cases of disagreements, PVD was consulted for arbitration. We used the Mendeley reference manager version 1.1.18 (Elsevier, Amsterdam, Netherlands) to identify and remove study duplicates.

2.2 Inclusion criteria

The systematic review and meta-analysis included studies reporting on T-cell function in adults (> 18 years) with CVDs and T2D. We excluded animal studies since we wanted to focus on human subjects. Other exclusions included books, letters, case reports, and reviews. Furthermore, we excluded studies that included participants using steatogenic medications or drugs that interfere with the immune system and patients with a known history of haematological malignancy.

2.3 Data Extraction and Quality Assessment

The data extraction, synthesis and quality assessment of included studies were carried out as previously described [19]. Briefly, the extracted data items

included; names of the authors, publication year, study design, study size, age, gender, types of CVD and main findings of each study. The risk of bias on the included studies was independently assessed by two reviewers (TMN and VM) and a third reviewer (PVD), was consulted in instances of disagreements using the modified Downs and Black checklist, which is suitable for both randomised and nonrandomised studies [20]. Furthermore, the quality of evidence across the selected studies was assessed by two independent reviewers (TMN and VM) using the Grading of Recommendations Assessment Development and Evaluation (GRADE) approach [21].

2.4 Statistical analysis

The mean and standard deviation was extracted or calculated using Hozo *et al.'s* method for each continuous effect measure. Pearson's chisquared test (Chi^2) and Higgin's I^2 statistics were used for the test for statistical heterogeneity. A random-effects model was used to generate pooled effect estimates when substantial heterogeneity existed ($I^2 > 50\%$). Effect sizes were interpreted according to Cohen's d method whereby a standardised mean difference of 0.2, 0.5 and 0.8 was equated to small, medium and large, respectively [22]. Moreover, a pvalue < .05 was considered statistically significant and interrater reliability was assessed for both the included studies and risk of bias using Cohen's kappa. A kappa value of < 0.00 was interpreted as a poor strength of agreement, 0.00–0.20 as slight agreement, 0.21–0.40 as fair agreement, 0.41–0.60 as moderate agreement, 0.61–0.80 as substantial agreement and 0.81–1.00 as perfect agreement [23].

3. Results

3.1 Selected studies

A total of 151 studies were identified and screened for eligibility. A total of fifteen studies (n = 15) met the inclusion criteria (Fig. 1). Of these, 10 studies reported on T-cell activation in individuals with T2D whilst the remaining 5 reported on T-cell function in nondiabetics with CVD (overall agreement 91.53%, kappa = 0.75). The primary search strategy identified a total 76 studies, of which 66 studies were excluded due to no full-texts availability (n = 13) and presented no clear study design (n = 9). The majority of the studies (n = 44) were excluded because they were not relevant to the topic of interest. There were only 10 studies, published between 2011 and 2019, that met the inclusion criteria and 9 of these were included in the quantitative analysis. On the other hand, the secondary CVD search strategy (concept 2) retrieved 75 studies and a total of 62 studies were excluded because they were not relevant to the topic of interest, 4 were reviews and the other 4 were due to study design which contained no suitable controls [24,25]. Therefore, a total of 5 studies published between 2011 and 2014 fulfilled the inclusion criteria on T-cell activation in CVD and were included in this review. Of these studies, only 3 were included in the quantitative analysis.

3.2 Study characteristics

All included studies were published in peer-reviewed journals and characteristics of included participants are shown in Tables 1 and 2. Briefly, this study comprised of a total of 2744 participants with a mean age of 59.77 ± 13.60 years and a male/female gender ratio of 3.5. The included studies comprised of 6 prospective cohort studies [13,26–29] and 9 cross-sectional studies [11,12,14,30–35]. In total, 1062 individuals had T2D, 321 were nondiabetics with CVD and 1361 were healthy controls. In addition, 118 (11%) with T2D were on treatment and 944 (89%) were not specified, while 543 (51%) had T2D and 519 (49%) had both T2D and CVDs (T2D + CVD). The CVDs were all grouped into macrovascular complications and included a total of 840 individuals of which 304 had acute coronary syndrome (ACS) [26,29,30,33,34], 48 had atherosclerotic macrovascular complication (AS) [30], 30 had CA [13], 282 had CAD [14,31,34,35], 83 had CHD [12] and 93 had unspecified CVDs [27,28,32].

3.3 Risk of bias assessment

The risk of bias for each study was assessed using the modified Downs and Black checklist [20]. The median score range of included studies was 12 (8–18) (Supplementary file 2). Seven of the studies were scored as fair (13–17 points) [14,26–29,32] and the rest poor (< 13 points) [11–13,30,31,33–35]. All the studies had low risk of reporting bias with a median of 6 (5–10) out of the possible score of 10 (overall agreement 83.89%, kappa = 0.68). The studies also had a relatively low risk of internal validity bias with a median of 3 (3–5) out of the possible score of 7 (overall agreement 75.08%, kappa = 0.50). All studies performed poor on the external validity (except 1 study) and selection bias domains with each a median of 0 (0–3) out of the possible score of 3 (overall agreement 77.04%, kappa = 0.74) and 1 (0–3) out of the possible score of 6 (overall agreement 88.34%, kappa = 0.77), respectively. The funnel plots showed perfect symmetry on included studies (Fig. 1S).

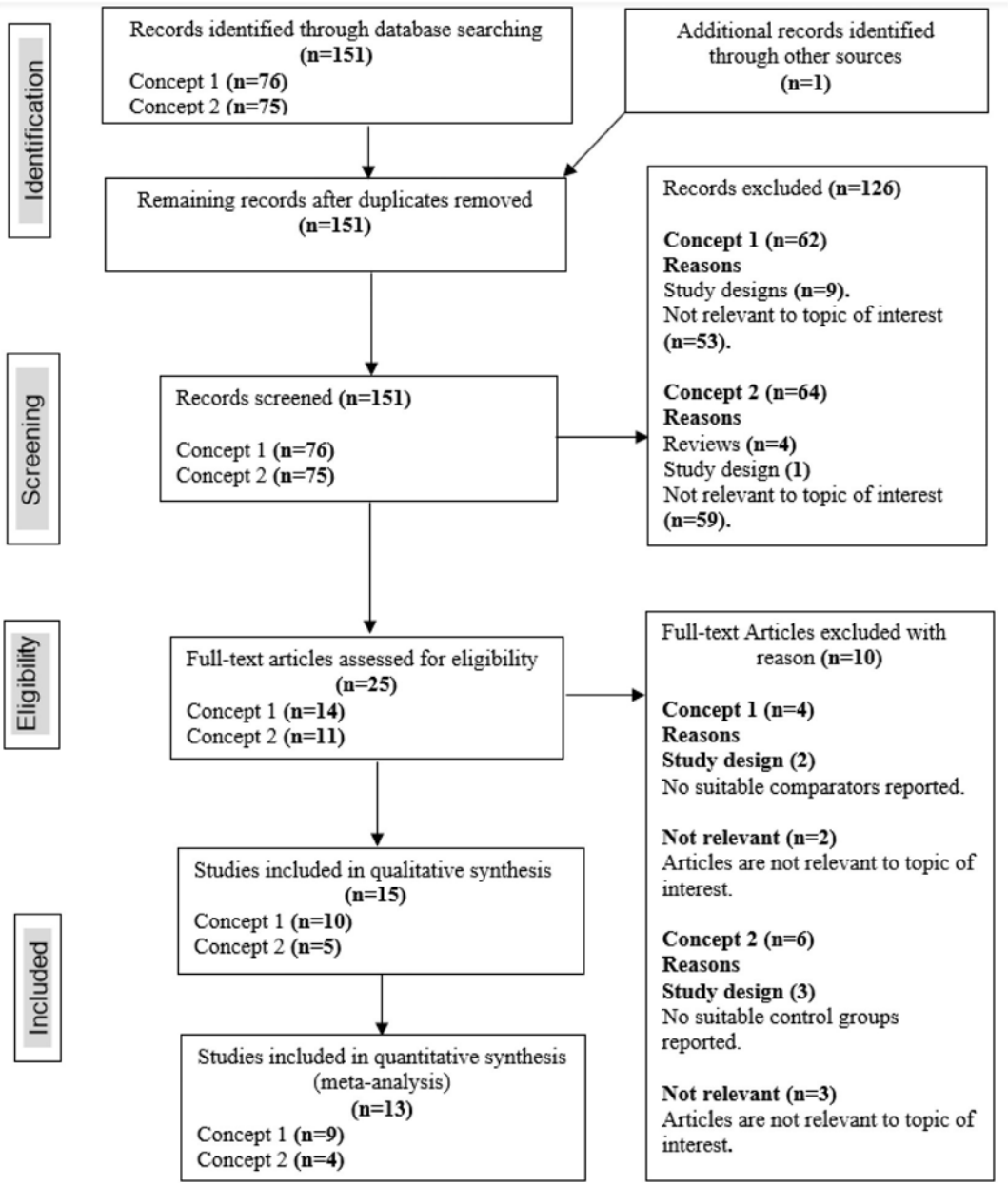

Fig. 1. Flow chart of study selection procedures.

3.4 Data synthesis of included studies

All included studies showed increased T-cell function in T2D, whereby 8 studies reported increased T-cell activation in T2D when compared to normoglycaemic controls [11,13,14,26,27,30–32]. Whilst 4 studies reported increased T-cell activation in nondiabetics with CVD when compared to controls [26,29,33,35]. Moreover, 5 studies reported increased pro-inflammatory T-helper subsets in individuals with T2D + CVD compared to controls [11,13,28,31,32], while 1 study reported on a reduced number of immunosuppressive Tregs [11]. Interestingly, 2 studies reported on a reduced number of anti-inflammatory T-helper subset and their cytokines [13,31] in both T2D and T2D + CVD groups compared to the controls.

3.4.1 Reported glucose metabolic profiles

Of the 10 included studies, 6 reported on glucose metabolic profiles between different groups [11,12,14,26,28,31]. Overall, the lowest mean body mass index (BMI) was reported in the control group (24.05 ± 3.32) when compared to both T2D (26.60 ± 4.02) and T2D + CVD (25.93 ± 3.35) groups.

Table 1
Characteristics of included studies on T-cell activation in individuals with T2D and CVD (n = 10).

Study	Country	Study design	Study size	Male, n (%)	Age (Years)	Risk of bias	Reported measures of immune activation/T-cell activation	Main findings
Rattik et al. (2019)[28]	Sweden	Cohort study	153 participants. (55 T2D; 54 T2D + CVD and 44 controls)	103 (67%)	73.71 ± 2.06	Fair	Effector memory T-cells and Tregs	Effector memory T-cells are potential biomarker for CVDs in individuals with T2D. Moreover, there was no difference in the proportion of Tregs between T2D and (T2D + CVD) group.
Gong et al. (2016)[14]	China	Cross sectional	359 participants. (76 T2D; 158 T2D + CAD and 125 controls)	293 (56.9%)	62.91 ± 11	Fair	IL-17, IL-22, IL-9 and IL-27	Serum IL-22 levels are elevated in T2D, coronary CAD and T2D-CAD comorbidity than controls. Furthermore, IL-22 protects endothelial cells from glucose and lysophosphatidylcholine-induced injury.
Wang et al. (2016)[13]	China	Cohort study	60 participants. (20 T2D; 30 T2D + CA and 10 controls)	23 (38.3%)	51.85 ± 17.62	Poor	Th1, Th2, Th17, INF- γ, IL-4 and IL-17	Angiotensin II promotes the development of CA in T2D patients via regulating the T-cells activities.
Eldor et al. (2015)[27]	Israel	Cohort study	89 participants. (23 T2D; 26 T2D + CVD and 40 controls)	Incalculable	Incalculable	Fair	%CD247 and CRP	The expression of CD247 in significantly reduced in individuals with T2D and is associated with the development of CVDs. Thus, CD247 is a potential diagnostic and prognostic marker for detecting disease progression and severity.
Olson et al. (2015)[32]	USA	Cross sectional	889 participants. (141 T2D; 13 T2D + CVD and 735 controls)	Incalculable	Incalculable	Fair	Th1, Th2, %CD4 naïve and %CD4 memory-cells	T-cells are involved in the chronic immune activation and suppression observed in T2D.
Zhao et al. (2014)[12]	China	Cross sectional	109 participants. (42 T2D and 67 T2D + CHD)	58 (53.23)	59.67 ± 13.76	Poor	hs-CRP, Th1, Th17, Th22 and percentage expression of CD4+ T-cells	Increased peripheral proinflammatory Th subsets contribute to the increased prevalence of diabetic cardiovasculopathy. Elevated Th subsets are also associated with increased CRP levels.
Madhumitha et al. (2014)[31]	India	Cross sectional	142 participants. (60 T2DM; 21 T2DM + CAD and 61 controls)	Not reported	48.29 ± 16.46	Fair	Th1 cytokines (IL-2, IL-12 and INF-γ) and Th2 cytokines (IL-4, IL-5 and IL-13)	The transition from T2D to T2D-CAD co-morbidity, is associated with strong downregulation of Th2 cytokines and upregulation of Th1 responses.
Shi et al. (2013)[30]	China	Cross sectional	173 participants. (42 T2DM; 48 T2DM + AS; 35 T2DM + ACS and 48 controls)	No reported	52.56 ± 10.25	Poor	$CD4^+CD28^-$, $CD4^+CD28^-PD\text{-}1^+$ and INF- γ	The upregulation of PD-1 is closely associated with the severity of diabetic atherosclerotic macrovascular diseases.
Mahmoud et al. (2013)[11]	Kuwait	Cross sectional	70 participants. (24 T2D; 16 T2D + CHD and 30 controls)	44 (62.9%)	50.41 ± 1.95	Poor	Th1, Th17, Treg and Expression of $CD4^+IFN\gamma^+$, $CD4^+TNF\text{-}\alpha^+$, $CD4^+IL\text{-}8^+$, $CD4^+IL\text{-}6^+$, $CD4^+IL\text{-}1??^+$ and $CD4^+IL\text{-}17^+$ T-cells	Hyperglycaemia and dyslipidaemia are associated with an increased inflammatory cytokine expression, suggesting the involvement of T cells in the development of T2D and CHD as its complication.
Giubilato et al. (2011)[26]	Italy	Cohort study	171 participants. (60 T2D; 51 T2D + ACS and 60 controls)	213 (74.5%)	62.44 ± 9.61	Fair	$\%CD4^+$ T-cells, $\%CD4^+CD28^-$ expression and hs-CRP	There is a higher prevalence of $CD4^+CD28^-$ T-cells in individuals with T2D. Furthermore, the T-cell subset is associated with poor hyperglycaemic control and the occurrence of first cardiovascular event

Abbreviations: ACS – acute coronary syndrome; AS – atherosclerotic macrovascular complication; CA – carotid atherosclerosis; CAD – coronary artery disease; CHD – Coronary heart disease; CVD – cardiovascular disease; hs-CRP – highly sensitive C-reactive Protein; IL – Interleukin; INF-γ – Interferon gamma; PD-1 – Programmed Cell death-1; Th – T helper; TNF- α – Tumour necrosis factor-alpha; Treg – regulatory T-cells; T2D – Type 2 diabetes mellitus.

Table 2
Characteristics of included studies on T-cell activation in nondiabetics with CVD (n = 5).

Study	Country	Study design	Study size	Male, n (%)	Age (years)	Risk of bias	Reported measures of immune activation/t-cell activation	Main findings
Emoto et al. (2014)[33]	Japan	Cross sectional	140 participants. (73 CAD and 67 controls)	118 (84.3%)	59.75 ± 9.49	Poor	%$CD4^+$, %$CD4^+CD28^-$ expression and hs-CRP	Individuals with CAD had increased percentage expression of $CD4^+CD428^-$ T-cells but decreased Tregs as well as Treg/non-Treg $CD4^+$ T-cells ratio when compared to controls. Moreower, CAD patients had increased hs-CRP levels compared to controls.
Hego et al. (2014)[29]	Italy	Cohort study	70 participants. (35 ACS and 35controls)	47 (67.1%)	63.5 ± 10.98	Fair	%$CD4^+$, %$CD4^+CD28^-$ expression and hs-CRP	Individuals with ACS had increased frequency of $CD4^+CD28^-$ T-cells compared to controls. Moreover, the inhibitory effect of CD31 on TCR signalling of $CD4^+$ and $CD4^+CD28^-$ T cells was reduced in ACS patients compared to controls.
Teo et al. (2013)[34]	Brazil	Cross sectional	66 participants. (20 ACS, 30 CAD and 16 controls)	47 (71.2%)	60.88 ± 8.82	Poor	$%CD4^+CD28^-$ expression, IFN-γ, TNF-α and CRP	There was no difference in the frequency of $CD4^+CD28^-$ T-cells between all groups. However, $CD4^+CD28^-$ T-cells were the main source of pro-inflammatory cytokines in CAD.
Dumitriu et al. (2012)[35]	England	Cross sectional	78 participants. (48 ACS and 30 controls)	50 (64.1%)	61.98 ± 13.33	Poor	$%CD4^+CD28^-$ expression, IFN-γ, and TNF-α	The frequency of $CD4^+CD28^-$ T-cells was higher in ACS compared to controls. In addition, the $CD4^+CD28^-$ T-cells expressed higher levels of the alternative co-stimulatory receptors (OX40 and 4-1BB) when compared to classical $CD4^+CD28^+$ T-cells.
Giubilato et al. (2011)[36]	Italy	Cohort study	175 participants. (115 ACS and 60 controls)	136 (77.7%)	60.97 ± 9.45	Fair	%$CD4^+$, %$CD4^+CD28^-$ expression and hs-CRP	ACS patients had increased prevalence of $CD4^+CD28^-$ T-cells when compared to controls.

Abbreviations: ACS – acute coronary syndrome; CAD – coronary artery disease; hs-CRP – highly sensitive C-reactive Protein; INF-γ – Interferon gamma; Th – T helper; TNF-α – Tumour necrosis factor-alpha; Treg – regulatory T-cells.

A meta-analysis between the T2D and the control group showed significant heterogeneity between included studies (Chi^2 = 12.31, I^2 = 76%, P = .06). The included studies reported on significantly higher BMI in individuals with T2D when compared to the control group ([MD = 2.25, 95% CI (1.41; 3.09), p< .00001]) (Fig. 2S). Relevant to HbA1c level, which is a measure of metabolic state of diabetes for the last three months, only 4 studies reported on increased HbA1c levels in the T2D compared to the control group ([MD = 2.06, 95% CI (1.42; 2.70), p < .00001]) [11,14,28,31]. However, there were significant levels of unexplained statistical heterogeneity amongst these 4 studies (Chi^2 = 113.25, I^2 = 97%, p < .00001) (Fig. 3S).

3.4.2 Reported effect measure of T-cell activation

Increased expression of the rare pro-atherogenic $CD4^+CD28^-$ T-cells was reported by 2 studies and was shown to be higher in T2D compared to controls, with a mean percentage of 7.85 ± 0.88 and 1.82 ± 0.45, respectively [26,30]. The pooled effect estimates showed a large effect size in percentage expression of $CD4^+CD28^-$ T-cells in individuals with T2D when compared to healthy controls ([MD = 4.02, 95% CI (−0.62; 8.65), p = .09) (Fig. 2A). Moreover, T2D and CVD comorbidity was significantly associated with increased circulating $CD4^+CD28^-$ T-cells, as the mean increased to 21.34 ± 12.47 compared to the T2D group ([MD = 11.44, 95% CI (8.27; 14.62), p < .00001]). However, substantial level of heterogeneity was present in these studies (Chi^2 = 2.03 and I^2 = 51%, p < .00001) (Fig. 2B). On the other hand, there was increased level of $CD4^+CD28^-$ T-cells in nondiabetic with CVD when compared to controls. ([MD = 2.16, 95% CI (0.23; 4.08), p = .03], Chi^2 = 48.85 and I^2 = 96%, p < .00001) (Fig. 2C). Interestingly, although this pooled estimate also revealed significant difference between the nondiabetics with CVD and control group, the overall mean difference (Z = 2.02) was of small effect size (0.2) when compared to that of T2D (Z = 7.06), medium effect size (0.7).

3.4.3 Reported effect measures of cardiovascular risks

3.4.3.1. Overall pooled estimates for cardiovascular risk. Pooled standard mean differences showed reduced CVD risk in controls compared to individuals with T2D ([SMD = −0.34, 95% CI (−0.78; 0.10), p = .13], Chi^2 = 466.36, I^2 = 96%, p < .00001) (Fig. 3). Notably, one of the included studies [11] showed significantly different study-level outcome in CVD risk profile. When the data from this study were omitted, there was a small effect size between T2D group and healthy controls ([SMD = 0.03, 95% CI (−0.30; 0.35), p = .87], Chi^2 = 191.96, I^2 = 92%, p < .00001). Moreover, pooled estimates showed an insignificant increased odds risk of CVD in individuals

with T2D when compared to controls ([OR = 0.94, 95% CI (0.45; 1.97), p = .87], $Chi^2 = 22.47$, $I^2 = 78\%$, p = .0004) (Fig. 5A).

As expected, odds risk of CVD was higher in nondiabetics with CVD group when compared to controls ([OR = 2.33, 95% CI (1.75; 3.09), p < .00001)], $Chi^2 = 43.01$, $I^2 = 88\%$, p < .00001 (Fig. 5B). However, due to substantial level of statistical heterogeneity in these pooled estimates in Figs. 3 and 4, a subgroup analysis based on the reported effect measure of cardiovascular risk was conducted.

3.4.3.2. Body mass index. Overall, data from the included 5 studies showed a lower BMI mean in T2D + CVD group (25.93 ± 3.35) when compared to the T2D group (26.60 ± 4.02) [11,12,14,28,31].

3.4.3.3. Total cholesterol. Five of the included studies [11,13,14,26,31] reported no significant difference in total cholesterol levels between the T2D group and controls ([SMD = 0.07, 95% CI (−0.74; 0.88), p = .87]) (Fig. 3). However, there was substantial level of statistical heterogeneity in these studies ($Chi^2 = 69.28$ and $I^2 = 94\%$, p < .00001). In addition, there was no difference in total cholesterol .levels in T2D and T2D + CVD groups ([SMD = −0.03, 95% CI (−1.01; 0.96), p = .96], $Chi^2 = 84.16$, $I^2 = 95\%$, p < .00001) (Fig. 4).

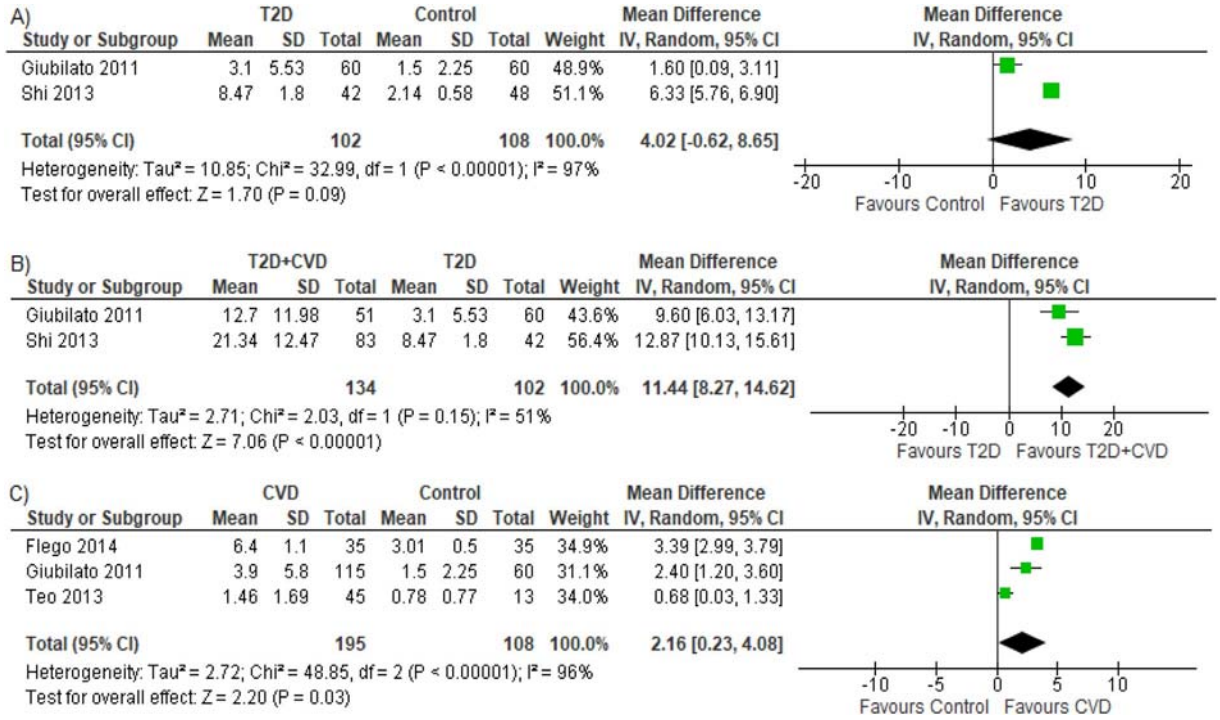

Fig. 2. T-cell activation measured by the expression of $CD4^+CD28^-$ T-cells in (A) T2D compared to controls; (B) in individuals with T2D + CVD compared to T2D; (C) in nondiabetics with CVD compared to controls

Similarly, there was no significant difference in total cholesterol levels between nondiabetics with CVD and healthy controls ([SMD = −0.17, 95% CI (−0.50; 0.15), p = .30], $Chi^2 = 3.36$, $I^2 = 41\%$, p = .19) (Fig. 4S).

3.4.3.4. High-density lipoprotein. Pooled estimates from 6 studies [11,13,14,26,28,31] revealed decreased high-density lipoprotein (HDL) levels in individuals with T2D when compared to controls ([SMD = −0.86, 95% CI (−1.65; −0.07), p = .03]). However, there were substantial levels of statistical heterogeneity in these studies ($Chi^2 = 94.68$ and $I^2 = 95\%$, p < .00001) (Fig. 3). Data from 7 included studies [11–14,26,28,31] showed a large effect size difference in HDL levels between the T2D (1.22 ± 0.45) and T2D + CVD (1.25 ± 0.51) groups ([SMD = −0.90, 95% CI (−1.82; 0.03), p = .06] $Chi^2 = 141.85$, $I^2 = 96\%$, p < .00001) (Fig. 4). In addition, there was a medium effect size difference in HDL levels of nondiabetics with CVD and control group ([SMD = −0.53, 95% CI (−1.14; 0.07), p = .08] $Chi^2 = 25.77$, $I^2 = 88\%$, p < .00001) (Fig. 4S).

3.4.3.5. Low-density lipoprotein. A total of 6 studies reported on decreased levels of low-density lipoprotein (LDL) in T2D when compared to the control group ([SMD = −1.18, 95% CI (−2.06;

−0.30), p = .009]). The included studies showed substantial levels of statistical heterogeneity (Chi^2 = 117.32 and I^2 = 96%, p < .00001) (Fig. 3). Notably, T2D + CVD group was significantly associated with increased LDL levels compared to T2D group ([SMD = 0.90, 95% CI (0.30; 1.50), p = .0003] Chi^2 = 51.44, I^2 = 90%, p < .00001) (Fig. 4). On the other hand, there was no significant difference in LDL levels between nondiabetics with CVD and control group ([SMD = −0.20, 95% CI (−0.56; 0.17), p = .29] Chi^2 = 9.65, I^2 = 69%, p = .02) (Fig. 4S).

3.4.3.6. C-reactive protein levels. A total of 621 participants from 6 studies were included in this analysis [12,26,27,29,34,35] and the results revealed that individuals with T2D + CVD had higher CRP mean levels (12.38 ± 17.22) when compared to both nondiabetics with CVD (5.75 ± 16.30) and controls (1.15 ± 1.14). Notably, pooled estimates showed a significant increase in CRP levels of nondiabetics with CVD when compared to controls ([SMD = 0.35, 95% CI (0.15; 0.54), p = .0005] Chi^2 = 1.45, I^2 = 0%, p = .69) (Fig. 4S). 3.4.3.7. Hypertension. Three studies reported an increased prevalence of hypertension in individuals with T2D + CVD (mean ratio 0.75), compared to T2D (mean ratio 0.63) and healthy controls (mean ratio 0.55) [14,26,28]. Individuals with T2D showed no association with the prevalence of hypertension when compared to the control group ([OR = 1.34, 95% CI (0.90; 1.99), p = .15]). There was no heterogeneity in the included studies (Chi^2 = 1.62 and I^2 = 0%, p = .45) (Fig. 5A). Hypertension was associated with the presence of known CVD and T2D (OR = 1.90, 95% CI (1.24; 2.91), p = .003], Chi^2 = 2.09 and I^2 = 4%, p = .35) (Fig. 5S). As expected, the prevalence of hypertension was associated with known cases of CVD when compared to controls (OR = 2.74, 95% CI (1.87; 4.02), p < .00001], Chi^2 = 44.34 and I^2 = 95%, p < .00001) (Fig. 5B).

3.4.3.8. Smoking. Three studies reported on smoking as a risk factor for CVDs [13,14,26]. There was no association between smoking in T2D and controls ([OR = 0.60, 95% CI (0.16; 2.31), p = .46]) (Fig. 5A). However, a substantial level of heterogeneity was present in these studies (Chi^2 = 8.87 and I^2 = 77%, p = .01). Similarly, there was no association between smoking in T2D and T2D + CVD groups ([OR = 1.91, 95% CI (0.57; 6.42), p = .30], Chi^2 = 9.83 and I^2 = 80%, p = .007) (Fig. 5S). Whereas smoking was associated with CVDs in nondiabetics when compared to controls (OR = 1.90, 95% CI (1.24; 2.91), p = .003], Chi^2 = 1.49 and I^2 = 0%, p = .47) (Fig. 5B). The main findings of this meta-analysis are presented in the summary of findings table (Table 3).

3.5 A narrative synthesis of included studies

3.5.1 Expression of Th subsets

Four of the included studies reported differences in Th subsets between T2D and control groups [11–13,32]. Of these, 1 study demonstrated no significant differences in the Th subsets between the T2D group and the control [32]. However, 2 reported increased expression of pro-inflammatory Th1 in T2D + CVD group when compared to T2D [12,13]. In addition, 2 studies revealed an upregulated expression of Th17 in individuals with T2D when compared to controls [11,13]. Moreover, 3 of these studies associated the presence of CVD in T2D with a further increase in Th17 expression [11–13].

One study reported a decreased expression of anti-inflammatory Th2 subset in T2D when compared to the control group [13]. The presence of a CVD in T2D further decreased the expression of Th2 [13]. The same study reported a similar pattern with Tregs whereby their expression was decreased in T2D when compared to controls. Furthermore, the presence of a CVD in T2D was associated with a further decrease in Tregs expression [13]. On the other hand, increased frequency of $CD4^+CD28^-$ T-cells was reported in individuals with CVD compared to controls in 4 of the included studies [26,29,33,35]. However, 1 study reported no difference in the expression of $CD4^+CD28^-$ T-cells between the CVD and control groups [34]. A meta-analysis could not be performed on T-cell subsets due to lack of data for statistical analysis.

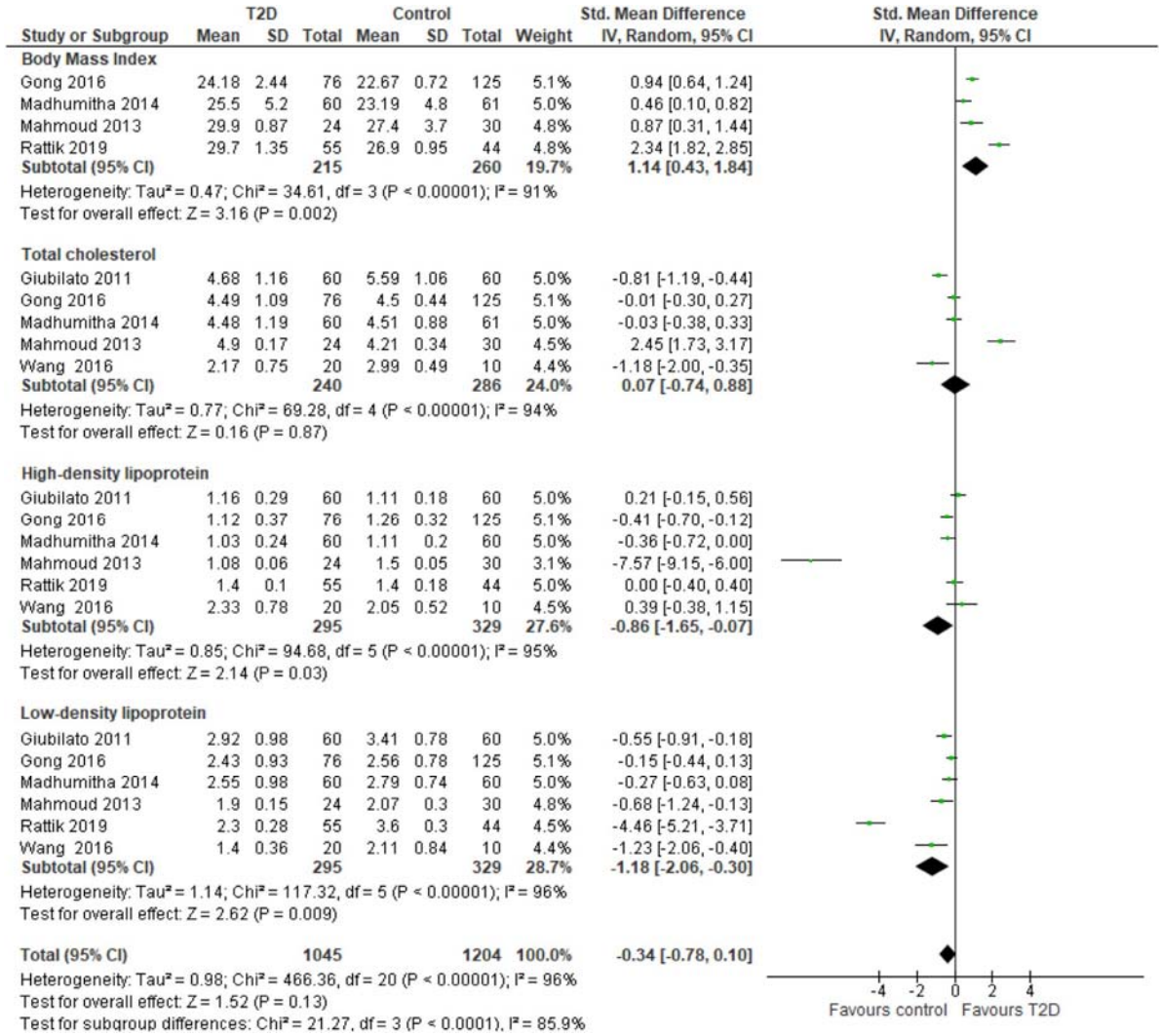

Fig. 3. Pooled estimates of cardiovascular risk in T2D compared to controls.

3.5.2 Pro-inflammatory cytokines

Increased circulating pro-inflammatory cytokines in T2D were reported in 5 of the 9 included studies [11,13,14,30,31]. Of these studies, 4 reported on increased interferon gamma (INF-γ) levels in T2D when compared to controls [13,14,30,31]. Interferon-gamma (INF-γ) is a proinflammatory signature cytokine for Th1 [36]. Furthermore, these studies associated the presence of CVD in T2D with a further increase in INF-γ levels. Although 3 studies reported on differences in interleukin (IL)-17 (a signature cytokine for Th17), only 2 reported increased expression of IL-17 in T2D when compared to controls [11,14]. The other study showed decreased IL-17 in T2D when compared to controls [13]. Nevertheless, all studies associated the presence of CVD in T2D with increased IL-17 levels when compared to both controls and T2D groups [11,13,14]. Two of the included studies reporting on T-cell activation in CVD, showed increased secretion of pro-inflammatory IFN-γ and TNF-α cytokines in individuals with CVD compared to controls [33,34]. A meta-analysis could not be performed on pro-inflammatory cytokines due to lack of data for statistical analysis hence these effect measures are reported narratively.

3.5.3 Anti-inflammatory cytokines

Two studies reported on Th_2 anti-inflammatory cytokines [13,31]. Increased levels of IL-4 were demonstrated in T2D compared to healthy controls, while Wang et al showed no significant difference between the 2 groups [13,31]. However, in 1 study, the presence of a CVD in T2D further decreased IL-4 levels when compared to both T2D and the control group [31]. No meta-analysis could be performed due to lack of data for statistical analysis.

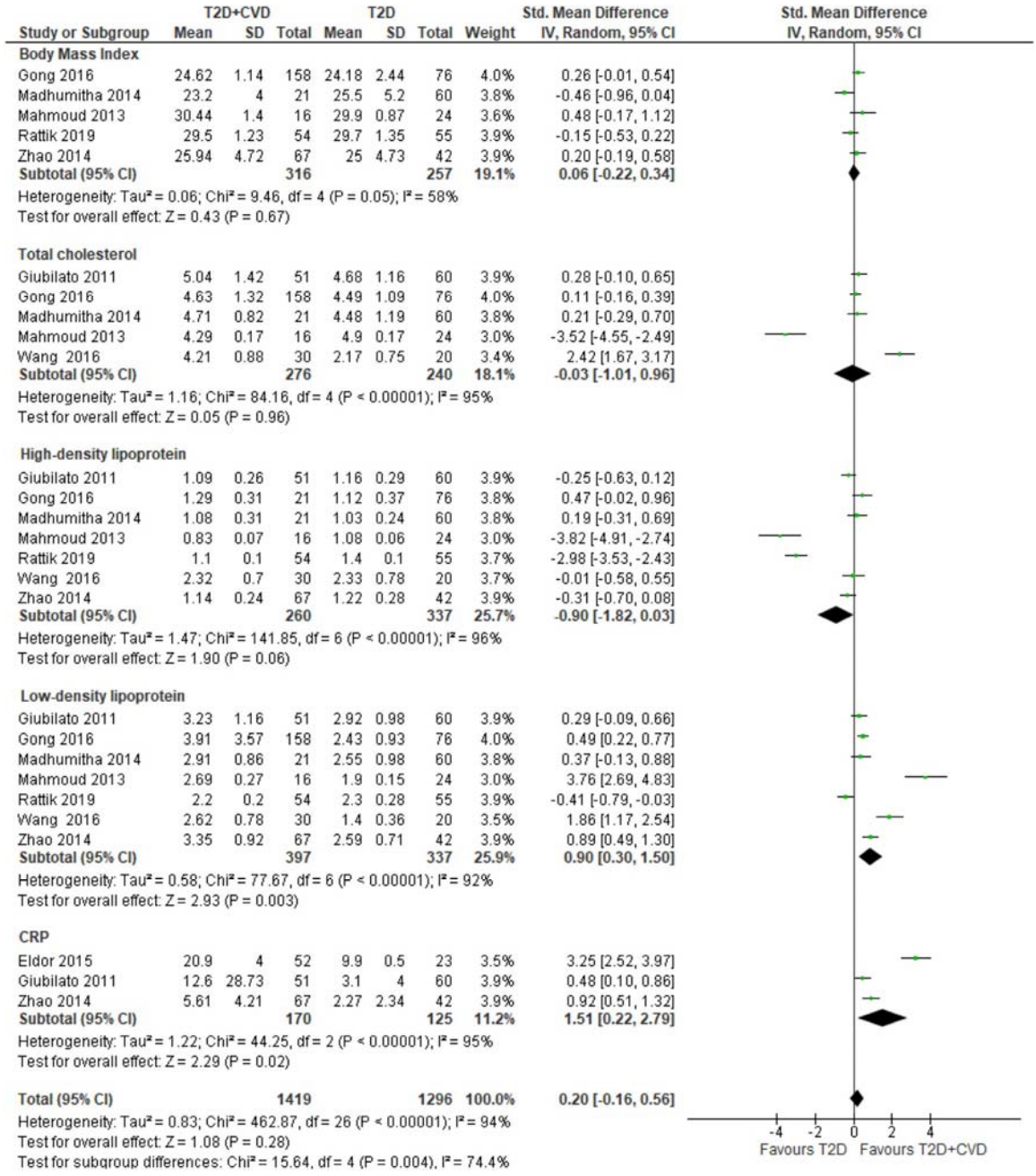

Fig. 4. Pooled estimates of cardiovascular risk in T2D compared T2D + CVD.

4. Discussion

This systematic review aimed at assessing available literature describing the role of T-cell activation and other markers of inflammation in the development of CVDs in T2D. Majority of included studies showed increased T-cell activation in individuals with T2D when compared to the control group. Furthermore, synthesised data suggested that individuals with T2D are at higher risk of developing CVD albeit the data was from observational studies. It was also clear that none of the included studies measured T-cell exhaustion. Moreover, T-cells activation is increased in nondiabetics with CVD when compared to controls.

Therefore, these findings suggest that increased T-cell activation is not unique to T2D but the degree of activation is exacerbated by the presence of T2D.

Increased Th_1 and Th_{17} subsets and loss of Tregs cells have been implicated in the pathogenesis of inflammatory disease [37–40]. Our synthesised data provides a comprehensive increased level of pro-inflammatory Th subsets in T2D, thus implicating increased inflammation and T-cell activation in the development of CVDs. Moreover, elevated pro-inflammatory cytokines and CRP levels reported in individuals with T2D further implicate chronic

inflammation as a link between T2D and increased risk of developing CVD.

Tregs have been reported to have a protective role against the development of CVDs. In that context, low levels of circulating Tregs and Th_2 have been associated with increased risk of acute coronary events [41,42]. Data synthesised in this review showed lower levels of Th_2 anti-inflammatory cytokines and a reduction in the number of Tregs in individuals with T2D compared to controls. Therefore, exacerbated inflammation with T-cell activation and decreased T-cell immune-suppressive potential may be associated with the development of CVDs in T2D.

