# UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Human Development and Health


# Effect of 18-Carbon *Cis* and *Trans* Fatty Acids on Inflammatory Responses of Cultured Human Endothelial Cells


by

**Carina Valenzuela Avendaño**


Thesis for the degree of Doctor of Philosophy

January 2020

# University of Southampton

## Abstract

FACULTY OF MEDICINE

Human Development and Health

Thesis for the degree of Doctor of Philosophy

**EFFECT OF 18-CARBON CIS AND TRANS FATTY ACIDS ON INFLAMMATORY RESPONSES OF CULTURED HUMAN ENDOTHELIAL CELLS**

by

Carina Valenzuela Avendaño

The endothelium plays a key role in maintaining vascular homeostasis by regulating vascular tone and permeability. Endothelial dysfunction is a pro-inflammatory state characterized by chronic activation of the endothelium, which leads to atherosclerosis and cardiovascular disease (CVD). Intake of *trans* fatty acids (TFAs) is associated with an increased risk of CVD and other chronic diseases and this risk is usually associated with industrial TFAs rather than ruminant TFAs; however the effect of TFAs on inflammation remains unclear. The aim of this project was to compare the inflammatory response in cultured EA.hy926 endothelial cells (ECs) exposed to different 18-carbon *trans* fatty acids (*trans* vaccenic acid (TVA), elaidic acid (EA), *cis* vaccenic acid, conjugated *cis*-9,*trans*-11 and *trans*-10, *cis*-12 linoleic acids (CLAs)) of ruminant and industrial origin and to two 18-carbon *cis* fatty acids (oleic and linoleic).

EA.hy926 cells remained viable at the different FA concentrations used, with the exception of CLA10,12 at 50 μM. In addition, the FAs tested were incorporated into ECs in culture in a dose dependent manner, and this incorporation was accompanied by the appearance of some metabolic products. EA increased production of most of the cytokines and adhesion molecules measured after inflammatory stimulation, while TVA caused their reduction in non-stimulated cells and showed a neutral effect in tumour necrosis factor (TNF)-α stimulated ECs. EA also showed some anti-inflammatory effects under basal conditions when used at low concentrations. In relation to the effect of the FAs on expression of inflammatory pathway genes, EA (50 μM) induced a significant upregulation of toll-like receptor (TLR)-4 and cyclooxygenase (COX)-2 gene expression in TNF-α stimulated cells. In contrast, TVA (1 μM) induced a significant downregulation of nuclear factor kappa B subunit 1 (NFκB1) in TNF-α stimulated cells. TVA (10 μM) also decreased the relative gene expression of COX-2 under basal conditions. EA (50 μM) induced a significant increase of THP-1 monocyte cell adhesion to ECs, both with and without TNF-α stimulation. In contrast, TVA (1 μM) reduced THP-1 monocyte adhesion, under basal conditions and after TNF-α stimulation, and decreased the level of surface expression of ICAM-1 when used at 50 μM.

In relation to the effects of the CLAs, CLA9,11 had neutral or anti-inflammatory effects in non-stimulated ECs, reducing the levels of monocyte chemoattractant protein 1, regulated on activation normal T expressed and secreted, interleukin (IL)-8 and IL-6. CLA10,12 exposure reduced the same cytokines in basal conditions, which the exception of IL-6, which was increased. In contrast, CLA10,12 increased the levels of most of the inflammatory mediators after TNF-α stimulation. CLA9,11 had null effects after inflammatory stimulation. Both CLAs increased NFκB gene expression under basal conditions, together with a reduction of peroxisome proliferator activated receptor α, COX-2 and IL-6 gene expression. Instead, both CLAs (1 μM) decreased the NFκB mRNA appearance after TNF-α stimulation, but only CLA9,11 maintained this downregulation at 10 μM. CLA10,12 had no effect on THP-1 adhesion while decreasing significantly the % of cells expressing ICAM-1 and also levels of ICAM-1 expression per cell when used at 10 μM. Although CLA9,11 did not have any effect on ICAM-1 cell surface expression, it reduced the monocyte adhesion to the EA.hy926 cell monolayer.