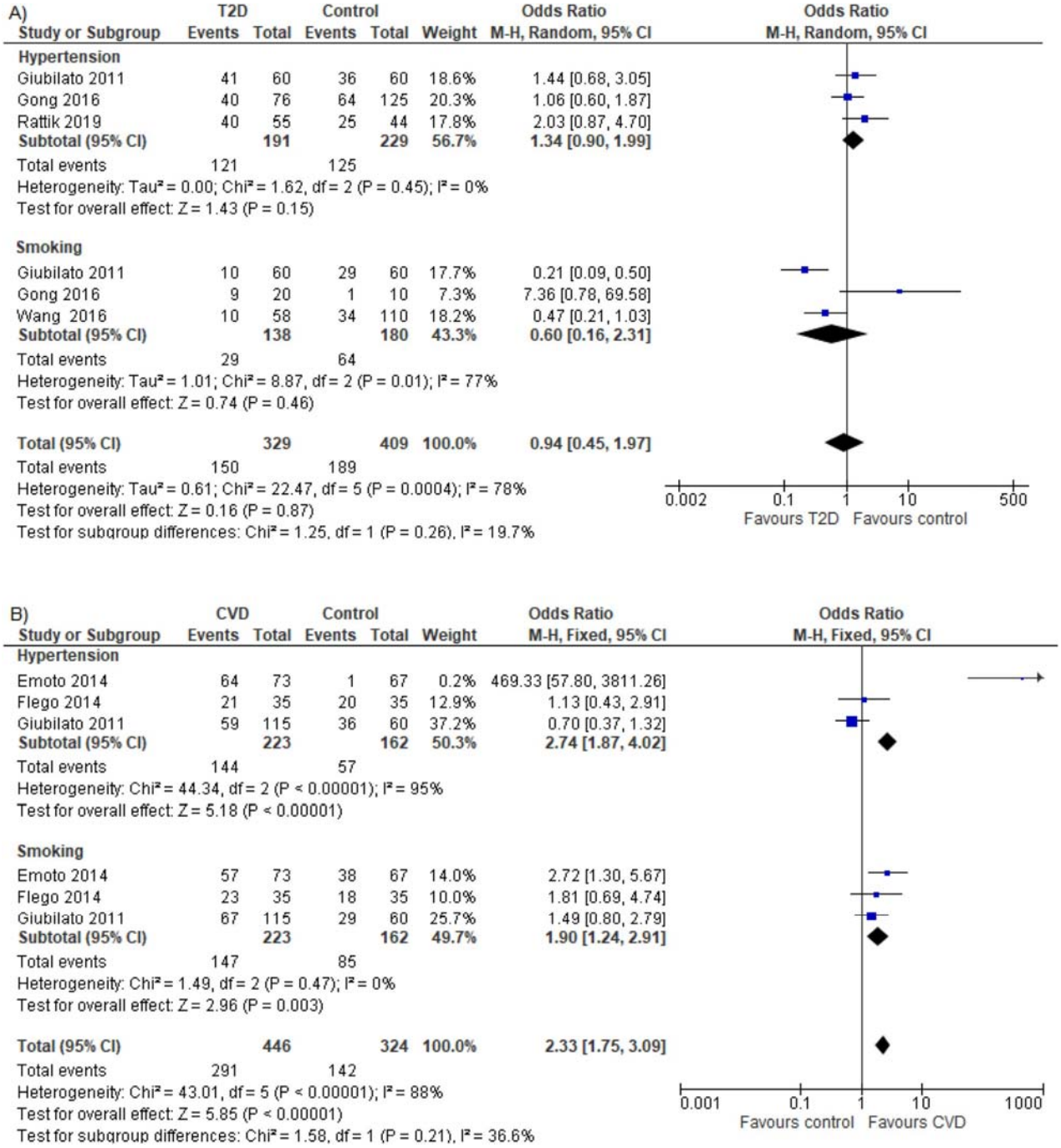

Fig. 5. The prevalence of cardiovascular risk factors in (A) T2D compared to healthy controls and in (B) nondiabetics with CVD compared to healthy controls.

Table 3
Summary of findings table.

T2D compared to control group

Patient or population: Adult individuals with T2D
Exposure: T2D
Comparison: Non-diabetic (control)

Outcomes	Anticipated absolute effects* (95% CI)		Relative effect (95% CI)	№ of participants (studies)	Certainty of the evidence (GRADE)	Comments
	Risk with Control	Risk with T2D				
T-cell Activation Scale from: 1.5 to 8.47%	The mean t-cell activation was **1.82%**	The mean t-cell activation in the exposure group was 4.02% higher (0.62 lower to 8.65 higher)	-	210 (2 observational studies)	⨁◯◯◯ LOW	
Cardiovascular Disease Risk Measured using HDL Scale from: 1.4 to 2.33 mmol/l	The mean for HDL was **1.41** mmol/l	The mean for HDL in the exposure group 0.86 mmol/l lower (−1.65 lower to −0.07 lower)	-	525 (5 observational studies)	⨁◯◯◯ LOW	
T-cell exhaustion Not measured	See comment	See comment	NE	-	See comment	None of the included studies measured T-cell exhaustion

***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). **CI:** Confidence interval; **MD:** Mean difference; **OR:** Odds ratio; **NE:** Not estimable

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 effect.

The $CD4^+CD28^-$ T-cells are a long-lived Th_1 subset that has both proatherogenic and plaque-destabilising properties [33]. However, unlike conventional Th_1 cells, these T-cells also express cytotoxic molecules (perforin and granzyme B) and are rarely found in healthy individuals [43]. Nonetheless, this subset has been implicated in the pathogenesis of various inflammatory disorders [33,44]. In chronic inflammation, the $CD4^+CD28^+$ T-cells lose the expression of CD28, a co-stimulatory marker and therefore making them insensitive to both suppression and apoptotic responses [44]. In addition, $CD4^+CD28^-$ T-cells release an abundant amount of pro-inflammatory cytokines and cytotoxic mediators which are responsible for tissue damage in the pathogenesis of inflammatory disorders such as CVD [33,43]. Data synthesised in this systematic review showed a high prevalence of $CD4^+CD28^-$ T-cells and increased pro-inflammatory cytokine release, in individuals with T2D as well as nondiabetics with CVD. Interestingly, although both these groups reported increased T-cell activation when compared to their respective control groups, the effect size was greater in individuals with T2D compared to those without [22]. Nonetheless, it is evident that T-cell activation is exacerbated by the presence of T2D and is implicated in the development of CVDs in T2D.

It is well-established that individuals with T2D have a higher cardiovascular risk and mortality rate when compared to their non-diabetic counterparts and are disproportionately affected with CVDs [45]. Hypertension is a well-established cardiovascular risk factor present in two-thirds of individuals with T2D [46]. Moreover, the co-existence of hypertension and T2D increases the risk of developing CVDs by almost four-fold when compared to controls [47]. Our study showed a significant association between hypertension and CVDs. Dyslipidaemia, another major risk factor of CVDs that is characterised by changes in both quality and quantity of lipoproteins plays a significant role in the development of atherosclerosis [48]. In that context, high and low levels of LDL and HDL were demonstrated to be closely associated with T2D [48,49]. In accordance with this, data synthesised from this review showed dyslipidaemia as a characteristic feature of T2D, while the presence of CVD was significantly associated with an increased degree of dyslipidaemia.

These findings support the notation that individuals with T2D are at a higher risk of developing CVD. Therefore, hypertension and lipid profiles (particularly HDL) may be used as good markers for cardiovascular risk stratification and potential therapeutic targets in CVDs.

To date, this is the first systematic review and meta-analysis that comprehensively assessed T-cell function in individuals with T2D and their association with increased risk of developing CVD. In addition, the evidence presented in this review indicates that T-cells may be a potential therapeutic target in the management of T2D, although these data were synthesised from observational studies. These findings pave the way for future studies to explore novel avenues in developing new drugs for both management and treatment of diabetes.

The limitations of the current systematic review include; a restricted number of studies investigating the role of T-cells in both T2D + CVDs. In addition, none of the included studies were from African regions, where there is increased urbanisation and risk of CVDs. Secondly, there was a high risk of bias in 8 of the included studies [11–13,30,31,33–35] and the cross-sectional nature of all the included studies was also a significant limitation. Thirdly, the included body of evidence was from observational studies and thus is of low quality. This consequence therefore lowered the certainty of associations between T-cell activation and cardiovascular risk in T2D. Further, randomised controlled trials studies with high-quality evidence and reduced risk of bias due to randomisation are needed to address this. Lastly, although several studies reported on the different anti-hyperglycaemic treatments used by individuals with T2D [11,26], there were insufficient study-level data to perform any sub-group analysis. Therefore, we could not ascertain the effect of anti-hyperglycaemic drugs on T-cell function and cardiovascular risk.

5. Conclusion

The evidence from the included studies showed that peripheral blood T-cells are activated in individuals with T2D or CVD. Moreover, there is increased cardiovascular risk in individuals with T2D. Notably, the transition from T2D to T2D + CVD co-morbidity is associated with exacerbated levels of T-cell activation and increased cardiovascular risk. This was indicated by increased levels of $CD4^{+}CD28^{-}$ T-cells, LDL, CRP and decreased HDL as well as the development of hypertension, leading to a poorer prognosis. In addition, increased T-cell activation in T2D is coupled with a decreased frequency of peripheral immunosuppressive Tregs, increased frequency of pro-inflammatory T- helper subsets and cytokines, including enhanced expression of T-cell negative co-stimulatory molecules. Therefore, a possible approach to reduce the risk of developing CVD in T2D is by modulating T-cell activation, which could be effective in alleviating immune suppression or inflammation. Furthermore, the use of interventions that target and alter CD4+ T-cell subpopulations in T2D could be beneficial in reducing the risk of developing CVD.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary files.

Declaration of Competing Interests

We declare no competing interests associated with this manuscript.

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Authors' contribution

TMN, PVD, VM and BBN conceptualised, designed and drafted this manuscript. All authors wrote and approved the final manuscript. TMN is the guarantor of the review.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https:// doi.org/10.1016/j.clim.2019.108313.

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CHAPTER 2.4: Systematic Review and Meta-analysis 2

A Systematic Review and Meta-Analysis n the Regulation of Programmed Cell Death-1on T-cells in Type 2 Diabetes

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Candidate's contribution: TMN (candidate) conceptualised, designed, and wrote this manuscript. In addition, the candidate appraised included studies, conducted data extraction, and performed all statistical analysis. TMN the guarantor of this review.

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Abstract

Aim: To assess T-cell exhaustion mediated by programmed cell death 1 (PD-1) pathway in patients living with type 2 diabetes (T2D).

Research design and methods: MEDLINE and ProQuest electronic databases were searched for eligible studies from inception up to February 2020. The risk of bias and the quality of evidence were independently assessed by two independent reviewers using the modified Newcastle-Ottawa Scale adapted for cross-sectional studies and the Grading of Recommendations Assessment, Development and Evaluation (GRADE) tool, respectively. The random effects model was used to calculate effect estimates.

Results: We identified 5 studies involving 380 participants which met the inclusion criteria. The pooled estimates showed elevated T helper cell exhaustion in patients with T2D in comparison to controls (MD: 2.57% [95% CI: -3.84, 8.97]; $I^2 = 100\%$, $p<0.00001$). Likewise, T2D patients had increased levels of cytotoxic T-cells exhaustion (MD: 3.09% [95% CI: -12.96, 19.14]; $I^2 = 100\%$, $p<0.00001$). Although the upregulation of PD-1 on T-cells did not affect glucose metabolism-related profiles, it was associated with inflammation and the development of cardiovascular disease.

Conclusion: In patients living with T2D, immune dysfunction is at least in part due to T-cell exhaustion mediated by the upregulation of PD-1 expression. Therefore, the use of immune checkpoint inhibitors as a therapeutic strategy may be of beneficial in restoring immune function in T2D patients.

Keywords: Chronic inflammation; Immune activation; Programmed cell death 1; T-cell exhaustion; Type 2 diabetes mellitus.

1. Introduction

Type 2 diabetes (T2D) is a low-grade inflammatory condition that is characterised by insulin resistance, hyperglycaemia and immune dysregulation.[1,2] Notably, impaired glucose tolerance and obesity drives chronic inflammation in T2D.[3,4] This can lead to an exacerbated activation of both the innate and adaptive immune systems.[5] In fact, immune responses mediated by T-cells play a pivotal role in maintaining immune homeostasis.[6] In chronic inflammatory conditions, increased expression of negative co-stimulatory molecules is known to promote T-cell exhaustion.[7]

It is acknowledged that negative co-stimulatory molecules such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death-1 (PD-1) are essential in inducing immune tolerance during T-cell maturation, and regulating T-cell effector functions.[8] However, persistent immune activation in patients with T2D may lead to aberrant expression of these markers and altered T-cell effector function.[9] Of the various inhibitory molecules, PD-1 has been identified as one of the most potent negative regulators of T-cell functions.[10] For instance, enhanced PD-1 signalling has been linked with cytotoxic ($CD8^+$) T-cell exhaustion in viral infections.[11] Recent evidence also suggests that PD-1 plays a major role in T-helper ($CD4^+$) cell exhaustion during chronic infections.[12,13] Notably, since T-cells are involved in the pathogenesis of T2D[14], there has been a great interest in understanding the impact of PD-1 in T-cell mediated inflammation and dysfunction in conditions of metabolic syndrome. Interestingly, we have progressively explored the detrimental effects linking increased levels of T-cell activation and pro-inflammatory T-cell subsets with low-grade inflammation in T2D.[15–17] To date, available literature on the expression of PD-1 on T-cells of T2D patients is inconclusive, with dysregulated expression of PD-1 linked to the progression of low-grade inflammation in conditions of impaired glucose tolerance.[18,19] Therefore, in this systematic review and meta-analysis, we aimed to assess T-cell exhaustion mediated by PD-1 expression in patients living with T2D.

2. Methods

This systematic review and meta-analysis was prepared according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines.[20] This study forms part of the registered protocol with the International prospective register of systematic reviews (PROSPERO), registration number: CRD42018099745. We conducted the qualitative and quantitative synthesis to answer the following questions;

Question 1: Are circulating T-cells exhausted in adult patients living with T2D?

Question 2: Does PD-1 receptor mediate T-cell exhaustion in T2D?

2.1 Search strategy

A comprehensive search was conducted on the MEDLINE electronic database and ProQuest grey literature, from inception up until the 20th of February 2020. The search was conducted by two

independent reviewers (TMN and BBN), whilst a third reviewer (PVD) was consulted for arbitration in cases of disagreements. The search strategy was adapted to MEDLINE databases without any language restrictions using medical subjects heading (MeSH) terms and keywords such as "programmed cell death-1", "T-cell exhaustion", "type 2 diabetes mellitus" and their respective synonyms and associated words or phrases. A detailed search strategy is provided as a supplementary file (Table 1S). In addition to scanning reference lists of retrieved studies, the ProQuest grey literature database was also searched for relevant studies. The Mendeley reference manager software (Elsevier, Amsterdam, Netherlands) was used to manage the reference list and to remove study duplicates.

2.2 Inclusion criteria and study selection

This systematic review and meta-analysis included studies that reported on the expression of PD-1 on T-cells of individuals with T2D. Reviews, books, editorials, letters and studies that reported PD-1 expression on other immune cells that are not T-cells were excluded. The studies identified by the search strategy were independently screened and selected by two reviewers (TMN and PVD) using the following pre-defined PECO: **Participants:** Adults ($\geq$18 years old); **Exposure:** T2D; **Comparator:** Healthy controls (normoglycaemics); **Outcome:** T-cell exhaustion. In cases of disagreements, a third reviewer, BBN was consulted for arbitration.

2.3 Data extraction

Two independent investigators (TMN and VM) extracted the data items using a pre-defined data extraction sheet. A third reviewer (PVD) was consulted for arbitration in instances of disagreements. The extracted data items included the names of the authors, year of publication, study design, age of participants, T-cell subsets that PD-1 expression was reported on and the main findings.

2.4 Risk of bias and quality assessment

Two reviewers (TMN and VM) independently assessed risk of bias in the included studies using the modified Newcastle-Ottawa Scale adapted for cross-sectional studies.[21] Briefly, the tool uses three domains namely, selection of study groups, comparability of the groups and outcome ascertainment to assess study quality. A study is considered unsatisfactory if the total score is $\leq$ 4, satisfactory (5-6), good (7-8) and very good (9-10). In cases of disagreements, a third reviewer (BBN) was consulted for arbitration. The same reviewers evaluated the quality of evidence using the Grading of Recommendations Assessment Development and Evaluation (GRADE) approach. [22]

2.5 Statistical analysis

Cohen's kappa scores were used to measure interrater reliability.[23] The mean and standard deviation for each continuous effect measure was extracted or calculated using *Hozo et al*. method.[24] In cases where standard deviations were not reported, the Cochrane guidelines were followed to estimate the values.[25]. Heterogeneity was quantitatively assessed using Higgin's I^2 index[26] and the random-effects model was

used[27] to calculate the pooled estimates. The effect estimates were reported using mean difference (MD) and 95% confidence interval (CI). A p-value < 0.05 was considered statistically significant. To evaluate the influence of each study on the overall effect size, a sensitivity analysis was conducted using the leave-one-out method. All statistical analysis was performed using REVMAN version 5.3 software (Cochrane Collaboration, Oxford, UK)

3. Results

3.1 Included studies

The search strategy identified a total of 12 citations and only 5 studies[9,18,19,28,29] met the inclusion criteria (overall agreement 95.45%, kappa = 0.88). A total of 5 studies were excluded at the abstract stage because 2 were reviews and 3 were not relevant to the topic of interest. Of the remaining 7 studies that were assessed for eligibility using full texts, 2 studies were excluded because there were not relevant to the topic of interest. As a result, a total of 5 studies were included in this systematic review and meta-analysis as indicated in Figure 1.

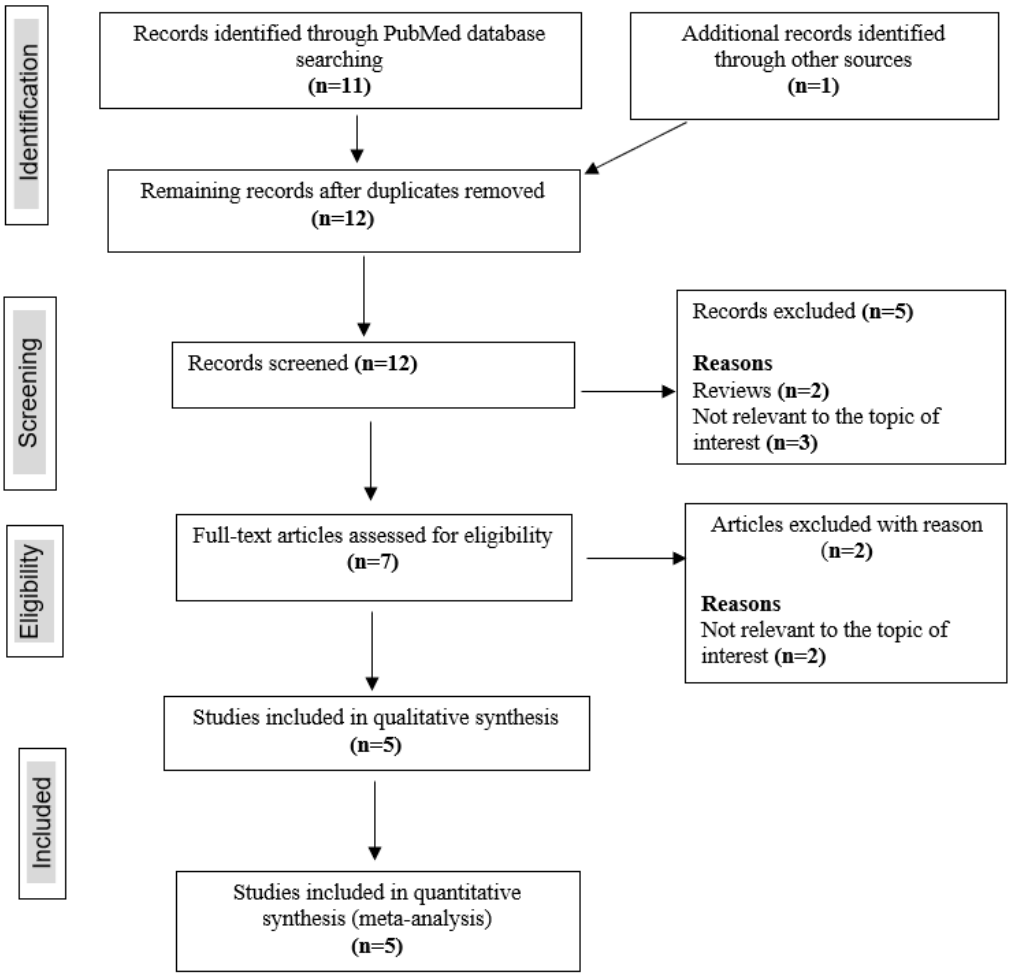

Figure 1: PRISMA diagram showing the study selection process

3.2 Study characteristics

The included studies were published between 2013 and 2019 and their characteristics are presented in Table 1. All citations were all cross-sectional studies comprising a total of 380 participants. Of these, 198 were T2D patients and 182 were healthy controls. The study population had an average age of 55.64 ± 9.09 and a male to female ratio of 0.75. Overall, the included studies reported on participants from China (n=3), Japan (n=1) and South Africa (n=1).

3.3 Risk of bias assessment and publication bias

The overall median score range of included studies was 7 (4-8), one study was scored as unsatisfactory (4 points)[28] and the rest good (7-8 points)[9,18,19,29] (Table 2S). Included studies had a selection median of 3 (2-4) out of possible 5 stars (overall agreement 100%, kappa = 1), comparability median of 2 (0-2) out of possible 2 stars (overall agreement 91.4%, kappa = 0.83) and outcome ascertainment median of 2 (2-3) out of 3 possible stars (overall agreement 93%, kappa = 0.87). We explored potential publication bias by visual inspection of funnel plots and they showed that publication bias was not likely amongst the included studies (Figure 1S).

Table 1: Characteristic features of included studies and the reported on the expression of programmed cell death 1 (PD-1) on T-cells in patients with type 2 diabetes (T2D)

Study	Country	Study size	Male, n (%)	Age (years)	T-cell subsets reported	Main findings
Shi et al., 2013[18]	China	90 participants (42 T2D and 48 controls)	Not reported	51.46 ± 10.68	$CD4^+CD28^-$	The expression of PD-1 on $CD4^+CD28^-$ T-cell subset was increased in T2D patients when compared to healthy controls. Moreover, the upregulation of PD-1 on these T-cells was associated with the development of atherosclerotic macrovascular diseases.
Fujisawa et al., 2015[28]	Japan	48 participants (19 T2D and 29 controls)	24 (50)	49 ± 11.82	$CD4^+$	The levels of PD-1 expression on T-helper cells were comparable between individuals with T2D versus healthy controls.
Jia et al., 2016[9]	China	130 participants (80 T2D and 50 controls)	70 (54)	61 ± 4.10	$CD4^+$ and $CD8^+$	The expression of PD-1 on both $CD4^+$ and $CD8^+$ was increased in T2D when compared to healthy controls. In addition, the upregulation of PD-1 on T-cells positively correlated with the levels of C-reactive protein (CRP), an inflammation marker.
Nyambuya et al., 2018[29]	South Africa	69 participants (34 T2D and 35 controls)	10 (14)	54.48 ± 4.45	$CD4^+$	There was no difference in the expression of PD-1 on T-cells in T2D patients and healthy controls. Moreover, there was no correlation between the expression of PD-1 on T-cell and glucose metabolic profile. However, T2D patients had increased levels of inflammation.
Sun et al., 2019[19]	China	43 participants (23 T2D and 20 controls)	20 (47)	57.47 ± 9.03	$CD4^+$ and $CD8^+$	$CD4^+$ and $CD8^+$ T-cells from individuals with T2D expressed lower levels of PD-1 when compared to healthy controls. However, no correlation was found between PD-1 expression and glucose metabolic profiles.

3.4 Glucose metabolic profiles

A total of 3 studies reported on glucose metabolism-related profiles of included participants. As expected, T2D patients had significantly increased fasting blood glucose levels (MD: 2.81mmol/L [95% CI: 0.28, 5.34]; $I^2 = 99\%$, p<0.00001) and glycated haemoglobin levels (MD: 2.57% [95% CI: -0.08, 5.23]; $I^2 = 100\%$, p<0.00001) (Figure 2S), thus indicating poor glucose control.

3.5 T-cell exhaustion mediated by increased PD-1 signalling pathway in t2d

Two studies [9,18] reported on increased expression of PD-1on $CD4^+$ and $CD8^+$ T-cells in T2D patients when compared to healthy controls. In contrast, two of the included studies[28,29] reported comparable levels, whereas 1 study [19] showed decreased levels of PD-1 expression on T-cells in T2D patients and controls. The pooled estimates showed increased PD-1 signalling on T helper cells (MD: 2.57% [95% CI: -3.84, 8.97]; $I^2 = 100\%$, p<0.00001) and cytotoxic T-cells (MD: 3.09% [95% CI: -12.96, 19.14]; $I^2 = 100\%$, p<0.00001) of T2D patients in comparison to controls (Figure 2). Thus, suggesting PD-1 mediated T-cell exhaustion in diabetic state.

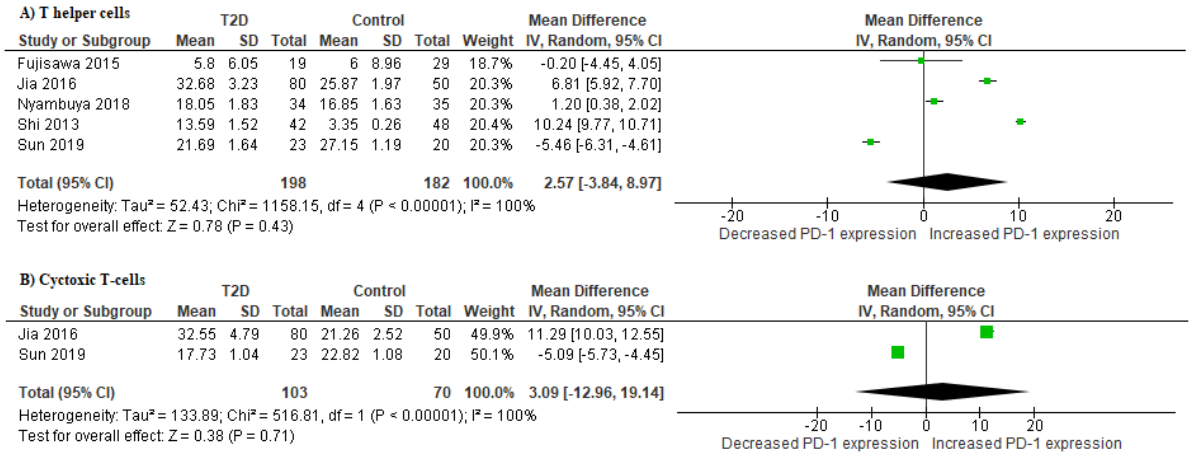

Figure 2: A comparison of mean difference of programmed cell death 1 (PD-1) expression on T helper cells (A) and cytotoxic T-cells (B) between T2D patients and healthy controls.

We performed a subgroup analysis based on extracted and computed values of PD-1 expression on T helper cells. Pooled estimates of studies where PD-1 expression values were extracted were lower (MD: 1.55% [-10.88, 13.99]; 100%, p <0.00001) than studies where the values were computed (MD: 4.00% [-1.50, 9.50]; $I^2 = 99\%$, p <0.00001) (Table 3S). To further investigate the sources of heterogeneity amongst the included studies and assess the robustness of the reported estimates, we conducted a sensitivity analysis and it revealed that sample type and risk of bias might be further sources of heterogeneity in the estimates of PD-1 mediated T-cell exhaustion. There was no difference on the levels of T-cell exhaustion between studies

reporting on whole blood samples and those on peripheral blood mononuclear cells. Moreover, the level of statistical heterogeneity remained substantial (Table 4S). The sensitivity analysis did not change the direction of the polled effect estimate, thus suggesting our findings to be robust. A qualitative synthesis of included studies in this review revealed that the upregulation of PD-1 expression on T-cells had no association with glucose metabolism[19,29], but was positively correlated with inflammation and the development of cardiovascular diseases (CVDs)[9,18]. A summary of findings is provided in Table 2.

Table 2: Summary of findings table

Type 2 diabetes compared to healthy controls

Patient or population: Adults (≥18 years of age)
Exposure: Type 2 diabetes mellitus (T2D)
Comparison: Healthy controls (normoglycaemics)

Outcomes	Absolute effects* (95% CI)		Relative effect (95% CI)	№ of participants (studies)	Certainty of the evidence (GRADE)	Comments
	Risk with control	Risk in T2D patients				
T-cell exhaustion Measured by PD-1 expression on CD4 T-cells	-	The mean level in the exposure group was 2.57 higher (-3.84 to 8.97)	-	380 (5 observational studies)	⊕⊕00 LOW	
T-cell exhaustion Measured by PD-1 expression on CD8 T-cells	-	The mean level in the exposure group was 3.09 higher (-12.95 lower to 19.14 higher)		242 (3 observational studies)	⊕⊕00 LOW	

***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).

CI: Confidence interval; **MD:** Mean difference; **OR:** Odds ratio; **NE**: Not estimable

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 effect

4. Discussion

The aim of this study was to assess T-cell exhaustion mediated by PD-1 expression in patients living with T2D. Although T-cell exhaustion is associated with conditions of impaired glucose tolerance [18,19], the regulation of PD-1 expression on T-cells of T2D patients remains elusive. In this study, pooled estimates showed that in T2D patients, PD-1 expression is upregulated when compared to healthy controls. Such findings are congruent with available evidence suggesting that PD-1 expression might promote

inflammation and the development of CVDs.[9,18] Taken together, these findings support the notion that T-cell exhaustion may be mediated by PD-1 signalling pathway in patients living with T2D.

PD-1 is a receptor belonging to the CD28 family that delivers a negative signal upon interacting with its two ligands programmed death ligand 1or 2 (PD-L1 or PD-L2).[10,30] Successful T-cell activation requires two signals to induce their effector functions, whereby the first primary signal is via the T-cell receptor (TCR) and the second is through CD28 co-stimulation.[31] The upregulation of PD-1 blocks the co-stimulatory signalling, resulting in immune suppression.[32] Patients living with T2D patients are known to present with abnormally increased levels of circulating interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-6 and IL-17.[33,34] In fact, these pro-inflammatory cytokines have the ability to induce PD-1 signalling by upregulating the expression of its ligands in tumour environments, autoimmunity or during chronic infections.[35] The activation of PD-1 signalling pathway dephosphorylates TCR signalling and zeta-chain-associated protein kinase (ZAP)70 by recruiting Src homology phosphatase (SHP)-1/2 which results in the dysregulation of a number of molecular mechanisms including; the insulin-dependent phosphoinositide 3-kinase/protein kinase B (PI3K/AKT); as well as the pro-inflammatory Janus kinase/signal transducers and activators of transcription 3 (JAK/STAT3) and nuclear factor kappa B (NF-κB) elements.[36] These pathways are essential for T-cell proliferation, activation and survival.[37–39] In particular, JAK/STAT and NF-κB pathways are the principal signalling mechanisms for a variety of cytokines and growth factors implicated in cell death[39,40], whilst the activation of PI3K/AKT is crucial for cell survival and proliferation, including improvements in glucose control [37,41]. Therefore, a pro-inflammatory state in T2D patients may induce the activation of PD-1 signalling leading to immune suppression and T-cell exhaustion.

The expression of PD-1 on T-cells and cytokine production is closely regulated in a physiological state. However, in a state of impaired glucose metabolism, it is apparent that PD-1 expression is dysregulated. For instance, exposure of murine T-cells to galactose or glucose substrates has been shown to enhance the expression of PD-1 in addition to increasing oxidative phosphorylation.[42] These findings suggest that PD-1 may play a significant role in regulating cellular metabolism, or could impact energy generating mechanisms such as the AMP-activated protein kinase (AMPK) pathway. To support this hypothesis, the anti-diabetic drug, metformin (is known to activate AMPK), has already been shown to enhance T-cell function by altering the PD-L1/PD-1 axis.[43] In agreement with the findings reported in this study, it seems that the levels of PD-1 expression are elevated on T-cells of T2D patients. This is especially true since the activation of PD-1 signalling in cultured T-cells from healthy donors altered energy metabolic pathways by inhibiting the uptake and utilisation of glucose whilst promoting fatty acid β-oxidation as a source of ATP.[44] Thus, providing further evidence that tight regulation of T-cell function is necessary for optimal glucose

metabolism. Therefore, in addition to the well-described chronic antigen stimulation and inflammation in T-cell exhaustion[7], increased expression of PD-1 and loss of effector function is also influenced by cellular glucose metabolism. As a result, this evidence highlights the need to target PD-1 signalling pathway as a therapeutic mechanism to improve metabolic functions in conditions of metabolic stress.

Chronic inflammation is implicated in the development of CVD in patients living with T2D.[18] In that context, T2D patients are at a two- to four-fold risk of developing CVD when compared to healthy controls.[45] Interestingly, increased levels of CRP, a sensitive systemic marker of inflammation has been associated with increased risk of developing CVDs in T2D patients.[46] The evidence synthesised in this study demonstrated increased levels of CRP in patients with T2D[9,29] which was associated with increased expression of PD-1.[9] Moreover, the upregulation of PD-1 on T-cells was consistent with the development of atherosclerotic macrovascular diseases.[18] The use of immune checkpoint inhibitors which block co-stimulatory signalling pathway are known to be effective in rejuvenating T-cell effector function in chronic infections and tumour environments [47]. However, this consequence has been correlated with the new-onset of impaired glucose homeostasis.[48] Therefore, the use of immune checkpoint inhibitors in T2D as a treatment strategy may have a double-edged sword effect whereby it improves T-cell mediated immune responses but further impairing glucose control, as reported elsewhere.[49] This emphasises the need to find a fine balance between improving T-cell function whilst enhancing glucose metabolism when targeting PD-1 signalling as a therapeutic strategy in T2D patients. The overall impact of PD-1 signalling on T-cell function is illustrated in Figure 3.

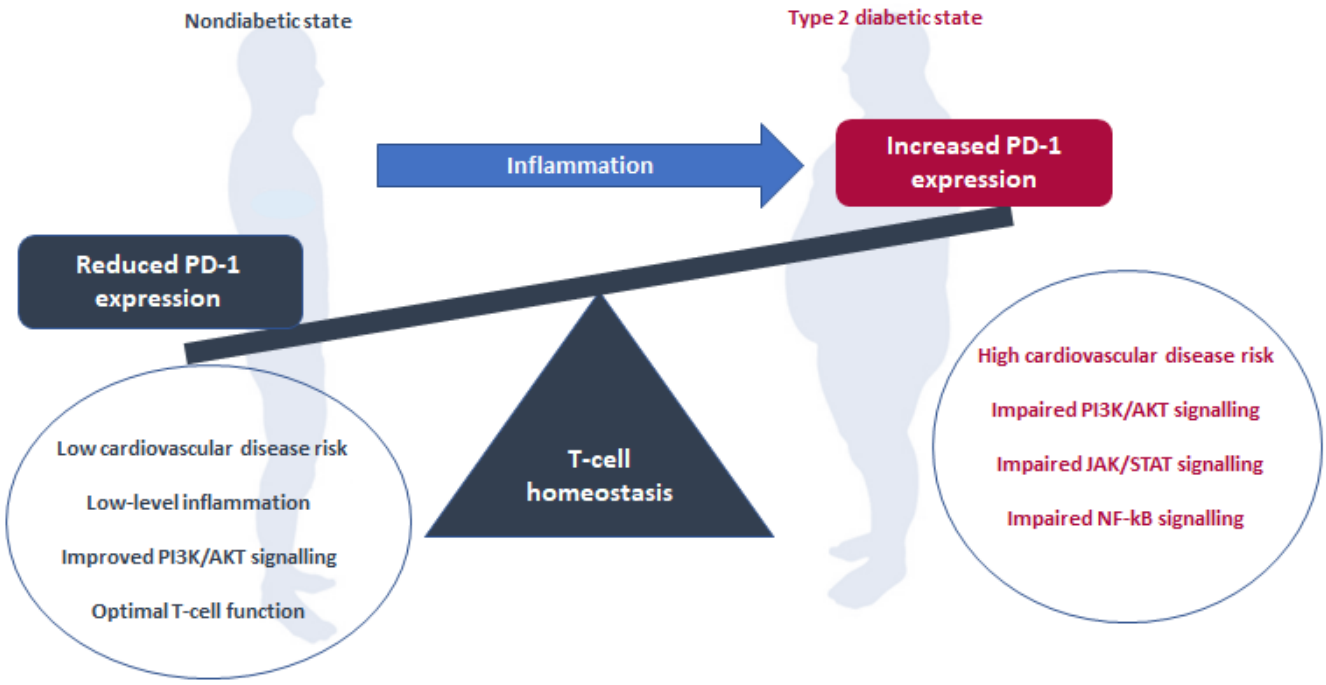

Figure 3: An overview of mechanisms that implicates programmed cell death-1 signalling and its modulatory effects on T-cell function. In brief, chronic immune activation in type 2 diabetes mellitus is known to increase cardiovascular risk and T-cell exhaustion, which is likely to be mediated by the upregulation of programmed cell death-1 (PD-1), a negative T-cell regulator. Thus, increased expression of PD-1 can alter glucose metabolism by inhibiting the actions of phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signalling. It further appears that PD-1 expression promotes T-cell proliferation and survival but inhibit their effector functions by upregulating the detrimental mechanisms such Janus kinase/signal transducers and activators of transcription (JAK/STAT) and nuclear factor kappa B (NF-κB) signalling pathways.

Overall, this systematic review and meta-analysis had a few limitations. Firstly, the number of included studies was low as well as the quality of evidence due to their cross-sectional nature. Secondly, there was substantial amount of unexplained statistical heterogeneity in this study. Lastly, the level of T-cell exhaustion is influenced by the duration of exposure to chronic inflammation, of which the majority of included studies did not report on disease duration. Nevertheless, our study has a unique strength in that to our knowledge, it is the first systematic review and meta-analysis to assess T-cell exhaustion in T2D. Moreover, the methodologies employed in this study were robust as indicated by high levels of inter-rater agreements. Results from sensitivity analysis indicated that the reported pooled effect sizes were not influenced by a single study, thus making the findings reported herein robust. Lastly, the current findings are important as they pave way for future therapeutic strategies to explore the use of immune checkpoint inhibitors in order to resuscitate immune responses mediated by T-cells. This will thus potentially correct the immune dysfunction observed in T2D patients.

In conclusion, low-grade inflammation in conditions of impaired glucose tolerance is associated with chronic immune activation and dysfunction, which collectively increases the risk of developing diabetes-associated cardiovascular complications. The evidence synthesised here suggests that immune dysfunction observed in T2D is in part due to T-cell exhaustion mediated by increased expression of PD-1. Therefore, the use of immune checkpoint inhibitors in rejuvenating the immune response in these patients may be an effective therapeutic strategy.

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Abbreviations

ATP: Adenosine triphosphate; **AMPK:** AMP-activated protein kinase; **CRP:** C-reactive protein; **CTLA-4:** cytotoxic T-lymphocyte-associated protein 4; **CVDs:** Cardiovascular diseases; **$CD4^+$:** T-helper cells; **$CD8^+$:** Cytotoxic T-cells; **IFN-γ:** Interferon gamma; **IL:** Interleukin; **JAK/STAT3:** Janus kinase/signal transducers and activators of transcription 3; **PD-1:** Programmed cell death-1; **PD-L1/2:** programmed death ligand 1/2; **PI3K/AKT:** phosphoinositide 3-kinase/protein kinase B; **MD:** mean difference; **T2D:** Type 2 diabetes mellitus; **TCR:** T-cell receptor; **(TNF-α:** Tumour necrosis factor alpha ; **NF-κB:** nuclear factor kappa B

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Obesity-related asthma in children is characterized by T-helper 1 rather than T-helper 2 immune response: A meta-analysis

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ABSTRACT

Background: Asthma is a chronic inflammatory condition characterized by T-helper (TH) 2 polarization. In children, the prevalence of obesity is associated with an increased incidence of asthma. Notably, obesity is linked with TH1-mediated inflammation and has been identified as a major risk factor for asthma.

Objective: To investigate the impact of obesity on TH1 (tumor necrosis factor a, interferon gamma, interleukin (IL)-6, IL-8) and TH2 (IL-4, IL-5, IL-10, IL-13) immune responses in children with asthma.

Methods: We searched the MEDLINE and gray literature electronic databases for eligible studies from inception up until April 2020. The quality of included studies and evidence was independently assessed by 2 reviewers. The random-effects model was used in this meta-analysis, and outcomes were reported as standardized mean difference (SMD) and 95% confidence interval (CI).

Results: Overall, 5 studies comprising 482 participants met the inclusion criteria. The meta-analysis revealed an increased TH2-mediated immune response in lean people with asthma compared with controls without asthma (SMD: -1.15 [95% CI: -1.93, 0.36]; $I^2 = 93\%$; $p^H < .001$). However, in obese people with asthma, there was polarization toward T_H1 immune response compared with lean people with asthma (SMD: -0.43 [95% CI: -0.79, -0.08]; $I^2 = 88\%$, $p^H < .001$).

Conclusion: This meta-analysis reveals that there are differences in immune responses mediated by T-helper cells in lean and obese children with asthma. Moreover, and not unique to asthma, obesity polarizes the immune response toward T_H1 rather than the classical T_H2. This could be an important aspect to understand to establish effective therapeutic targets for obese children with asthma.

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Introduction

Chronic respiratory diseases (CRDs) are ranked among the top 5 noncommunicable diseases that are currently posing the greatest health care burden and contributing significantly to increased mortality globally.[1] Among these CRDs is asthma, a chronic inflammatory disorder that is characterized by airway obstruction mediated by various inflammatory immune cells, including inflammatory leukocytes.[2,3] Recently, it was estimated that asthma affects 339 million people worldwide, with a mortality rate of more than 356,000 deaths annually.[4] This burden may be attributed to increased prevalence of childhood obesity, a major risk factor for asthma,[5] and comorbidities such as type 2 diabetes mellitus and cardiovascular diseases.[6] Of note, systemic inflammation has been identified as a common characteristic feature among these comorbidities[7,8] and has led to renewed interest in understanding the pathoimmunologic aspects of asthma.

Immunoglobulin E (IgE)-mediated asthma is categorized as a type 1 hypersensitivity response that is broadly classified as either eosinophilic or noneosinophilic[9] with exacerbated T-helper 2 (T_H2) immune responses mediated by its cytokines interleukin (IL)-4, IL5, and IL-13.[10] Hence, T_H2 polarization is regarded as a hallmark of asthma pathogenesis. Interestingly, although T_H2-mediated responses in people with asthma have been characterized,[10-12] the clinical relevance of these findings remain controversial. This is partly due to the variable immunological responses in asthma therapy and the lack of clear mechanisms and factors that are independent of $T_H2$emediated responses, as described elsewhere,[13,14] particularly, in cases of comorbidity with other disorders such as obesity.[15] Notably, obesity has been associated with disease severity and reduced efficacy of asthma treatment, which is further affected by age-dependent factors in children.[16,17] However, the pathophysiological mechanisms of obesity-related asthma remain poorly understood. Here, we hypothesize that these detrimental phenomena are partially a consequence of altered T-helper cell responses induced by obesity. This is in conjunction with an exacerbated proinflammatory response in obesity being driven by T_H1 cells, which are characterized by increased release of its cytokines, that is, interferon gamma (IFN-g), tumor necrosis factor (TNF)-a, and IL-6, as reported elsewhere.[18,19] Likewise, these findings have been confirmed in obesity-related asthma,[20-22] albeit others still suggested the T_H2 polarization hallmark.[23]

Although previous systematic reviews and meta-analysis have investigated the broad associations between obesity and asthma,[24-26] none have assessed the impact of obesity on immune responses mediated by T_H1 and T_H2 in asthma.

Therefore, in this study, we aimed to investigate and establish whether obesity alters T_H1 (TNF-a, IFN-g, IL-6,IL-8)and T_H2 (IL-4,IL-5,IL-10,IL-13) immune responses in asthma. The selected cytokines are known to mediate respiratory inflammation[27] and affect asthma control in obesity-asthma conundrum.[28]

Methods

This meta-analysis was prepared according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines and performed to address the following questions:

Question 1: Is the immune system of lean children with asthma polarized toward a proinflammatory T_H1 or anti-inflammatory T_H2 response?

Question 2: Does obesity alter these T-helper cell responses in obese children with asthma when compared with their lean counterparts?

Search Strategy

A comprehensive search was conducted from the inception of MEDLINE electronic database and ProQuest gray literature up until April 2020. The search was conducted by 2 independent reviewers (TMN and BBN), whereas a third reviewer (PVD) was consulted for arbitration in cases of disagreements. The search strategy comprised Medical Subject Heading and text words, such as obesity, asthma, T_H1 and T_H2 cells, and their respective synonyms. In addition to gray literature search, scanning reference lists of retrieved studies was also applied to identify relevant studies. No language restrictions were applied in the search strategy, and the Mendeley reference manager software (Elsevier, Amsterdam, Netherlands) was used to manage the reference list and remove study duplicates.

Study Selection

Studies were independently screened and selected by 2 reviewers (TMN and B.B.N.) using a predefined inclusion and exclusion criteria. Briefly, this meta-analysis only included studies that reported on both T_H1 and T_H2 immune responses in children with asthma (aged 18 years). Moreover, the studies were only included if participants were categorized as either obese or lean. Reviews, books, editorials, letters, and studies that included adult participants (>18 years) were excluded. In addition, studies that did not compare obese and lean people with asthma or lean people with asthma vs lean healthy controls were excluded from this study.

Data Extraction

Two independent investigators (TMN and VM) carried out the data extraction process using a predefined data extraction sheet. In cases of disagreements, BBN was consulted for arbitration. The primary aim of the study was to assess T_H1 and T_H2 function in obese children with asthma. The extracted data items from each study included the names of the authors, year of publication, study design, age of participants, effect measures of T_H1 and T_H2 function (cytokines), and the main findings.

Outcomes

The outcomes of this meta-analysis were T_H1 and T_H2 immune responses that were reported as standardized mean difference (SMD).

Quality Assessment

The quality of evidence was assessed using the Grading of Recommendations Assessment Development and Evaluation[29] approach by 2 independent reviewers, TMN and VM. In cases of disagreements, a third reviewer, PVD, was consulted for arbitration. Furthermore, the quality of included studies was assessed using the modified Newcastle-Ottawa Scale adapted for cross-sectional studies.[30] The tool uses the star system to appraise studies based on the following 3 domains: selection of study groups, comparability of the groups, and outcome ascertainment. A score of 0 to 4 is rated as unsatisfactory, 5 to 6 as satisfactory, 7 to 8 as good, and 9 to 10 as very good.

Statistical Analysis

All statistical analyses were performed using REVMAN version 5.3 software (Cochrane Collaboration, Oxford, United Kingdom). In addition, heterogeneity was quantitatively assessed using Higgin's I^2 index.[31] In cases of substantial heterogeneity ($I^2 > 50\%$) in the pooled effects estimate, the random-effects model was used. The fixed-effects model was used in cases of low heterogeneity ($I^2 < 50\%$).[32] Statistical significance of heterogeneity was reported as p^H. SMD and 95% confidence interval (CI) that account for the differences in the reported effect measures were used to measure the effect size of an outcome. Moreover, Cohen's method was used to interpret the calculated effect estimate, whereby an SMD of 0.2, 0.5, and 0.8 was equated to small, medium, and large, respectively.[33] A P value less than .05 was considered statistically significant. A sensitivity analysis was conducted to evaluate the influence of each study on the overall effect size using the leave-one-out method. The interrater reliability was evaluated for both the included studies and risk of bias by means of Cohen's kappa. A kappa value of less than 0.00 was taken as poor strength of agreement, 0.00 to 0.20 as slight agreement, 0.21 to 0.40 as fair agreement, 0.41 to 0.60 as moderate agreement, 0.61 to 0.80 as substantial agreement, and 0.81 to 1.00 as perfect agreement.[34]

Results

Study Selection and Data Synthesis

Overall, 369 studies were identified and independently screened for eligibility by 2 reviewers. A total of 352 studies were excluded from the screening stage because 65 were reviews and 287 were not relevant to the topic of interest. The full texts of the remaining 17 studies were then assessed for eligibility, and a total of 5 studies were excluded owing to their study design. In the excluded studies, individuals with asthma were not grouped as either lean or obese. In addition, 6 studies were excluded due to the reported T-helper cells subtypes other than T_H1 and T_H2. One study was also excluded owing to limited data values of effect measures reported,[21] and only 5 studies[20,22,23,35,36] met the inclusion criteria as shown in Figure 1 (overall agreement 99.73%, kappa = 0.89). The interrater agreement in the study selection process was perfect. The meta-analysis was split into 2 major comparisons, one which compared T_H1 and T_H2 functions in lean people with asthma vs controls, whereas the other focused on lean vs obese people with asthma.

Characteristics of Included Studies

All included studies were published in peer-reviewed journals between 2012 and 2016, and the detailed study-level characteristic features are described in Table 1. Briefly, all studies were cross-sectional studies involving a total of 482 participants with an age range of 7-18 years from United States and Mexico. Of these, 109 were controls and 373 had asthma, of which 190 were lean and 185 were obese. The reported cases of asthma were based on the physician's diagnosis and medical electronic records. Obesity was defined as having a body mass index (BMI) greater than the 95th percentile for age and sex, whereas lean was defined as having a BMI less than the 85th percentile.[37] For the participants of this study whose average age was approximately 9 years, obesity was defined as BMI greater than 21.1 kg/m^2 and lean as BMI less than 18.6 kg/m^2. However, for 15-year-old participants, obesity was defined as BMI greater than 26.8 kg/m^2 and lean as BMI less than 23.4 kg/m^2.

Quality Assessment

All included studies were of good quality and had a median score range of 7 (6-7) out of a possible score of 10 (eTable 1). Four of the studies were rated as good[20,22,35,36] and the remainder as satisfactory.[23] Briefly, included studies had a selection

median of 3 (3-4) of possible 5 stars (overall agreement 100%, kappa = 1), comparability median of 2 (0-2) of possible 2 stars (overall agreement 70%, kappa = 0.6), and outcome ascertainment median of 2 (2-3) of 3 possible stars (overall agreement 100%, kappa = 1).

Publication Bias

Potential publication bias was explored using visual inspection of funnel plots. However, owing to lower number of studies than recommended, we could not effectively assess publication bias (eFig 1). Therefore, there may be potential publication bias among the included studies.

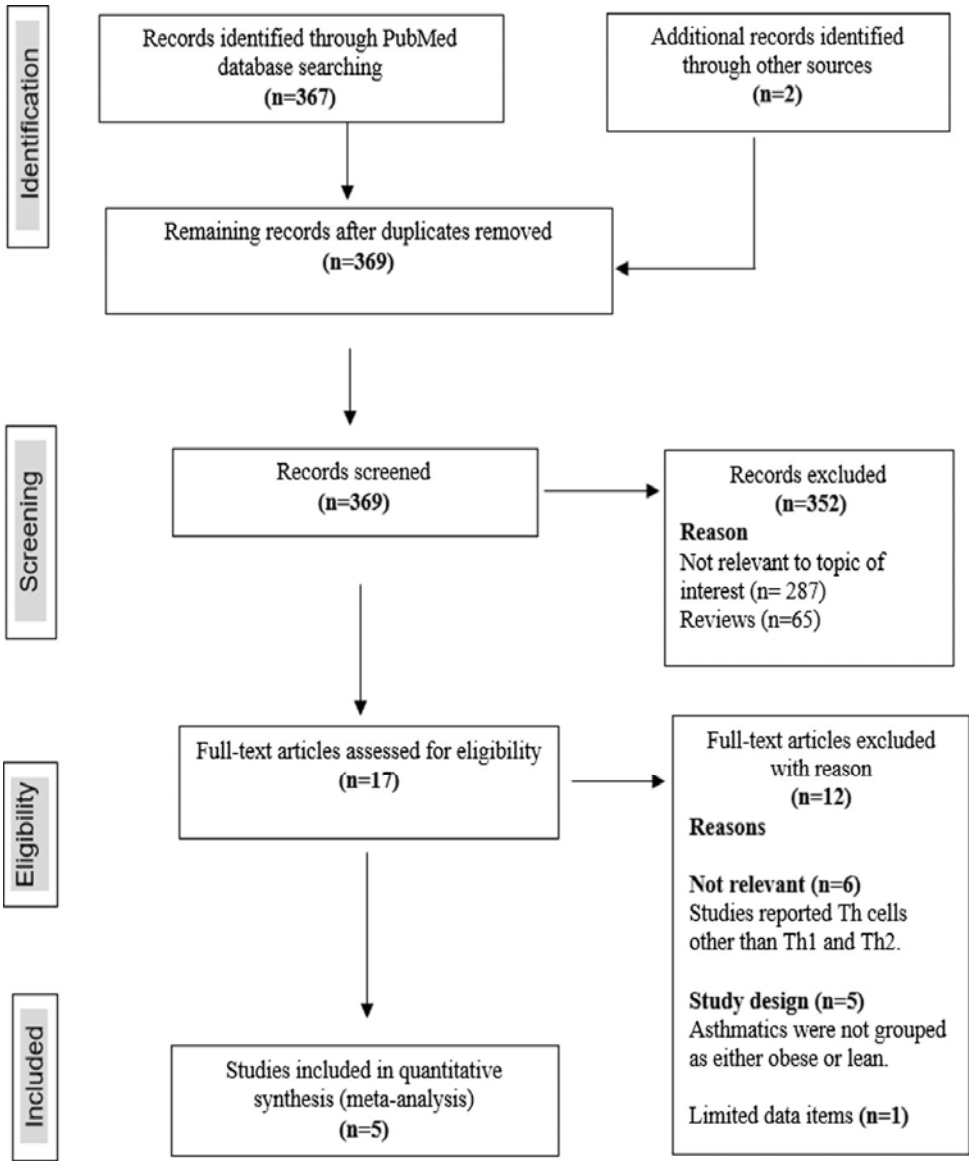

Figure 1. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses flow diagram illustrating the study selection process.

Table 1

Characteristics of Included Studies on $T_H1/2$ Cell Function in Obesity-Related Asthma in Children (n = 5)

Study	Country	Study design	Study size	Asthma diagnosis	BMI (kg/m^2)	Age (y)	T_H cell effect measures	Main findings
Rastogi et al[20]	United States	Cross-sectional	90 participants (30 lean people with asthma; 30 obese people with asthma and 30 controls)	Physician diagnosis based on the NAEPP guidelines	Obese people with asthma (28.4) Lean people with asthma (17.3)	9.23 1.3	Serum T_H1 cytokines IL-6, TNFa, and IFN-g and Serum T_H2 cytokines (IL-4, IL-5, and IL13).	Pediatric obesity-associated asthma is marked by T_H1 and not T_H2 polarization. In addition, poor pulmonary function is associated with increased T_H1 cytokines in obese people with asthma.
Youssef et al[36]	Egypt	Cross-sectional	70 participants (25 lean people with asthma; 25 obese people with asthma and 20 controls)	Physician diagnosis based on GINA guidelines	Obese people with asthma (25.1) Lean people with asthma (18.6)	8.61 2.8	Serum T_H1 cytokine (IFN-g) and T_H2 cytokine (IL-4).	Obesity-associated asthma in children is characterized by T_H1 and not T_H2 polarization. Moreover, elevated levels of IFNg in obese people with asthma were associated with poor pulmonary function and disease severity.
Sanchez-Zauco et al[23]	Mexico	Cross-sectional	65 estimated participants (23 lean people with asthma; 19 obese people with asthma and 23 controls)	Physician diagnosis	Obese people with asthma (23.9) Lean people with asthma (17.6)	9.17	Serum T_H1 cytokines (IL-2, IFNg, and TNF-a) and Serum T_H2 cytokines (IL-4 and IL-10).	Obese people with asthma have decreased $T_H1$emediated immune response when compared with lean people with asthma and controls. In addition, T_H2 cytokines are increased in lean people with asthma when compared with obese people with asthma and controls.
Rastogi et al[22]	United States	Cross-sectional	114 participants (39 lean people with asthma; 39 obese people with asthma and 36 controls)	Physician diagnosis and medical records	Obese people with asthma (33.2) Lean people with asthma (22.2)	15.9 1.7	Serum T_H1 cytokines (IL-2, IL-6, TNF-a and IFN-g) and Serum T_H2 cytokines (IL-4, IL10, and IL-13).	$T_H1/2$ cytokine ratio is higher in obese people with asthma when compared with lean people with asthma. Moreover, the high $T_H1/2$ ratio directly correlated with metabolic abnormalities and poor pulmonary function.
Lautenbacher et al[35]	United States	Cross-sectional	143 participants (71 lean people with asthma and 72 obese people with asthma)	Physician diagnosis and medical records	Not reported	(7-18)	Serum T_H1 cytokines (IFN-g, TNF, IL-6, and IL-8) and Serum T_H2 cytokines (IL-4, IL-5 and IL-13).	T_H1 cytokines are increased in obese people with asthma vs lean people with asthma. In addition, increased T_H1 function is associated with poor pulmonary function. T_H2 cytokines are comparable across all 3 groups except for IL13, which is high among lean people with asthma.

Abbreviations: BMI, body mass index; GINA, Global Initiative for Asthma; IL, interleukin; IFN, interferon; NAEPP, National Asthma Education and Prevention Program; T_H, T-helper; TNF, tumor necrosis factor.

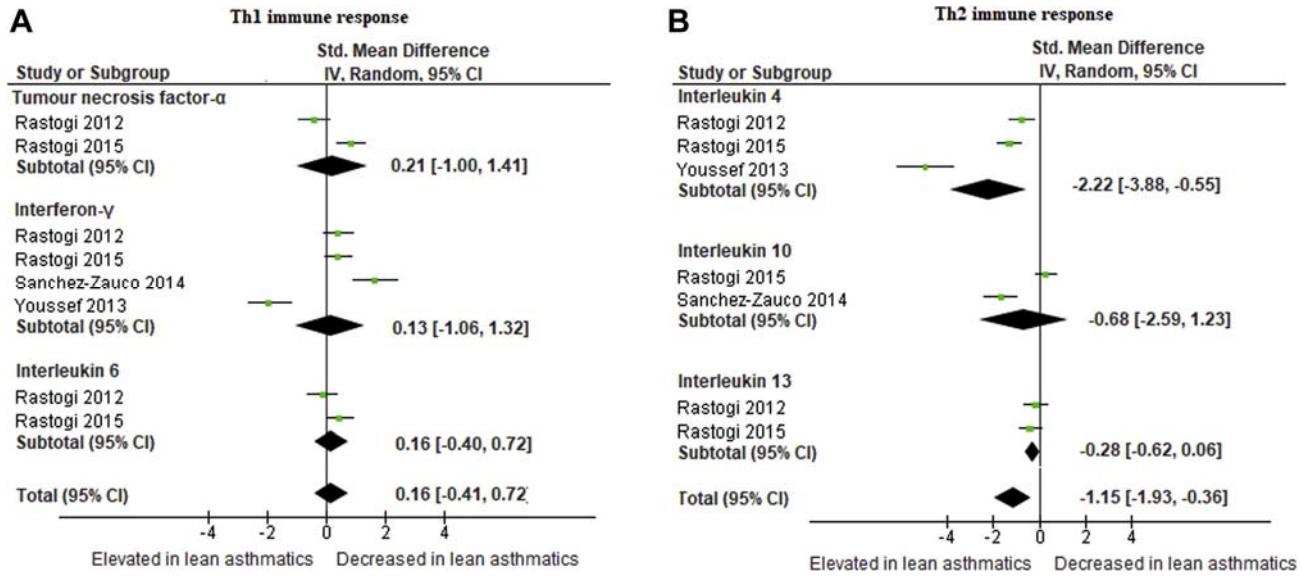

Figure 2. A comparison of standardized mean difference of T_H1- and T_H2-mediated responses between lean people with asthma and healthy controls. 95% CI, 95% confidence interval; T_H1, T-helper 1; T_H2, T-helper 2.

$T_H1/2$ Immune Responses in Lean People With Asthma

Overall pooled estimates revealed a T_H1-weighted imbalance in lean people with asthma compared with the controls without asthma (SMD: 0.16 [95% CI: 0.41, 0.72]; I^2 = 89%; p^H < .001) (Fig 2A). Owing to substantial levels of statistical heterogeneity in the reported effect estimate, we performed a subgroup analysis to explore sources of heterogeneity based on the reported effect measures of T_H1 response. The test for subgroup differences revealed no significant subgroup effect (P = 1.00) (eTable 2), suggesting that the observed differences in T_H1 immune response are not modified by varying effect measures reported. However, the pooled estimates support a T_H2 weighted response in lean children with asthma compared with controls (SMD: 1.15 [95% CI: 1.93, 0.36]; I^2 = 93%; p^H < .001) (Fig 2B). Nonetheless, due to high statistical heterogeneity among the included studies and differences in the reported effect measures of the T_H2 responses, we performed a subgroup analysis based on the reported effect measures of T_H2 function.

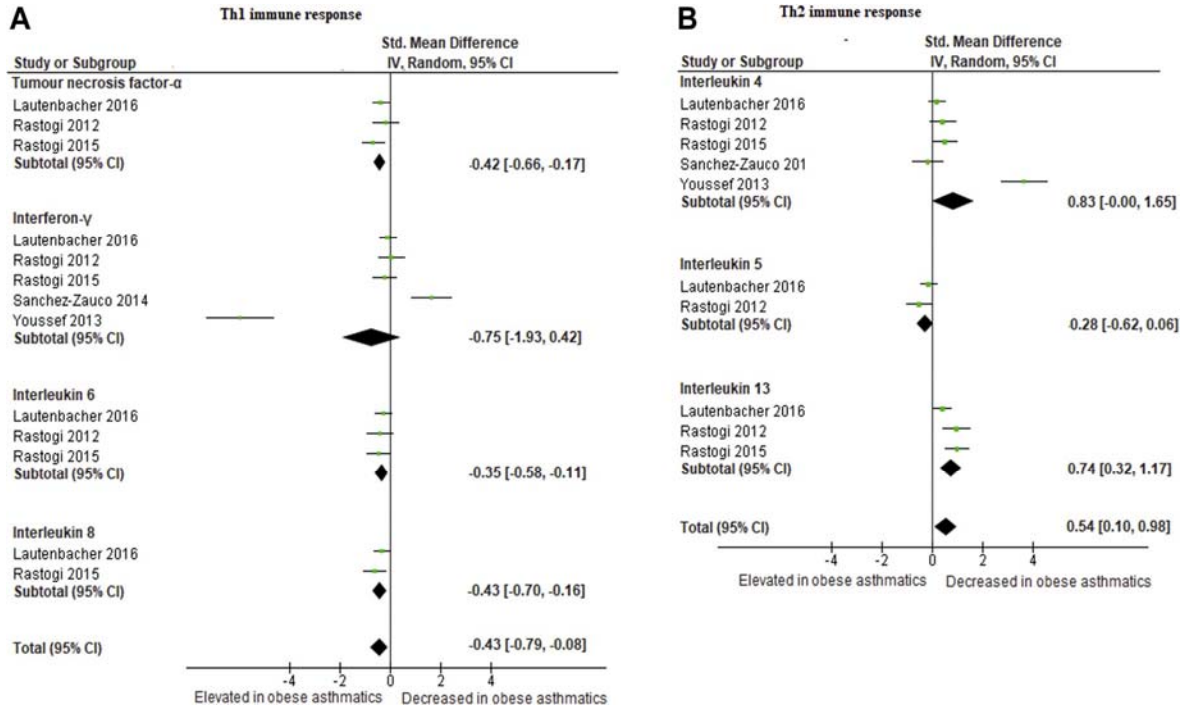

Figure 3. A comparison of standardized mean difference of T_H1- and T_H2-mediated responses between lean and obese people with asthma. 95% CI, 95% confidence interval; T_H1, T-helper 1; T_H2, T-helper 2.

Subgroup analysis of T_H2 immune response

The test for subgroup differences revealed a statistically significant subgroup effect (P = .08) (eTable 2). Thus, the different cytokines assessed as effect measures modified the overall effect of T_H2 immune response in lean people with asthma in comparison with controls. However, there were substantial levels of unexplained heterogeneity between included studies (I^2 = 60.6%). Interestingly, there was a large effect size in IL-4 levels between lean people with asthma and controls (SMD: 2.22 [95% CI: 3.88, 0.55]; I^2 = 95%; p^H < .001). Moreover, IL-13 levels were higher in lean people with asthma compared to controls (SMD: 0.28 [95% CI: 0.62, 0.06]; I^2 = 0%; p^H = .50), and there was a medium effect size in IL-10 levels (SMD: 0.68 [95% CI: 2.59, 1.23]; I^2 = 95%; p^H < .001).

Obesity-Related Asthma Is Characterized by T_H1 and Not T_H2 Polarization

Interestingly, unlike in the lean counterparts, immune responses in obese individuals with asthma are T_H1 weighted. The pooled estimates revealed that T_H1 cytokine levels are increased in obese people with asthma compared to lean people with asthma (SMD: 0.43 [95% CI: 0.79, 0.08], I^2 = 88%; p^H < .001) (Fig 3A). However, pooled estimates of T_H2 immune-mediated responses were decreased in obese individuals with asthma compared to lean people with asthma (SMD: 0.54 [95% CI: 0.10, 0.98]; I^2 = 89%; p^H < .001) (Fig 3B). Nonetheless, due to substantial statistical heterogeneity, we performed a subgroup analysis based on the reported effect measures.

Subgroup analysis of T_H2-Mediated immune response

The test for subgroup differences revealed a statistically significant subgroup effect (P < .001) with substantial levels of unexplained heterogeneity (I^2 = 87.7%) (eTable 3). Pooled estimates of IL-4 and IL-13 levels were lower in obese people with asthma compared with lean people with asthma (SMD: 0.83 [95% CI: 0.00, 1.65]; I^2 = 92%%; p^H = .44) and (SMD: 0.74 [95% CI: 0.32, 1.17]; I^2 = 64%; p^H = .06), respectively. In contrast, IL-5 levels were higher in obese people with asthma vs lean counterparts (SMD: 0.28 [95% CI: 0.62, 0.06]; I^2 = 27%; p^H = .24).

Sensitivity Analysis

To investigate the levels of high unexplained sources of heterogeneity and to ascertain whether our findings were influenced by any single study, we performed a sensitivity analysis based on the guidelines followed to diagnose asthma (eTable 4). Heterogeneity among the included studies remained high in both reported outcomes involving lean and obese people with asthma. We repeated the meta-analysis by leaving out the study by Youssef et al[36] due to its peculiarly large effect size in the reported outcomes. The findings remained robust except for the magnitude of T_H2 immune response, which changed from a medium (0.54 [0.10, 0.98]) to a small (0.29 [0.02, 0.59]) effect size (eTable 4).

Discussion

The primary aim of this meta-analysis was to determine whether obesity alters T_H1 and T_H2 immune responses in children with asthma. As expected, the pooled effect estimates revealed increased T_H2-mediated immune responses in lean people with asthma. However, in obesity-related asthma, there is polarization toward T_H1 immune response, which is associated with poor pulmonary function.[20,22,35] Therefore, the overall findings from this meta-analysis suggest that obesity, although not unique to asthma, aggravates T_H1-mediated immune responses in obesity-related asthma.

T-helper cells are involved in the initiation and mediation of airway inflammation in asthma,[11,12] by exacerbating cytokine release and action. Notably, cytokines play a pivotal role in generating a cytokine milieu that promotes the differentiation of naive T-helper cells into T_H2 cells.[2] As a consequence, T_H2 effector responses are aggravated coupled with increased secretion of cytokines such as IL-4, IL-5, and IL-13. Our findings revealed that both IL-4 and IL-13 levels were increased in lean people with asthma compared with both controls and obese people with asthma. Interestingly, IL-4 and IL-13 are central in mediating T_H2 immune responses and also share a common receptor that orchestrates B-cell activation and IgE antibody secretion.[38,39] Moreover, IL-13 is involved in airway inflammation and obstruction by activating proinflammatory macrophages and increasing mucus production.[40] Overall, these findings are supporting evidence of T_H2-mediated immune response in lean children with asthma.

In our previous work, we reported that in the context of T-helper cells, obesity-induced inflammation is mediated by increased T_H1 and T_H17 responses coupled with decreased T_H2 and regulatory T cells.[41] These findings prompt us to postulate that obesity-related asthma may be a different entity of this disease because obesity is a low-grade inflammatory state characterized by increased adiposity and activation of various inflammatory pathways.[42,43] As such, increased activation and release of proinflammatory cytokines and adipokines promotes systemic inflammation, which has been linked to hypersensitivity reactions.[44,45] The meta-analysis revealed that obese individuals with asthma have increased $T_H1$mediated immune response.

Furthermore, obesity worsens the severity of asthma in children, in part by increasing IL-5 and eosinophil-mediated inflammation as previously reported.[46] IL-5 signaling can promote IL-4-induced B-cell activation and class switch to IgE-synthesizing plasma cells, resulting in marked increase in serum IgE levels and activation of eosinophils.[47,48]

In fact, elevated levels of IgE promote the activation of eosinophils through increased IgE receptor signaling[48,49] on their cell surface subsequent to eosinophilia, thus highlighting the direct association between IL-5 and IgE levels in asthma[50] and their association with poor pulmonary function. Interestingly, in this study, the levels of IL-5 were higher in obese people with asthma than in lean counterparts. Therefore, we postulate that therapies targeting IL-5 activity may be less effective if the therapeutic doses used in obese people with asthma are similar to those prescribed for lean people with asthma. Collectively, these findings suggest that, although obesity-related asthma is characterized by T_H1 polarization, the levels of IL-5 are still increased. Therefore, therapies that inhibit IL-5 activity, such as mepolizumab combined with corticosteroids, which reduce T-cell activation while promoting the differentiation of regulatory T cells,[51] maybe of therapeutic benefit and improve the response to treatment in obese people with asthma. Nevertheless, beyond the T_H1/T_H2 paradigm, other mechanisms involving additional T-cell subsets, such as CD8, T_H9, T_H17, T_H22, and follicular T-helper cell subsets have to be studied in detail for their role in asthma pathogenesis in obese children, as reviewed elsewhere.[51,52]

In addition, exacerbated inflammation in obese people with asthma has also been attributed to increased levels of leptin and TNF-a which were directly associated with enhanced activation of eosinophils in these patients.[53] Likewise, low levels of vitamin D concomitant to increased risk of respiratory infections in obese children with asthma have also been described.[15] This may in part be attributed to impaired immunomodulatory effects of vitamin D on $T_H1/2$ and T_H17 immune responses.[54,55] In addition to these factors, asthma outcomes are significantly influenced by socio-economic distal factors. For instance, poor communities are most likely to have unhealthy residential environments,[56] which together with genetics and wheezing episodes at preschool age[57] predisposes these children to develop asthma. Moreover, these societies are associated with high levels of stress, violence, and poor access to medical care, which increases morbidities.[56] Obesity-related asthma, however, is more driven by increased sedentary lifestyle and unhealthy eating that are most prevalent in developed countries.[58] Congruent to this, 60% of the included studies were from those with high income,[20,22,35] and the remaining studies were from low- to middle-income countries.[23,36]

The limitations of the current review include lack of standardization of asthma diagnosis, which was based on physician diagnosis and electronic records. This was a challenge because the approaches used potentially varied significantly, making it difficult to assess the validity of studies and to compare their findings. Moreover, people with asthma were generally grouped and not categorized based on their immunologic phenotypes (eosinophilic or noneosinophilic). This was a major limitation because different asthma subtypes present with unique immunologic features. Together with differences in disease duration among the participants, the heterogeneous nature of asthma and reported T_H2 effect measures, which were pooled cytokines with antagonistic effect, may potentially be the source of unexplained heterogeneity among the included studies, particularly in the analysis between lean and obese people with asthma. Finally, critical population features, such as social and economic demographics, which heavily influence the interaction between obesity and asthma in children were not reported in the included studies. Ethnicity was reported in 3 of the included studies,[20,22,35] which involved an approximately equal number of Hispanics and African American children. The remaining 2 studies[23,36] did not report on this aspect, and this limits the generalizability of our findings. However, our study highlights the evidence gap that needs to be explored to better understand the disease pathology in white people because they may not be represented in these patient cohorts.

Despite these limitations, our study has significant strengths. First, and to the best of our knowledge, this is the first meta-analysis to explore the effect of obesity on T_H1 and T_H2 immune responses in children with asthma. This is of special interest to understand the T_H1/T_H2 paradigm in modulating an adaptive immune response, especially in relation to the development of metabolic complications, as recently reviewed.[59] Second, the study selection and quality assessment processes employed in this study were robust and comprehensive as indicated by high levels of interrater reliability. Moreover, the reported effect sizes were consistent, and the sensitivity analysis demonstrated the robustness of the presented findings. Finally, the findings of this study provide insights into future asthma therapies to consider the impact of obesity on the immune response, which we have shown to be completely different when compared with the lean counterparts, despite sharing the same condition.

In conclusion, the evidence synthesized in this meta-analysis reveals that there are differences in immune responses mediated by T-helper cells in lean and obese children with asthma. In addition, although not unique to asthma, obesity polarizes the immune response toward T_H1 rather than the classical T_H2. Therefore, asthma therapies that particularly target immune responses should consider the underlying obese state of these patients for an effective treatment outcome.

Supplementary Data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.anai.2020.06.020.

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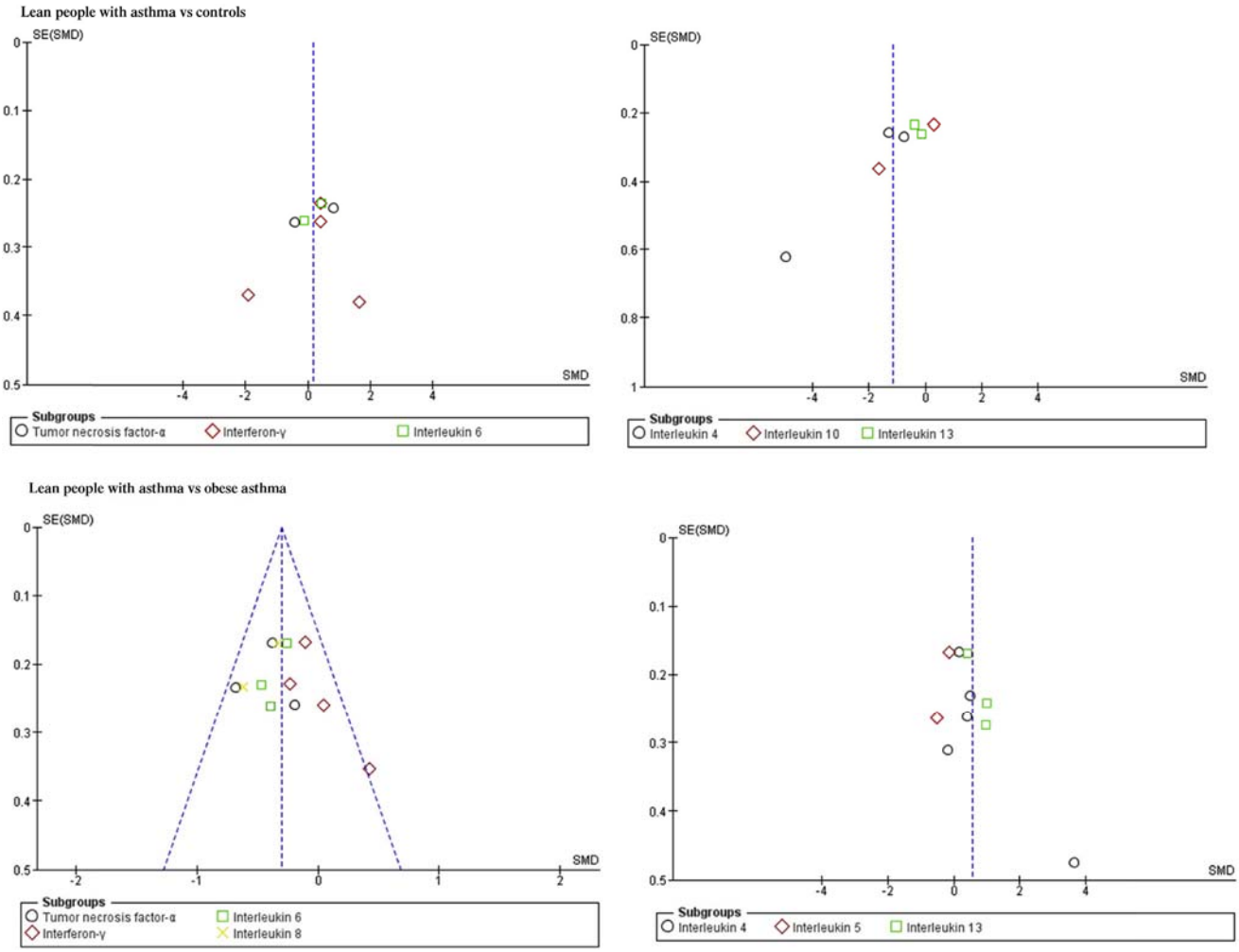
Supplementary Data



eFigure 1. Funnel plots reporting publication bias. SMD, standardized mean difference.

eTable 1

Quality Assessment of Included Studies Using the Modified Newcastle-Ottawa Scale for Cross-Sectional Studies (n = 5)

Study and year	Selection					Comparability		Outcome			Total quality score	Rating
	Representativeness of the sample	Selected group of users	Sample size	Diagnose	Average	Confounders	Score	Assessment methods	Statistical test	Average of		
Rastogi, 2012	0	0	0	***	3	**	2	*	*	2	7	Good
Lautenbacher, 2016	0	0	0	***	3	**	2	*	*	2	7	Good
Rastogi, 2015	0	0	0	***	3	**	2	*	*	2	7	Good
Youssef, 2013	0	*	0	***	4	0	0	**	*	3	7	Good
Sanchez-Zauco, 2014	0	*	0	***	4	0	0	*	*	2	6	Satisfactory

* represents the number of scores awarded per domain. eTable 2

Forest Plot Text Data for T_H1/T_H2 Immune Responses in Lean Individuals With Asthma

Outcome	Effect measure	Study	Control			Lean individuals with asthma			Weight	Random effects	I^2, p_H
			Mean	SD	Total	Mean	SD	Total		SMD [95% CI]	
T_H1 immune response	Tumor necrosis factor-a	Rastogi, 2012	1.01	0.33	30	1.21	0.59	30	12.7%	0.41 [0.92, 0.10]	
		Rastogi, 2015	0.65	0.43	36	0.29	0.44	39	12.9%	0.82 [0.35, 1.29]	
		Subtotal			66			69	25.6%	0.21 [L1.00, 1.41]	92%, <.001
	Interferon gamma	Rastogi, 2012	1.1	0.66	30	0.72	1.15	30	12.7%	0.40 [0.11, 0.91]	
		Rastogi, 2015	0.64	1.01	36	0.27	0.81	39	13.0%	0.40 [0.06, 0.86]	
		Sanchez-Zauco, 2014	25.7	7.8	23	13.0	7.4	16	11.4%	1.63 [0.89, 2.37]	
		Youssef, 2013	20.0	9.5	20	40.8	11.3	25	11.5%	1.94 [2.66, 1.22]	
		Subtotal			109			110	48.6%	0.13 [L1.06, 1.32]	94%, <.001
	Interleukin 6	Rastogi, 2012	0.88	1.1	30	1.03	1.11	30	12.7%	0.13 [0.64, 0.37]	
		Rastogi, 2015	0.03	0.59	36	0.19	0.39	39	13.0%	0.44 [0.02, 0.90]	
		Subtotal			66			69	25.7%	0.16 [L0.40, 0.72]	63%, .10
		Total			241			248	100%	0.16 [L0.41, 0.72]	89%, <.001
		Test for subgroup differences									(P = 1.00), 0%
T_H2 immune response	Interleukin 4	Rastogi, 2012	0.27	0.82	30	0.48	1.11	30	14.8%	0.76 [1.28, 0.23]	
		Rastogi, 2015	1.1	0.5	36	1.76	0.5	39	14.9%	1.31 [1.81, 0.81]	
		Youssef, 2013	10.0	2.2	20	89.0	20.9	25	11.4%	4.95 [6.17, 3.73]	
		Subtotal			86			94	41.1%	L2.22 [L3.88, L0.55]	95%, <.001
	Interleukin 10	Rastogi, 2015	0.21	0.71	36	0.04	0.49	39	15.0%	0.28 [0.18, 0.73] 1.67	
		Sanchez-Zauco, 2014	0.3	0.3	20	14.9	11.7	23	14.0%	[2.38, 0.97]	
		Subtotal			56			62	29.1%		
	Interleukin 13	Rastogi, 2012	0.79	1.08	30	0.96	1.12	30	14.8%	0.68 [2.59, 1.23]	
		Rastogi, 2015	0.34	0.88	36	0.6	0.35	39	15.0%	0.15 [0.66, 0.35]	95%, <.001
		Subtotal			66			69	29.9%	0.39 [0.85, 0.07]	
										L0.28 [L0.62, 0.06]	0%, .50
		Total			208			225	100.0%	1.15 [1.93, 0.36]	93%, <.001
		Test for subgroup differences									(P = .08), I^2 = 60.6%

Abbreviations: CI, confidence interval; T_H1, T-helper 1; T_H2, T-helper 2; SMD, standardized mean difference.
Bold values are the pooled effect size of the cytokine reported.

eTable 3

Forest Plot Text Data for T_H1/T_H2 Immune Responses in Obese Individuals With Asthma

Outcome	Effect measure	Study	Lean individuals with asthma			Obese individuals with asthma			Weight	Random effects	I^2, p_H
			Mean	SD	Total	Mean	SD	Total		SMD [95% CI]	
T_H1 immune 2016 response	Tumor necrosis factor-a	Lautenbacher,	0.68	0.69	71	0.92	0.57	72	8.5%	0.38 [0.71, 0.05]	
	Interferon-g	Rastogi, 2012	1.21	0.59	30	1.31	0.43	30	7.8%	0.19 [0.70, 0.32]	5%, .35
		Rastogi, 2015	0.29	0.44	39	0.6	0.46	39	8.0%	0.68 [1.14, 0.22]	
		Subtotal			140			141	24.3%		
		Lautenbacher, 2016	0.46	0.99	71	0.56	0.87	72	8.6%	0.42 [0.66, 0.17]	
		Rastogi, 2012	0.72	1.15	30	0.68	0.85	30	7.8%	0.11 [0.43, 0.22]	
		Rastogi, 2015	0.27	0.81	39	0.47	0.89	39	8.1%	0.04 [0.47, 0.55] 0.23	
		Sanchez-Zauco, 2014	13.0	7.4	16	4.2	1.3	17	6.3%	[0.68, 0.21]	
										1.64 [0.84, 2.44]	
		Youssef, 2013	40.8	11.3	25	203.0	36.0	25	4.0%	5.98 [7.33, 4.64]	
		Subtotal			181			183	34.7%	-0.75 [-1.93, 0.42]	96%, <.001

	Interleukin 6	Lautenbacher, 2016	0.32	0.98	71	0.58	0.98	72	8.6%	0.26 [0.59, 0.07]	
		Rastogi, 2012	1.03 0.19	1.11	30	1.41	0.74	30	7.8%	0.40 [0.91, 0.11]	
		Rastogi, 2015		0.39	39	0.05	0.6	39	8.0%		
		Subtotal	0.78		140			141	24.4%	0.47 [0.92, 0.02] 0.35	0%, .75
	Interleukin 8	Lautenbacher, 2016	0.18	1.08	71	1.12	0.95	72	8.5%	[0.58, 0.11] 0.33 [0.66,	
		Rastogi, 2015		0.74	39	0.59	0.55	39	8.0%	0.00] 0.62 [1.08, 0.17]	
		Subtotal			110			111	16.6%		
		Total			571			576	100.0%	0.43 [0.70, 0.16]	2%, <.001
		Test for subgroup differences								0.43 [0.79, 0.08]	88%, <.001
T_H2 immune response	Interleukin 4	Lautenbacher, 2016	0.51	0.77	71	0.38	0.77	72	10.9%	0.17 [0.16, 0.50]	P = .89, I^2 = 0%
		Rastogi, 2012	0.48	1.11	30	0.06	0.87	30	10.0%	0.42 [0.10, 0.93]	
		Rastogi, 2015	1.76	0.5	39	1.42	0.8	39	10.3%	0.50 [0.05, 0.96]	
		Sanchez-Zauco, 2014	107.7	23.4	23	112.4	30.3	19	9.5%	0.17 [0.78, 0.44]	
		Youssef, 2013	89.0	20.9	25	30.0	8.2	25	7.6%	3.66 [2.73, 4.59]	
		Subtotal			188			185	48.3%	0.83 [0.00, 1.65] 0.16	92%, <.001
		Lautenbacher, 2016 0.4			71			72	10.9%	[0.48, 0.17] 0.52 [1.04,	
		Rastogi, 2012	0.11	0.68	30			30	10.0%	0.01]	
	Interleukin 5	Subtotal		1.05	101		0.72	102	20.8%		27%, .24
		Lautenbacher, 2016	0.37		71	0.29 0.4	0.87	72	10.8%	0.28 [0.62, 0.06]	
	Interleukin 13			0.96		0.03	0.75			0.39 [0.06, 0.72]	
		Rastogi, 2012	0.96	1.12	30	0.03	0.89	30	9.9%	0.97 [0.43, 1.50]	
		Rastogi, 2015	0.6	0.35	39	0.1	0.62	39	10.2%	0.98 [0.51, 1.45]	
		Subtotal			140			141	30.9%	0.74 [0.32, 1.17]	64%, .06
		Total			429			428	100.0%	0.54 [0.10, 0.98]	89%, <.001
		Test for subgroup differences									P < .001, I^2 = 87.7%

Abbreviations: CI, confidence interval; T_H1, T-helper 1; T_H2, T-helper 2; SMD, standardized mean difference. eTable 4

Sensitivity Analysis of T_H1 and T_H2 Immune Responses in Obese Individuals With Asthma Based on the Guidelines Followed in Diagnosing Asthma

Outcomes	Asthma diagnosis guidelines followed	Number of studies	Omitted studies	SMD [95% CI]	I^2 (%), p^H value	Overall effect: Z (P value)
T_H1 immune response	All	5[1-5]	None	0.43 [0.79, 0.08] 0.18	88% (p^H <.001) Not	2.37 (.02)
	NAEPP	1[1]	4 [2-5]	[0.48, 0.11] 5.98 [7.33,	applicable	1.21 (.22)
	GINA	1[5]	4 [1-4]	4.64] 0.25 [0.51, 0.01]	Not applicable	8.74 (< .001)
	Unspecified	3[2,3,5]	2[1,5]	0.24 [0.45, 0.03]	73% (p^H <.001)	1.88 (.06)
			After removal of Youssef et al, 2013[5]		65% (p^H = .01)	2.26 (.02)
T_H2 immune response	All	5 [1-5]	None	0.54 [0.10, 0.98]	89% (p^H < .001)	2.42 (.02)
	NAEPP	1[1]	4[2-5]	0.28 [0.56, 1.13]	Not applicable	0.66 (.51)
	GINA	1[5]	4[1-4]	3.66 [2.73, 4.59]	Not applicable	7.71 (< .001)
	Unspecified	3 [2-4]	2[1,5]	0.29 [0.03, 0.61]	74% (p^H = .002)	1.75 (.08)
			After removal of Youssef et al[5]	0.29 [0.02, 0.59]	77% (p^H < .001)	1.85 (.06)

Abbreviations: CI, confidence interval; T_H1, T-helper 1; T_H2, T-helper 2; SMD, standardized mean difference.