The results suggest that TVA has some anti-inflammatory properties, while EA enhances the response to an inflammatory stimulus. In addition, both CLAs showed similar effects under basal conditions, acting generally in a neutral or modest anti-inflammatory way. In contrast, after TNF-α stimulation, CLA10,12 showed some pro-inflammatory effects. This suggests that the EC responses to some FAs may change depending on the presence of inflammation. These findings suggest differential effects induced by the TFAs tested, in general fitting with the idea that industrial TFAs and ruminant TFAs can have different and perhaps opposing biological actions.

# Table of Contents

# Table of Tables

# Table of Figures

Table of Figures

Table of Figures

# Research Thesis: Declaration of Authorship

| | |
|---|---|
| Print name: | Carina Valenzuela |
| Title of thesis: | Effect of 18-carbon *cis* and *trans* fatty acids on inflammatory responses of cultured human endothelial cells |

I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as:

   Valenzuela CA, Baker EJ, Miles EA, Calder PC. Eighteen-carbon *trans* fatty acids and inflammation in the context of atherosclerosis. Prog Lipid Res. 2019 Oct;76:101009. Doi: 10.1016/j.plipres.2019.101009. Epub 2019 Nov 2.

Signature:

Date:

09/01/2020

# Acknowledgements

Firstly, I want to thank my supervisors Professor Philip Calder and Dr. Elizabeth Miles, for giving me the opportunity to be part of their research group, for their kindness when I made mistakes, their encouragement when I did well and for all their help, guidance and support throughout this journey.

My appreciation to the *Comisión Nacional de Investigación Científica y Tecnológica* CONICYT (del Ministerio de Educación, Chile, Programa de Formación de Capital Humano Avanzado) and the Universidad de Valparaiso, Escuela de Nutrición (in particular to Marcela Alviña), for granting me the opportunity to undertake this challenge and all of the experiences that came with it.

To my dear friend and personal lab-HAM mentor, Ella Baker, many many thanks. For the generosity in sharing your knowledge, your support, for keeping me motivated through the ups and downs in these 4 years. Thanks to you I lived my best PhD!

I also would like to thank all members of the research group that I was luckily part of. My appreciation to all of them which were accessible to help me whenever I needed them. Big thanks to my friend Helena, to Lucinda, Annette, Caroline, Stefania and Chris. Special thanks to Melissa Doherty, for all her technical support in the Molecular lab. Many thanks to other friends that have help me with lab techniques, Camelia and Giorgia.

This adventure wouldn't have been the same without the close friends and chosen family I have made here in Southampton: Reyna, Felix, Rubi, Angela, Martina, Mia, Priscila, Ale, Yann, Lalo, Paola, Astrid, Camila, Leire, Gabi and Juraj. All my appreciation for you my dear friends, I feel very thankful that our paths met, for your love, support and patience.

Muchas gracias a los mentores que han sido un ejemplo para mí, por su apoyo y generosidad para compartir sus conocimientos, y por haber despertado en mi la motivación por investigar y seguir la carrera académica. Profes Erik Díaz, Héctor Toledo y en particular a mi queridísimo Profesor Miguel Llanos, quien ha sido un gran maestro para mi y que al igual que los otros profes, es un excelente académico y una gran persona. Gracias por creer en mí Profes.

Quiero agradecer a l@s amig@s que me han apoyado y entregado su cariño desde Chile: Cintillo, Xime, Jose, Coty, Andrea, Pancho, Karla, Pauli y Paola. Muchas gracias a todos por mantenerse presentes a la distancia en estos 4 años. Los quiero mucho!

Finalmente, quiero agradecer a mi familia, a mis hermanos Gabriel y Diego, mis sobrin@s, mis tí@s Melicha y Cristian, mi primo Leo, mi Papá Lulo, mi tata Juan y mi nona Luzmi, por su apoyo y por ser parte esencial de quien soy y de mis logros. En especial quiero agradecer a mi Mamá Cecilia, por ser un modelo de esfuerzo y fuente de inspiración para mí. Gracias por apoyarme siempre, te amo Mamá.

Many thanks to all of you! / Muchas gracias a todos ustedes!