3.

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CHAPTER 2.6: Systematic Review 2

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Review article

The impact of metformin and aspirin on T-cell mediated inflammation: A systematic review of in vitro and in vivo findings

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ABSTRACT

Chronic inflammation and hyperglycaemia are well-established aspects in the pathogenesis of type 2 diabetes mellitus (T2D), including the progression of its associated complications such as cardiovascular diseases (CVDs). In fact, emerging evidence shows that dysfunctional immune responses due to dysregulated T-cell function aggravates CVD-related complications in T2D. However, there is a lack of specific therapeutic interventions that protect patients with diabetes who are at risk of heart failure. Metformin and aspirin are among the leading therapies being used to protect or at the very least slow the progression of CVD-related complications. The current review made use of major electronic databases to identify and systematically synthesise emerging experimental data on the impact of these pharmacological drugs on T-cell responses. The quality and risk of bias of include evidence were independently assessed by two reviewers. Overwhelming evidence showed that both metformin and aspirin can ameliorate T-cell mediated inflammation by inducing regulatory T-cells (Tregs) polarisation, inhibiting T-cell trafficking and activation as well as signal transducer and activator of transcription (STAT)3 signalling. As a plausible mechanism to mediate T-cell function, metformin showed enhanced potential to regulate mechanistic targets of rapamycin (mTOR), STAT5 and adenosine-monophosphate-activated protein kinase (AMPK) signalling pathways. Whilst aspirin modulated nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kB) and co-stimulatory signalling pathways and induced T-cell anergy. Overall, synthesised data prompt further investigation into the combinational effect of metformin and aspirin for the management of T2D-related cardiovascular complications.

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Introduction

Type 2 diabetes (T2D) is a low-grade chronic inflammatory condition that is distinguished by abnormally elevated blood glucose levels, insulin resistance and chronic immune activation [1–3]. Immune dysfunction as a consequence of chronic inflammation is a well-described feature associated with the progression of cardiovascular diseases (CVDs) in T2D patients, with substantial evidence implicating dysfunctional immune response mediated by T-cell activation during the pathogenesis of this process [4,5]. In fact, exacerbated activation of T helper (Th)1, $CD4^+$ and $CD8^+$ T-cells in T2D, together with reduced levels of Th2 and regulatory T-cells (Tregs) has been shown to greatly accelerates inflammation and insulin resistance [6]. Once activated, T-cells can proliferate and release pro- or anti-inflammatory cytokines that either activate or inhibit signalling pathways in immune cells [7–9]. Therefore, regulation of T-cell signalling is important in modulating immune responses, which could be essential in attenuating T2Dassociated complications if optimally controlled.

Chronic immune activation is a hallmark of T2D and increasing evidence has demonstrated that currently used oral glucose lowering and anti-inflammatory drugs such as metformin and aspirin can modulate immune responses and attenuate inflammation-related complications by regulating T-cell function [9–12]. For instance, in addition to its anti-hyperglycaemic properties, metformin suppresses hepatic glucose production through the activation of adenosine-monophosphate-activated protein kinase (AMPK) [13,14]. Metformin attenuates pro-inflammatory processes by downregulating the signal transducer and activator of transcription (STAT)3 and mechanistic target of rapamycin (mTOR) activity [15,16]. However, some studies have reported discordant findings on the effect of metformin on inflammation or T-cell function [11,17]. Therefore, the impact of metformin on T-cell mediated inflammation needs further clarity.

Apart from metformin, aspirin has been another drug target increasingly studied for its therapeutic benefits against CVDs and T2D-related complications [18–20]. Aspirin is a nonsteroidal anti-inflammatory drug that is known to act by blocking cyclooxygenase activity, leading to the attenuation of T-cell activation [21]. Moreover, aspirin inhibits the activation of pro-inflammatory nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kB) and STAT3 signalling in both normal physiology and inflammatory conditions [10,22,23]. However, other studies have reported on the negative effect of aspirin in regulating T-cell function [24,25]. Thus, it is necessary to enhance our understanding on the impact of metformin and aspirin on T-cell function in T2D. To the best of our knowledge, there are no updated reviews available that have systematically synthesised and comprehensively reported on the impact of metformin or aspirin on T-cell function in connection to metabolic diseases. Therefore, such evidence is critically explored in the current review, including implicated mechanisms that link T-cell activation and aggravation of inflammation in T2D or related metabolic complications.

Methods

This systematic review was prepared using the Preferred Reporting Items for Systematic Review and Meta-analysis (PRISMA) guidelines [26] and it forms part of a bigger study investigating T-cell function in T2D which is registered with the international prospective register of a systematic review (PROSPERO), registration number: CRD42018099745.

This systematic review was performed to address the following questions;

Question 1: Does metformin or aspirin alter T-cell function?

Question 2: How do these drugs modulate immune response mediated by T-cells in a physiological state and chronic inflammation? To achieve this, we used the following PICO process;

P – T-cells from individuals with or without inflammatory diseases and animal models of inflammation I – Metformin or aspirin

C – Treatment naïve participants (In vitro-untreated cells and in vivo participants not on aspirin or metformin) O – T-cell activation.

2.1. Search strategy

A comprehensive search was conducted from inception up to the 31st of January 2020, using Cochrane Library, Embase and PubMed electronic databases as well as grey literature by two independent reviewers (TMN and SRN). In cases of disagreements, a third reviewer (PVD) was consulted for arbitration. Two search strategies were independently applied to identify relevant studies, one for metformin and the other for aspirin. The search strategies were adapted to the databases without language restrictions using medical subjects heading (MeSH) terms and keywords such as “aspirin”, “inflammation”, “metabolic syndrome”, “metformin”, “T-cells”, “type 2 diabetes mellitus” and their respective synonyms and associated words or phrases. A detailed PubMed search strategy is provided in Table 1S.

2.2. Study selection

This review included both human and animal studies that reported on the effects of metformin and aspirin on T-cell function in disease models of T2D or metabolic syndrome as well as in normal physiology. However, reviews, books, editorials, letters and studies on cancer and infectious diseases were excluded from this study.

Two independent reviewers (TMN and SRN) identified eligible studies with the help of third reviewer (BBN).

2.3. Data extraction

The aim of the study was to systematically assess both human and animal studies that reported on the effects of metformin and aspirin on T-cell function in normal physiology and experimental models of T2D and metabolic syndrome. Briefly, extracted data items included; names of the authors, year of publication, study design, experimental model used, interventions used and dosage, combinational or comparative therapy, and main findings of each study. To manage extracted information including identifying and removing study duplicates, the Mendeley reference manager version 1.19.4-dev2 software (Elsevier, Amsterdam, Netherlands) was used.

2.4. Quality assessment and risk of bias

The quality and risk of bias of included studies were assessed by two independent reviews (SRN and KM) with the help of a third reviewer (VM) in cases of disagreements, as previously described [27]. Briefly, the modified Downs and Black checklist [28] and the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines [29] were used for human and animal studies, respectively. The Downs and Black checklist has 26 questions relating to the four domains namely; reporting bias, external validity, internal validity and selection bias against which the included human studies were appraised against. The overall total score of each study was rated as excellent if it was (24–27 points) good if (19–23 points), fair if (13–18 points) and poor if (≤12 points). On the other hand, the ARRIVE checklist has 20 questions and four domains namely; introduction, methods, results and discussion was used in animal studies. A study is considered to have met the minimum criteria if it scores a minimum of 10 points and contains most elements or aspects required for publication.

Results

3.1. Study selection and characteristics of included studies

Overall, a total of 250 studies were identified and screened for eligibility whereas only 31 met the inclusion criteria (overall agreement 91.53%, kappa = 0.75) (Fig. 1) and these articles were published between 1975 and 2019. Of those that met the criteria, 14 studies reported on the impact of metformin as an intervention. Briefly, the metformin search strategy retrieved 63 studies of which 49 were excluded as 6 were reviews whilst the other 43 were not relevant [30–33].

The included studies reporting on the effect of metformin comprised of 4 human and 10 animal studies. Moreover, the included human studies consisted of 4 observational studies and 1 randomized control trial.

The aspirin search strategy identified a total of 187 studies of which 156 were excluded due to study design models (n = 41) and 101 were not relevant to the topic of interest whilst 28 were reviews. As a result, 17 studies were included, of which 10 were human studies and 7 were animal studies reporting on the impact of aspirin on T-cell function. The included studies reporting on aspirin were in vitro experimental studies, whereby cells from humans were collected and cultured in the presence of aspirin (n = 9) except for 1 study which was a non-randomized control trial, where participants were first given aspirin and then the cells were collected and analysed.

3.2. Quality and risk of bias of included studies

The modified Downs and Black guidelines checklist with 26 questions was used to appraise studies reporting on human

related outcomes by two independent reviewers. All included studies were published in peer reviewed journals. For included human studies reporting on the effect of metformin (n = 4), the median score range was 13 (11–15) out of a possible score of 27 across all four domains. Of these, three studies scored fair (13–15 points) and one poor (11 points). Overall, all included studies had a lower risk of reporting bias with a median of 7 (6–8) out of a possible score of 10 (overall agreement 81.43%, kappa = 0.63). In addition, the studies had a low selection bias with a median of 4 (3–4) out of a possible score of 6 (overall agreement 95.24%, kappa = 0.91). However, the studies performed poor on internal and external validity bias domain with median 3 (1–4) out of a possible score of 7 (overall agreement 83.67%, kappa = 0.72) and 0 out of a possible score of 3 (overall agreement 95.24%, kappa = 0.91), respectively (Table 2S).

All included animal studies reporting on the impact of metformin in this review met the minimum requirements for publication using the ARRIVE guidelines checklists with 20 questions. Briefly, the median score range of all included metformin studies was 16.5 (13–18) out of a possible score of 20. Moreover, the included studies scored high in all four domains with a median of 4 (4–4) out of a possible score of 4 (overall agreement 100%, kappa = 1); 7.5 (5–8) out of a possible score of 9 (overall agreement 88.89%, kappa = 0.76); 2 (1–3) out of a possible score of 3 (overall agreement 87.50%, kappa = 0.75) and 3 (3–3) out of a possible score of 3 (overall agreement 90%, kappa = 0.8) in the introduction, methods, results and discussion domains, respectively (Table 3S).

On the other hand, all human studies reporting on the impact of aspirin were scored as poor except for one study [25] with a median score of 9.5 (6–13). In addition, the studies performed poor in all domains except for reporting bias where they showed relatively low risk with a median score of 5.5 (4–9) (overall agreement 84%, kappa = 0.68) (Table 4S). However, animal studies assessing the efficacy of aspirin were of relatively good quality with a median score of 15 (10–15). These studies scored high in all domains with a median of 4 (4–4) out of a possible score of 4 in the introduction domain and 6 (1–7) out of a possible score of 9 in the methods domain. In addition, the included studies had a median range score of 2 (1–2) out of a possible score of 3 in the results and 3 (3–3) out of a possible score of 3 in the discussion domain. The inter-rater reliability was scored as; perfect for introduction (overall agreement 100%, kappa = 1) and methods (overall agreement 92.1%, kappa = 0.84) domains. Moreover, results agreement (overall agreement 75%, kappa = 0.75) and discussion (overall agreement 80.95%, kappa = 0.62) domains were scored as substantial (Table 5S). All included studies were published in peer review journals

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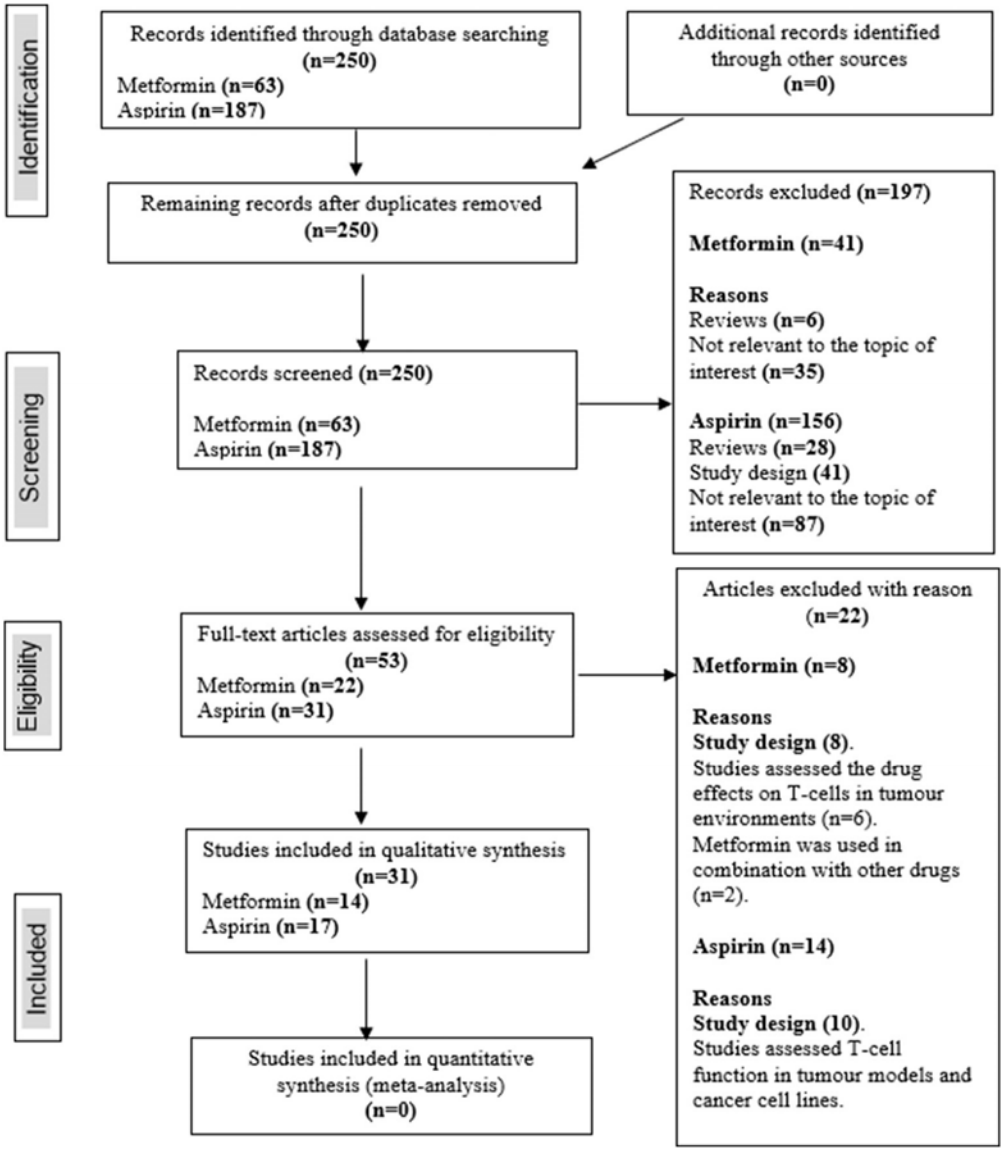

Fig. 1. PRISMA diagram indicating the study selection process.

Table 1

Ex vivo studies reporting on the role of metformin in modulating T-cell responses (n = 4).

Author, year	Experimental model	Metformin dosage	Effect on T-cells/main findings
Solano et al., 2008 [36]	Cultured T-cells were isolated from BALB/c mice lymph nodes	10 or 100 μM for 4 h	Metformin treatment dose dependently suppressed proliferation, reduced viability and induced apoptosis of T-cells when compared to the untreated group. In addition, metformin increased the levels of antioxidant molecule, glutathione and diminished lipid peroxidation in comparison to the untreated group.
Kang et al., 2013 [37]	Cultured T-cells were drawn from mice axillary lymph nodes.	0.5; 1; 5 and 10 mM for 3 days	Metformin treatment dose dependently decreased the number of Th17 and downregulated Signal transducer and activator of transcription (STAT)3 phosphorylation through adenosine monophosphate-activated protein kinase (AMPK) pathway.
Zarrouk et al., 2014 [13]	Cultured T-cells were isolated from OT-I TCR transgenic and AMPK^{null} mice spleens or lymph nodes	10 mM up to 72 h	Metformin treatment reduced the expression of activation marker CD25, adhesion molecule CD69 and amino acid transporter CD98 on cultured T-cells compared to control group. In addition, metformin treated T-cells showed decreased glucose uptake, but increased lactate output compared to controls. Moreover, metformin inhibited mechanistic target of rapamycin (mTOR) activity on CD8$^+$ T-cells and blocked T-cell blastogenesis and proliferation independent of AMPK.
Son et al., 2014 [15]	CD4$^+$ T-cells were isolated from mice spleen and cultured under Th17 polarizing conditions for 3 days	1 mM for 1 h for 3 days	Metformin treatment reduced the number of Th17 cells and increased Tregs. Furthermore, it suppressed osteoclastogenesis by inhibition of mTOR and STAT3 mediated by activated AMPK.

3.3. In vitro evidence on the impact of metformin on T-cell function

In total, only four studies reported on the in vitro effects of metformin (Table 1). Briefly, cultured T-cells isolated from BALB/c lymph nodes treated with metformin suppressed proliferation, reduced viability and induced apoptosis of T-cells when compared to the untreated group dose dependently [34]. In addition, metformin increased the levels of the antioxidant molecule, glutathione and diminished lipid peroxidation in comparison to the untreated group. In addition, in cultured T-cells from mice axillary lymph nodes [35], metformin treatment also dose-dependently decreased the number of Th17 and downregulated STAT3 phosphorylation through AMPK pathway. The latter plays a major role in energy regulation [35] and remains an interesting mechanism explored to assess the therapeutic capabilities of metformin [14].

Furthermore, in cultured T-cells isolated from OT-I TCR transgenic and AMPK-null mice spleens or lymph nodes [13], metformin treatment could reduce the expression of the activation marker CD25, the adhesion molecule CD69 and amino acid transporter CD98 on cultured T-cells compared to control group. Moreover, metformin treated T-cells showed decreased glucose uptake, but increased lactate output compared to controls. These effects were linked with the inhibition of mechanistic target of rapamycin (mTOR) activity on CD8$^+$ T-cells, T-cell blastogenesis and proliferation independent of AMPK. Consistently, others demonstrated that metformin treatment reduced the number of Th17 cells and increased Tregs, whilst suppressing that of mTOR and STAT3 mediated by activated AMPK [15]. Interestingly, the overall in vitro evidence supports the notion that in addition to regulating AMPK, metformin inhibits proliferation of T-cells in general [36] and ameliorates inflammation by reducing the number of Th17 whilst promoting the proliferation of Tregs [15,37].

3.4. In vivo evidence on the impact of metformin on T-cell mediated function

The majority of studies assessing the impact of metformin on T-cell function were those reporting on in vivo experimental models (Table 2). In fact, summarised evidence assessed the therapeutic effects of this blood glucose lowering drug on T-cell responses through various animal models. Notably, these models were predominantly mice exposed to various factors to induce a pathological state, including exposure to collagen-induced arthritis in DBA1/J mice, concanavalin A-induced hepatitis in BALB/c mice, inflammatory bowel disease in C57BL/6 mice, autoimmune encephalomyelitis in C57BL/6 mice, diet-induced obesity in C57BL/6 J mice, and systemic lupus erythematosus in Roquinsan/san mice (Table 2). Collectively, these experimental models represent a chronic inflammatory state which is essential in investigating T-cell mediated responses.

The findings using these experimental models demonstrated that metformin treatment at 100 or 150 mg/kg for 16 days could significantly attenuate autoimmune arthritis, concomitant to reducing levels of Th17 cells and pro-inflammatory cytokines, including

tissue necrosis factor (TNF)-α and interleukin (IL)-1 [35]. At a much lower metformin dose (50 mg/kg daily for 13 weeks), others showed [16] that metformin could alleviate arthritis in mice by reducing autoantibody expression and joint inflammation. These effects were concomitant to decreasing Th17, mTOR and STAT3 signalling, whilst increased the number of Tregs and AMPK activity. Although they did not look at AMPK, others showed that metformin treatment at 5 mg/mouse for 9 weeks attenuated autoimmune arthritis by modulating Th17/Tregs ratio, consistent to reducing the number of Th17 and $CD4^{+}pSTAT3^{+}$ T-cells in C57BL/6 mice [15].

In BALB/c mice with hepatitis [17], metformin treatment at 200 mg/kg from 24 h could exacerbate inflammation-induced liver injury by enhancing activation of $CD4^{+}$ T-cells, dendritic cells and macrophages. These results were concomitant to its effect in increasing lymphocytes infiltration into the liver and the secretion of serum levels of pro-inflammatory cytokines, tumour necrosis factor (TNF)-α and interferon (IFN)-γ and IL-17 from $CD4^{+}$ T-cells. Furthermore, it was evident that metformin treatment at 50 mg/kg daily for 16 days could ameliorate inflammatory bowel disease by reducing inflammation through inhibiting Th17 proliferation, STAT3 and mTOR signalling [46]. Here, metformin also increased the number of Tregs, including the expression levels of AMPK and STAT5 on $CD4^{+}$ T-cells.

In vivo evidence also showed that besides impacting arthritis or hepatitis to regulate T-cell function, metformin could affect encephalomyelitis and lupus erythematosus [39,41], conditions characterised with an abnormal T-cell response. For example, Sun and colleagues [47] demonstrated that metformin treatment at 100 mg/kg/ day for 30 days attenuated encephalomyelitis by reducing the number of Th17 and increasing that of Tregs. In addition, stimulated metformin treated T-cells exhibited reduced IL-17 and enhanced IL-10 and transforming growth factor-β secretion. Treatment also inhibited mTOR signalling but enhanced AMPK activity. Alternatively, Lee and coworkers [49] reported that metformin treatment at 5 mg/d for 3 weeks could ameliorate systemic lupus erythematosus, mainly by reducing the number of Th17 cells and $CD4^{+}$ $ICOS^{+}$ follicular Th cells, as well as elevating Tregs numbers and AMPK activity. Moreover, relevant to the metabolic syndrome, metformin, used at 10 mg/kg or 50 mg/kg daily for 14 weeks, dose dependently reduced body weight and improved both lipid and glucose metabolism. In addition, treatment reduced the number of Th17, whilst raising the levels of Tregs, IL-17 mRNA, and increasing that of Foxp3 in diet-induced obese (DIO) mice [48].

3.5. Evidence from clinical studies on the impact of metformin on T-cell mediated function

There are currently a few studies that have assessed the effects of metformin in regulating T-cell function in human subjects. However, Table 2 lists four clinical studies that have reported on the current subject, with two findings being cross-sectional, and each of the remaining being a randomised control trial and a cohort study. The summarised results from cross-sectional studies were those done on with T2D or diabetics at risk of developing abdominal aortic aneurysm [11,51]. For instance, Dworacki and co-workers [51] demonstrated that metformin treatment at 500–2550 mg daily for 6 months improved thymic output by elevating the number of recent thymic emigrants T-cells ($CD45^{+}CD3^{+}RO^{-}RA^{+}$) and mature $CD4^{+}$ T-cells when compared to treatment naïve T2D patients. Although the therapeutic benefit was observed, others [11] showed the use of metformin at a similar dose did not reduce inflammation in individuals with or at risk of developing abdominal aortic aneurysm. In addition, the same study showed that metformin treatment did not affect any change in the frequency of Th17 and Tregs in individuals with diabetes. Moreover, there was no difference in the levels of both pro- and anti-inflammatory cytokines between individuals with diabetes on metformin treatment versus those who were untreated. Consistently, evidence from a randomized clinical trial making use of metformin at 1500 mg daily for 6 months, showed that treatment did not reduce cardiovascular risk as the frequency of both proatherogenic $CD4^{+}CD28^{null}$ and $CD4^{+}$ T-cells in individuals with hyperinsulinemia and polycystic ovary syndrome [50]. However, supporting the beneficial effects reported by Dworacki and co-workers [51], the cohort study, reporting on the use of metformin at 500 mg for 3 months, demonstrated that this biguanide could alleviate Behcet's disease clinical symptoms and significantly reduced inflammation, by increased number of Tregs and reduced that of Th17 [12].

Table 2
In vivo studies reporting on the role of metformin in modulating T-cell responses (n = 12).

Author, year	Experimental model	Metformin dosage	Effect on T-cells/main findings
Kang et al., 2013 [37]	Collagen-induced arthritis DBA1/J mice model	100 or 150 mg/kg daily for 16 days in vivo	Both doses of metformin significantly attenuated autoimmune arthritis, decreased levels of T helper (Th)17 cells and pro-inflammatory cytokines, tissue necrosis factor (TNF)-α and interleukin (IL)-1.
Volarevic et al., 2014 [17]	Concanavalin A induced hepatitis in male BALB/c mice model	200 mg/kg thrice a day from 24 h before and after concanavalin A administration	Metformin exacerbated inflammation-induced liver injury by enhancing activation of $CD4^+$ T-cells, dendritic cells and macrophages. In addition, metformin treatment increased lymphocytes infiltration into the liver and the secretion of serum levels of pro-inflammatory cytokines, TNF-α and interferon (IFN)-γ and IL-17 from $CD4^+$ T-cells. However, metformin treatment had no effect on the percentage of both $CD8^+$ and $CD4^+$ $CD25^+Foxp3^+$ regulatory T-cells (Tregs).
Son et al., 2014 [15]	Collagen-induced arthritis male C57BL/6 mice model	5 mg/mouse 3 times a week for 9 weeks after first immunization in vivo	Metformin treatment attenuated autoimmune arthritis by modulating Th17/Tregs ratio. In addition, the treatment reduced the number of Th17 and $CD4^+pSTAT3^+$ T-cells (STAT3 is required for pathogenic Th17 differentiation and antagonizes the development of Tregs) compared to controls. However, the number of $CD4^+CD25^+Foxp3^+$ Tregs and $CD4^+pSTAT5^+$ T-cells (STAT5 enhances differentiation of Tregs) was increased in the treatment group compared to controls.
Lee et al., 2015 [38]	Inflammatory bowel disease induced C57BL/6 mice model	50 mg/kg daily for 16 days after inflammatory bowel disease induction	Metformin treatment ameliorated inflammatory bowel disease by reducing inflammation through inhibiting Th17 proliferation, STAT3 and mTOR signalling. Metformin also increased the number of Tregs, including the expression levels of AMPK and STAT5 on $CD4^+$ T-cells.
Sun et al., 2016 [39]	Autoimmune encephalomyelitis induced female C57BL/6 mice model	100 mg/kg/day for 30 days	Metformin treatment attenuated encephalomyelitis by reducing the number of Th17 and increasing that of Tregs. In addition, stimulated metformin treated T-cells exhibited reduced IL-17 and enhanced IL-10 and transforming growth factor-β secretion. Treatment also inhibited mTOR signalling but enhanced AMPK activity.
Kim et al., 2016 [40]	Diet-induced obese C57BL/6 mice	10 mg/kg or 50 mg/kg daily for 14 weeks	Metformin dose dependently reduced body weight and improved both lipid and glucose metabolism. In addition, treatment reduced the number of Th17, whilst raising that of Tregs in obese mice. Moreover, treatment decreased mRNAs of IL-17 and increased that of Foxp3.
Lee et al., 2017 [41]	Systemic lupus erythematosus male *Roquin*$^{san/san}$ mice model	5 mg/d for 3 weeks	Metformin treatment ameliorated systemic lupus erythematosus, reducing the number of Th17 cells and $CD4^+$ $ICOS^+$ follicular Th cells. In addition, treatment increased the number of Tregs and AMPK activity, but inhibited mTOR and STAT3 signalling in T-cells.
Kim et al., 2018 [16]	Collagen-induced arthritis male DBA/1 J mice model	50 mg/kg daily for 13 weeks post immunization	Metformin alleviated the development of arthritis by reducing autoantibody expression and joint inflammation. Furthermore, metformin decreased Th17, mTOR and STAT3 signalling. Moreover, it increased the number of Tregs and AMPK activity when compared to control group.

Author, year	Experimental model	Human	Studies
Moro et al., 2013 [42]	Individuals with hyperinsulinemia and polycystic ovary syndrome	1500 mg daily for 6 months	Treatment with metformin did not reduce cardiovascular risk as the frequency of both proatherogenic $CD4^+CD28^{null}$ and $CD4^+$ T-cells remained unchanged post treatment when compared to the baseline levels.
Dworacki et al., 2015 [43]	Individuals with T2D	500–2550 mg daily	Metformin treatment increased thymic output by elevating the number of recent thymic emigrants naïve T-cells ($CD45^+$ $CD3^+$ RO^- RA^+) and mature $CD4^+$ T-cells when compared to drug naïve T2D patients. Interestingly, thymic output of metformin treated group was the same as the non-diabetic control group.
Wang et al., 2018 [11]	Individuals with diabetes and overt or at risk of developing abdominal aortic aneurysm.	500–2550 mg daily for 6 months	Treatment with metformin did not reduce inflammation in individuals with or at risk of developing abdominal aortic aneurysm. In addition, metformin treatment did not affect any change in the frequency of T helper (Th)17 and regulatory T-cells (Tregs) in individuals with diabetes. There was also no difference in the levels of both pro- and anti-inflammatory cytokines between individuals with diabetes on metformin treatment and those not.
Yong et al., 2019 [12]	Individuals with Behcet's disease	500 mg 2 or 3 times per day for 3 months	Metformin treatment alleviated Behcet's disease clinical symptoms and significantly reduced inflammation. In addition, treatment with metformin increased number of Tregs and reduced that of Th17.

3.6. Molecular mechanisms implicated in the regulatory effect of metformin on T-cell mediated function

Currently, it is accepted that immune homeostasis is maintained by a delicate balance between anti- and pro-inflammatory T-cell subsets. In brief, Th17 and Th1 are considered to be pro-inflammatory subsets, whilst Tregs and Th2 as anti-inflammatory effector cells [44]. It is well known that Th17 cells secrete IL-17, IL-21 and IL-22 whilst Th1 cells can release IFN-γ, interleukin IL-2 and TNF-α; and Tregs produce IL-10, IL-35 and transforming growth factor (TGF)-β as their signature cytokines [45–47]. Subsequently, T-cell subset ratio as well as the cytokines they release directly modulates immune responses. Therefore, the circulating number of Th17 and Tregs is important in controlling inflammation. However, current evidence shows that in metabolic disorders such as T2D, Th ratio is skewed towards the pro-inflammatory subset resulting in aggravated pro-inflammatory response [48]. Overall, evidence synthesised from included in vitro studies that reported of the effects of metformin supported its inhibitory effects of proliferation of T-cells in general [36]. This was consistent with amelioration of inflammation by reducing the number of Th17 whilst promoting the proliferation of Tregs [15,37].

Activated T-cells can upregulate the levels of markers such as CD25 (IL-2R) and CD98 including adhesion molecules like CD69 that play a role in proliferation and trafficking [49,50]. Interestingly, evidence from this review showed that metformin can inhibit the expression of CD25, CD69 and CD98 on cultured T-cells from T-cell receptor (TCR) transgenic mice [13]. Overall, these findings suggest that metformin can inhibit T-cell activation and promotes T-cell unresponsiveness in chronic inflammatory conditions. Importantly, it is also evident that metformin can modulate T-cell function at least in part via AMPK/ STAT/mTOR regulatory mechanisms.

The anti-inflammatory pharmacodynamics of metformin are centred on its ability to activate AMPK, a major cellular regulator of glucose and lipid metabolism [14,35]. In relation to T-cell function, AMPK can suppress mTOR signalling and its downstream target STAT3 (which is important in Th17 differentiation) whilst enhancing Tregs differentiation via STAT5 signalling [51]. Certainly, the included studies demonstrated that the anti-inflammatory effects of metformin can be induced via the modulation of mTOR and STAT3/5 signalling pathways on T-cells in various animal models of chronic inflammation [15,16,37–39,41]. For example, metformin can inhibit or interfere with mTOR and STAT3 signalling, but can also enhance STAT5 activity through AMPK activation [15,16,37–39,41]. This could be the possible explanation for the reported inflammation ameliorative effects of metformin in Behcet's disease [12]. Further suggesting that there is a strong connection between T-cell function and regulation of energy metabolism, especially in conditions of metabolic syndrome since AMPK acts as an energy sensor and modulator, as reported elsewhere [14,35].

3.7. In vitro evidence on the impact of aspirin on T-cell mediated function

Aspirin is one of the widely used anti-inflammatory agents being investigated for its prophylactic effects against diabetes associated complications. A total of twelve studies reported on the ex vivo impact of aspirin on modulating T-cell mediated function (Table 3). The prominent ex vivo models used included isolation of T-cells from neutrophilic asthma-induced C57BL6 mice or orthodontic relapse-induced Sprague-Dawley rats, as well as T-cells derived from healthy volunteers, or those with phytohaemagglutinin and Sjogren's syndrome (Table 3). The major findings from these studies demonstrated that apart from inhibiting cyclooxygenase activity as its anti-inflammatory mechanism, aspirin could block NF-kB signalling by inhibiting the activation of IkB kinase (IKK) [52]. Interestingly, besides STAT signalling, immunological modulation can be induced by transcriptional activation of NF-kB in response to stimuli from stressed cells, cytokines, infections and free radicals [53]. Transcriptional factor NF-κB is known to aggravate inflammation by inducing the expression of genes that encode for the production of pro-inflammatory cytokines and chemokines [52]. Therefore, activated NF-kB signalling remains the prime mechanisms to be explored to understand the impact of aspirin on T-cell function and its regulation of pro-inflammatory markers in conditions of pathology.

Findings from included ex vivo studies using cultured cells showed that aspirin had no cytotoxic effects on T-cells. The summarised evidence showed that aspirin could inhibit the expression of B7, CD40 and MHC class II expression whilst up-regulating that of immunoglobulin-like transcription-3 and programed cell death 1(PD-1) ligand on dendritic cells in a dose-dependent manner in vitro [54–57]. Interestingly, stimulation of T-cells by aspirin treated dendritic cells in these studies induced poor Th1 cell proliferation and their cytokine release [55,56]. However, aspirin showed enhanced potential to induce the production of Tregs which expressed the same transcriptional regulator Foxp3 levels as those of non-treated dendritic cells [55,56]. Therefore, the anti-inflammatory effect of aspirin on T-cell function could at least in part be mediated by its inhibitory effects on antigen presenting cells and co- stimulatory signalling. Nevertheless, consistent with metformin, it appears aspirin also displays inhibitory effect on T-cell trafficking and function. In that context, aspirin dose dependently inhibited the expression of integrins and intercellular adhesion molecule-1 on endothelial cells as well as L-selectin on T-cells and their activation and

transmigration thereof in ex vivo culture [58–60]. Thus, suggesting that aspirin offers cardio-protection at least in part, through the inhibition of adhesion molecules essential for T-cell trafficking.

3.8. In vivo evidence on the impact of aspirin on T-cell mediated function

Briefly, only eight studies reported on the impact of aspirin on T-cell function, inclusive of two randomized controlled trials, as displayed in Table 4. BALB/c mice, double transgenic male rats harbouring human renin and angiotensinogen genes (dTGR), asthma-induced C57BL/6 mice, and neutrophilic asthma induced C57BL/6 mice were the predominant animal models used to assess the impact of aspirin on T-cell function.

Through the exploration of these experimental models, Muller and colleagues [23] demonstrated that the use of aspirin at 25 or 600 mg/kg for 3 weeks could reduce infiltration of both $CD4^+$ and $CD8^+$ T-cells into damaged heart and kidney vessels. Importantly, the low dose of aspirin significantly reduced $CD4^+$ T-cells with a slight effect on $CD8^+$ T-cells, whilst blocking the activation of NF-κB signalling. Alternatively, Javeed and colleagues [64] showed that treatment with 6 mg/kg or 60 mg/kg/day for 4 weeks could dose-dependently reduce the frequency of circulatory $CD4^+$ T-cells including thymocytes but enhanced that of functional regulatory T-cells (Tregs) in dTGR rats. Moon and coworkers [8] demonstrated that 18 mg/kg for 4 days of aspirin could significantly increase eosinophil infiltration by enhancing the production of Th2 cytokine downstream mediator, eotaxin. Moreover, Th17 and the levels of IL-17 cytokine were decreased in asthma-induced C57BL6 mice. The same authors [10] also showed that aspirin treatment at 18 mg/kg daily for 3 days could consistently inhibit Th17 airway inflammation by blocking IL-17 and IL-6 positive feedback in neutrophilic asthma-induced C57BL6 mice.

Interestingly, the beneficial effects of aspirin in effectively modulating T-cell mediated inflammation were also demonstrated by others [62], whereby its use at 9 mg/kg/day over 40 days could dose-dependently increase the number of Tregs in heart transplanted SpragueDawley rats. On the other hand, Liu and colleagues [9] showed that aspirin treatment with 300 mg/kg/day for 10 days could reduce the frequency of $CD4^+$ T-cells and inhibit orthodontic relapse of tooth movement in Sprague-Dawley rats. To collaborate in vivo findings, only two nonrandomised clinical trials reporting on the impact of aspirin on T-cell function could be retrieved (Table 4). Whereby, Crout and coworkers [24] reported that administration of aspirin at 900 mg/five times daily for 4 days could significantly suppress lymphocytes transformation (blastogenesis) without any effect on the proportions of T-cells. Another study [25] showed that treatment with aspirin at 1500 mg/daily for two weeks did not induce genetic toxicity in T-cells nor did it influence DNA synthesis and repair of T-cell lymphocytes in individuals with soft-tissue injury.

3.9. Molecular mechanisms implicated in the regulatory effect of aspirin on T-cell mediated function

Evidence from this review showed that inhibition of NF-kB signalling by aspirin could block the differentiation and function of $CD4^+$ and $CD8^+$ T-cells in normal physiology [9,24,56,61], whilst promoting activation and differentiation of Tregs. In contrast, only one study reported that inhibition of NF-kB signalling by aspirin had no effect on the levels of Th1 and Th2 associated cytokines (IFN-γ, IL-2 and IL-13) [22]. Overall, these findings suggest that the anti-inflammatory effects of aspirin are not only limited to NF-kB signalling but may involve other mechanisms such as Janus kinase (JAK)-STATs signalling pathway (Table 3). In fact, further synthesis of data showed that like metformin, aspirin blocks Th17 polarisation that is induced by IL-6, in a dose-dependent manner concomitant to decreased STAT3 signalling in lipopolysaccharide-induced mice [10].

T-cell receptors are important primary signal transducers during the activation of T-cells. However, this signalling alone is not sufficient to successfully activate T-cells, hence secondary co-stimulatory signal mediated by co-stimulatory molecules such as CD28 and CD40L is required [64–66]. Once activated, T-cells can now carry out their effector functions in a subtype specific manner. In this review, included studies demonstrated that aspirin can modulate T-cell mediated inflammation through the inhibition of co-stimulatory signals and the upregulation of negative regulatory molecules which collectively induce T-cell. T-cell anergy is a hyporesponsive state of T-cells that occurs due to inadequate activation signalling.

Interestingly, like metformin, aspirin treatment reduced the frequency of Th1 and Th17 cells as well as $CD8^+$ T-cells, including proinflammatory cytokines in response to aspirin treatment in animal models of inflammation [8,9]. Although aspirin could enhance the differentiation of Th2 and Tregs [8,10,23,62,63], the exact mechanisms that leads to decreased pro-inflammatory T-cell subsets and increased anti-inflammatory subsets remain unclear. Therefore, we speculate that it is most likely in part, due to the modulatory effects of aspirin on the JAK-STAT signalling pathway, as demonstrated elsewhere [67,68].

Table 3
Ex vivo studies reporting on the role of aspirin in modulating T-cell responses (n = 12).

Author, year	Experimental model	Aspirin dosage	Effect on T-cells/main findings
Hackstein et al., 2001 [57]	Male C57BL/6 mice	0.5; 1 or 2.5 mM up to 7 days	Aspirin inhibited the expression of T-cell co-stimulatory molecules ligands (CD40, B7-1 and B7-2) and major histocompatibility complex (MHC) class II on dendritic cells in a dose-depended manner. In addition, the dendritic cells poorly stimulated naïve T-cell proliferation and reduced the secretion of interleukin (IL)-2.
Moon et al., 2013 [10]	Neutrophilic asthma induced C57BL/6 mice model	1;10; 100 or 1000 nM for 3 days	Th17 polarisation induced by IL-6 was inhibited by aspirin in a dose-dependent manner. The suppression of Th17 function by aspirin correlated with decreased expression of acetyl-signal transducer and activator of T-cells (STAT)3 (downstream signalling of IL-6).
Liu et al., 2017 [9]	Orthodontic relapse induced male Sprague-Dawley rats	100 μg/mL for 48 h	Aspirin treatment significantly suppressed the differentiation of Th1 and $CD4^+$ T-cells and significantly inhibited the release of Th1 associated cytokines (tumour necrosis factor (TNF)-α and IFN-γ).
Crout et al., 1975 [24]	T-cells from treated individuals were activated with phytohaemagglutinin	900 mg five times daily for 4 days	Aspirin treatment did not change T-cell proportions nor their viability in culture compared to baseline levels.
Mazzeo et al., 1998 [61]	Culture T-cells were isolated from healthy volunteers	1; 5 or 10 mmol/L for 1 h	Treatment with aspirin dose-dependently inhibited IL-12 secretion and Th1 differentiation via the inhibition of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kB).
Gerli et al., 1998 [58]	Cultured T-cells were isolated from individuals with Sjogren's Syndrome	30; 300 or 600 μg/mL for 0.5–2.5 h	Aspirin and dose-dependably inhibited the adhesion and transmigration of T-cells in the same manner by suppressing the activation of integrin. In addition, although aspirin increased the activation of protein kinase C in T-cells, it did not increase their proliferation nor interleukin (IL)-2 synthesis.
Voisard et al., 2001 [59]	Human coronary in vitro model. $CD4^+$ T-cells were isolated from healthy volunteers	1; 2; 5 or 10 mmol/L up to 18 h	Aspirin (5 and 10 mmol/L doses) reduced the expression of intercellular adhesion molecule-1 on human coronary endothelial cells, dose dependently. Furthermore, it inhibited the adherence of $CD4^+$ T-cells to the activated endothelial cells by half.
Gerli et al., 2001 [60]	Cultured T-cells were isolated from individuals with Sjogren's Syndrome	30; 300 or 600μg/ml up to 2 h	Aspirin treatment significantly reduced T-cell adhesion to activated endothelial cells in a dose-dependent manner. Moreover, the expression of adhesion molecule L-selectin on T-cells was reduced dose dependently.
Cianferoni et at., 2001 [22]	Cultured T-cells were isolated from healthy blood donors	0.001 or 1 mM for 15 min	Aspirin significantly reduced IL-4 secretion and RNA expression in $CD4^+$ T-cells. However, this was independent of reduced NF-kB activation but dependent on reduced IL-4 promoter activity. In addition, aspirin did not affect the expression of interferon gamma (IFN-γ), IL-2 and IL-13.
Buckland et al., 2006 [55]	Cultured blood cells were isolated from healthy volunteers	2.5 mM for 5 days	Aspirin suppressed NF-κB signalling pathway and the expression of B7 costimulatory molecule but upregulated immunoglobulin-like transcript-3 and programed cell death 1 ligand (PD1-L) on dendritic cells. In addition, aspirin treated dendritic cells induced anergy in responder T-cells and reduced their secretion of IFN-γ. Interestingly, stimulation with aspirin-treated dendritic cells induced the production of regulatory T-cells (Tregs) that are $CD25^{hi}$ and express the transcriptional regulator Foxp3.
Buckland et al., 2006 [54]	Blood cells were isolated from healthy volunteers	2.5 mM for 5 days	Aspirin-treated dendritic cells inhibited the expression of T-cell co-stimulatory molecules ligands, CD40, B7-1 and B7-2 molecules on their cell surface. In addition, the dendritic cells dose dependently inhibited T-cell response by inducing the upregulation of T-cell activation co-inhibitor, immunoglobulin-like transcript-3. Moreover, although naïve $CD4^+$ T-cells produced significant levels of IFN-γ when exposed to aspirin-treated dendritic cells, they were hypoproliferative.
Hernandez et al., 2007 [56]	Cultured blood cells were isolated from healthy volunteers	0.5; 2; 3 or 5 mM for 5 days	Dendritic cells treated with aspirin decreased the expression of T-cell costimulatory molecules ligands (CD40 and B7-1) expect B7-2. Moreover, the treated dendritic cells weakly activated T-cells which produced reduced IL-2 and IFN-γ compared to untreated controls. Interestingly, Tregs generated from the blockage of NF-κB in both aspirin-treated and non-treated dendritic cells exhibited similar Foxp3 mRNA expression.

Table 4
In vivo studies reporting on the role of aspirin in modulating T-cell responses (n = 8).

Author, year	Study type	Experimental model	Aspirin dosage	Effect on T-cells/main findings
Muller et al., 2001 [23]	Animal	Double transgenic male rats harbouring human renin and angiotensinogen genes (dTGR) and Sprague-Dawley rats	25 or 600 mg/kg per day for 3 weeks	Both low and high dose aspirin reduced infiltration of both $CD4^+$ and $CD8^+$ T-cells into damaged heart and kidney vessels. Importantly, the low dose of aspirin significantly reduced $CD4^+$ T-cells with a slight effect on $CD8^+$ T-cells. In addition, high dose aspirin reduced infiltration of all cells and inhibited the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) signalling.
Javeed et al., 2009 [63]	Animal	Female BALB/c mice	6 or 60 mg/kg per day for 4 weeks	Aspirin dose-dependently reduced the frequency of circulatory $CD4^+$ T-cells including thymocytes but enhanced that of functional regulatory T-cells (Tregs).
Moon et al., 2010 [8]	Animal	Asthma induced C57BL/6 mice model	18 mg/kg for 4 days post immunization	Treatment with aspirin only significantly increased eosinophil infiltration by enhancing the production of Th2 cytokine downstream mediator, eotaxin. T helper (Th17) and its cytokine interleukin (IL)-17 were decreased and associated with an up-regulation of allergen-specific IL-13 production from T-cells in aspirin-treatment.
Moon et al., 2013 [10]	Animal	Neutrophilic asthma induced C57BL/6 mice model	18 mg/kg daily for 3 days post immunization	Aspirin treatment inhibited Th17 airway inflammation by blocking IL-17 and IL-6 positive feedback.
Zhu and Gao 2015 [62]	Animal	Wistar and Sprague-Dawley rats	9 mg/kg/day from day 3 to 40 post heart Transplantation	Aspirin-treatment duration dependently increased the number of Tregs in heart transplanted Sprague-Dawley rats.
Liu et al., 2017 [9]	Animal	Orthodontic relapse induced male Sprague-Dawley rats	300 mg/kg/day for 10 days post procedure in vivo	Aspirin treatment reduced the frequency of $CD4^+$ T-cells and inhibited orthodontic relapse of tooth movement.

Author, year	Study type	Experimental model	Human	Studies
Crout et al., 1975 [24]	Non-randomized controlled trial	Healthy individuals volunteered to ingest aspirin for 4 days	900 mg five times daily for 4 days	Aspirin ingestion significantly suppressed lymphocytes transformation (blastogenesis). However, it did not affect the proportions of T-cells.
Ozkul et al., 1996 [25]	Non-randomized controlled trial	Individuals with soft-tissue injury	1500 mg/day for 2 weeks	Treatment with all aspirin did not induce genetic toxicity in T-cells nor did it influence DNA synthesis and repair of T-cell lymphocytes.

Table 5
Experimental evidence on the combinational use of metformin and aspirin, and its impact on T-cell function.

Author, year	Study type	Experimental model	Dosages	Effect on T-cells/main findings
Gillani et al., 2017 [72]	Single blinded randomized controlled trial	Type 2 diabetes (T2D) patients	Metformin 850mgand aspirin 100 mg once daily for 12 months	Combinational use of metformin and aspirin significantly improved glucose tolerance. Moreover, it reduced cardiovascular risk by four-fold in comparison to the metformin group only through reduction of low density lipoprotein and Total cholesterol whilst increasing high density lipoprotein.
Hassan et al., 2016 [76]	In vivo	Streptozotocin induced diabetic rats	Metformin 100 mg/kg and aspirin 100 mg/kg for 10 days	Combinational treatment showed a significant improvement in glucose control but did not reduce cardiovascular disease (CVD) risk.
Ford et al., 2015 [73]	In vivo	Diet-induced obese (DIO) C57BL/6 mice	Metformin 2.5 g/kg and salicylate 1 g/kg for 5 weeks	Cultured mouse hepatocytes in combination of Metformin and salicylate significantly inhibited lipogenesis in a dose dependent manner and activated adenosine-monophosphate-activated protein kinase (AMPK). Moreover, treatment reduced lipids levels and improved insulin sensitivity.
	In vitro	Human hepatocytes	Metformin 0.1 mM and salicylate 0.3 mM for 1 h	Combinational use of the drugs activated AMPK and inhibited lipogenesis, thus reducing CVD risk. Moreover, it improved glucose control.
Mahlangu et al., 2020 [74]	In vivo	DIO C57BL/6 mice	Metformin + ASA (3 mg/kg) for 6 weeks	Combinational treatment ameliorated inflammation by elevating Th2 associated cytokines whilst reducing Th1 associated ones.

3.10. The potential benefits of combining metformin and aspirin to improve T-cell function

At present, it is acknowledged that metformin is a drug of choice for T2D, a condition that is characterised by hyperglycaemia and chronic inflammation. The therapeutic properties by which metformin controls blood glucose levels have been partially described, with its inhibitory effect of hepatic glucose production by activating AMPK and blocking fructose-1-6-bisphosphatase having been some of its prominent mechanisms of action [69]. However, although an exacerbated immune activation has been identified in conditions of T2D [1,70], such information had not been precisely scrutinised to inform on the regulatory effect of this biguanide on T-cell function. Interestingly, beyond blocking hepatic glucose production, emerging evidence summarised in the current study supports the beneficial effects of metformin in improving immune function, in part through effective modulation of T-cell function [15,16,71]. However, due to the rapid rise in metabolic diseases, including T2D and linked cardiovascular complications, there is an increasing need to understand whether combining metformin with other therapies like aspirin could be even more beneficial in alleviating such complications.

Indeed, due to its established anti-inflammatory effects and active use to manage cardiovascular complications [18–20], there is an interest to understand whether combining metformin and aspirin could be more effective in modulating inflammatory conditions like T2D. Thus, in addition to synthesising and informing on the impact of aspirin on T-cell regulation, including associated pathophysiological mechanisms, the current study explored the modulatory effects of combining metformin and aspirin on T-cell function. Table 5 summarizes some preliminary studies that have examined the combination effects of these drugs against metabolic complications. Results from a clinical trial showed that combinational use of metformin and aspirin was more effective in improving glucose tolerance and reducing cardiovascular risk by lowering total cholesterol and low density lipoprotein when compared to the use of metformin as a monotherapy [72]. In addition, the combinational use of these drugs was effective as primary prevention strategy in T2D patients at risk of developing CVDs [72]. Consistently, in rodent models, combination treatment significantly improved glucose control but did not reduce CVD risk in streptozotoc-induced diabetic mice.

Elsewhere, Ford and co-workers reported that combining metformin and aspirin treatment could significantly reduce cardiovascular risk by inhibiting lipogenesis in DIO mice and human hepatocytes, and these results were consistent with activation of AMPK activity [73]. Recently, our group demonstrated that combining metformin and a low dose aspirin could ameliorate elevated inflammation by increasing Th2 associated cytokines whilst reducing Th1 linked cytokines, such as IFN-γ in DIO mice [74]. Such information is of interest since effective regulation of Th1/Th2 cytokine ration could be a vital aspect to manage T2D and its linked abnormalities, as recently reviewed [75]. Nevertheless, although preliminary studies support the beneficial effects of combining metformin and aspirin to mitigate metabolic complications (Table 5), information on how this therapy impacts T-cell function remains relatively unknown. Thus, in addition to establishing the safe use of combining both these agents, further studies are necessary to improve our understanding on the synergistic effects of metformin and aspirin against inflammation and linked complications, as other have reported no effect on cardiovascular risk [76].

3.11. Impact of dose and time on the effects of metformin and aspirin on T-cell mediated function

The impact of metformin on T-cell function, in different experimental settings, was shown to be dose-dependent [37,40]. For instance, ex vivo experimental models, which directly assessed the therapeutic effects of metformin on T-cell function on cultured cells isolated from mice showed that doses between 1 and 10 mM, from 4 h to 3 days, were predominantly used (Table 1). For aspirin, cells isolated from mice or rats, tested doses ranged from 0.5 to 2.5 mM, from 48 h up to 7 days (Table 3); however human derived primary cells were exposed to concentrations reaching a maximum of 10 mM, for up to 5 days (Table 3). Although ex vivo culture provides the benefits of directly assessing the therapeutic effects of metformin or aspirin on T-cell function by eliminating other interfering factors, primary cultured cells have various limitations such as acknowledged difficult in maintaining the phenotypic changes during culture, as well as short term treatment period, since primary cells cannot survive long in culture [77]. However, summarised evidence in Tables 1 and 3 remains crucial in providing necessary information on dose and time section for future studies assessing the impact of these drugs in other experimental settings.

Except for two studies reporting on the use of metformin at 5 mg/d for 3 or 9 weeks [15,41], the majority of findings assessed its therapeutic dose of 10, 50, 100 or 200 mg/kg daily, for various time points (Table 2). However, 200 mg/kg was only used for a shorter time interval (24 h), whilst 50 mg/kg was predominantly employed in most studies [17,38,40], with time points ranging from 16 days to 13 weeks. In any case, most experimental models of metabolic syndrome predominantly use metformin doses between 100 and 250 mg/kg in rodents [78,79], whilst the variation in dose-response observed in the current study could explain the different conditions being explored to assess T-cell function (Table 2). This further highlights the pleiotropic effects of metformin [80], being able to affect T-cell function, dose-dependently in various in vivo models.

Relevant to aspirin, five studies investigated the effects of this compound on T-cell function in different experimental models using mice, whilst one study was on Sprague-Dawley rats (Table 4). In mice, the lowest aspirin dose used was 6 mg/kg per day for 4 weeks [63], whilst the highest was 600 mg/kg per day for 3 weeks [23]. Like metformin, aspirin is shown to positively regulate T-cell function at various doses and interventions period, with these effects obviously impacted by the disease model being used. For example, aspirin dose of 18 mg/kg for 4 days for example was predominantly used to assess T-cell function in asthma-induced C57BL/6 mice [8,10]. Whereas in Sprague-Dawley rats, a dose of 300 mg/kg/day for 10 days post procedure was found to be effective at reducing the frequency of $CD4^+$ T-cells [9].

The current findings were limited by the relatively few studies assessing the direct effect of metformin or aspirin on T-cell function in individuals who administered these drugs orally. However, most studies focused on ex vivo effects, treating isolated T-cells from human subjects with various doses of treatment compounds, as described above. However, by using effective doses with rodent models, formulas for dose extrapolation from animal to human could a viable strategy to further confirm the activity of metformin and aspirin on human subjects, as discussed elsewhere [81].

Discussion

Hyperglycaemia-induced inflammation has been linked with chronic immune activation in individuals with T2D [82]. Moreover, chronic inflammation mediated by T-cell activation has been associated with the development of T2D associated complications such as CVDs [83–85]. As a result, various pharmacological drugs that aim to eliminate these symptoms and to prevent or at the very least slow the development of its complications are being explored. Metformin, a glucose lowering drug, is currently being used as the first-line medication for the treatment of T2D. However, this drug offers very limited cardio-protection albeit the increased risk of cardiovascular complications in these patients [86]. Consequently, anti-inflammatory drugs such as aspirin are

explored for their beneficial effects if used in combination with metformin to offer cardio-protection [72]. Although both metformin and aspirin have been reported to ameliorate inflammation, their impact on T-cell function is not well understood. Therefore, it remains important investigate this phenomenon.

Overall, the current systematic review supports evidence that metformin modulates T-cell function beyond conditions of autoimmune disease, in part via AMPK/STATs signalling. Interestingly, cytokines can activate JAK which in turn phosphorylates and activates various members of STAT family [89].

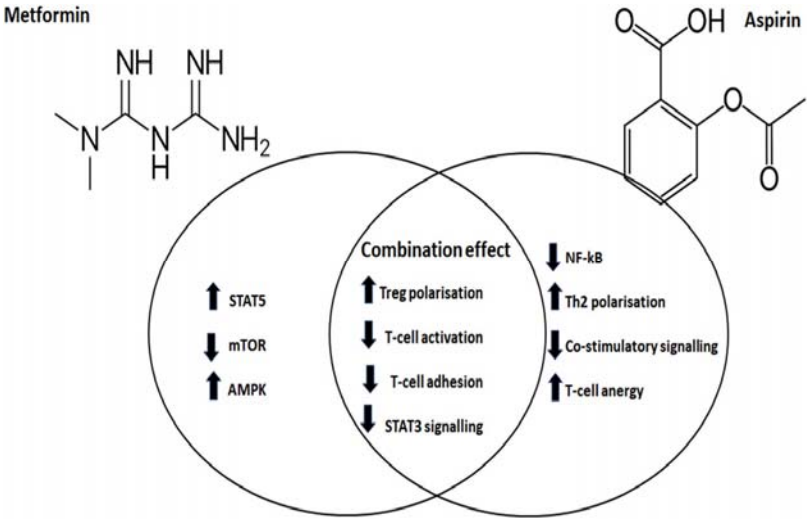

Fig. 2. An overview of some therapeutic mechanisms involved in the regulation of T-cell function in response to aspirin and metformin treatment. Synthesised evidence from included studies showed that both metformin and aspirin can ameliorate T-cell mediated inflammation in part by inducing regulatory T-cells (Treg) polarisation, inhibiting T-cell trafficking and activation as well as signal transducer and activator of transcription (STAT)3 signalling. As a plausible mechanism to mediate T-cell function, metformin showed enhanced potential to regulate mechanistic targets of rapamycin (mTOR), STAT5 and adenosinemonophosphate-activated protein kinase (AMPK) signalling pathways. Whilst aspirin modulated nuclear factor kappa-light chain-enhancer of activated B-cells (NF-kB) and co-stimulatory signalling pathways and induced T-cell anergy.

Briefly, the modulation of circulating number of Th17 and Tregs is important in controlling inflammation and the balance of the ratio thereof is crucial in regulating the immune response [87]. However, in autoimmune diseases or metabolic disorders such as T2D, this ratio is skewed towards the pro-inflammatory subset resulting in exacerbated inflammation [84,88]. Interestingly, evidence presented here demonstrated that metformin can alleviate T-cell mediated inflammation by inhibiting the activation and differentiation of Th17, whilst promoting that of Tregs. For example, metformin was able to inhibit STAT3 activation which is required for the differentiation of Th17 whilst promoting STAT5 signalling, which is required for Treg differentiation [15,38]. Therefore, we propose that the mechanism of action for metformin on T-cell regulation may be in part due to STAT3 competing for the same binding locus on pro-inflammatory IL-17 promoter region with STAT5, as previously described [51]. In fact, overwhelming evidence from included studies support the notion that metformin ameliorates inflammation by modulating T-cell function via STATs signalling. This is supported by some included studies showing different modulatory effects of metformin on T-cell function in different experimental models of inflammation. Whereby, metformin reduced the expression of activation and adhesion markers such as CD25 and CD69, respectively [11,17], which are all essential in T-cell effector function.

Activated STATs then translocate to the nucleus where they act as transcription factors during gene transcription and cell proliferation [90]. Subsequently, T-cell cytokines can modulate the JAK-STAT signalling pathway and polarise the immune response to either a pro- or anti-inflammatory state [91]. Similar to metformin, aspirin can modulate T-cell function by targeting the STAT pathway. For instance, it could inhibit polarisation of Th17 induced by IL-6 via the blockage of STAT3 activity in chronically inflamed mice [10]. However, data presented in this review suggests that the inhibitory effect of aspirin is to a larger extent via signalling blockage of NF-kB [23,24,56,61]. It is well-established that the activation of NF-kB signalling promotes the differentiation of Th1 and Th17, in part by modulating TCR signalling and the release of cytokines such as IL-12 and IL-6 [92,93]. Therefore, synthesised evidence here showed that aspirin has the ability to block NF-kB signalling leading to inhibition of the proliferation of pro-inflammatory T-cell subsets [23,24,56,61], whilst promoting the differentiation of Tregs [56]. However, the exact role of NF-kB signalling in Treg differentiation and function still remains to be further explored. On the other hand, others have suggested that the modulatory effect of aspirin on inflammation could be its ability to induce T-cell anergy via the inhibition of co-stimulatory molecules [54–57]. In addition, like metformin, aspirin inhibited the expression of adhesion molecules essential for their trafficking [58–60].

Overall, these findings suggest that the anti-inflammatory effects of aspirin are mainly in part via the modulation of NF-kB and STAT3 signalling. Of interest, some preliminary findings are already supporting the beneficial effects of
combining metformin and aspirin to ameliorate inflammation and improve metabolic function, as summarised in Table 5. For instance, results from a previous clinical trial support the beneficial effects of combination therapy in reducing CVD-risk in those with T2D [71]. Whereas, recent findings from our group also clearly demonstrated an improved modulation of Th1/Th2 cytokine responses with combinational treatment in DIO mice [73]. These results suggest further exploration of the combined use of metformin and aspirin to improve our understanding of how such therapy mitigates inflammation in conditions of impaired metabolism.

Overall, this study is not without limitations. For instance, included metformin studies reported both positive and negative modulatory effects on T-cell function. This may be attributed to differences in the disease models or experimental dose used by studies summarised in the current review. In addition, some of the included studies only reported an increase [94] or decrease of $CD4^+$ T-cells [23], of which subset analysis would have given a better picture that best describes the exact modulatory effects. Therefore, analysis of T-cell subsets, particularly Th17/Tregs in future studies may help address this shortcoming. Alternatively, the majority of human studies reporting on the therapeutic effect of aspirin were on cultured cells from healthy individuals, of which findings need further confirmation in clinical settings. Therefore, future studies need to include T-cells from individuals with metabolic diseases or animal models. In addition, there was a variation in dose selection for both drugs that would bring the best modulatory effect on T-cell function and this aspect should be addressed in future studies. Lastly, evidence from included human studies looking at both metformin and aspirin were of low quality, mostly because these were not randomized controlled trials. Despite these limitations, our study has a unique strength in that, to the best of our knowledge, it is the first systematic review to comprehensively assess the impact of metformin and aspirin on T-cell function. Importantly, albeit both drugs induced diverse therapeutic effects in modulating T-cell function, our study revealed that their mechanism of action may overlap in inhibiting STAT3 signalling and the expression of activation and adhesion molecules as summarised in Fig. 2. Therefore, these findings pave way for future therapeutic studies to target these pathways bearing in mind the negative effects associated with both these drugs [95,96].

Concluding remarks

Chronic inflammation promotes the development of T2D and its associated complications such as CVD. Results synthesised in this review support the notion that apart from improving glucose metabolism, metformin can also ameliorate T-cell mediated inflammation by altering Th17/Treg ratio and inhibiting mTOR/STAT signalling. Alternatively, it appears the cardio-protective effect of aspirin is not only limited to its ability to inhibit cyclooxygenase activity, but it also modulates T-cell function. In that context, it seems aspirin can modulate T-cell activation and function by downregulating the expression of co-stimulatory molecules and inhibiting NF-kB and STAT3 signalling. Overall, the current evidence supports that the combinational use of metformin and aspirin can be an effective therapeutic strategy to reduce the progression of patients with T2D. However, such a hypothesis needs an in depth exploration in both in vitro experiments and clinical settings of T2D. Moreover, the safe doses and side effects that may arise from using dual-therapy are yet still to be determined.

Abbreviations

AMPK adenosine-monophosphate-activated protein kinase
CVDs cardiovascular diseases
IFN-γ interferon gamma
IL interleukin
JAK Janus kinase
mTOR mechanistic target of rapamycin
NF-kB nuclear factor kappa-light-chain-enhancer of activated B-cells
PRISMA Preferred Reporting Items for Systematic Review and Meta-Analysis
PROSPERO prospective register of a systematic review
Tregs regulatory T-cells
Stat signal transducer and activator of transcription
T2D type 2 diabetes mellitus
Th T helper cells
TNF-α tumour necrosis factor alpha

Author contributions

TMN, PVD and BBN conceptualised, designed and drafted the review. All authors, including SRN, KB, VM and LT wrote and approved the final manuscript.

Declaration of competing interest

Authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2020.117854.

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Prologue

The following chapter (Experimental article 1) aimed at assessing whether T-cell dysfunction in metabolic disorders is mediated by Fas (CD95) and/or Programmed cell death-1 (PD-1). Moreover, to determine if there is any association between metabolic disorders and T-cell dysfunction. Using a diet-induced obesity mouse model of glucose intolerance, we report weight gain following high-fat diet-feeding (HFF) which was associated with poor glucose control, hypercholesterolemia, exacerbated inflammation, and immune activation. Most importantly, HFF induced an upregulation of Fas expression and had no influence on PD-1 levels. Thus, highlighting the fact that metabolic dysregulations in the early stages of obesity may drive the pathogenesis of metabolic T-cell disorders, a process partially mediated by the aberrant expression of Fas. It therefore remains important to explore therapeutic strategies that target Fas-FasL axis in individuals that are obese and at risk of developing metabolic disease-related complications such as cardiovascular disease and type 2 diabetes.

CHAPTER 3: Experimental Article 1

Diet-Induced Obesity Promotes the Upregulation of Fas Expression on T-cells

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Abstract

Objective: To assess whether T-cell dysfunction in metabolic disorders is mediated by Fas and Programmed cell death-1 (PD-1). In addition, we aimed to determine if there is any association between metabolic disorders and T-cell dysfunction.

Design: We used a diet-induced obesity mouse model of glucose intolerance. A total of 12 male C57BL/6 mice were randomised into either a high-fat diet (HFD) or low-fat-diet group for 8 weeks (n=6/group). Changes in body weights were monitored on a weekly basis. While the lipid, glucose, and haematological profiles, as well as FAS (CD95) and PD1 expression on T-cells immunophenotype were measured after 8 weeks of HFD-feeding.

Results: The HFD-fed group had a higher percentage weight gain (29.17%) in comparison to the LFD-fed group (21.74%) after the 8-week period. In addition, the HFD group had increased fasting glucose and glucose excursion following a 2-hour postprandial period. Furthermore, total cholesterol levels were elevated in the HFD group when compared to the LFD group ($p<0.05$). Notably, the absolute white cell count was significantly increased in the HFD-fed group which was concomitant with levels of peripheral neutrophils ($p<0.05$). However, the monocytes and basophils counts were comparable between the two diets groups ($p>0.05$). Interestingly, HFD-feeding was associated with reduced percentage of circulating lymphocytes ($p=0.0116$) and an elevated expression of Fas on T-cells ($p<0.0001$). Moreover, elevated levels of Fas were directly associated with body weights ($r=0.93$, $p=0.0333$). No associations were found between Fas expression and dyslipidaemia or fasting blood glucose levels ($p>0.05$). Lastly, the expression of PD-1 on T-cells was comparable between the two diet groups ($p=0.1822$) and no associations were found with increased Fas expression ($p>0.005$).

Conclusion: Immune activation, dyslipidaemia and poor glucose control in the early stages of obesity may drive the pathogenesis of metabolic T-cell disorders. Importantly, T-cell dysfunction in obesity is partially mediated by an upregulation of Fas which is independent of dyslipidaemia and hyperglycaemia.

Keywords: Diet-induced obesity; Fas; metabolic disorders; programmed cell death-1; T-cell dysfunction

1 Introduction

The prevalence of obesity has rapidly increased over the years [1], with more than two-thirds of individuals with obesity at high risk of developing the metabolic syndrome and cardiovascular disease (CVD) [2, 3]. Obesity is strongly associated with metabolic dysfunction and chronic T cell activation [4, 5]. This is mainly attributed to dysregulated cytokines and adipokines secretions which activate signalling pathways such as the Janus kinases (JAK)/ signal transducer activator of transcription (STAT) that modulate insulin signalling [6, 7]. For instance, exacerbated levels of interleukin (IL)-6 and leptin in obesity result in the downstream activation of STAT3 signalling [8, 9], which is closely associated with insulin resistance [10]. The latter has been attributed to blockage of insulin signalling transduction induced by an upregulation of suppressor of cytokine signalling 3 expression in obesity [11]. In our group, we previously described the involvement of T-cells in obesity-induced immune activation, insulin resistance, and impaired glucose control [7] . In fact, the former is strongly associated with T-cell dysfunction [12] mediated by increased expression of regulatory markers such as Fas (CD95) and programmed cell death -1 (PD-1)[13, 14].

The binding of the Fas ligand (FasL) to its counter-receptor results in downstream activation of caspase 8 and activation-induced cell death [15]. However, alternative research has also reported anti-apoptotic signalling modulated by the Fas/FasL axis [16]. In particular, Fas signalling provides co-stimulatory transductions during T-cell activation [17]. Thus, its aberrant expression may drive alterations in regulatory mechanisms of T-cell responses as previously reported [16]. An upregulation of Fas expression on $CD8^+$ T-cells is directly associated with an increase in body mass index (BMI) in individuals with obesity [13]. Although, others observed a downregulation of Fas expression on $CD4^+$ T-cells in obese individuals with poor glucose control [18]. Despite these reported inconsistences, it is apparent that there is a close relationship between metabolic disorders and aberrant Fas expression on T-cells. However, there is no clear understanding on whether the T-cell dysfunction mediated by Fas in metabolic disorders is driven by poor glucose control, obesity or dyslipidaemia.

Likewise and part from its well characterised negative inhibitory effect, PD-1 is also essential in co-stimulatory signalling that promotes T cell activation upon binding to its ligand (PD-L1 and PD-L2) [19]. In particular, chronic T-cell activation can induce T-cell exhaustion, which is characterised by an upregulation of PD-1 [20]. The activation of PD-1/PD-L axis results in transduction of negative co-stimulatory signal that inhibits T-cell activation [21]. Notably, the upregulation of PD-1 is consistent with loss of T-cell effector function in a mouse model of diet-induced obesity (DIO) [22]. In contrast, the expression of PD-1 on T-cells in individuals with poor glucose control was not associated with any glucose profiles [18, 23], with others even reporting its downregulation in individuals with type 2 diabetes mellitus [24].

Therefore, using a mouse model of DIO, we aimed at assessing whether T-cell dysfunction in metabolic disorders is mediated by increased expression of Fas and PD-1. Moreover, to determine if there are any associations between poor glucose control or dyslipidaemia and the expression of the T-cell regulators.

2. Methods and materials

2.1 Animal handling

Male C57BL/6 mice were purchased and housed in a cage at the Biomedical Research Unit at University of KwaZulu-Natal (UKZN) in a controlled environment. The animals were exposed to a controlled 12-hour light/dark cycle at a temperature range of 23-25 ^{0}C and relative humidity of approximately 50%. The mice received standard laboratory food and water *ad libitum*. All animal procedures were carried out in accordance with UKZN Animal Research Ethics Committee (AREC) protocol (AREC/086/016).

2.2 Study design

In this DIO model, a total of 12 six-week-old male C57BL/6 mice were randomly allocated into two diet groups (n=6/group). These comprised of a low-fat diet (LFD, 10% energy from fat, Research Diets #D12450J) and high-fat diet (HFD, 60% energy from fat, Research Diets #D12492). The animals were allowed to a 2-week acclimatisation period and the body weights were measured on a weekly basis for 8 weeks (Figure 1). Haematological parameters, glucose and lipids profiles were measured after 8 weeks of HFD or LFD feeding.

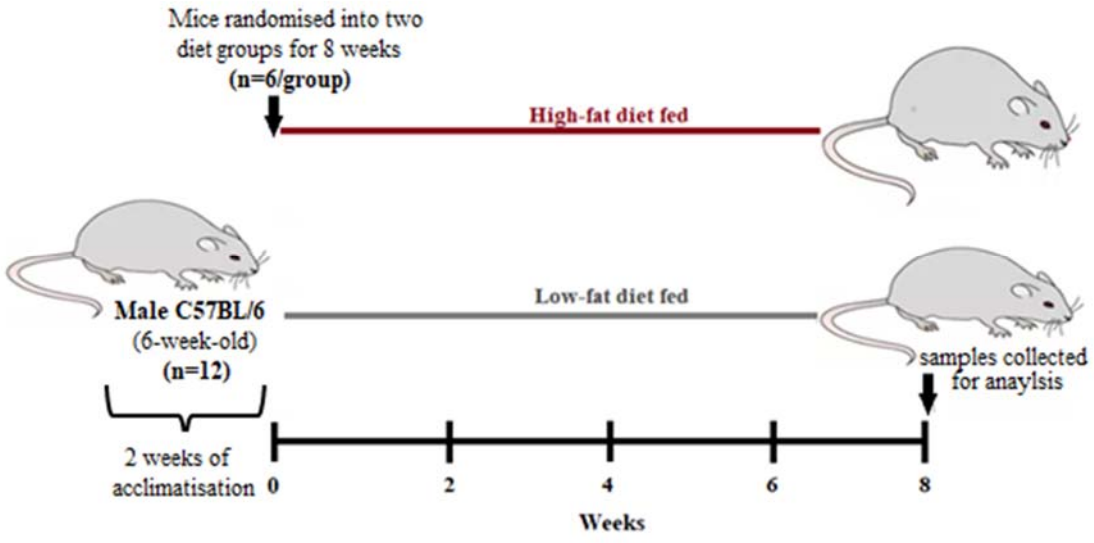

Figure 1: Experimental design. A total of 12 six-week-old male C57BL/6 mice were used in this experiment. Briefly, the mice were randomly allocated into two diets groups comprising of high-fat diet and low-fat diet for 8 weeks (n=6/group). The weights were measured weekly, while the postprandial glucose levels, haematological parameters and blood lipid profiles were measured on the 8th-week post diet-feeding.

2.3 Measurements of metabolic profiles and haematological parameters

Glucose plasma concentrations were performed using the OneTouch select glucometer (LifeScan Inc, CA, USA) and the 2 hour oral postprandial glucose test was performed as previously described [25]. In order to determine the lipid profiles, total cholesterol, high-density lipoprotein (HDL-cholesterol and low-density lipoprotein (LDL)-cholesterol were measured using a mouse specific enzyme-linked immunosorbent assay kit (Abcam, MA, USA), according to the manufacturer's instructions. All haematological parameters were measured using Beckman Coulter AcT5 Diff (Beckman Coulter, Miami, USA).

2.4 Measurements of Fas and PD-1 levels on T-cells

The IMag™ Mouse T Lymphocyte Enrichment Set-DM (BD biosciences, NJ, USA) was used as per manufacturer's instructions to isolate T lymphocytes from whole blood. In order to determine T-cell dysfunction in this DIO model, we enumerated the levels of Fas and PD1 expression on $CD3^+$ T-cells. Briefly, isolated T lymphocytes were stained using the following monoclonal antibodies (mAbs) were used to assess the expression of Fas and PD-1 on T-cells. Anti-mouse CD3-FITC (clone 17A2) and CD95-APC (clone J43) mAbs were obtained from BioLegends, CA, USA; whilst PD-1-BV421 (clone J43) mAb was acquired from Beckton Dickinson (BD biosciences, NJ, USA). Flow cytometry analysis was performed using a BD FASCanto II (BD biosciences, NJ, USA), and data was analysed using the FlowJo version 10.6.2 analysis software (BD biosciences, NJ, USA).

2.5 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6 software (GraphPad Software Inc, CA, USA). The Kolmogorov-Smirnov test with Dallal-Wilkinson-Lilliefor P-value was performed to test for normality. An unpaired student *t-test* was performed for parametric data and reported as mean ± standard error. While, non-parametric data were log-transformed to meet the assumptions of normality prior to statistical analysis. The Man Whitney *U* test was used for non-parametric data, and the results were reported as median interquartile range [IQR]. Correlations were performed using Pearson (for parametric data) or Spearman rank (non-parametric data) coefficients. A p-value of < 0.05 was considered as statistically significant.

3. Results

3.1 High-fat diet feeding impaired metabolic function in mice

In order to induce obesity, the mice were fed a HFD for a period of 8 weeks (Figure 2A). As expected, the HFD-fed group had increased percentage weight gain (29.17%) in comparison to the LFD-fed group (21.74%). Furthermore, the HFD-fed group had significantly elevated levels of fasting blood glucose ($p=0.007$) after the 8-week HFD-feeding period (Figure 2B). Moreover, the HFD-fed group had a larger

postprandial area under the curve (AUC) when compared to the LFD-fed group (p=0.0029) (Figure 2C). In order to assess dyslipidaemia in our DIO model, the lipid profiles were measured, and the total cholesterol levels were significantly increased in the HFD-fed group when compared to the LFD-fed group (p=0.0079) (Figure 2D). However, LDL-cholesterol and HDL-cholesterol were comparable between the two diet groups (P>0.05) (Table 1).

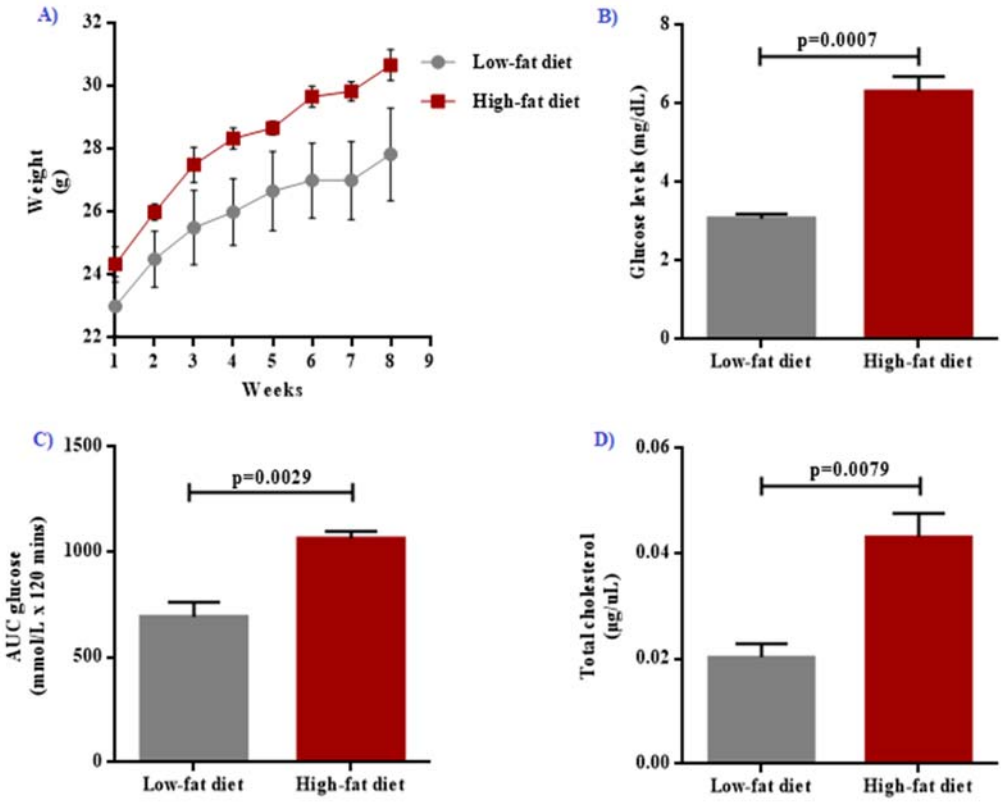

Figure 2: Metabolic changes after 8-weeks of diets feeding (n=6/group). Panel A demonstrates changes in animal body weights over 8 weeks, whilst panel B, C and D illustrates fasting glucose, area under curve (AUC) in 2-hour postprandial glucose test and total cholesterol levels, respectively. Data presented as mean and standard error (SE).

3.2 Haematological changes following high-fat diet feeding

HFD-feeding significantly increased white cell count (p=0.0096) and neutrophils (p=0.0177) in comparison to the LFD-fed group (Table 1). However, the levels circulating lymphocytes were significantly reduced following HFD-feeding when compared to the LFD-fed group (p=0.0116), while all other haematological parameters were comparable following 8-weeks of HFD or LFD feeding (Table 1).