# Definitions and Abbreviations

ALA, α-linolenic acid;

ANOVA, analysis of variance;

ARA, arachidonic acid;

BMI, body mass index;

cDNA, complementary deoxyribonucleic acid;

CHD, coronary heart disease;

CLA, conjugated linoleic acid;

CLAs, conjugated linoleic acids;

CLA9,11, cis-9, trans-11 CLA;

CLA10,12, trans-10, cis-12 CLA;

COX-2, cyclooxygenase 2;

CRP, C-reactive protein;

CREB, cyclic-AMP response element binding protein;

CSF, cerebrospinal fluid;

CVA, *cis* vaccenic acid;

CVD, cardiovascular disease;

C/EBP, CCAAT-enhancer binding protein;

DG, diacylglycerol;

DHA, docosahexaenoic acid;

DNA, deoxyribonucleic acid;

DMEM, Dulbeccos's Modified Eagle's Medium;

DMSO, dimethyl sulfoxide;

EA, elaidic acid;

ECs, endothelial cells;

ELISA, Enzyme-Linked ImmunoSorbent Assay;

EPA, eicosapentaenoic acid;

FAs, fatty acids;

FAME, fatty acid methyl ester;

FAMEs; fatty acid methyl esters;

GC, gas chromatography;

HAECs, human aortic endothelial cells;

HCASMCs, human coronary arterial smooth muscle cells;

HDL, high density lipoproteins;

HMECs, human microvascular endothelial cells;

HUVEC, human umbilical vein endothelial cells;

ICAM-1, intercellular adhesion molecule-1;

IFN, interferon;

IκB, inhibitor of NFκB;

IL, interleukin;

iTFA, industrialized *trans* fatty acids;

LA, linoleic acid;

LDL, low density lipoproteins;

LED, light-emitting diode;

LFA1, lymphocyte function-associated antigen 1;

LPS, lipopolysaccharide;

MCP-1, monocyte chemoattractant protein 1;

MMPs, matrix metalloproteinases;

mRNA, messenger RNA;

MUFA, monounsaturated fatty acids;

NAFLD, non-alcoholic fatty liver disease;

NEFA, non-esterified fatty acid;

NEMO, NFκB essential modulator;

NFκB, nuclear factor kappa-beta;

NO, nitric oxide;

n-3, omega 3;

n-6, omega 6;

OA, oleic acid;

oxLDL; oxidised low density lipoprotein;

PAI-1, plasminogen activator inhibitor-1;

PBMC, peripheral blood mononuclear cell;

PE, phycoerythrin;

PECAM-1, platelet endothelial cell adhesion molecule 1;

PG, prostaglandin;

PHSO, partially hydrogenated soybean oil;

PHVF, partially hydrogenated vegetable fat;

PLs, phospholipids;

PPAR, peroxisome proliferator activated receptor;

PRRs, pattern recognition receptors;

PTGS2, protaglandin-endoperoxide synthase 2;

PUFA, polyunsaturated fatty acids;

RANTES, regulated on activation, normal T cell expressed and secreted;

RCTs, randomized controlled trials;

RNA, ribonucleic acid;

ROS, reactive oxygen species;

rTFA, ruminant *trans* fatty acids;

RT-PCR, real time polymerase chain reaction;

SFA, saturated fatty acids;

SMCs, smooth muscle cells;

SOD-2, superoxide dismutase 2;

sTNF-R, soluble tumour necrosis factor α receptor;

TAG, triacylglycerol;

TFAs, *trans* fatty acids;

TIRAP, MyD88-adapter–like (Mal)/toll/IL-1 receptor homology domain–containing adapter protein;

TLRs, toll-like receptors;

TNF-α, tumour necrosis factor alpha;

TNF-RI, tumour necrosis factor receptor 1;

TRAIL, TNF related apoptosis-inducing ligand;

TRAM, TRIF-related adapter molecule;

TRIF, toll/IL-1 receptor homology domain–containing adapter inducing IFN-β;

TVA, *trans*-vaccenic acid;

VEGF, vascular endothelial growth factor;

VCAM-1, vascular adhesion molecule-1;

WAT, white adipose tissue.

# Chapter 1 Introduction

Low-grade chronic Inflammation is a feature of obesity, and has been related to insulin resistance and atherosclerosis. The vascular endothelium plays a key role in maintaining vascular homeostasis by regulating vascular tone and permeability. Endothelial dysfunction is a pro-inflammatory state characterized by chronic activation of the endothelium, which leads to atherosclerosis and cardiovascular disease (CVD).

Intake of *trans* fatty acids (TFAs) is associated with an increased risk of CVD and other chronic diseases; however the effect of TFAs on inflammation remains controversial. The research described in this thesis explores the effects of 18-carbon TFAs on endothelial cell inflammation.

This chapter will provide the background to understand the current knowledge in this matter and explain why an endothelial cell model will be used to study the effect of different types of TFAs on their inflammatory responses in relation to cardiovascular disease development.

## 1.1 Fatty acids

Lipids are essential components of cell membranes, playing varied roles in mediating and controlling an extensive array of cellular activities including membrane structure and organization, metabolic and gene regulation, protein structure and function, energy production and signalling pathways. They have been associated with immune and inflammatory responses, cell proliferation, mediation of apoptosis and also have been linked to the pathogenesis of many diseases, including CVD, diabetes, cancer and neurodegenerative disorders (Calder, 2004).

Lipids consist of a large number of organic compounds, including fatty acids (FA), monoacylglycerols (MG), diacylglycerols (DG), triacylglycerols (TAG), phospholipids (PL), eicosanoids, resolvins, docosanoids, sterols, sterol esters, carotenoids, vitamins A and E, fatty alcohols, hydrocarbons and wax esters. These lipids and their FA components are widely dispersed in nature. Dietary fat includes all the lipids in plant and animal tissues that are eaten as food. The most common fats (solid) or oils (liquid) are glycerolipids, which are largely composed of TG. The TG are accompanied by minor amounts of PL, MG, DG and sterols/sterol esters. FA constitute the main components of many of these lipid

entities and are required by humans as a source of energy and for metabolic and structural activities (FAO, 2010).

Different foods contain different amounts of fat and different types of FA, and these may be affected by processing, storage, and cooking methods. The majority of FAs consumed in the diet are available to the bloodstream through efficient processes of digestion and absorption. FAs can also be synthesized in the human body, either from non-lipid precursors such as glucose or from other FA; exceptions to this are the essential FAs (i.e. linoleic and α-linolenic acids (LA, ALA)), which must be obtained from the diet (Calder, 2015a).

### 1.1.1 Structure and geometry of FA

Fatty acids are carboxylic acids with hydrocarbon chains ranging from 2 to 30 carbons long, with a carboxyl group at one end and a methyl group at the other. Based on the chain length, FA are classified as short chain (2–6 carbon atoms), medium chain (8–12 carbon atoms), and long chain (14 or more carbon atoms). The most common dietary FAs are also classified into three broad classes according to the degree of unsaturation; saturated fatty acids (SFA) do not contain double bonds in the hydrocarbon chain, monounsaturated fatty acids (MUFA) have one double bond and polyunsaturated fatty acids (PUFA) have two or more double bonds. In general, these FAs have an even number of carbon atoms and have unbranched structures. The double bonds of naturally occurring unsaturated FAs usually have a *cis* orientation, meaning that the hydrogen atoms attached to the double bonds are on the same side (FAO, 2010). On the other hand, TFAs have one or more double bonds in the *trans* geometric configuration, meaning that the carbon chain extends from the opposite side of the double bond and this spatial variance provides them with different physical, chemical and biological properties when compared to their *cis* isomers (Figure 1).