Table 1: Characteristics of mice after a 8-week feed on low-fat diet versus high-fat diet (n=6/group)

Parameter	Low-fat diet (n=6)	High-fat diet (n=6)	p-value
Body weight (g)*	1.38 ± 0.12	1.47 ± 0.01	**<0.0001**
Fasting glucose (mg/dL)	3.08 ± 0.11	6.30 ± 0.39	**0.0007**
Area under the curve (mmol/L x 120 min)	692.70 ± 67.82	1062 ± 35.22	**0.0029**
Lipid profiles	0.020 [0.014-0.023]	0.043 [0.039-0.048]	**0.0079**
Total cholesterol (µg/uL)	0.114 ± 0.048	0.091 ± 0.004	0.6611
HDL-cholesterol (µg/uL)	0.152 ± 0.025	0.093 ± 0.003	0.0803
LDL-cholesterol (µg/uL)			
White cell indices	4.42 ± 0.47	9.26 ± 1.13	**0.0096**
White cell count (10^3/µL)	7.80 ± 0.47	11.04 ± 0.90	**0.0177**
Neutrophils (%)	89.90 ± 0.46	86.06 ± 1.09	**0.0116**
Lymphocytes (%)	1.84 ± 0.09	2.6 ± 0.35	0.0928
Monocytes (%)	0.4 ± 0.11	0.2 ± 0.03	0.1440
Basophils (%)			
Red cell indices	7.03 ± 0.27	6.52 ± 0.44	0.3575
Red cell count (10^6/µL)	27.13 ± 0.94	26.13 ± 1.03	0.4933
Haemoglobin (g/dL)	30.24 ± 1.29	27.44 ± 2.01	0.2809
Haematocrit (%)	43.00 [43.00-43.50]	42.00 [41.00-43.00]	0.1190
Mean cell volume (fL)			
Platelet indices	572.00 ± 124.60	888.60 ± 73.80	0.0680
Platelet count	5.47 ± 0.23	5.42 ± 0.13	0.8553
Mean platelet volume (fL)			
T-cell markers	40.23 ± 3.92	84.88 ± 4.49	**<0.0001**
% expression of Fas on $CD3^+$ T-cells	0.59 ± 0.20	1.23 ± 0.39	0.1822
% expression of PD-1 on $CD3^+$ T-cells			

***:** log transformed data. Results expressed as mean ± standard error and median interquartile range

3.3 Expression of CD95 and PD-1 on T-cells

In order to assess T-cell dysfunction in obesity and poor glucose control, we measured the expression Fas and PD-1 on T-cells following the 8-weeks of HFD-feeding (Table 1). Notably, there was a significant increase in the expression of Fas on T-cells in the HFD-fed group (84.88 ± 4.49) when compared to the LFD-fed group (40.23 ± 3.92), p<0.0001(Figure 3A). However, PD-1 expression was comparable between the two groups (p=0.1822) (Figure 3H).

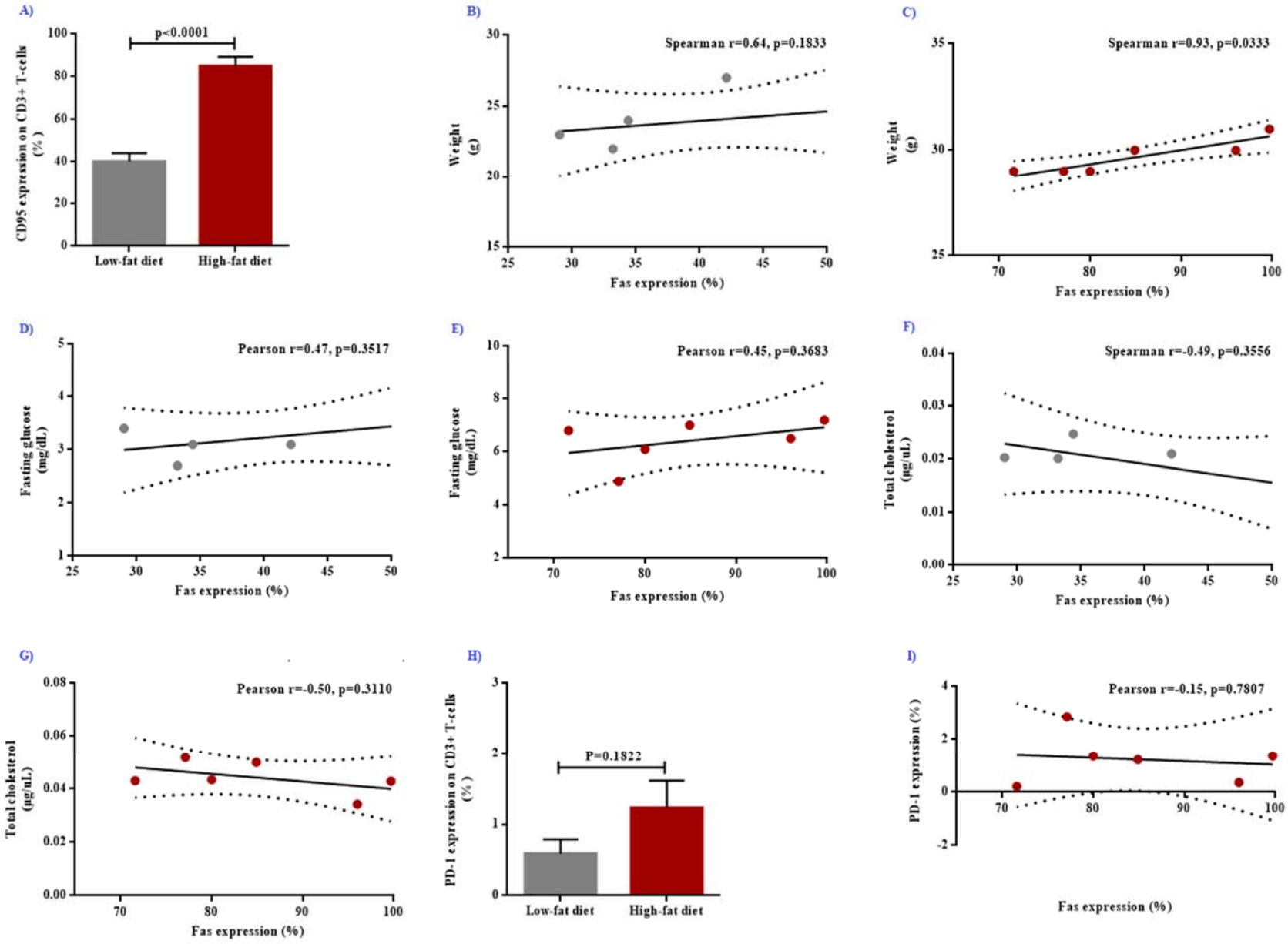
A)
p<0.0001
CD95 expression on CD3+ T-cells (%)
Low-fat diet
High-fat diet
B)
Spearman r=0.64, p=0.1833
Weight (g)
Fas expression (%)
C)
Spearman r=0.93, p=0.0333
Weight (g)
Fas expression (%)
D)
Pearson r=0.47, p=0.3517
Fasting glucose (mg/dL)
Fas expression (%)
E)
Pearson r=0.45, p=0.3683
Fasting glucose (mg/dL)
Fas expression (%)
F)
Spearman r=-0.49, p=0.3556
Total cholesterol (µg/mL)
Fas expression (%)
G)
Pearson r=-0.50, p=0.3110
Total cholesterol (µg/mL)
Fas expression (%)
H)
P=0.1822
PD-1 expression on CD3+ T-cells (%)
Low-fat diet
High-fat diet
I)
Pearson r=-0.15, p=0.7807
PD-1 expression (%)
Fas expression (%)

Figure 3: Fas and programmed cell death 1 (PD-1) expression on $CD3^+$ T-cells after the 8-week of HFD and LFD feeding (n=6/group). The high-fat diet (HFD)-fed group had increased levels of Fas expression when compared to the low-fat diet (LFD)-fed group **(A)**. The levels of Fas on T-cells were independent of weight **(B)**, fasting plasma glucose **(D)** and total cholesterol **(F)** in the LFD group, as well as fasting glucose **(E)** and total cholesterol **(G)** in the HFD group ($p>0.05$). However, Fas expression was directly associated with body weight in the HFD group ($r=0.93$, $p=0.0333$) **(C)** whereas the expression of PD-1 on T-cells was comparable between the LFD-fed and HFD-fed groups ($p>0.05$) **(H)**. Fas and PD-1 expression on T-cells reported as mean ± standard error and the correlations are presented as either Pearson r or Spearman r 95% confidence interval.

3.4 Associations between Fas-mediated T-cell dysfunction and metabolic disorders

Obesity is strongly characterised by poor glucose control and dyslipidaemia [3, 26]. To assess whether there is any association between Fas expression and metabolic disorders, we assessed the relationship between Fas expression on T cells and body weight, fasting blood glucose and total cholesterol levels. We found a positive correlation between Fas expression and body weights (Spearman $r=0.95$, $p<0.0001$), fasting plasma glucose (Pearson $r=0.92$, $p<0.0001$) and total cholesterol (Pearson $r=0.76$, $p=0.0039$) (data not shown). We further performed a sub-group analysis based on the different study diets (HFD and LFD) and in the HFD group, the levels of Fas expression on T cells increased with changes in body weight in the HFD-fed mice only (Spearman $r=0.93$, $p=0.0333$) (Figure 3C). No significant associations were found with fasting plasma glucose (Figure 3D and E) and total cholesterol (Figure 3F and G) in both diets and weight in the LFD-fed group (Figure 3B). Correlation analysis between Fas and PD-1 expression also yielded no significant results in both LFD-fed (Pearson $r=-0.38$, $p=0.4633$) and HFD-fed (Pearson $r=-0.15$, $p=0.7807$) groups (Figure 3I).

4. Discussion

The aim of this study was to assess the expression Fas and PD-1 on circulating T cells in obesity using a DIO mouse model. In this model, 8-week HFD feeding induces long term glucose impairment, dyslipidaemia and weight gain [27, 28]. Interestingly, these changes are analogous with characteristic features of metabolic syndrome in humans [29], whereby poor glucose control and increased total cholesterol have been reported in obese adults [30]. In our study, both lipid and glucose metabolism were altered following HFD-feeding. Notably, when assessing the lipid profiles, only the total cholesterol levels were elevated in the HFD-fed group whereas LDL-cholesterol and HDL-cholesterol remained comparable between the two diet groups. The discordant cholesterol results may be attributed to increased triglyceride levels in obesity which together with aberrant cholesterol levels predisposes obese individuals to CVDs [3].

It is established that leucocytosis is an indicator of immune activation and is closely associated with inflammation. In previous studies, obesity was positively associated with increase in white cell count [31, 32], whereby an increase in BMI was associated with neutrophilia [31]. Likewise, our results showed that HFD-fed mice gained weight and had significantly elevated white cell count, which was

indicative of a pro-inflammatory state in obesity. This may suggest that leucocytosis in obesity is mainly driven by increased proliferation of neutrophils. Obesity-related leucocytosis is associated with dyslipidaemia, which is characterised by increased total cholesterol and LDL-cholesterol and low HDL-cholesterol [33]. Notably, increased white cell count has been directly associated with aberrant cholesterol levels in patients with metabolic syndrome [34]. Overall, our findings seem to suggest that increased immune activation and dyslipidaemia may be responsible for the pathogenesis of metabolic syndromes in individuals with obesity.

Fas is one the increasingly explored proteins for its role in immune activation [16]. Apart from mediating apoptotic cell death, Fas signalling also induces other non-apoptotic activities regulated by members of the tumour necrosis factor receptor superfamily. These include activation and proliferation of leucocytes [35], which is well-described in patients with metabolic disorders and experimental models of obesity and non-alcoholic fatty liver disease [13, 26, 36, 37]. Where in obese individuals, increased expression of Fas on monocytes, neutrophils and T-cells was associated with the activation pro-inflammatory pathways and differentiation of immune cells in conditions of metabolic disease [13, 26, 36, 38]. Interestingly, blockage of Fas signalling can attenuate obesity-induced adipose tissue inflammation by inhibiting IL-6 whilst promoting IL-10 secretion [26]. Subsequently, IL-10 can inhibit Fas expression and its signalling through the activation of FLICE-like inhibitory protein (FLIP) [39]. In our study, we observed increased Fas expression on T-cells and low lymphocyte count in the HFD-fed group. Since the dual effect of Fas-FasL axis is depended on cellular context [15], this may suggest that FAS-mediated pro-apoptotic signalling could explain the reduction in lymphocytes count in the HFD-fed group. Lastly, also as one of the important regulators of immune activation, we report on comparable levels of PD-1 expression on T-cells between the HFD-fed and LFD-fed group. However this is in contrast to previous studies [22, 40, 41], whereby the upregulation of PD-1 was reported in patients with obesity. The difference in the findings may be attributed to the immunological responses attributed to obesity-induced inflammation, since the upregulation of PD-1 in T-cell exhaustion is strongly linked with a chronic inflammatory state [42]. However, to be certain, different experimental models must be explored to assess the expression levels of PD-1 on T-cells in conditions of metabolic syndrome.

Our study had a few limitations. We did not assess the expression of Fas and PD-1 on T-cell subsets which would have provided insight to the expression of these regulatory markers. However, a previous study [13] showed that the expression of Fas on $CD4^+$ T-cells were comparable between obese and lean individuals. We therefore opted to assess the expression of these regulatory markers on the major T-cell lineage. Lastly, we did not determine whether the upregulation of Fas expressions is directly associated with increased activation of Fas signalling. Future studies need to investigate both these aspects to unveil and understand the mechanisms mediated by Fas in T-cell dysfunction.

5. Conclusion

Obesity is characterised by dyslipidaemia, increased immune activation and T-cell dysfunction. Most importantly, altered T cell function is partially mediated by an upregulation of Fas which is independent of dyslipidaemia and hyperglycaemia. Therefore, therapeutic strategies that target Fas-FasL axis may be of benefit for patients with obesity who are also at risk of developing metabolic disease-related complications such as CVD and type 2 diabetes mellitus.

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Authors' contributions

TMN, PVD and BBN conceptualised, designed the study and drafted the manuscript. TMN and BBN performed formal analysis, methodology and validation as well as visualization. All authors wrote, reviewed, edited and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Prologue

The next chapter (Experimental article 2) focuses on the modulatory effects of low-dose aspirin (LDA) and its combination with metformin (LDA+Met) on metabolic profiles, inflammation, immune activation and the expression of T-cell function markers using a diet-induced inflammation mouse model of pre-diabetes. In this manuscript, the model was associated with impaired glucose tolerance, hypercholesterolemia, increased levels of pro-inflammatory cytokines and decreased expression of regulatory marker (CD69) on T-helper cells. Notably, treatment with LDA+Met and not LDA only, exhibited successful modulatory effects in normalising glucose control, ameliorating inflammation and most importantly, improving T-cell functions. Thus, suggesting that the alleviation of inflammation, together with the lowering of glucose levels in type 2 diabetes mellitus may be an effective strategy to improve T-cell function in these patients.

CHAPTER 4: Experimental Article 2

The aberrant expression of CD69 on peripheral T-helper cells in diet-induced inflammation is ameliorated by low-dose aspirin and metformin treatment

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Abstract

Chronic inflammation in patients with type 2 diabetes (T2D) is associated with T-cell dysfunction. We evaluated changes in metabolic profiles, inflammation status and the expression of T-cell function markers following high-fat diet (HFD)-feeding. In addition, we assessed the modulatory effects of treatment with low-dose aspirin (LDA) and its combination with metformin (LDA+Met) on these parameters. Notably, HFD-feeding (HFF) induced metabolic disorders and aggravated inflammation. Most importantly, it was associated with decreased expression of CD69 on T-helper cells but had no effect on the expression of programmed cell death 1 (PD-1). Treatment with LDA monotherapy had no effect on the measured parameters. However, its combination with metformin ameliorated the levels of inflammation and up-regulated the expression of CD69 although it had no therapeutic effect on the levels of PD-1 expression. Therefore, alleviating inflammation and lowering glucose levels in T2D may be an effective strategy to improve T-cell function in these patients.

Keywords: CD69; PD-1; T-cell regulation; inflammation; low-dose aspirin; metformin; type 2 diabetes mellitus.

1. Introduction

Chronic inflammation and an altered cytokine milieu are associated with T-cell dysfunction and the pathogenesis of metabolic disorders [1]. Obesity is a major risk-factor of several metabolic diseases and is characterised by low-grade inflammation in non-communicable disease such as type 2 diabetes (T2D) [2–4]. Approximately 13% of individuals who are obese develop T2D, and over two-thirds of patients with T2D are obese [7]. In addition to T2D, obesity comorbidities include cardiovascular disease (CVD) and immune dysfunction [5,6].

Chronic immune activation induces an aberrant expression of T-cell regulatory markers such as CD69 and programmed cell death 1 (PD-1), which may result in T-cell dysfunction. For instance, individuals with obesity express lower CD69 expression on $CD4^+$ and $CD8^+$ T-cells, which is associated with reduced secretion of T-helper (T_H) cytokines [6]. In contrast, increased levels of CD69 expression on $CD4^+$ and $CD8^+$ T-cells in obesity have been reported [7,8]. Similarly, contradictory findings on the levels of PD-1, a negative regulator of T-cell function has been described in patients with T2D, [12, 13]. Notably, the expression of CD69 on T-cell subsets is directly associated with adiposity [7]. However, evidence on the expression of these markers in pre-diabetes or overt T2D remains elusive. Despite the inconsistencies in the levels of CD69 and PD-1 expression on T-cells, it is evident that the levels of expression of these receptors are altered in obesity and a state of impaired glucose tolerance.

Hyperglycaemia is a hallmark of T2D which remains a crucial therapeutic drug target in the management of patients with T2D. Metformin is the widely used glucose-lowering drug that is well-characterised for the ability to improve glucose metabolism. In addition, the anti-inflammatory properties of metformin have been suggested through indirect mechanisms [9]. We previously described how metformin and aspirin, a well-known anti-inflammatory drug ameliorate T-cell mediated inflammation [10]. Although these modulatory effects were reported as monotherapy, we explored whether combinational use of metformin and low-dose aspirin (LDA) would further ameliorate inflammation and improve T-cell function in metabolic disorders. Therefore, using a diet-induced inflammation model of impaired glucose tolerance, we evaluated changes in metabolic profiles, inflammatory status and T-cell function following a high-fat diet (HFD)-feeding. We further assessed the modulatory effects of short-term treatment with LDA or its combination with metformin (LDA+Met) on the levels of generalised immune activation and the expression of CD69 and programmed cell death-1 (PD-1) on T_H cells.

2. Methods and materials

2.1 Animal handling

Eighteen six-week-old male C57BL/6 mice (n=18) were purchased and kept at the Biomedical Research Unit at the University of KwaZulu-Natal. The animals were housed in a controlled environment whereby they were exposed to a 12-hour light and dark cycle and unlimited water and food supply

throughout the experiment. The study was conducted in accordance with the National Society of Medical Research & the National Institutes of Animal Care and use of Laboratory Animals of the National Academy of Science. The animals were kept separately in cages (n=6/cage) based on their respective diets and treatment groups. The study protocol was approved by the University of KwaZulu-Natal Animal Research Ethics Committee (AREC), protocol number: AREC/086/016.

2.2 Study design

The animals were acclimatised to the environment over 2 weeks. Thereafter, the study experiments were carried out in two stages involving 16-week- and 24-week-old mice, respectively (Figure 1).

Experiment one

In this phase of the study, we aimed to instigate diet-induced inflammation through HFD-feeding (HFF). Briefly, the mice were randomised into either a low-fat diet (LFD) containing 10 Kcal% derived from fat (19 g% Protein, 67 g% carbohydrates, 4 g% fat, Research Diets #D12450J) (n=6) or HFD constituting 60 Kcal% derived from fat (26 g% Protein, 26 g% carbohydrates, 35 g% fat, Research Diets #D12492) (n=12). The animal body weights were monitored on a weekly basis. After eight weeks of diet feeding, venous blood was drawn from the lateral tail vein into serum separator tubes (SST) and ethylenediaminetetraacetic acid EDTA microtainer tubes (BD Bioscience, USA). Baseline levels of glucose and lipid profiles, haematological indices, cytokine levels and T-cell parameters were then measured from the blood samples.

Experiment two

The second phase aimed to assess the modulatory effects of LDA and metformin on the parameters measured at baseline, particularly inflammation indices and T-cell function markers. Following eight weeks of HFD-feeding, the animals were randomised into two treatment groups (n=6/group). These comprised of a LDA (3 mg/kg) and a LDA and metformin (150 mg/kg) (LDA+Met) combinational group. The treatments were administered daily via oral gavage for a period of 6 weeks. The animal body weights were measured weekly, and at the end of treatment period on week 14, blood was drawn, and changes in metabolic profiles, inflammation and expression of T-cell markers were determined.

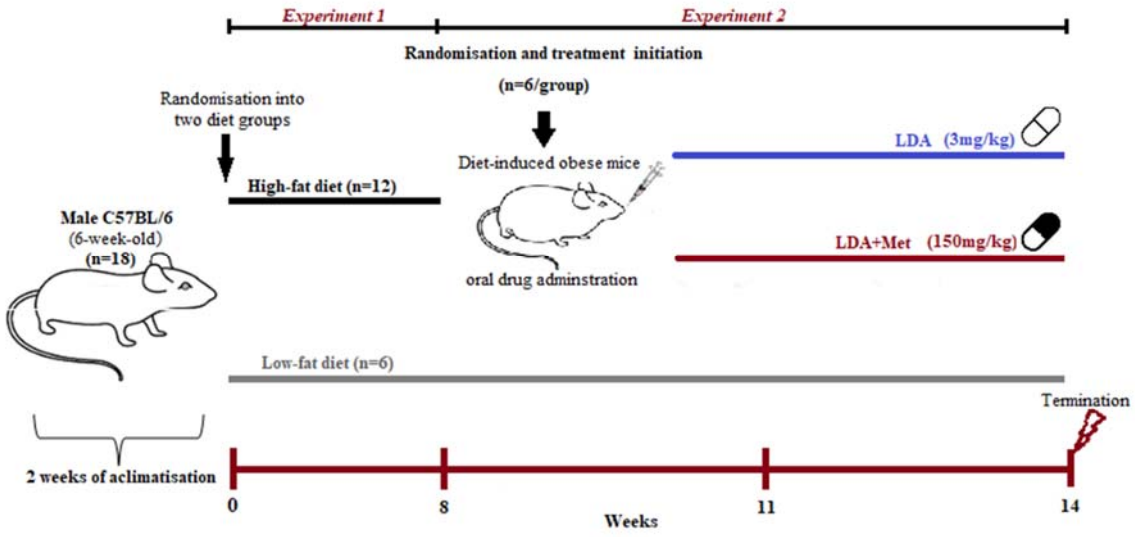

Figure 1: Study design involving two experimental stages. Briefly, in experiment 1, a total of 18 six-week-old male C57BL/6 mice were acclimatised to the environment for 2 weeks and were thereafter randomised into either a high-fat diet (n=12) or low-fat diet (n=6) groups for a total of 14 weeks. Experiment 2 was initiated at week 8, whereby high-fat diet-fed mice were randomised into a short-term treatment with either low-dose (LDA) aspirin or its combination with metformin (LDA+Met) over 6 weeks (n=6/group).

2.3 Measurements of metabolic parameters and haematological indices

The fasting serum insulin levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Thermo Fisher, Massachusetts, USA) following an 8 hour fasting period. Fasting plasma glucose concentrations were determined using the OneTouch select glucometer (LifeScan Inc, CA, USA), and the 2-hour postprandial oral glucose tolerance test was performed as previously described [11]. The lipid profiles were assessed by measuring the levels of total cholesterol, high-density lipoprotein (HDL)-cholesterol and low-density lipoprotein (LDL)-cholesterol using a mouse-specific ELISA kit (Abcam, MA, USA), as per manufacturer's instructions. The haematological parameters were determined using Beckman Coulter AcT5 Diff (Beckman Coulter, Miami, USA) whilst T_H cell associated cytokines (IL-6, TNF-α, IL-2 and IL-17A) were measured using the BioLegend, LEGENDplex™ Mouse Th Cytokine kit (BD Biosciences, San Diego, USA).

2.4 Measurements of CD69 and PD-1 levels on T_H cells

In order not to interfere with T-cell functionality, we negatively selected T lymphocytes from whole blood using the IMag™ Mouse T Lymphocyte Enrichment Set-DM (BD biosciences, NJ, USA), as per manufacturer's instructions. To determine the effect of HFF and the modulatory effects of short-term treatment with LDA and metformin on T-cell function, we measured the levels of CD69 and PD-1 expression on $CD4^+$ T-cells. The following monoclonal antibodies were used to enumerate activated T_H cells; anti-mouse CD4 PE-Cy7 (clone GK1.5), CD69 APC-Cy7 (clone 812S3) and PD-1 BV421 (clone

MHI4), and were purchased from Beckton Dickinson (BD biosciences, NJ, USA). A detailed gating strategy for the measurements of CD69 and PD-1 on $CD4^+$ T-cells is illustrated in Figure 2. The BD FACS CANTO II flow cytometer (Becton Dickson, NJ, USA) was used for data acquisition and FlowJo version 10.6.2 analysis software (BD biosciences, NJ, USA) for data analysis.

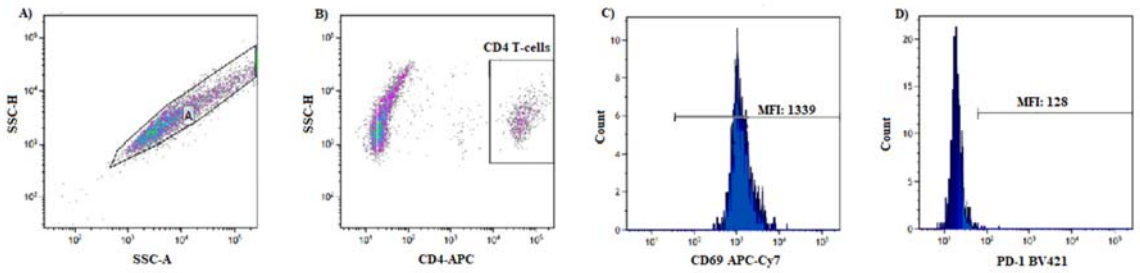

Figure 2: An overview of the gating strategy used in this current study. Panel **(A)** illustrates gating of singlets using side scatter (SSC) area and height, while panel **(B)** shows the identification of T-helper cells using $CD4^+$ monoclonal antibody. Panel **C** and **D** illustrate the measurement of CD69 and programmed cell death-1 (PD-1) expression on T-helper cells, respectively. The gates were set using an unstained sample.

2.5 Statistical analysis

The Kolmogorov-Smirnov test with Dallal-Wilkinson-Lillie was performed for normality testing. For parametric data, the mean differences between the LFD- and HFD-fed groups were assessed using unpaired student *t-test* and were reported as mean ± standard error. The Man Whitney U test was used for non-parametric data and reported as the median and interquartile range [IQR]. Comparisons across the diet and treatments groups were assessed using a Two-way analysis of variance (ANOVA). A posthoc Tukey's multiple comparisons test was performed if the F-value reached statistical significance ($p<0.05$). The Kruskal-Wallis test, followed by a Dunn's posthoc test, was used for non-parametric data. All cytokine data were log-transformed to meet the assumptions of normality prior to statistical analysis and reported as mean ± standard error. A p-value of < 0.05 was considered statistically significant. The GraphPad Prism version 6 software (GraphPad Software Inc, CA, USA) was used for all statistical analysis.

3. Results

3.1 Baseline metabolic profiles after 8-weeks of feeding

The weight gain in the HFD-fed group spiked in the first three weeks (range of 8-14%) whist in the LFD-fed group it was much lower, ranging from 2-7%. Overall, the HFD-fed group had a slightly increased percentage weight gain (15%) when compared to the LFD-fed group (11%) over the 8-week period (Figure 3a). In order to determine changes in glucose control, we measured insulin and fasting plasma glucose levels. The HFD-fed group had significantly elevated fasting glucose levels ($p<0.0001$) (Table 1) and an increased 2-hour postprandial area under the curve (AUC) ($p<0.0001$) (Figure 3b). Moreover, the levels of insulin in the HFD-fed group were markedly elevated ($p=0.0182$) in comparison

to the LFD-fed group (Figure 3c). To evaluate a state of dyslipidaemia following 8-weeks of diet feeding, we measured the lipid profiles. Total cholesterol (TC) levels were significantly elevated in the HFD-fed group (p=0.022) (Figure 3d), while the HDL-c levels were comparable between the two groups (p=0.4332) (Figure 3e). Interestingly, the LDL-c were significantly lower in the HFD-fed group when compared to the LFD-fed group (p=0.0311) (Figure 3f).

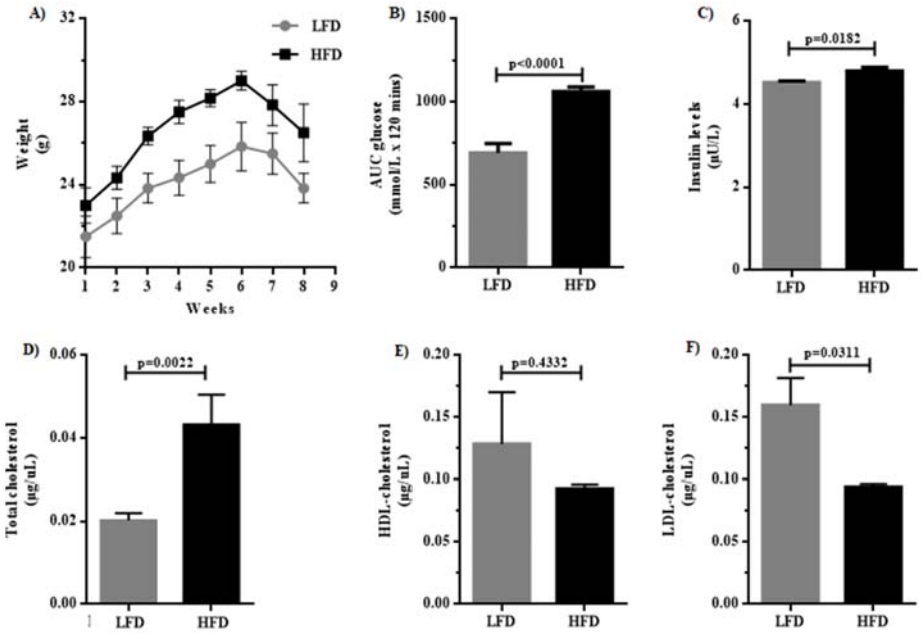

Figure 3: Changes in metabolic profiles following 8-week diet feeding. Figure (**A)** illustrates body weight changes over the 8 weeks of being on a high-fat diet (HFD), or low-fat diet (LFD) whilst panel **(B)** and **(C)** shows comparisons of the area under curve (AUC) in a 2-hour oral glucose test and insulin levels, respectively. Figure **(D)**, **(E)** and **(F)** shows differences in total cholesterol, high-density lipoprotein (HDL)-cholesterol and low-density lipoprotein (LDL)-cholesterol, respectively. All results are presented as mean ± standard error except for total cholesterol levels which are reported as median, interquartile range.

3.2 Elevated levels of inflammation following 8 weeks of HFD-feeding

In order to determine the levels of generalised immune activation, we measured complete blood counts and inflammatory cytokines. The results demonstrated that HFF was associated with an increased total white cell count (WCC) (p=0.008) but had no effect on lymphocyte, red blood cell (RBC) and platelet counts (Table 1). In addition, HFF induced an elevation of IL-6 (p<0.0001), TNF-α (p=0.0312) and IL-17A (p<0.0001) but had no effect on IL-2 (p=0.8124). Moreover, HFD-feeding downregulated the expression of CD69 (p=0.0009) on $CD4^+$ T-cells without altering the levels of PD-1 (p=0.6408) (Table 1).

Table 1: Baseline characteristics of mice fed a low-fat diet (LFD) versus high-fat diet (HFD) after 8 weeks

Parameter	LFD (n=6)	HFD (n=6)	p-value
Bodyweight (g)	23.83 ± 0.70	26.50 ± 1.38	0.1272
Insulin (µU/L)	4.52 ± 0.04	4.80 ± 0.09	**0.0182**
Fasting blood glucose (mg/dL)	3.23 ± 0.18	6.42 ± 0.34	**< 0.0001**
Area under the curve (mmol/L x 120 min)	692. ± 55.38	1062 ± 28.75	**< 0.0001**
Lipid profiles (µg/uL)			
Total cholesterol	0.02 [0.02-0.02]	0.09 [0.09-0.10]	**0.0022**
HDL-cholesterol	0.128 ± 0.042	0.093 ± 0.004	0.4332
LDL-cholesterol	0.160 ± 0.022	0.094 ± 0.003	**0.0311**
Haematological parameters			
White cell count (10^3/µL)	4.88 ± 0.39	8.31 ± 0.56	**0.0008**
Lymphocytes (%)	90.53 ± 0.82	89.55 ± 0.60	0.3567
Red cell count (10^6/µL)	7.15 ± 0.25	6.64 ± 0.38	0.2904
Platelet count (10^3/µL)	783.5 [626.3-864.0]	709.5 [515.0-735.3]	0.1797
Inflammatory cytokines **(pg/mL)**			
Interleukin-6	1.25 ± 0.02	2.01 ± 0.18	**< 0.0001**
Tumour necrosis factor-α	1.54 ± 0.19	2.09 ± 0.02	**0.0312**
Interleukin-2	1.880 ± 0.03	1.90 ± 0.06	0.8124
Interleukin-17A	0.94 ± 0.03	2.28 ± 0.15	**< 0.0001**
Expression of activation markers on CD4+ T-cells (MF)I			
CD69	1270 ± 50.60	1019 ± 18.39	**0.0009**
PD1	119.90 ± 3.96	123.80 ± 7.04	0.6408

MFI: Mean fluorescence intensity. Results expressed as mean ± standard error and median interquartile range

3.3 Changes in metabolic profiles post 6-week treatment with LDA or its combination with metformin

To measure the effect of short-term LDA and LDA+Met treatment on metabolic profiles, we assessed changes in body weights, insulin, glucose levels and cholesterol levels. The Kruskal-Wallis test showed that there were significant changes in the body weights ($K_{(3)} = 14.79$, p=0.0020) and insulin levels ($K_{(3)} = 9.43$, p=0.0241) across the experimental groups (Table 2). The Dunn's posthoc analysis showed an increase in body weight of the HFD-fed group [28.50 (24.75-30.25)] in comparison to the LFD-fed group [24.00 (22.75-24.25)], (p=0.0292). However, there were no significant reduction in body weights in the LDA- (p>0.9999) or LDA+Met-treated (p>0.9999) groups when compared to the untreated HFD-fed group. Although HFF did not alter the insulin levels, the LDA+Met-treated group [4.53 (4.50-4.57)] had significantly lower levels in comparison to the LDA-treated group [4.76 (4.61-5.26)], (p=0.0475) (Table 2). The two-way ANOVA showed that fasting blood glucose levels differed significantly across the experimental groups ($F_{(3, 15)} = 31.13$, p<0.0001). Tukey's posthoc analysis showed that treatment with LDA (3.48 ± 0.35) or LDA+Met (3.22 ± 0.32) significantly lowered the levels of fasting blood glucose when compared to the untreated HFD-fed group (6.42 ± 0.34), (p<0.0001) (Table 2).

Assessment of lipid profiles revealed comparable levels of HDL-c ($F_{(3, 15)} = 0.2623$, p=0.8515) and LDL-c ($F_{(3, 15)} = 3.004$, p=0.0636) across all experimental groups. Notably, the levels of TC were different across the experimental groups ($K_{(3)} = 9.13$, p=0.0276) and post-hoc analysis showed elevated levels in the HFD-fed group [0.09 (0.09-0.10)] when compared to the LFD group [0.02 (0.02-0.02)],

(p=0.0479) (Table 2). However, in comparison to the HFD-fed group, treatment with LDA [0.020 (0.01-0.07)], (p=0.0607) or LDA+Met [0.02 (0.02-0.10)], (p=0.6148) did not alter the TC levels.

3.4 LDA and metformin ameliorate immune activation and T-cell mediated inflammation

Analysis of haematological parameters showed significant changes in total WCC ($F_{(3, 15)} = 19.71$, $p<0.0001$) and lymphocyte counts ($F_{(3, 15)} = 9.063$, $p=0.0011$) across the experimental groups (Table 2). A posthoc analysis showed that HFF markedly increased total WCC ($p<0.0001$) in comparison to the LFD-fed group. Notably, treatment with LDA+Met significantly lowered the WCC when compared to the untreated HFD-fed group (p=0.0095). However, LDA treatment did not affect the total WCC (p=0.0771) (Table 2). Importantly, although HFF did not alter lymphocyte counts, treatment with LDA+Met reduced the lymphocyte count when compared to the untreated HFD-fed group (p=0.0264). Lymphocytes count remained comparable after treatment with LDA only. Platelets ($F_{(3, 15)} = 1.226$, $p=0.3349$) and RBC counts ($K_{(3)} = 6.034$, $p=0.1100$) remained comparable across all experimental groups (Table 2).

Analysis of all cytokine profiles showed that there were significant differences in the levels of IL-6 ($F_{(3, 15)} = 15.19$, $p<0.0001$), TNF-α ($F_{(3, 15)} = 4.641$, $p=0.0173$), IL-2 ($F_{(3, 15)} = 19.72$, $p<0.0001$) and IL-17A ($F_{(3, 15)} = 28.41$, $p<0.0001$) across all experimental groups. A post-hoc analysis revealed that treatment with LDA+Met significantly reduced IL-6 (p=0.0002), TNF-α (p=0.0465), IL-2 (p=0.0001) and IL-17A ($p<0.0001$) in comparison to the untreated HFD-fed group (Table 2, Figure 4a-d). However, LDA treatment did not significantly lower the cytokine levels except IL-17A (0.0259) (Figure 4d).

Table 2: Modulatory effects of low-dose aspirin and metformin treatment on metabolic profiles, inflammation and T-cell function

Parameter	LFD (n=6)	HFD (n=6)	LDA (n=6)	LDA+Met (n=6)	p-value
Body weight (g)	24.00 [22.75-24.25]	28.50 [24.75-30.25]	27.00 [26.75-27.00]	29.00 [28.50-29.75]	**0.0020**
Insulin (µU/L)	4.66 [4.52-4.76]	4.83 [4.57-8.10]	4.76 [4.61-5.26]	4.53 [4.50-4.57]	**0.0241**
Fasting blood glucose (mg/dL)	3.23 ± 0.18	6.42 ± 0.34	3.48 ± 0.35	3.22 ± 0.32	**<0.0001**
Lipid profiles (µg/uL)					
Total cholesterol	0.02 [0.02-0.02]	0.09 [0.09-0.10]	0.02 [0.01-0.07]	0.02 [0.02-0.10]	**0.0276**
HDL-cholesterol	0.128 ± 0.042	0.093 ± 0.004	0.112 ± 0.024	0.120 ± 0.044	0.8515
LDL-cholesterol	0.160 ± 0.022	0.094 ± 0.003	0.157 ± 0.031	0.084 ±0.017	0.0636
Haematological parameters					
White cell count (10^3/µL)	4.88 ± 0.39	10.14 ± 0.76	8.30 ± 0.23	7.54 ± 0.61	**<0.0001**
Lymphocytes (%)	90.53 ± 0.82	87.42 ± 1.22	86.98 ± 0.22	82.26 ± 1.89	**0.0011**
Red cell count (10^6/µL)	7.12 [6.80-7.77]	6.66 [5.35-7.16]	7.52 [7.09-7.91]	7.55 [7.32-7.74]	0.1100
Platelet count	764.30 ± 48.95	805.6 ± 85.81	942.00 ± 36.37	829.60 ± 75.31	0.3349
Inflammatory cytokines					
Interleukin-6 (pg/mL)	1.25 ± 0.02	2.00 ± 0.07	1.40 ± 0.17	1.28 ± 0.04	**<0.0001**
TNF-α (pg/mL)	1.43 ± 0.16	2.11 ± 0.06	1.80 ± 0.18	1.53 ± 0.21	**0.0173**
Interleukin-2 (pg/mL)	1.12 ± 0.02	2.09 ± 0.09	1.95 ± 0.20	1.17 ± 0.09	**<0.0001**
Interleukin-17A (pg/mL)	0.94 ± 0.03	2.16 ± 0.03	1.66 ± 0.21	0.95 ± 0.04	**<0.0001**
Expression of activation markers on $CD4^+$ T-cells (MFI)					
CD69	1230 [1171-1311]	1134 [1110-1186]	1308 [1214-1365]	1399 [1355-1435]	**0.0016**
PD1	127.7 [110.5-135.3]	116.8 [104.9-131.6]	110.0 [68.75-111.6]	109.5 [104.9-122.0]	0.1199

Results expressed as mean, ± standard error or median, interquartile range. **LFD:** Low-fat diet, **HFD:** high-fat diet, LDA: Low-dose aspirin, LDA+Met: combination of low-dose aspirin and metformin, **AUC:** Area under the curve, **TNF- α:** Tumour necrosis factor- α. **MFI:** Mean fluorescence intensity

3.5 Combined LDA and metformin treatment is associated with increased expression of CD69 on T_H cells

Metabolic disorders are associated with chronic immune activation mediated by dysregulated T-cell function. To evaluate T-cell function in diet-induced inflammation model, we measured the expression of CD69 and PD-1 on $CD4^+$ T-cells and the impact of short-term treatment with LDA or LDA+Met on their expression. Notably, there were significant changes in the levels of CD69 expression following treatment ($K_{(3)} = 15.29$, p=0.0016) (Table 2). The posthoc analysis showed elevated CD69 expression on T_H cells in the LDA+Met-treated group [1399 (1355-1435)] but not LDA [1308 (1214-1365)] (p=0.1487) when compared to the untreated HFD-fed group [1134 (1110-1186)], (p=0.0010) (Table 2, Figure 4e). There were no differences in the expression of PD-1 on T_H cells across all experimental groups ($K_{(3)} = 5.836$, p=0.1199) (Table 2, Figure 4f).