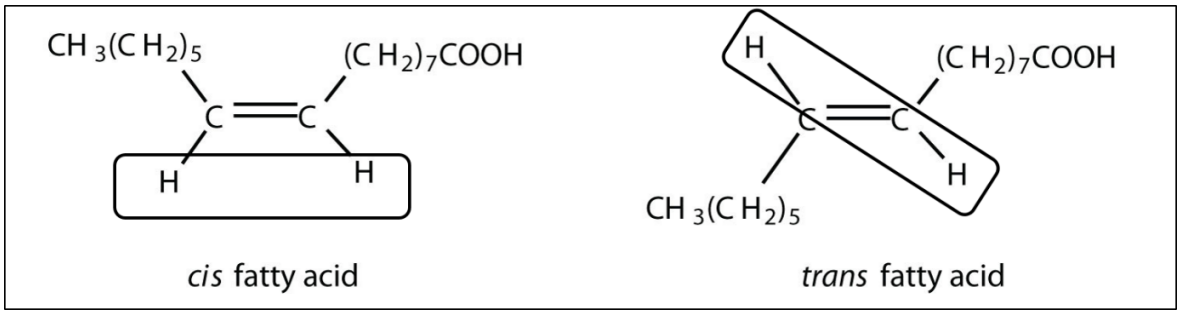
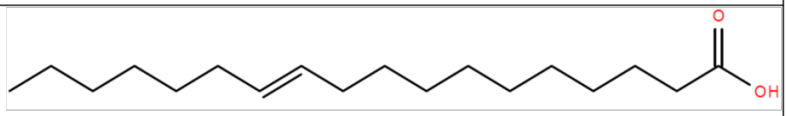
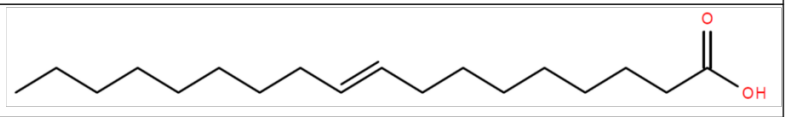
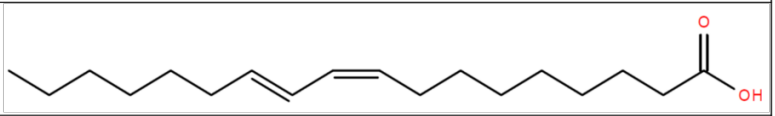
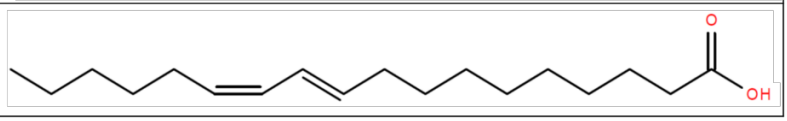



Figure 1.1: *Cis* and *trans* spatial configuration of fatty acids.

### 1.1.2 TFA structure and metabolism

TFAs may be produced naturally through metabolism or may be produced through industrial processing. TFAs from natural and industrial sources have different structures; the main 18-carbon TFAs used in this project are shown in table 1.1. Ruminant biohydrogenation of unsaturated FAs produces mainly *trans*-vaccenic acid (TVA; *trans*-11 18:1) and *cis*-9, *trans*-11 conjugated linoleic acid (CLA9,11) as intermediates during bacterial fermentation. In contrast, during catalytic hydrogenation of vegetable oils, ethylenic double bonds progressively disappear by being saturated by hydrogen. Before disappearing, their positions can shift along the FA chain (positional isomerization) and/or their geometry can change from *cis* to *trans* configuration and back, leading to various TFAs in the final product, although the main one is elaidic acid (EA; *trans*-9 18:1).

Table 1.1: Chemical structure of the 18-carbon TFAs of interest in this project

| TFA | Systematic Name | Structure |
|---|---|---|
| *trans* vaccenic acid; *trans*-11 18:1; TVA | *trans*-11-octadecenoic acid |  |
| Elaidic acid; *trans* 9 18:1; EA | *trans*-9-octadecenoic acid |  |
| Rumenic acid; *cis* 9, *trans* 11 conjugated linoleic acid; CLA9,11 | 9Z,11E-octadeca-9,11-dienoic acid |  |
| *trans* 10, *cis* 12 conjugated linoleic acid; CLA10,12 | 10E,12Z-octadeca-10,12-dienoic acid |  |