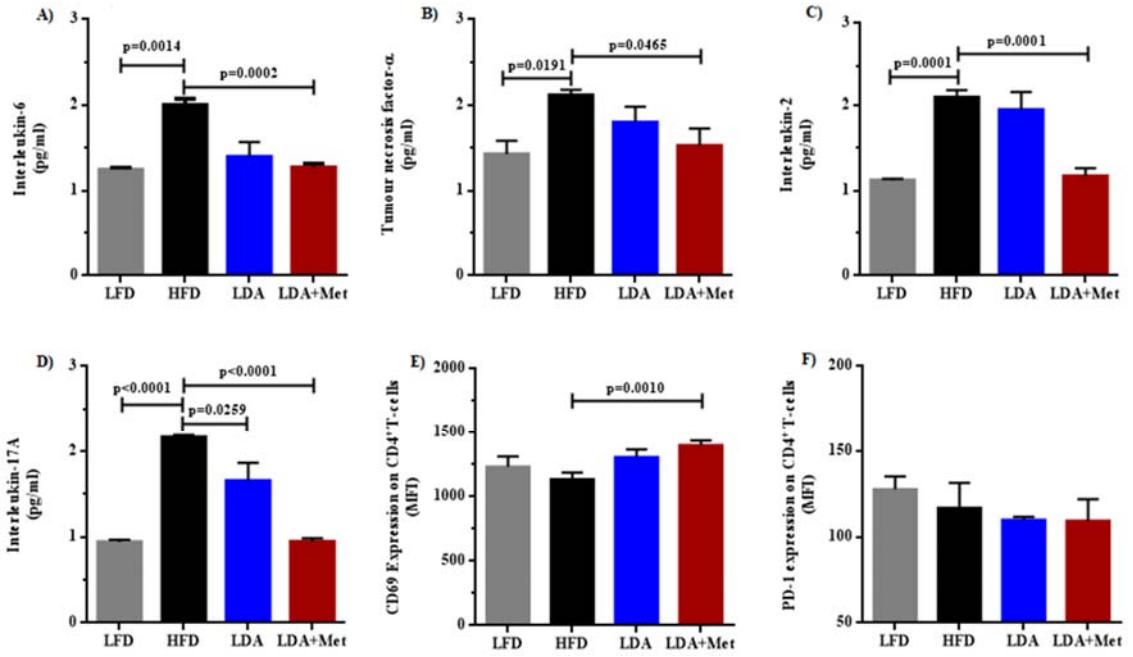

Figure 4: Modulatory effect of low-dose aspirin (LDA) or its combination with metformin on inflammation and T-cell function. High-fat diet (HFD)-feeding induced an upregulation of interleukin (IL)-6, tumour necrosis factor (TNF)-α, IL-2 and IL-17A. Interestingly, short-term combinational treatment with low-dose aspirin and metformin (LDA+Met) reduced all the pro-inflammatory cytokine levels **(A-D)**. Expression of T-cell regulatory markers revealed that HFD-feeding downregulated the expression of CD69 on $CD4^+$ T-cells which were upregulated post-treatment with LDA+Met but not LDA **(E)**. The expression of programmed cell death 1 (PD-1) however, remained comparable across all diets and treatment groups **(F)**. All results are presented as mean ± standard error except for the expression of CD69 and PD-1, which are reported as median, interquartile range.

4. Discussion

In this study, we used a HFD-induced inflammation model to evaluate metabolic abnormalities associated with inflammation and T-cell dysfunction. In addition, we assessed the modulatory effects of short-term treatment with LDA or its combination with metformin on inflammation and the expression of CD69 and PD-1 on T_H cells. Notably, our results demonstrated that HFF induced a state of glucose intolerance, hyperinsulinaemia, hypocholesteraemia and inflammation. These pathological changes are associated with the early phase of metabolic syndrome, which is characterised by insulin resistance, glucose intolerance and inflammation that is independent of body weights gain [12]. Interestingly, HFF was associated with decreased CD69 expression on T_H cells but had no effect on the expression of PD-1 in metabolic disorder.

Insulin resistance and obesity are closely associated with hypercholesterolemia [13,14]. In fact, hyperinsulinaemia in obesity and T2D promotes the synthesis of very-low LDL-c and inhibits its removal by blocking lipoprotein lipase activity [15]. In turn, lipid-induced insulin resistance in skeletal muscle impairs insulin receptor signalling and glucose transporter type 4 (GLUT4) translocation [16].

In this study, we report on increased TC levels with no changes in HDL-c and LDL-c levels following HFF, and no therapeutic benefit of both treatment regimens in normalising cholesterol levels. Therefore, we speculate that the hypercholesterolemia observed may be driven by hypertriglyceridemia. Consequently, therapies that target to reduce triglyceride levels may be of great benefit in lowering cardiovascular risk in patients with T2D as previously described [17]. Notably, both drugs improved glucose metabolism and the efficacy of LDA treatment which may be attributed to its ability to alleviate inflammation and thereby improving insulin sensitivity in T2D [18].

Obesity-related inflammation is characterised by an elevation in leucocyte counts, indicative of generalised immune activation [19]. Therefore, reduction in WCC and lymphocytes following treatment with LDA+Met highlights the amelioration of generalised immune activation. Obesity-related inflammation is associated with the polarisation of TH cells towards the pro-inflammatory T_H1 and T_H17 subsets [20]. In this study, we report an elevation of all T_H1 and T_H17 associated cytokines that we measured (IL-2, IL-6, IL-17 and TNF-α) following HFF. The binding of IL-2 to its receptor (CD25) modulates T_H cell differentiation and function via the Janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathways [21]. In that context, the elevation of IL-2 following HFF may promote the differentiation of T_H cells into pro-inflammatory effector cell subsets which may result in an increase of IL-6, IL-17 and TNF-α cytokines. Interestingly, treatment with LDA+Met normalised IL-2 levels which may have induced the differentiation of regulatory T-cells (T_{regs}) through the activation of JAK/STAT5 signalling [21,22]. T_{regs} via IL-10 inhibit T_H1 and T_H17 function, hence the decrease in the associated cytokines.

The activation of the T-cell receptor induces an immediate upregulation of CD69 on T-cells which gradually decline over days [23]. This highlights its essential role as an immuno-regulator receptor that modulate inflammatory responses mediated by T_H cells [24]. In our study, the downregulation of CD69 following HFD-feeding was concomitant with increases in $T_H1$and T_H17 associated cytokines. Whereby, CD69 blocks sphingosine 1 phosphate receptor-1 (S1P1) signalling, which favours the differentiation of T_H1 and T_H17. Briefly, the binding of S1P1 to its receptor activates the mammalian target of rapamycin (mTOR) complex and JAK2/STAT3 signalling pathway which promotes polarisation of pro-inflammatory T_H1 and T_H17 subsets whilst inhibiting T_{regs} [25]. Notably, the expression of CD69 on activated T-cells induces degradation of the S1P1 receptor in addition to blocking its signalling via the JAK3/STAT5 which favours T_{regs} polarisation [25,26]. Interestingly, treatment with LDA+Met was associated with an increased expression of CD69, and this was congruent with reductions in T_H1 and T_H17 cytokines. This change may be attributed to increased JAK3/STAT5 signalling, which polarises T_H cells towards T_{regs}. Although LDA is a well-acknowledged anti-inflammatory drug, it did not affect the expression of CD69 and T_H cytokine release, and its efficacy was only enhanced when combined with metformin. This may be due to the inhibitory effect of

metformin mTOR and STAT3 signalling [27,28]. Although PD-1 is a well characterised negative regulator of T-cells, in our study, it remained unchanged in both experiments, and this may be due to the immunological responses in the early to intermediate phases of obesity-induced inflammation since the upregulation of PD-1 in T-cell exhaustion is strongly linked with a prolonged pro-inflammatory state [29]. Collectively, these findings suggest that T-cells dysfunction in metabolic disorders may be mediated by aberrant expression of CD69.

Our study had a few limitations as we did not assess whether the upregulation of CD69 following treatment directly enhanced its signalling pathway. Future studies are required to address this aspect. Lastly, some of the reported T_H cell cytokines were measured in serum and could have been influenced by other immune cells other than T-cells which also excrete these cytokines.

5. Conclusion

HFD-induced inflammation is associated with impaired glucose tolerance, hypercholesterolemia and T-cell dysfunction. Notably, only combined treatment with LDA and metformin normalised glucose control, ameliorated inflammation and improved T-cell function. Nevertheless, both treatment regimens did not normalise the levels of cholesterol. Therefore, alleviating inflammation and lowering glucose levels in T2D may be an effective strategy to improve T-cell function in these patients.

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Authors' contributions

TMN, PVD and BBN conceptualised, designed the study and drafted the manuscript. TMN and BBN performed formal analysis, methodology and validation as well as visualisation. All authors wrote, reviewed, edited and approved the final manuscript.

Declaration of Interest

None.

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Prologue

The subsequent chapter (Experimental article 3) aimed at assessing the modulatory effect of metformin and fluvastatin, a statin on glucose, haemtological and lipid profiles in pre-diabetic state. Here, we report the development of a pre-diabetic state flowing high-fat diet feeding. Notably, although treatment with metformin or fluvastatin did not exhibit any immune-modulatory effects, they both lowered the cholesterol levels and ratios. However, the reduction magnitude was larger in the fluvastatin group when compared to the metformin group. Thus, these findings highlight the enhanced cardio-protective effects of statins through the inhibition of atherosclerosis. Therefore, its usage in patients with T2D, particularly those with dyslipidaemia maybe of great benefit in lowering the risk of developing diabetes-associated CVD.

CHAPTER 5. Experimental Article 3

The Modulatory Effects of Short-term Treatment with Metformin and Fluvastatin on Glucose and Lipid Metabolism

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

Status: Submitted to Innate Immunity Journal

Abstract

Objective: To assess the impact of metformin and fluvastatin treatment on glucose, haematological and lipid profiles in a pre-diabetic state.

Design: A total of 21 male six-weeks old C57BL/6 mice were used in this diet-induced mouse model of pre-diabetes. In the first phase of the experiment, all mice were high-fat diet (HFD)-fed over an 8-week period to induce a pre-diabetic state. Thereafter, the animals were randomised into a short-term treatment with either metformin or fluvastatin over a 6-week period (n=7/group) in the second phase of the experiment. Changes in body weights were monitored on a weekly basis, whilst glucose, haematological and lipid profiles were measured following treatment.

Results: HFD-feeding (HFF) was associated with the development of a pre-diabetic state. Treatment with metformin or fluvastatin was associated with a lower body weights and area under curve (AUC) in a 2-hour oral glucose test when compared to the untreated HFD-fed group ($P<0.05$). Notably, both treatments had no influence on the measured haematological parameters when compared to the untreated HFD-fed group ($p>0.05$). However, the levels of total cholesterol (TC) ($K_{(2)} = 11.66$, $p=0.0004$), low-density lipoprotein cholesterol (LDL-c) ($F_{(2, 10)} = 42.20$, $p<0.0001$) and high-density lipoprotein cholesterol (HDl-c) ($F_{(2, 10)} = 4.451$, $p=0.0415$) were different across all groups. The Dunn's posthoc analysis showed that treatment with metformin [0.030 (0.017-0.032)], $p=0.0093$ or fluvastatin [0.030 (0.028-0.031)], $p=0.0093$ significantly lowered TC when compared to the untreated HFD group [0.120 (0.107-0.139)]. The Tukey's posthoc analysis showed a reduction in the levels of LDL-c following treatment with metformin (0.049 ± 0.003), $p<0.0001$ or fluvastatin (0.055 ± 0.009), $p<0.0001$ treatment. In addition, short-term treatment with metformin (0.043 ± 0.005) but fluvastatin (0.057 ± 0.004) lowered HDL-c when compared to the untreated HFD-fed group (0.059 ± 0.003), $p=0.0415$. The calculated cholesterol ratios differed significantly across the groups ($F_{(2, 10)} = 25.16$, $p=0.0001$). Whereby, treatment with either metformin (0.652 ± 0.114), $p=0.0004$ or fluvastatin (0.525 ± 0.040), $p=0.002$ was associated with lower cholesterol ratios when compared to the untreated HFD-group (2.039 ± 0.232).

Conclusion: Fluvastatin significantly lowers cardiovascular risk by lowering cholesterol levels in a pre-diabetic state although it does not render any immune-modulatory effects. Therefore, its combinational use with metformin in patients with T2D, particularly those with dyslipidaemia maybe of great benefit in lowering the risk of developing diabetes-associated CVD.

Keywords: Cardiovascular risk; cholesterol; pre-diabetes; metformin; fluvastatin

1. Introduction

Cardiovascular disease (CVD) are a common co-morbidity in patients living with type 2 diabetes (T2D) [1,2]. Diabetes-associated CVD are mediated by obesity, chronic inflammation, insulin resistance and altered lipid metabolism [3,4]. Most importantly, the latter process is strongly associated with increased risk of developing accelerated atherogenesis in diabetes due to dyslipidaemia [2,5,6]. Consequently, drugs such fluvastatin, a statin that lowers cholesterol levels are being used in patients at risk of developing CVD [7]. Although, statins have an outstanding efficacy in lowering the levels of low-density lipoprotein cholesterol (LDL-c) in general [7,8], they seem to be less effective the primary/secondary prevention of CVD since their use is closely associated with increased risk of developing diabetes [9,10]. Like metformin, the first choice glucose lowering drug used in T2D, fluvastatin is also associated with immune suppression [11–14].

In addition to modulating glucose and inflammation, metformin also influences lipid metabolism, particularly the cholesterol pathways [15]. However, the exact pharmacological mechanisms of action are still controversial. Despite its effective anti-hyperglycaemic and anti-inflammatory properties, patients with T2D on metformin are still at high risk of developing CVD [16]. Consequently, its combinational use with statins that offer substantial cardio-protective benefits through the amelioration of inflammation and lowering of cholesterol levels are being explored [17,18]. Hence, it is encouraged to initiate statin therapy in patients with T2D without any overt CVD [19]. However, data on the efficacy of fluvastatin in poor glucose control is very scarce. Although both metformin and fluvastatin modulate glucose and lipid metabolism, it remains important to understand the mono-therapeutic impacts of these drugs on metabolic and immunological profiles in order to effectively design a combinational treatment regimen with high efficacy.

2. Methods and materials

2.1 Animal handling

A total of twenty-eight six-week-old male C57BL/6 mice (n=21) were purchased and housed in a controlled environment at the Biomedical Research Unit at the University of KwaZulu-Natal. The mice were exposed to a 12-hour light/dark cycle and received food and water *ad libitum* throughout the experiments. All experimental procedures were conducted in accordance with the National Society of Medical Research & the National Institutes of Animal Care and use of Laboratory Animals of the National Academy of Science and received ethical clearance from the University of KwaZulu-Natal Animal Research Ethics Committee (AREC) [protocol number: AREC/081/018D].

2.2 Study design

The animals were subjected to a 2-week acclimatation period and were kept in separate cages (n =7/group). The study experiments were carried out in two phases (Figure 1).

Experiment phase 1

The first phase of the study aimed at inducing obesity through high-fat diet (HFD)-feeding using a 60 Kcal% derived from fat (26 g% Protein, 26 g% carbohydrates, 35 g% fat, Research Diets #D12492) (n=28). The animal body weights were measured and monitored on a weekly basis. Following 8-weeks of being on HFD, venous blood was drawn from the lateral tail vein into serum separator tubes (SST) and ethylenediaminetetraacetic acid EDTA microtainer tubes (BD Bioscience, USA). The blood samples were then used to measure glucose and haematological indices as well as lipid profiles.

Experiment phase 2

The second phase of the experiment aimed at assessing the modulatory effects of metformin and statin on glucose, haematological and lipid profiles measured at baseline (phase 1). After 8-weeks of HFD-feeding (HFF), the mice were randomised into two treatment groups comprising of metformin (150 mg/kg) and statin (20 mg/kg) (n=7/group). The drugs were administered daily via oral gavage for a period of 6-weeks. Following the completion of short-term treatment, blood was drawn through the lateral tail vein.

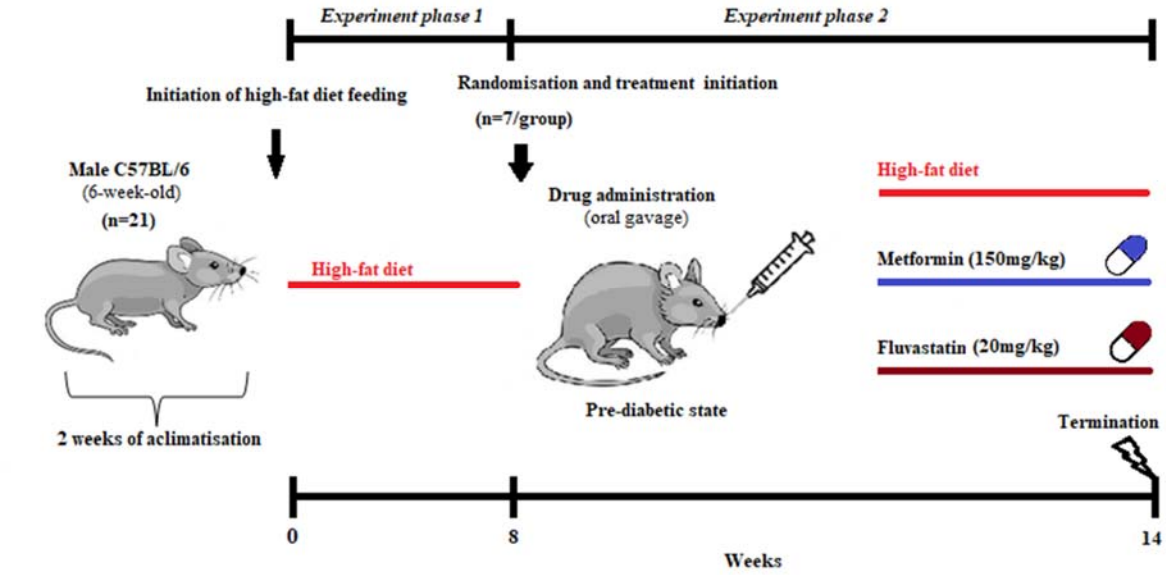

Figure 1: Study design involving two experimental phases. ***Experiment phase* 1:** Twenty-one six-week-old male C57BL/6 mice were acclimatised to the environment for 2 weeks prior to the initiation of high-fat diet feeding. ***Experiment phase 2:*** Following the end of week 8, the animals were then randomised into a short-term treatment with either metformin or fluvastatin over a 6-week period (n=7/group).

2.3 Measurements of glucose, haematological and lipid indices

Fasting plasma glucose concentrations were determined using the OneTouch select glucometer (LifeScan Inc, CA, USA) whilst the 2-hour postprandial oral glucose tolerance test was performed as previously described [20]. The levels of TC, HDL-c and LDL-c were measured using a mouse-specific ELISA kit (Abcam, MA, USA) as per manufacturer's instructions. The haematological parameters were determined using Beckman Coulter AcT5 Diff (Beckman Coulter, Miami, USA).

2.4 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 assessed using unpaired student *t-test* and were reported as mean ± standard error for parametric data. Non-parametric data was analysed using the Man Whitney U test and reported as the median and interquartile range [IQR]. The One-way analysis of variance (ANOVA) was used compare differences across the diet and treatments groups. A posthoc Tukey's multiple comparisons test was performed if the F-value reached statistical significance ($p<0.05$). For non-parametric data, the Kruskal-Wallis test followed by a Dunn's posthoc test was used. A p-value of < 0.05 was considered statistically significant. The GraphPad Prism version 8 software (GraphPad Software Inc, CA, USA) was used for all statistical analysis.

3. Results

3.1 Changes in body weights and glucose profiles following short-term treatment with metformin or fluvastatin

To measure the effect of short-term metformin and fluvastatin treatment on metabolic profiles, we assessed changes in body weights and glucose levels. The Kruskal-Wallis test showed that there were significant changes in the body weights ($K_{(2)} = 13.72$, $p<0.0001$) across the experimental groups (Table 1). The Dunn's posthoc analysis showed lower body weights in metformin [22.00 (21.00-23.00)], p=0.0064 or fluvastatin [22.00 (20.00-23.000)], p=0.0026 treated groups in comparison to the HFD-fed group [30.00 (29.00-30.00)].

The one-way ANOVA showed that fasting blood glucose levels did not statistically differ across the experimental groups ($F_{(2, 12)} = 3.430$, p=0.0663) (Figure 2b, Table 1). However, although not statistically significant, metformin lowered the fasting blood glucose levels whilst fluvastatin increased the levels when compared to the untreated HFD-fed group. The AUC varied significantly across all experimental groups ($F_{(2, 12)} = 16.80$, p=0.0003) (Table 1). The Tukey's multiple comparisons test showed a significant reduction in the AUC following treatment with metformin (803.8 ± 25.81), p=0.0007 or fluvastatin (855.9 ± 55.39), p=0.0010 in comparison to the untreated HFD-fed group (959.4 ± 28.47) (Figure 2c).

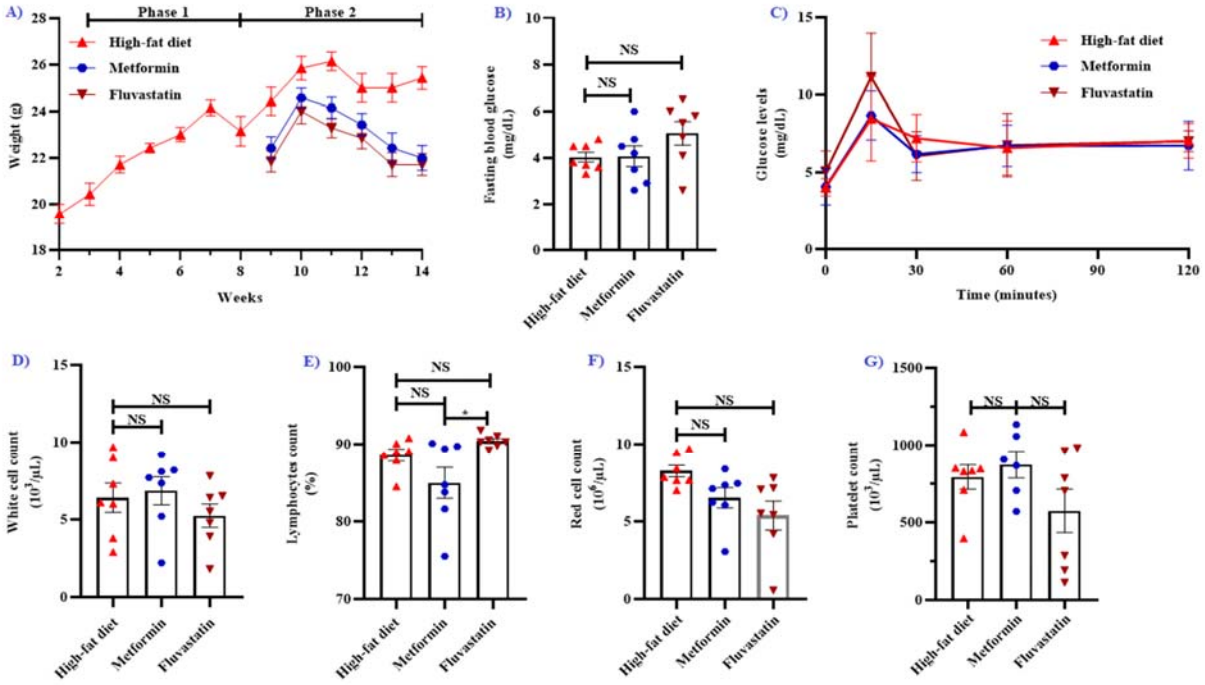

Figure 2: Changes in metabolic profiles following a short-term 6-week treatment. Figure (**A)** illustrates body weight changes over the 14-week period, whilst panel **(B)** and **(C)** shows comparisons of fasting blood glucose levels and the area under curve (AUC) in a 2-hour oral glucose test, respectively. Figure **(D), (E), (F)** and **(G)** shows differences in white cell count, lymphocyte differential count, red cell count and platelet count, respectively. All results are presented as mean ± standard error**.** NS: non-significant, *p<0.05

Table 1: Modulatory effects of metformin and fluvastatin on inflammation, lipid, and glucose control

Parameter	High-fat diet (n=7)	Metformin (n=7)	Fluvastatin (n=7)	p-value
Body weight (g)	30.00 [29.00-30.00]	22.00 [21.00-23.00]	22.00 [20.00-23.00]	**<0.0001**
Fasting glucose (mg/dL)	6.37 ± 0.28	4.07 ± 0.44	5.05 ± 0.50	0.0663
AUC (mmol/Lx 120 min)	959.4 ± 28.47	803.8 ± 25.81	855.9 ± 55.39	**0.0003**
Haematological profiles				
White cell count (10^3/µL)	6.40 ± 0.95	6.85 ± 0.90	5.24 ± 0.75	0.5106
Lymphocytes (%)	88.57 ± 0.77	84.97 ± 2.02	90.41 ± 0.31	**0.0180**
Red cell count (10^6/µL)	8.26 ± 0.38	6.52 ± 0.65	5.38 ± 0.93	0.0526
Platelet count	793.1 ± 78.44	1022 ± 165.2	574.3 ± 139.7	0.0752
***Lipid profiles* (µg/µL)**				
Total cholesterol	0.120 [0.107-0.139]	0.030 [0.017-0.032]	0.030 [0.028-0.031]	**0.0004**
LDL-cholesterol	0.115 ± 0.003	0.049 ± 0.003	0.055 ± 0.009	**<0.0001**
HDL-cholesterol	0.059 ± 0.003	0.043 ± 0.005	0.057 ± 0.004	**0.0415**
Cholesterol ratio	2.039 ± 0.232	0.652 ± 0.114	0.525 ± 0.040	**0.0001**

Results expressed as mean, ± standard error or median interquartile range. **AUC:** Area under the curve

3.2 Treatment with fluvastatin or metformin lowers cardiovascular risk in a pre-diabetes

The haematological parameters showed no significant changes in total WCC ($F_{(2, 12)} = 0.7113$, p=0.5106), RBC counts ($F_{(2, 12)} = 3.802$, p=0.0526) and platelet counts ($F_{(2, 12)} = 3.236$, p=0.0752) (Figure 2d/f/g, Table 1). In

contrast, lymphocyte ($F_{(2, 12)} = 5.716$, p=0.0180) differed across the groups (Table 1). A posthoc analysis showed that the metformin treated group (84.97 ± 2.02) had a lower lymphocyte count in comparison to the fluvastatin treated group (90.41 ± 0.31), p=0.0155 (Figure 2e). In order to determine the cardiovascular risk in a pre-diabetic state, we measured the levels of cholesterol in the respective treatment groups. Notably, the levels of TC ($K_{(2)} =$ 11.66, p=0.0004), LDL-c ($F_{(2, 10)} = 42.20$, p<0.0001) and HLD-c ($F_{(2, 10)} = 4.451$, p=0.0415) levels were different across all groups (Table 1, Figure 3a-c). The Dunn's posthoc analysis showed that treatment with metformin [0.030 (0.017-0.032)], p=0.0093 or fluvastatin [0.030 (0.028-0.031)], p=0.0093 significantly lowered TC when compared to the untreated HFD group [0.120 (0.107-0.139)] (Figure 3a). The Tukey's posthoc analysis showed a reduction in the levels of LDL-c following treatment with metformin (0.049 ± 0.003), p<0.0001 or fluvastatin (0.055 ± 0.009), p<0.0001 treatment (Figure 3b). In addition, short-term treatment with metformin (0.043 ± 0.005) but fluvastatin (0.057 ± 0.004) lowered HDL-c when compared to the untreated HFD-fed group (0.059 ± 0.003), p=0.0415 (Table 1, Figure3c). Cholesterol ratios, calculated by dividing TC with HDL-c, differed significantly across the groups ($F_{(2, 10)} = 25.16$, p=0.0001) (Table 1). The Tukey's posthoc test showed that treatment with either metformin (0.652 ± 0.114), p=0.0004 or fluvastatin (0.525 ± 0.040), p=0.002 was associated with lower cholesterol ratios when compared to the untreated HFD-group (2.039 ± 0.232) (Table 1, Figure 3d).

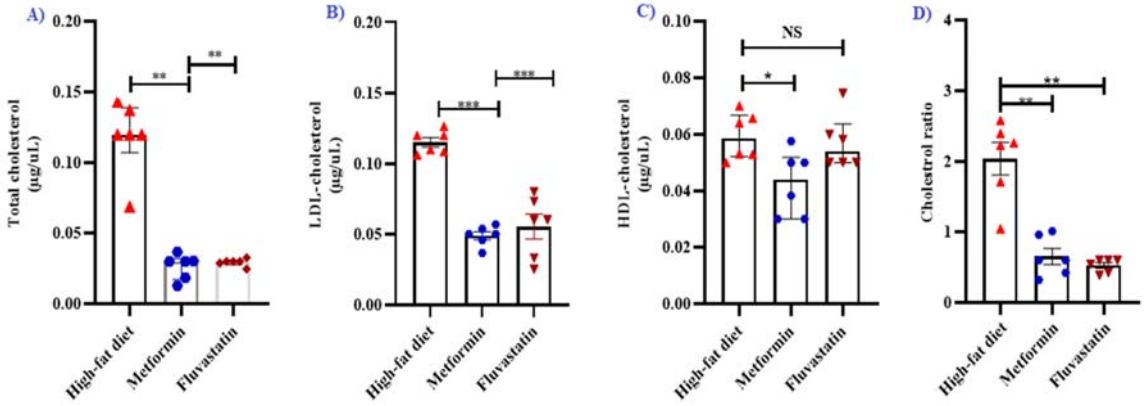

Figure 3: Changes in cholesterol levels following a 6-week short-term treatment. Figure (**A)** and **(B)** shows a significant reduction in total-cholesterol and low-density-lipoprotein cholesterol levels following treatment with metformin or fluvastatin, respectively. Figure (**C)** shows a reduction in high-density-lipoprotein cholesterol following metformin treatment. Figure **(D)** shows a decrease in cholesterol ratio following treatment with metformin or fluvastatin. Results expressed as mean, ± standard error or median interquartile range. ***p<0.0001, **p<0.001, *p<0.05.

4. Discussion

The aim of this study was to assess the modulatory effects of metformin and fluvastatin on glucose and haematological indices as well as lipid profiles using a diet-induced mouse model of pre-diabetes. In

this model, HFF is known to induce long term weight gain, impair glucose control and dyslipidaemia [21,22]. Notably, these changes are congruent with characteristic features of T2D in humans. As expected, treatment with metformin improved glucose control. However, short-term treatment with metformin or fluvastatin did not ameliorate inflammation but corrected dyslipidaemia by lowering TC and LDL-c levels.

Abnormal elevation of free fatty acid flux, secondary to insulin resistance in T2D drives the pathogenesis of diabetic dyslipidaemia [2]. The altered lipid metabolism in this state is characterised by exacerbated levels of triglycerides, Tc, LDL-c coupled with reduced levels of HLD-c [5]. Similarly, our pre-diabetes model showed hypercholesterolemia following HFF which was ameliorated by treatment with metformin and fluvastatin. Although both drugs lowered the cholesterol levels, the cholesterol ratio was lower in the fluvastatin group. This finding further highlights the efficacy of statins in lowering cholesterol levels. Fluvastatin is an inhibitor of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, an enzyme that catalyses the conversion HMG-CoA to mevalonate, in the synthesis of cholesterol and other isoprenoids [23]. Due to its inhibitory effects on isoprenoids, statins have the potential to regulate host homeostasis through the modulation of a broad range of transduction signalling. Most importantly, their modulation of immune responses [24]. For instance, statins inhibit the transcription factor NF-kB, the release of pro-inflammatory cytokines and the activation of peripheral mononuclear cells, including T-cells [12,25,26]. Our findings however, showed no immune-modulatory effects of fluvastatin and its no influence on normalising glucose control further highlights the need of its combinational use with metformin in a hyperglycaemic state.

The limitation of this current study was we did not assess the effect of combining metformin and statin on the parameters we measured. Nonetheless, pre-clinical studies have shown enhanced anti-atherosclerotic effects statin and metformin combinational [24]. Future clinical studies are required to confirm this promising combination and care must be taken in considering the impact of statins on lowering insulin secretion and glucose transporter type 4 (GLUT4) expression [27,28]. The resulting impaired glucose uptake and insulin resistant in adipose tissue, muscle and the liver may have attributed to fluvastatin group in our diet-induced model of pre-diabetes had the highest fasting glucose levels in comparison to the other groups.

5. Conclusion

Although fluvastatin did not exhibit any immune-modulatory properties in our model of pre-diabetes, it was significant in lowering the cholesterol levels and thus reducing the risk of developing atherosclerosis. Therefore, its usage in patients with T2D, particularly those with dyslipidaemia maybe of great benefit in lowering the risk of developing diabetes-associated CVD.

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Authors' contributions

TMN, PVD and BBN conceptualised, designed the study and drafted the manuscript. TMN and BBN performed formal analysis, methodology and validation as well as visualisation. All authors including VM wrote, reviewed, edited and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CHAPTER 6: General discussion

Chronic inflammation in pre-diabetes and the early development of type 2 diabetes (T2D), drives the pathogenesis of cardiovascular disease (CVD)-related complications [1–3]. Although chronic immune activation is closely associated with immune dysfunction in T2D, the exact role played by T-cells in the pathogenesis and progression of these diabetes-associated complications is not fully understood. We therefore studied the role of activated T-cells in pre-diabetes, an inflammatory state that precedes T2D. In addition, we explored the modulatory effects of low-dose aspirin (LDA), a well-known anti-inflammatory drug, and its combination with metformin, the first line oral glucose lowering drug (LDA+Met) on metabolic profiles, inflammation, immune activation, and T-cell function. Firstly, we explored published literature reporting on T-cell function in obesity, T2D and diabetes-associated complications (CVD and asthma) and performed a number of systematic reviews and meta-analyses. We further, critically synthesised evidence on the modulatory effects of aspirin and metformin on T-cell mediated responses in both physiological and chronic inflammatory state. Thereafter, we conducted a series of experiments, assessing T-cell function and inflammation in the early pathogenesis of T2D, using a high-fat diet (HFD)-induced mouse model of pre-diabetes.

6.1 Increased cardiovascular risk in T2D driven by hypercholesterolemia

Patients with T2D are at two-fold risk of developing CVD when compared to healthy controls [4]. The elevated cardiovascular risk is attributed to exacerbated levels of inflammation and immune activation in these patients [5,6]. Notably, all these attributes are associated with altered lipid metabolism, particularly elevated cholesterol levels, which leads to inflammation-induced atherogenesis [7,8]. Whereby, vascular inflammation promotes the accumulation of cholesterol within arterial smooth muscle cells and macrophages, resulting in the formation of foam cells [9]. The consequent hypercholesterolemia is a risk factor for the development of CVD. Notably, current treatment drugs targets the lowering of cholesterol as a therapeutic strategy to reduce cardiovascular risk [10–13]. In this study, HFD-feeding of mice for a period of 8-weeks was associated with hypercholesterolemia, which was characterised by increased levels of total cholesterol (Tc) and low-density lipoprotein (LDL)-c without changes in high-density lipoprotein (HDL)-c levels. Notably, these changes are congruent with those seen in patients with T2D [13,14]. In fact, our meta-analysis [15] involving clinical studies showed increased Tc with decreased HDL-c in patients with T2D is congruent with increased cardiovascular risk. Thus, highlighting the role of aberrant cholesterol levels in increasing the risk of developing CVD in patients with T2D. In our study although LDA monotherapy did not render any therapeutic benefit in modulating lipid metabolism in our mouse model of pre-diabetes, short-term treatment with LDA+Met, metformin or statin significantly lowered Tc and LDL-c levels in comparison to the untreated HFD-fed group. Notably, statin had the lowest cholesterol ratio in comparison to other drugs, thus highlighting its efficacy in lowering cholesterol levels and reducing cardiovascular risk in patients with T2D, as reported in a previous meta-analysis of randomised clinical trials [16].

6.2 Implications of low-grade inflammation and chronic immune activation in T2D

It is now well-established that chronic immune activation drives the pathogenesis and progression of diabetes-associated complications [5,6]. Recent studies have shown that these processes are partially mediated by T-cells [17,18]. Whereby, there is a polarisation of T-helper (T_H) cells into pro-inflammatory T_H1 and T_H17 subsets and a decrease in the levels of immunosuppressive regulatory T-cells (T_{regs}) and T_H2 subset [19–21]. The binding of cytokines released by the activated T-cell to their complementary receptors activates the pro-inflammatory signalling pathways such as Janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) that are crucial in modulating the differentiation of T_H cells [22]. In this study, we reported an elevation of interleukin (IL)-2, IL-6, IL-17, and tumour necrosis factor (TNF)-α following HFD-feeding in mice. Notably, increased IL-2 signalling can induce the differentiation of T_H cells towards the pro-inflammatory subsets, which leads to a pro-inflammatory milieu characterised by the elevated levels of T_H1 and T_H17-associated cytokines [22,23]. In our study, the significant reduction in all measured cytokines following short-term treatment with LDA+Met may be attributed to the normalised levels of IL-2, which initiates the activation of JAK/STAT5 signalling that promotes the differentiation of T_{regs} [22,23]. Therefore, the subsequent secretion of IL-10 by activated T_{regs} could have inhibited T_H1 and T_H17 responses, which resulted in the decrease of their associated cytokines (TNF-α, IL-6 and IL-17A) that were measured. Obesity a major risk factor for T2D that is associated with an elevated exacerbating inflammation and immune activation [24,25]. In our study, HFD-feeding was associated with leucocytosis and lymphocytosis which was resolved following treatment with LDA+Met. Overall, these findings demonstrate the efficacy of LDA+Met in downregulating T-cell mediated inflammation and immune activation. Future studies looking at the modulatory effects of LDA+Met on the JAK/STAT signalling are required to confirm the proposed hypothesis.

6.3 The successful modulatory effects of LDA+Met on altered T-cell function during early development of T2D

The qualitative and quantitative synthesis of available evidence showed increased levels of T-cell activation that were associated with an increased expression of negative co-stimulatory molecules in patients with T2D [15,26,27]. The upregulation of Fas and its subsequent binding to the Fas ligand (FasL) initiates the Fas/FasL signalling pathway which modulates both apoptotic and anti-apoptotic activities [28,29]. In that context, Fas provides a co-stimulatory signal that is essential for successful activation of T-cells [30]. Although contradictory findings have been reported on the expression of Fas, it is apparent that there is a close relationship between metabolic disorders and the aberrant Fas expression on T-cells [31–33]. Similarly, here we report enhanced Fas expression on T-cells following HFD-feeding which increased with body weights and was independent of glucose levels or lipid profiles. Taken together, this may suggest that T-cell dysfunction in poor glucose control is partially mediated by an upregulation of Fas which is independent of dyslipidaemia and hyperglycaemia.

Therefore, therapeutic strategies that modulate the Fas-FasL axis may be of great benefit, particularly in obese individuals who are at risk of developing CVD and T2D.

Furthermore, altered myocardial function in patients with T2D is associated with aggravated activation of T-cells [18,19,21,34]. Our meta-analysis showed an increase in the frequency of $CD4^{+}CD28^{-}$ T-cells [15], a long-lived T_H1 subset that has both pro-atherogenic and plaque-destabilising properties in patients with T2D [35]. Unlike the conventional T_H1 cells, these T-cells are resistant to both suppressive and apoptotic responses due to their loss of CD28 [36]. This highlights the importance of co-stimulatory signals in regulating T-cell activation and function. Therefore, the modulated expression of activation and inhibitory receptors is important in maintaining immune homeostasis. The activation of the T-cell receptor induces an upregulation of CD69 which gradually declines over days [37]. In this study, HFD-feeding resulted in the downregulation of CD69 expression which was concomitant with increased levels of $T_H1$and T_H17 associated cytokines. Notably, treatment with LDA+Met was associated with an increased expression of CD69, and this was congruent with reductions in T_H1 and T_H17 cytokines. This change may be due to increased JAK3/STAT5 signalling, which polarises T_H cells towards T_{regs} [38]. Even though LDA did not modify the expression of CD69 and T_H cytokine release, its efficacy was only enhanced when combined with metformin. This may be attributed to the inhibitory effect of metformin on mTOR and STAT3 signalling [39,40]. Future studies need to investigate and determine if there is any association between these signalling pathways and the expression of CD69 on T-cells.

Chronic T-cell activation can induce T-cell exhaustion, a state characterised by an upregulation of PD-1 [41]. Upregulated PD-1 signalling transduces a negative co-stimulatory signal that inhibits T-cell activation [42]. Notably, the upregulation of PD-1 is closely associated with loss of T-cell effector function in a mouse model of diet-induced obesity (DIO) [43]. In patients with T2D, contradictory findings on the expression of PD-1 on T-cells have been described [12, 13]. Surprisingly, in our study, the levels of PD-1 were comparable between the HFD and LFD group and following treatment. This unexpected finding may be attributed to the immunological responses in the early to intermediate phases of obesity-induced inflammation. A prolonged pro-inflammatory state is associated with an upregulation of PD-1 on the surface of exhausted T-cells [44]. In fact, our meta-analysis showed increased levels of PD-1 on T-cells in patients with T2D (Chapter 2.4). Thus, supporting the hypothesis that T-cells in patients in T2D are exhausted, and the process is mediated by increased PD-1 transduction. Therefore, the use of immune checkpoint inhibitors may be an effective therapeutic strategy in restoring T-cell effector functions in patients with T2D.

6.4 Conclusions and future perspectives

This current study described elevated levels of T-cell activation and dysfunction in a pre-diabetic state, that is characterised by poor glucose control, altered lipid metabolism, and exacerbated inflammation.

Notably, our pre-diabetes mouse model showed that this was in part mediated by the aberrant expression of T-cell regulatory markers, particularly Fas and CD69. Moreover, it was associated with increased body weight, levels of immune activation, and the release of T_H1 and T_H17 associated cytokines. We also demonstrated altered lipid metabolism marked by hypercholesterolemia. These findings highlighted the link between inflammation, immune activation, altered lipid metabolism and poor glucose control during early development of T2D. Interestingly, these findings mirrored those found in patients with T2D. The systematic reviews and meta-analyses conducted showed that patients with T2D have increased levels of T-cell activation and risk of developing CVD. The synthesised data also showed there is increased infiltration of pro-inflammatory T_H subsets in patients with T2D, which is coupled with decreased levels of the anti-inflammatory T_H subsets. Lastly, low-grade inflammation and persistent activation stimuli was associated with the upregulation of PD-1, thus suggesting that the circulating T-cells in patients living with T2D are exhausted. Therefore, continuous exploration of therapies that alleviate inflammation and modulate T-cell function are of critical importance in lowering the incidence of T2D-associated complications in these patients.

The findings of this study also showed that the combinational use of LDA and metformin can be an effective therapeutic strategy in alleviating inflammation and improving T-cell function. In our experimental model of pre-diabetes, it was apparent that the combination therapy effectively modulates the expression of regulators of T-cell activation and their associated transcription factors. This is in line with preliminary results from patients, indicating that combinational use of metformin and aspirin is more effective in improving glucose tolerance and reducing cardiovascular risk when compared to the use of metformin as a monotherapy [45]. On the other side, the dosage and side effects that may arise from using dual therapy are still unclear as reported elsewhere [46]. It is also important to note that although rejuvenating T-cell functions in diet-driven metabolic disturbances maybe beneficial in restoring T-cell effector functions and reducing the susceptibility risk to infections, such approach might exacerbate the level of T-cell mediated inflammation. Therefore, a delicate balance needs to be reached when using therapies that modulate T-cell responses in T2D.

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APPENDICES

CHAPTER 2.1

Additional file 1

PRISMA-P (Preferred Reporting Items for Systematic review and Meta-Analysis Protocols) 2015 checklist: recommended items to address in a systematic review protocol*

Section and topic	Item No	Checklist item	Reported on page (P)
ADMINISTRATIVE INFORMATION			
Title:			
Identification	1a	Identify the report as a protocol of a systematic review	P 1
Update	1b	If the protocol is for an update of a previous systematic review, identify as such	N/A
Registration	2	If registered, provide the name of the registry (such as PROSPERO) and registration number	P 3
Authors:			
Contact	3a	Provide name, institutional affiliation, e-mail address of all protocol authors; provide physical mailing address of corresponding author	P 1
Contributions	3b	Describe contributions of protocol authors and identify the guarantor of the review	P 11
Amendments	4	If the protocol represents an amendment of a previously completed or published protocol, identify as such and list changes; otherwise, state plan for documenting important protocol amendments	N/A
Support:			
Sources	5a	Indicate sources of financial or other support for the review	P 11
Sponsor	5b	Provide name for the review funder and/or sponsor	N/A
Role of sponsor or funder	5c	Describe roles of funder(s), sponsor(s), and/or institution(s), if any, in developing the protocol	N/A
INTRODUCTION			
Rationale	6	Describe the rationale for the review in the context of what is already known	P 4
Objectives	7	Provide an explicit statement of the question(s) the review will address with reference to participants, interventions, comparators, and outcomes (PICO)	P 7
METHODS			
Eligibility criteria	8	Specify the study characteristics (such as PICO, study design, setting, time frame) and report characteristics (such as years considered, language, publication status) to be used as criteria for eligibility for the review	P 7
Information sources	9	Describe all intended information sources (such as electronic databases, contact with study authors, trial registers or other grey literature sources) with planned dates of coverage	P 8

Search strategy	10	Present draft of search strategy to be used for at least one electronic database, including planned limits, such that it could be repeated	P 8
Study records:			
Data management	11a	Describe the mechanism(s) that will be used to manage records and data throughout the review	P 8
Selection process	11b	State the process that will be used for selecting studies (such as two independent reviewers) through each phase of the review (that is, screening, eligibility and inclusion in meta-analysis)	P 8
Data collection process	11c	Describe planned method of extracting data from reports (such as piloting forms, done independently, in duplicate), any processes for obtaining and confirming data from investigators	P 8
Data items	12	List and define all variables for which data will be sought (such as PICO items, funding sources), any pre-planned data assumptions and simplifications	P 8
Outcomes and prioritization	13	List and define all outcomes for which data will be sought, including prioritization of main and additional outcomes, with rationale	P 7
Risk of bias in individual studies	14	Describe anticipated methods for assessing risk of bias of individual studies, including whether this will be done at the outcome or study level, or both; state how this information will be used in data synthesis	P 9
Data synthesis	15a	Describe criteria under which study data will be quantitatively synthesised	P 9
	15b	If data are appropriate for quantitative synthesis, describe planned summary measures, methods of handling data and methods of combining data from studies, including any planned exploration of consistency (such as I^2, Kendall's τ)	P 9
	15c	Describe any proposed additional analyses (such as sensitivity or subgroup analyses, meta-regression)	P 10
	15d	If quantitative synthesis is not appropriate, describe the type of summary planned	
Meta-bias(es)	16	Specify any planned assessment of meta-bias(es) (such as publication bias across studies, selective reporting within studies)	P 9
Confidence in cumulative evidence	17	Describe how the strength of the body of evidence will be assessed (such as GRADE)	P 10

*** It is strongly recommended that this checklist be read in conjunction with the PRISMA-P Explanation and Elaboration (cite when available) for important clarification on the items. Amendments to a review protocol should be tracked and dated. The copyright for PRISMA-P (including checklist) is held by the PRISMA-P Group and is distributed under a Creative Commons Attribution Licence 4.0.**

From: Shamseer L, Moher D, Clarke M, Ghersi D, Liberati A, Petticrew M, Shekelle P, Stewart L, PRISMA-P Group. Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015: elaboration and explanation. BMJ. 2015 Jan 2;349(jan02 1):g7647.

Additional file 2: Search Strategy ran 06 June 2018

Concept 1: Diabetes mellitus	**Synonyms to be searched (MeSH or Text words)**
PubMed (hits=114 230) "Diabetes mellitus, Type 2"[Mesh]	Diabetes mellitus
	Glucose metabolism disorders
	Hyperglycaemia
	Metabolic diseases
	Metabolic syndromes

Concept 2: T-lymphocytes	**Synonyms to be searched (MeSH or Text words)**
PubMed (hits=114 230) "T-lymphocytes"[Mesh]	T-cells
	Th1/Th2 cells
	$CD4^+$
	$CD8^+$

Concept 3: Cardiovascular Diseases	**Synonyms to be searched (MeSH or Text words)**
PubMed (hits=2 191 229) "Cardiovascular diseases"[Mesh]	Heart diseases
	Vascular diseases

Combined Concept 1, 2 and 3 (PubMed hits = 51)

CHAPTER 2.2 Supplementary files

Table 1S: Quality assessment of included animal studies in the review using ARRIVE guidelines

	Introduction				Methods									Results				Discussion			
Author (year)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**Quality Score**
Kintscher et al., 2008	+	+	+	+	+	+	-	+	+	-	-	+	+	-	-	+	-	+	+	+	**14**
Winer et al., 2009	+	+	+	+	+	-	+	+	+	+	+	+	+	-	-	+	-	+	+	-	**15**
Rocha et al., 2009	+	+	+	+	+	-	+	+	-	-	+	+	+	-	-	-	-	+	+	+	**13**
Nishimura et al., 2009	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	**18**
Feuerer et al., 2009[5]	+	+	+	+	+	-	+	+	-	-	+	+	+	-	-	+	-	+	+	+	**14**
Zúñiga et al., 2010	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	+	**16**
Yang et al., 2010	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	+	**16**
Strissel et al., 2010	+	+	+	+	+	+	-	+	+	-	-	+	+	-	+	+	-	+	+	+	**15**
Miller et al., 2010	+	+	+	+	-	+	+	+	-	-	-	+	+	-	+	+	-	+	+	+	**14**
Deiuliis et al., 2011	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	-	+	+	+	**17**
Priceman et al., 2013	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	+	**16**
Morris et al., 2013	+	+	+	+	+	+	+	+	-	-	-	+	+	-	+	+	-	+	+	+	**15**
Montes et al., 2013	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+	**17**
Jiang et al., 2013	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	-	+	+	+	**17**
Deng et al., 2013	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	-	+	+	+	**17**
Zhong et al., 2014	+	+	+	+	+	+	+	+	-	+	-	+	+	-	+	+	-	+	+	+	**16**
Yi et al., 2014	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	**18**
Wolf et al., 2014	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	+	**16**
Fabrizi et al., 2014	+	+	+	+	-	+	+	+	+	-	-	+	+	-	+	+	-	+	+	+	**15**
Chatzigeorgiou et al., 2014	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	+	**16**
Poggi et al., 2015	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	**18**
Han et al., 2015	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	+	**16**
Liu et al., 2017	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+	-	+	+	+	**16**
Chen et al., 2017	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	+	**16**

Study quality items are: (1) Title (2) Abstract (3) Background (4) Objectives (5) Ethical statement (6) Study design (7) Experimental procedures (8) Experimental animals (9) Housing and husbandry (10) Sample size (11) Allocating animals to experimental group (12) Experimental outcomes (13) Statistical methods (14) Baseline data (15) Numbers analysed (16) Outcomes and estimation (17) Adverse events (18) Interpretation/scientific implications (19) Generalizability/translation and (20) Funding.

Table 2S: Quality assessment of human studies included in the review using Blacks and Downs

Author	Domain	Kintscher	Zeyda	Yang	Fabbrini	Deng	Zhong	McLaughlin	Fabrizi	Dalmas	Deiuliis	Travers
00REPORTING BIAS	1	1	1	1	1	1	1	1	1	1	1	1
	2	1	1	1	1	1	1	1	1	1	1	1
	3	1	1	1	0	0	0	0	0	1	1	1
	4	0	0	0	0	0	0	0	0	0	0	0
	5	1	0	0	0	1	0	0	0	0	0	1
	6	1	1	1	1	1	1	1	1	1	1	1
	7	1	1	1	1	1	1	1	1	1	1	1
	8	0	0	0	0	0	0	0	0	0	0	0
	9	0	0	0	0	0	0	0	0	0	0	0
	10	1	1	0	0	0	1	1	0	0	1	1
	Score	**7**	**6**	**5**	**4**	**5**	**5**	**5**	**4**	**5**	**6**	**7**
EXTERNAL VALIDITY	11	0	0	0	0	0	0	0	0	0	0	0
	12	0	0	0	0	0	0	0	0	0	0	0
	13	0	0	0	0	0	0	0	0	1	0	0
	Score	**0**	**0**	**0**	**0**	**0**	**0**	**2**	**0**	**1**	**0**	**0**
INTERNAL VALIDITY	14	0	0	0	0	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	0	0	0	0	0
	16	1	1	1	1	1	1	1	1	1	1	1
	17	0	0	0	0	0	0	0	0	1	0	0
	18	1	1	1	1	1	1	1	1	1	1	1
	19	0	0	0	0	0	0	0	0	0	0	0
	20	1	1	1	1	1	1	1	1	1	1	1
	Score	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**4**	**3**	**3**
SELECTION BIAS	21	1	1	1	1	1	1	1	1	1	1	1
	22	0	0	0	0	0	0	1	0	1	1	0
	23	0	0	0	0	0	0	1	0	0	0	0
	24	0	0	0	0	0	0	0	0	0	0	0
	25	0	0	0	0	0	0	0	0	0	0	0
	26	0	0	0	0	0	0	0	0	0	0	0
	Score	**1**	**1**	**1**	**1**	**1**	**1**	**3**	**1**	**2**	**2**	**1**
	Overall Score	**11**	**10**	**9**	**8**	**9**	**9**	**13**	**8**	**12**	**11**	**11**

Study quality items are: (1) Is the hypothesis/aim/objective of the study clearly described? (2) Are the main outcomes to be measured clearly described in the Introduction or Methods section? (3) Are the characteristics of the patients included in the study clearly described? (4) Are the interventions of interest clearly described? (5) Are the distributions of principal confounders in each group of subjects to be compared clearly described? (6) Are the main findings of the study clearly described? (7) Does the study provide estimates of the random variability in the data for the main outcomes? (8) Have all important adverse events that may be a consequence of the intervention been reported? (9) Have the characteristics of patients lost to follow-up been described? (10) Have actual probability values been reported? (11) Were the subjects asked to participate in the study representative of the entire population from which they were recruited? (12) Were those subjects who were prepared to participate representative of the entire population from which they were recruited? (13) Were the staff, places, and facilities where the patients were treated, representative of the treatment the majority of patients receive? (14) Was an attempt made to blind study subjects to the intervention they have received? (15) Was an attempt made to blind those measuring the main outcomes of the intervention? (16) If any of the results of the study were based on "data dredging", was this made clear? (17) In trials and cohort studies, do the analyses adjust for different lengths of follow-up of patients, or in case-control studies, is the time period between the intervention and outcome the same for cases and controls? (18) Were the statistical tests used to assess the main outcomes appropriate? (19) Was compliance with the intervention/s reliable? (20) Were the main outcome measures used accurate (valid and reliable)? (21) Were the patients in different intervention groups (trials and cohort studies) or were the cases and controls (case-control studies) recruited from the same population? (22) Were study subjects in different intervention groups (trials and cohort studies) or were the cases and controls (case-control studies) recruited over the same period of time? (23) Were study subjects randomized to intervention groups? (24) Was the randomized intervention assignment concealed from both patients and health care staff until recruitment was complete and irrevocable? (25) Was there adequate adjustment for confounding in the analyses from which the main findings were drawn? (26) Were losses of patients to follow-up taken into account?

CHAPTER 2.3 Supplementary files

Table 1S: MEDLINE **s**earch strategy

Metformin - concept 1
("metformin"[MeSH Terms] AND ("t-lymphocytes"[MeSH Terms] OR "t-lymphocytes"[All Fields] OR "t cells"[All Fields])
(n=63)

Aspirin – concept 2
("aspirin"[MeSH Terms] AND ("t-lymphocytes"[MeSH Terms] OR "t-lymphocytes"[All Fields] OR "t cells"[All Fields])
(n=187)

Concept 1 + concept 2 = 250 hits

Table 2S: Risk of bias of metformin human studies using the modified Black and Downs checklist (n=4)

Author		Dworacki 2015	Moro 2013	Wang 2018	Yong 2019
Reporting bias	1	1	1	1	1
	2	1	1	1	1
	3	1	1	1	1
	4	1	1	1	1
	5	1	1	1	0
	6	1	1	0	1
	7	1	1	1	1
	8	0	1	0	0
	9	0	0	0	0
	10	0	0	1	0
Total		**7**	**8**	**7**	**6**
External validity	11	0	0	0	0
	12	0	0	0	0
	13	0	0	0	0
Total		**0**	**0**	**0**	**0**
Internal validity	14	0	0	0	0
	15	0	0	0	0
	16	0	0	0	0
	17	1	1	1	0
	18	1	1	1	0
	19	0	1	0	0
	20	1	1	1	1
Total		**3**	**4**	**3**	**1**
Selection bias	21	1	1	1	1
	22	1	1	1	1
	23	1	1	1	1
	24	0	0	0	0
	25	0	0	0	0
	26	0	0	0	1
Total		**3**	**3**	**3**	**4**
Overall score		**13**	**15**	**13**	**11**

Table 3S: Risk of bias of included animal studies using metformin using ARRIVE guidelines (n=10)

	Introduction					Methods									Results					Discussion				
Author	**1**	**2**	**3**	**4**	**Total**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**Total**	**14**	**15**	**16**	**17**	**Total**	**18**	**19**	**20**	**Total**	**Overall score**
Kang 2013	+	+	+	+	**4**	+	+	+	+	-	+	+	+	-	**7**	-	-	+	-	**1**	+	+	+	**3**	**15**
Kim 2016	+	+	+	+	**4**	+	+	+	+	+	-	+	+	+	**8**	+	+	+	-	**3**	+	+	+	**3**	**18**
Kim 2018	+	+	+	+	**4**	+	+	+	+	+	-	+	+	+	**8**	+	-	+	-	**2**	+	+	+	**3**	**17**
Lee 2015	+	+	+	+	**4**	+	+	+	+	+	+	-	+	+	**8**	+	-	+	-	**2**	+	+	+	**3**	**17**
Lee 2017	+	+	+	+	**4**	+	+	+	+	-	-	-	+	-	**5**	-	-	+		**1**	+	+	+	**3**	**13**
Solano 2008	+	+	+	+	**4**	+	+	+	+	+	+	-	+	+	**8**	-	+	+	-	**2**	+	+	+	**3**	**17**
Son 2014	+	+	+	+	**4**	+	-	+	+	-	-	-	+	+	**5**	-	-	+	-	**1**	+	+	+	**3**	**13**
Sun 2016	+	+	+	+	**4**	+	+	+	+	+	+	+	+	-	**8**	+	-	+	-	**2**	+	+	+	**3**	**17**
Volarevic	+	+	+	+	**4**	+	-	+	+	+	-	+	+	+	**7**	-	+	+	-	**2**	+	+	+	**3**	**16**
Zarrouk 2014	+	+	+	+	**4**	+	+	-	+	+	+	-	+	+	**7**	-	+	+	-	**2**	+	+	+	**3**	**16**

Table 4S: Risk of bias of human studies reporting on aspirin using the modified Black and Downs checklist (n=10)

Author		Buckland 2006	Buckland 2006(1)	Cianferoni 2001	Crout 1975	Gherli 1198	Gherli 2001	Hernandez 2007	Mazzeo 1998	Ozkul 1996	Voisard 2001
Reporting bias	1	1	1	1	1	1	1	1	1	1	1
	2	1	1	1	1	1	1	1	1	1	1
	3	0	0	0	1	0	0	1	0	1	0
	4	1	0	1	1	1	1	1	1	1	1
	5	0	0	0	0	1	0	0	0	1	0
	6	1	1	1	1	1	1	0	1	1	1
	7	1	1	1	1	1	1	1	0	1	0
	8	0	0	0	0	0	1	0	0	1	0
	9	0	1	0	0	0	1	0	0	1	0
	10	0	0	0	0	0	0	1	0	0	0
Total		**5**	**5**	**5**	**6**	**6**	**7**	**6**	**4**	**9**	**4**
External validity	11	0	0	0	0	0	0	0	0	0	0
	12	0	0	0	0	0	0	0	0	0	0
	13	0	0	0	0	0	0	0	0	0	0
Total		**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Internal validity	14	0	0	0	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	0	0	0	0
	16	0	0	0	0	0	0	0	0	0	0
	17	1	1	1	1	1	0	0	1	1	0
	18	1	1	1	1	1	1	1	0	0	1
	19	0	1	1	0	1	1	0	0	0	0
	20	1	1	1	1	1	0	1	0	1	1
Total		**3**	**4**	**4**	**3**	**4**	**2**	**2**	**1**	**2**	**2**
Selection bias	21	0	0	0	1	1	0	0	1	1	0
	22	0	0	1	0	0	0	0	0	1	1
	23	0	0	0	0	0	0	0	0	0	1
	24	0	0	0	0	0	0	1	0	0	0
	25	1	0	0	0	0	0	0	0	0	0
	26	1	0	0	0	0	0	0	0	0	0
Total		**2**	**0**	**1**	**1**	**1**	**0**	**1**	**1**	**2**	**2**
Overall score		**10**	**9**	**10**	**10**	**11**	**9**	**9**	**6**	**13**	**8**

Table 5S: Risk of bias of included animal studies reporting on aspirin using ARRIVE guidelines (n=7)

	Introduction					Methods										Results					Discussion				
Author	**1**	**2**	**3**	**4**	**Total**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**Total**	**14**	**15**	**16**	**17**	**Total**	**18**	**19**	**20**	**Total**	**Overall score**
Hackstein 2001	+	+	+	+	**4**	-	-	-	+	-	-	-	-	-	**1**	+	-	+	-	**2**	+	+	+	**3**	**10**
Javeed 2009	+	+	+	+	**4**	+	-	+	+	+	-	-	+	+	**6**	-	+	+	-	**2**	+	+	+	**3**	**15**
Liu 2017	+	+	+	+	**4**	+	+	+	-	-	-	-	+	+	**5**	-	-	+	-	**1**	+	+	+	**3**	**13**
Moon 2010	+	+	+	+	**4**	+	+	+	+	-	-	-	+	+	**6**	-	+	+	-	**2**	+	+	+	**3**	**15**
Moon 2013	+	+	+	+	**4**	+	+	+	+	-	-	-	+	+	**6**	-	+	+	-	**2**	+	+	+	**3**	**15**
Muller 2001	+	+	+	+	**4**	+	-	+	-	-	+	+	+	+	**6**	-	-	-		**1**	+	+	+	**3**	**13**
Zhu 2015	+	+	+	+	**4**	+	+	+	-	+	-	+	+	+	**7**	-	-	+	-	**1**	+	+	+	**3**	**15**

Supplementary file 1

CHAPTER 2.4 Supplementary files

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	P1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	P2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	P3
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	P3
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	P4
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	P4
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	P4
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	P4
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	P4
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	P4
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	P5
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	P5
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	P5
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	P5

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	P5
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	N/A
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	P5
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	P6
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	P6
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	P6
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	P11
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	P6
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	N/A
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	P19
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	P21
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	P22
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	P22

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit: **www.prisma-statement.org**.

Supplementary file 2
Downs and Black checklist

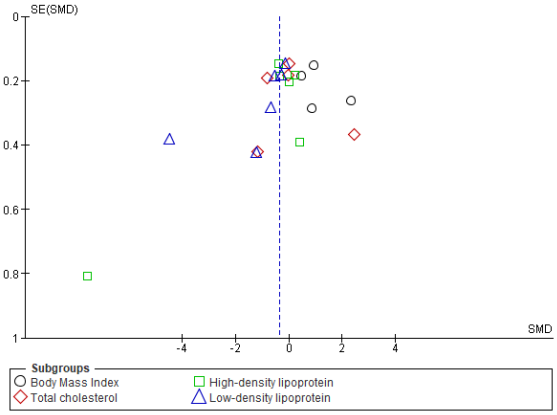
Quality of included T2D studies **(n=10)**

Author	Domain	Gong	Wang	Mahmoud	Zhao	Madhumitha	Shi	Giubilato	Olson	Eldor	Rattik
REPORTING BIAS	**1**	1	1	1	1	1	1	1	1	1	1
	2	1	1	1	1	1	1	1	1	1	1
	3	1	1	1	1	1	1	1	1	1	1
	4	0	0	0	0	0	0	0	0	0	0
	5	2	0	0	0	0	0	2	0	1	1
	6	1	1	1	1	1	1	1	1	1	1
	7	1	1	1	1	1	1	1	1	1	1
	8	0	0	0	0	0	0	1	0	0	0
	9	0	0	0	0	0	0	1	0	0	0
	10	1	1	0	1	1	0	1	1	1	1
	Score	**8**	**6**	**5**	**6**	**6**	**5**	**10**	**6**	**7**	**7**
EXTERNAL VALIDITY	**11**	0	0	0	1	0	0	0	1	0	1
	12	0	0	0	0	0	0	0	1	0	0
	13	0	0	0	0	0	0	0	1	0	0
	Score	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**3**	**0**	**1**
INTERNAL VALIDITY	**14**	0	0	0	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	0	0	0	0
	16	1	1	1	1	1	1	1	1	1	1
	17	0	0	0	0	0	0	1	0	1	0
	18	1	1	1	1	1	1	1	1	1	1
	19	0	0	0	0	0	0	1	1	1	0
	20	1	1	1	1	1	1	1	1	1	1
	Score	**3**	**3**	**3**	**3**	**3**	**3**	**5**	**4**	**5**	**3**
SELECTION BIAS	**21**	1	0	0	1	1	1	0	1	1	1
	22	1	0	0	1	0	0	0	0	1	1
	23	0	0	0	0	0	0	0	0	0	0
	24	0	0	0	0	0	0	0	0	0	0
	25	0	0	0	0	0	0	0	0	0	0
	26	0	0	0	0	0	0	0	0	0	0
	Score	**2**	**0**	**0**	**2**	**1**	**1**	**0**	**1**	**2**	**2**
	Overall score	**13**	**9**	**8**	**12**	**10**	**9**	**15**	**14**	**14**	**13**

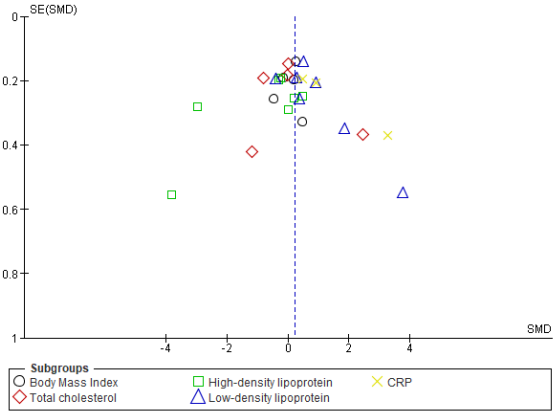
Quality of included CVD studies **(n=5)**

Author	Domain	Giubilato	Dumitriu	Teo	Flego	Emoto
REPORTING BIAS	**1**	1	1	1	1	1
	2	1	1	1	1	1
	3	1	1	1	1	1
	4	0	0	1	1	0
	5	2	0	1	1	1
	6	1	1	1	1	1
	7	1	1	1	1	1
	8	1	0	0	1	0
	9	1	0	0	1	0
	10	1	0	1	1	0
	Score	**10**	**5**	**8**	**10**	**6**
EXTERNAL VALIDITY	**11**	0	0	0	0	0
	12	0	0	0	0	0
	13	0	0	0	0	0
	Score	**0**	**0**	**0**	**0**	**0**
INTERNAL VALIDITY	**14**	0	0	0	0	0
	15	0	0	0	0	0
	16	1	1	1	1	1
	17	1	0	0	1	0
	18	1	1	1	1	1
	19	1	0	0	1	0
	20	1	1	1	1	1
	Score	**5**	**3**	**3**	**5**	**3**
SELECTION BIAS	**21**	0	0	1	1	1
	22	0	0	0	1	0
	23	0	0	0	0	0
	24	0	0	0	0	0
	25	0	0	0	1	0
	26	0	0	0	0	0
	Score	**0**	**0**	**1**	**3**	**1**
	Overall score	**15**	**8**	**12**	**18**	**10**

Supplementary file 3



Funnel plot of figure 3 pooled estimates of cardiovascular risk in T2D compared to controls



Funnel plot of figure 4S pooled estimates of cardiovascular risk in T2D compared to T2D+CVD

Figure 1S: Funnel plot of cardiovascular risk T2D compared to controls showing no publication bias symmetry.

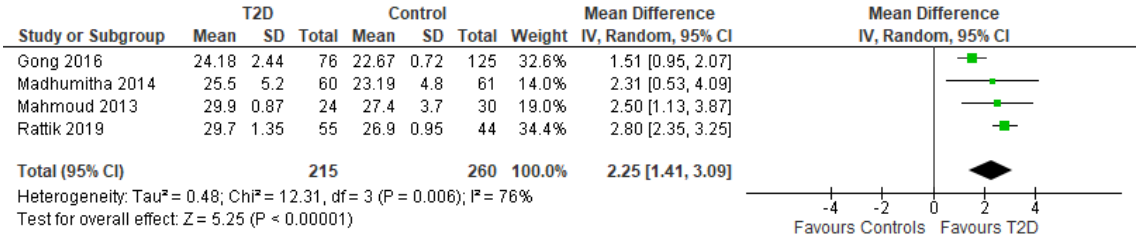


Figure 2S: BMI in T2D compared to controls

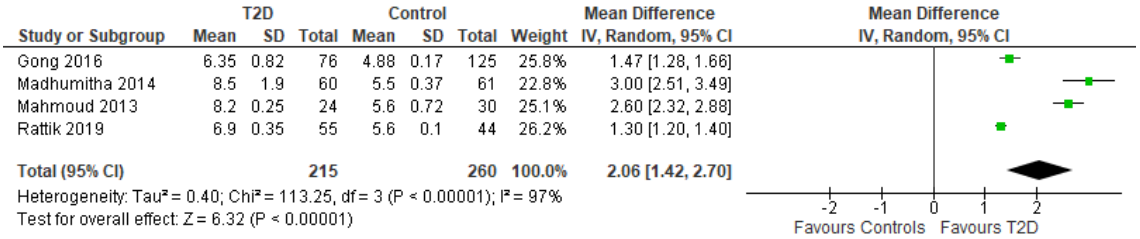


Figure 3S: Hb1Ac in T2D compared to controls

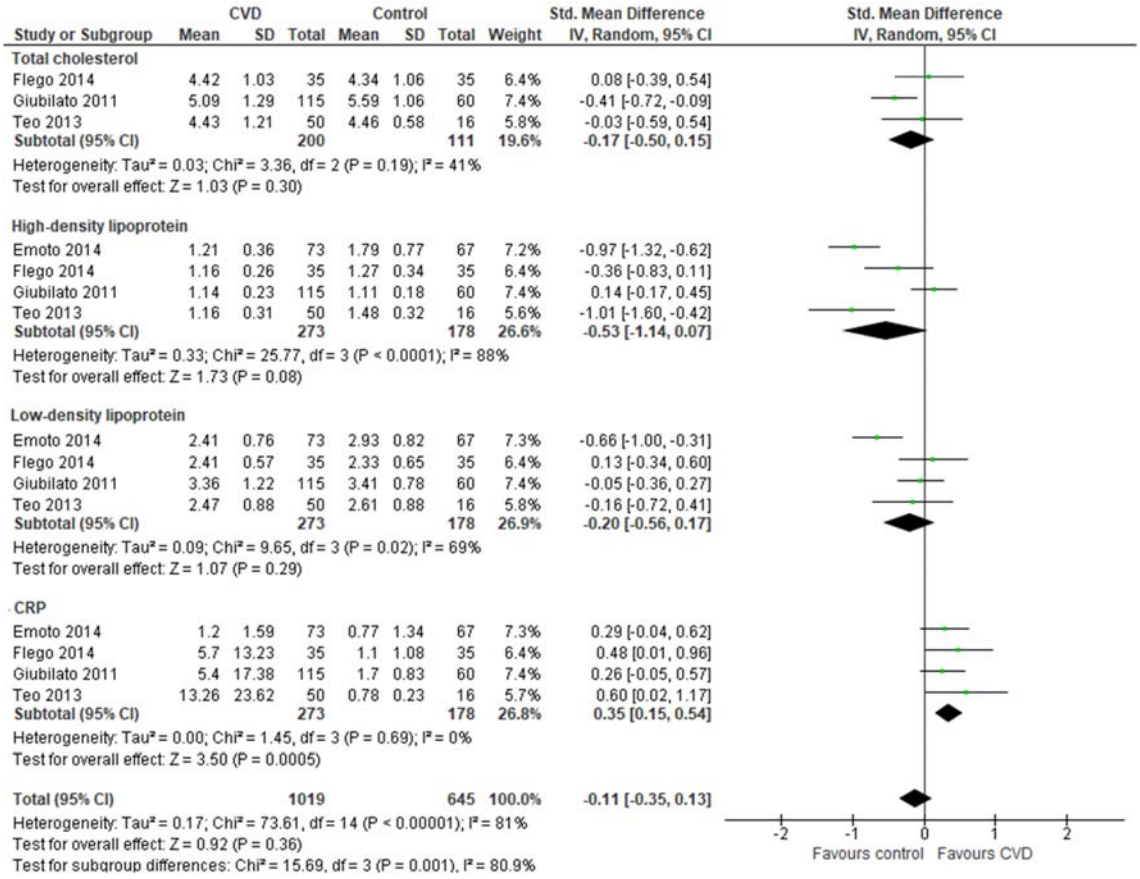


Figure 4S: Pooled estimates of cardiovascular risk in CVD compared controls.

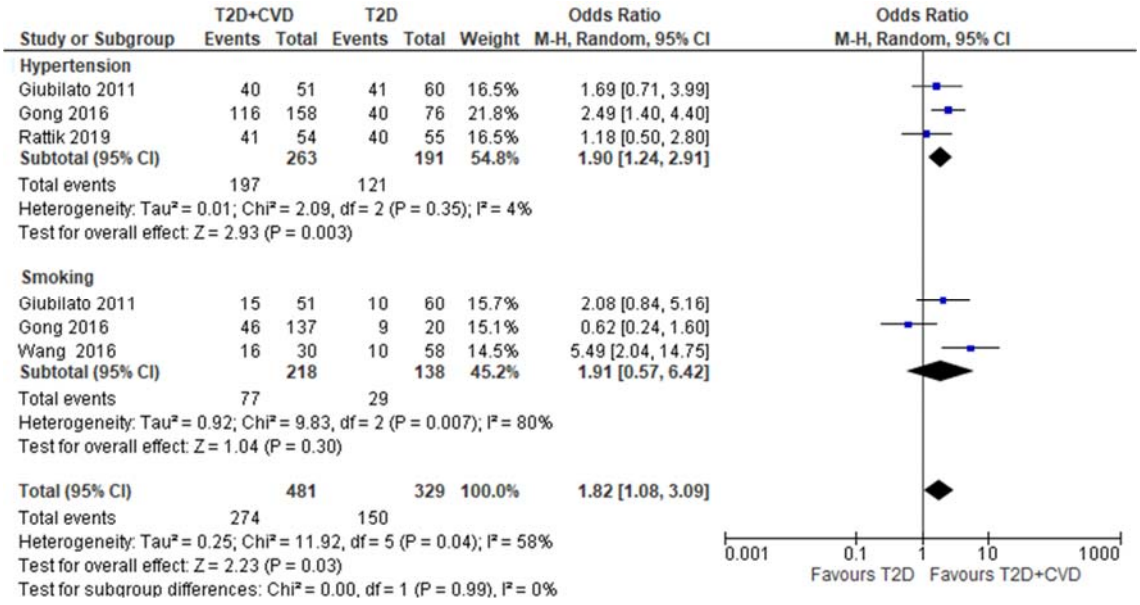


Figure 5S: The prevalence of CVD risk factors in T2D compared to T2D+CVD.

CHAPTER 2.5 Supplementary files

Supplementary Figures and Tables

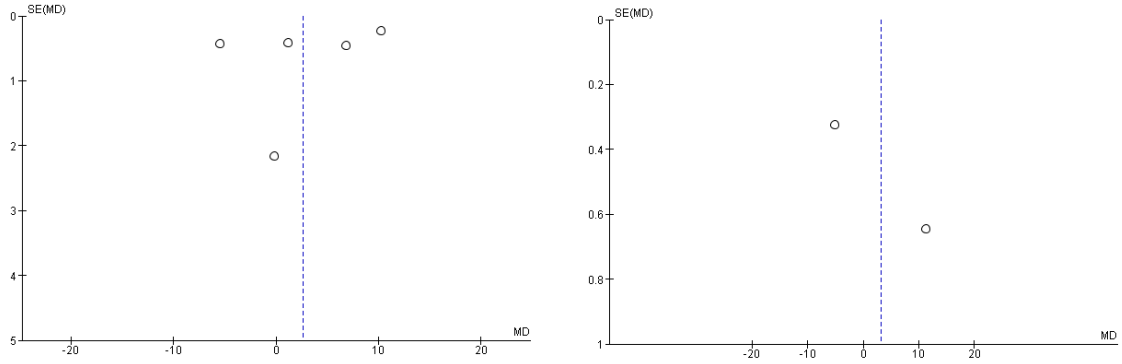


Figure 1S: Symmetrical forest plots indicating no publication bias

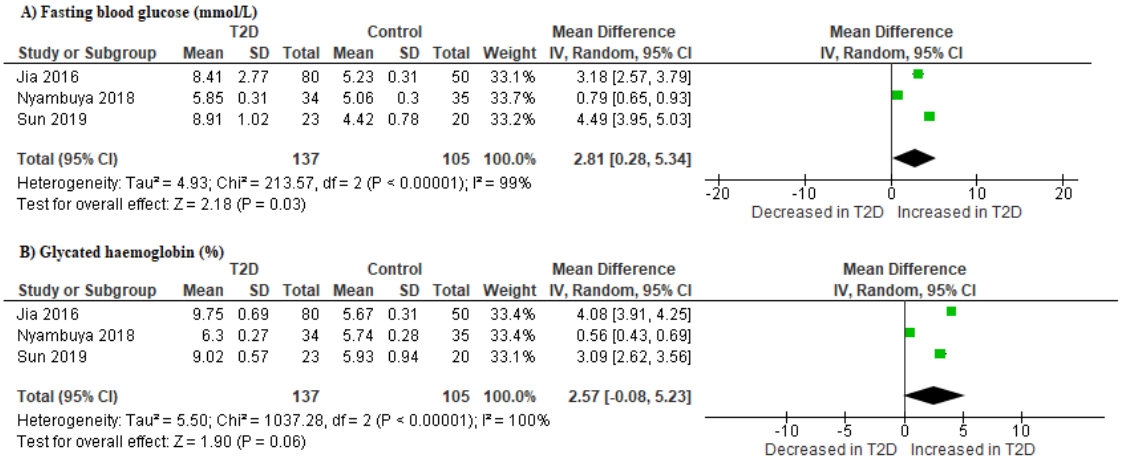


Figure 2S: Pooled estimates of glucose metabolic profiles indicating poor glucose control in T2D patients

Table 1S: PubMed search strategy

"Diabetes Mellitus, Type 2"[Mesh] AND (programmed[All Fields] AND ("death"[MeSH Terms] OR "death"[All Fields]) AND 1[All Fields]) AND "humans"[MeSH Terms] **21 hits**

Table 2S: Quality assessment of included studies using the modified Newcastle-Ottawa Scale for cross-sectional studies (n=5)

Study and year	Selection				Average	Comparability	Outcome			Total quality score	Rating
	Representativeness of the sample	Selected group of users	Sample size	Diagnose		Confounders	Assessment of methods	Statistical test	Average		
Shi 2013 [1]	0	0	0	***	**3**	**	*	*	**2**	**7**	**Good**
Fujisawa 2015 [2]	0	0	0	**	**2**	0	*	*	**2**	**4**	**Unsatisfactory**
Jia 2016 [3]	0	*	0	***	**4**	**	*	*	**2**	**8**	**Good**
Nyambuya 2018 [4]	0	*	0	***	**4**	*	*	*	**3**	**7**	**Good**
Sun 2019 [5]	0	*	0	**	**3**	**	*	*	**2**	**7**	**Good**

Table 3S: A subgroup analysis based on extracted and computed values of PD-1expression on T helper cells

Risk of bias	Number of studies	%MD [95% CI]	I^2 (%), p^H-value
All	5 [1–5]	2.57 [-3.84, 8.97]	100% (p <0.00001)
Extracted values	3 [1, 2, 5]	1.55 [-10.88, 13.99]	100% (p <0.00001)
Computed values	2 [3, 4]	4.00 [-1.50, 9.50]	99% (p <0.00001)

Table 4S: Sensitivity analysis of all included studies that reported on PD-1 expression on T-cells based on sample type and T-cell subset

Outcomes	Parameter	Number of studies	Omitted studies	MD [95%CI]	I^2 (%), p^H-value	Overall effect: Z, p-value
PD-1 expression on T helper cells	**All**	5 [1–5]	None	2.57 [-3.84, 8.97]	100% (p <0.00001)	0.78 (p = 0.43)
	Sample type					
	Whole blood	2 [3, 4]	3 [1, 2, 5]	4.00 [-1.50, 9.50]	99% (p <0.00001)	1.43 (p = 0.15)
	Peripheral blood mononuclear cells	3 [1, 2, 5]	2 [3, 4]	1.55 [-10.88, 13.99]	100% (p <0.00001)	0.24 (p = 0.81)
				0.61 [-5.30, 6.52]		
	T helper subtype		1 [1]			
	CD4	4 [2–5]		10.24 [9.77, 10.71]	99% (p <0.00001)	0.20 (p = 0.84)
			4 [2–5]			
	$CD4^+CD28^-$	1 [1]			Not applicable	43.11 (p <0.00001)

Reference

1. Shi B, Du X, Wang Q, Chen Y, Zhang X (2013) Increased PD-1 on CD4+CD28- T cell and soluble PD-1 ligand-1 in patients with T2DM: Association with atherosclerotic macrovascular diseases. Metabolism 62(6):778–785. https://doi.org/10.1016/j.metabol.2012.12.005
2. Fujisawa R, Haseda F, Tsutsumi C, et al (2015) Low programmed cell death-1 (PD-1) expression in peripheral CD4+ T cells in Japanese patients with autoimmune type 1 diabetes. Clin Exp Immunol 180(3):452–457. https://doi.org/10.1111/cei.12603
3. Jia Y, Zhao Y, Li C, Shao R (2016) The Expression of Programmed Death-1 on CD4+ and CD8+ T Lymphocytes in Patients with Type 2 Diabetes and Severe Sepsis. PLoS One 11(7):1–12. https://doi.org/10.1371/journal.pone.0159383
4. Nyambuya T, Davison GM, Hon G, Kengne A, Erasmus R, Matsha T (2018) T-cell Activation and Dysfunction in Hyperglycaemia. Med Technol South Africa 32(1):24–27
5. Sun P, Jin Q, Nie S, et al (2019) Unlike PD-L1, PD-1 is downregulated on partial immune cells in type 2 diabetes. J Diabetes Res 2019(March):1–8. https://doi.org/10.1155/2019/5035261

Ethical clearance

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

16 August 2018

Dr Bongani Brian Nkambule (52541)
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Dr Nkambule,

Protocol reference number: AREC/086/016
Project title: Investigating chronic inflammation and immune function in type 2 diabetes

Full Approval – Renewal Application

With regards to your renewal application received on 23 July 2018. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted with the following conditions:

CONDITIONS:

- The study must use the collected tissue and blood samples from the study. No additional animals are needed for this study.

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 16 August 2019.

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]

.....

Prof S Islam, PhD
Chair: Animal Research Ethics Committee

Cc Academic Leader Research: Dr Michelle Gordon
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Anita Engelbrecht
Cc BRU – Dr Linda Bester

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