In a *trans* double bond, the two hydrogen atoms bound to the carbon atoms that form the double bond are located on opposite sides of the carbon chain. Unlike the *cis* isomeric configuration, the double bond angle of TFAs is smaller and the acyl chain is more linear, resulting in a more rigid and straight molecule with a higher melting point. The spatial structure of TFAs is between that of saturated FAs and *cis* unsaturated FAs (Valenzuela & Morgado, 1999). Consequently, the presence of *trans* double bonds causes the physical properties of the FA to be more similar to those of a saturated rather than an unsaturated FA. As with *cis* FAs, TFAs can be metabolized by oxidation, elongation, and desaturation processes (Chardigny et al, 2007). There is evidence that TVA can be converted into CLA9,11 in different human, mouse and ruminant tissues (e.g. adipose tissue, mammary glands) (Griinari et al, 2000; Mosley et al, 2006a; Mosley et al, 2006b; Santora et al, 2000; Turpeinen et al, 2002), while the metabolism of EA has been related to an increase in 18:3 TFAs (Krogager et al, 2015), and other unknown FAs. CLAs can also be desaturated and elongated to conjugated 18:3, 20:3 and 20:4 isomers (Banni et al, 2001; Juaneda & Sebedio, 1999; Sebedio et al, 1997).

TFAs can also be stored in adipose tissue and incorporated into membrane lipids, which would make the membranes less fluid and may influence membrane protein function and interactions and lipid raft formation, in turn affecting cell signalling processes (Calder, 2015a).

### 1.1.3 Sources, intake and recommendations of TFA

As indicated earlier, there are two main sources of TFAs in the diet. Quantitatively the main source of TFAs is usually partially hydrogenated vegetable oils; the process of hydrogenation is used to convert liquid oils into solid or semisolid fats, for example to produce margarine (Emken, 1984). The development of the hydrogenation process in the early 20th century led to the introduction of significant amounts of these iTFAs into the Western diet. During the course of the 20th century, the production of partially hydrogenated vegetable oils increased steadily because of their low cost, long shelf life, and suitability for commercial frying (Ascherio & Willett, 1997). The intermediate melting point of iTFAs provides favourable characteristics to food, such as texture and palatability, and greater stability (Ganguly & Pierce, 2015). Additionally, their use was extended during the last part of 20th century as food manufacturers needed a replacement to butter due

to health recommendations to reduce saturated fat and cholesterol intake (Remig et al, 2010). EA is the most predominant iTFA. Oleic acid (OA, *cis*-9 18:1 or 18:1n-9) is the most common monounsaturated fatty acid found in many vegetable oils. When exposed to partial hydrogenation, oleic acid changes its configuration and becomes EA. While EA is the prime form of iTFA, other fatty acids in *trans* form can also be found in processed food products (i.e. *trans* isomers of 18:2, 18:3 and 16:2) (Ganguly & Pierce, 2015). Some of the commonly eaten foods with a high amount of iTFAs are fried foods, fast foods, pastries, margarines, shortenings, cake mixes and many frozen meals and packaged foods (Ganguly & Pierce, 2015). Another way to contribute TFAs to the diet is through cooking processes, such as high temperature frying of vegetable oils as in fried processed and fast food (Tsuzuki et al, 2010). Trace amounts of TFAs are also produced during the process used to deodorize or refine vegetable oils (Tasan & Demirci, 2003).

The second main source of TFAs is the naturally occurring TFAs formed by bacterial isomerases capable of converting the double bonds of polyunsaturated fats in plants to a *trans* configuration in the stomachs of ruminant animals. These rTFAs are found in grass grazing sheep and cattle. Therefore, meats of these animals as well as milk and milk products (cheese and butter) contain rTFAs (Ganguly & Pierce, 2015). TFAs in ruminant milk and meats represent about 2% to 6% of the total fat content (Ratnayake et al, 2009). The major contributor within this kind of TFA is TVA, which constitutes 50–80% of all rTFAs (Field et al, 2009). CLAs are also found in ruminant products and are identified by two double bonds within the aliphatic chain separated by a single bond in between (Ganguly & Pierce, 2015). Rumenic acid (*cis*-9, *trans*-11 CLA; CLA9,11) accounts for 70-80% of the total CLA content in dairy and meat products. The amount of *trans*-10, *cis*-12 CLA (CLA10,12) is less than 5% of total CLA and less than 0.1% of total fat content (Kuhnt et al, 2016). It is estimated that in North America 10-25% of total TFAs consumed are from ruminant sources (Allison et al, 1999; Elias & Innis, 2002; Harris et al, 2012) , although this could be higher in some countries in Europe, like Denmark or Germany (Kuhnt et al, 2016).

It seems that the greatest contributor of TFAs in the Western diet is processed food products due to the high concentration of TFAs in partially hydrogenated vegetable oils, as high as 30 to 50%, compared to only 5-6% in dairy and ruminant meat products (Mendis et al, 2008).

The National Diet and Nutrition Survey performed in 2008-2009 in the United Kingdom showed an intake of less than 2 g/day of TFAs for all age groups (0.8% of daily energy intake for adults), reporting a decreased intake compared to the previous surveys of 1997 and 2000 (Pot et al, 2012). However, this survey did not assess the intake from takeaway food from independent outlets, and so might have underestimated TFA intake. The Framingham Offspring cohort, which consists of older, predominately white, American adults, also reported a decreased intake of TFAs over time (1991 – 1998), from 1.6% to 1.2% of daily energy intake, probably due to a reduction in margarine consumption (Vadiveloo et al, 2014).

Regarding the intake of natural TFAs, Hulshof and colleagues described an intake <2 g/day of rTFAs in all Western European countries in the TRANSFAIR study, also reporting that the main contribution of TFAs in the diet was from industrial sources (Hulshof et al, 1999).

In the 1990s, the iTFA intake reported in the US population was between 5 and 10 g/day, with an upper limit of 20 g/day, and that corresponded to approximately 2–5% of total energy in the diet (Allison et al, 1999; Enig et al, 1990). More recently, a decreased intake of iTFAs in the USA has been reported: going from 4.6 g/day in 2003 to 1.3 g/day in 2010, as a result of legislation and food labelling (Doell et al, 2012). Nevertheless, there are people who still show high intakes of iTFAs due to high consumption of processed and fast food, ranging from 3.5 to 12.5% of daily energy intake (Kris-Etherton et al, 2012).

Data on TFA intake usually rely on estimates of food consumption and the use of appropriate food composition tables. The estimates of food consumption need to be detailed given that the content of iTFAs in apparently similar foods may vary considerably in the same country and from time to time, depending on market prices of fats, and on the disposition of the food industry to reduce the content of iTFAs in their products. On the other hand, concentrations of rTFAs in ruminant products also vary in relation to the food given to animals and therefore with the seasons (Jakobsen et al, 2006). However, this variation in gram per serving is much lower than the variation observed for iTFAs. As a consequence, TFA intake estimations should be interpreted with caution and preferably be supported by analysis of food portions and measurements of biomarkers for intake, such as TFAs in human plasma or tissues like erythrocytes, subcutaneous fat, and human milk (Stender et al, 2008).

Another problem with estimating the amount of dietary TFAs consumed occurs because of the differences in food labelling legislation between countries. For example, the presence of small amounts of TFAs in oils and hydrogenated or partially hydrogenated food products could generate an intake above the recommended limits and this could be exacerbated by labelling rules. In the USA, the Food and Drug Administration allows products containing <0.5 g of *trans* fat per serving to indicate a contribution of 0 g of *trans* fat. If a person consumes several products that contribute almost 0.5 g of *trans* fats per serving during the day, they could easily exceed the recommendation of less than 1% of daily energy intake (approximately 2 g of TFAs in a 2000 Kcal/day based diet), while labels indicate that they are free of *trans* fats (Ringseis et al, 2006).

Until recently the European Union did not have legislation regulating the content of *trans* fats in food products or specific requirements about their labelling. In April 2019, the European Commission decided to limit the amount of iTFA in food products to 2 g per 100 g of fat. The deadline to comply with the European regulation is 1st April 2021, after which products with higher amounts of iTFA will not be allowed to be placed on market (EUCommission, 2019). It is still unclear if the exact amount of iTFAs will be required to be included in the nutrition label of pre-packed products. Meanwhile, several European countries have made voluntary agreements with the food industry to reduce the amount of TFAs in foods; the United Kingdom is one of them.

Due to the overall negative effects of TFAs on health, it is currently recommended that these fatty acids contribute <1% of daily energy intake; in fact their poor health associations suggest that TFA intake should be as low as possible (2002; Eckel et al, 2007; Kris-Etherton et al, 2007; Lichtenstein et al, 2006). It is important to note that these recommendations do not distinguish the type of TFA (ruminant versus industrial), when there is evidence that the health effects of each type could be different (see later sections).

### 1.1.4 Concentration of TFA in human tissues

The observational studies that first described the health risks associated with TFAs measured consumption through food intake surveys, mainly focused on partially hydrogenated vegetable oils, but there are many problems with establishing TFA intake accurately, as described in the previous section. Instead, TFA levels in tissues or in plasma

may be good biomarkers of dietary intake. Nevertheless, there are not many studies describing TFA levels in representative samples of populations in different health conditions, and because of that the interpretation of these data are difficult, with no reference values of normal or desirable TFA levels to compare (Abdelmagid et al, 2017).

Table 1.2 shows a summary of the studies published reporting absolute concentrations of plasma 18-carbon TFAs in healthy subjects. The mean plasma concentration of EA, the main iTFA of the diet, was 32.5 µM in young Canadian adults measured in 2010 (Abdelmagid et al, 2017) and 13.5 µM in middle age adults from the USA, measured between 2009 and 2010 (Vesper et al, 2017). In contrast, the average levels of TVA were lower in young compared to the middle aged adults (9.41 vs 18.2 µM, respectively), which reflects differences in food intake patterns, with a lower intake of dairy and higher intake of processed foods in the group of younger adults.

Table 1.2: Selection of reported 18-carbon TFA concentrations in human plasma

| TFA | Concentration (µM) (mean ± SD) | Subjects, sample and methods | Reference |
|---|---|---|---|
| EA | 16.5 ± 11.3 | 826 healthy young Canadian males and females ethno-culturally diverse (Caucasian, East Asian and South Asian Canadians)<br>Age : 22.6 ± 2.5 y<br>BMI : 22.8 ± 3.4 kg/m$^2$<br>61 FAs (C14 – C24) measured in plasma total lipids by gas chromatography (fasting blood samples) | (Abdelmagid et al, 2015) |
| TVA | 14.0 ± 8.1 | | |
| CLA9,11 | 14.4 ± 6.2 | | |
| CLA10,12 | 4.3 ± 2.5 | | |
| EA | 32.5 ± 22.1 | 1294 healthy young Canadian males and females, part of the cross-sectional Toronto Nutrigenomics and Health Study, recruited between<br>September 2004 and November 2010.<br>Age: 22.7 ± 2.5 y<br>BMI: 22.9 ± 3.5 kg/m$^2$<br>TFAs measured in plasma total lipids by gas chromatography (fasting blood samples)<br>*Values shown correspond to 2010 cohort (n=197) | (Abdelmagid et al, 2017) |
| TVA | 9.41 ± 6.14 | | |
| CLA9,11 | 17.5 ± 9.9 | | |
| CLA10,12 | 6.3 ± 4.3 | | |
| EA | 33.4[a] → 13.5[b] | [a]1999-2000: 1613 subjects, mean age 42 y, mean BMI=26.7 kg/m$^2$<br>[b]2009-2010: 2462 subjects, mean age 46 y, mean BMI=27.8 kg/m$^2$<br>Adult US population, multi ethnic.<br>4 major TFAs measured (palmitoelaidic, TVA, EA and linoelaidic acid) by gas chromatography coupled with mass spectrometry (fasting blood samples). | (Vesper et al, 2017) |
| TVA | 37.9[a] → 18.2[b] | | |

Abbreviations: TFA, trans fatty acid; EA, elaidic acid; TVA, trans vaccenic acid; CLA9,11, cis-9, trans-11 conjugated linoleic acid; CLA10,12, trans-10, cis-12 conjugated linoleic acid; BMI, body mass index; FAs, fatty acids.

In relation to CLAs, both studies in Canadian young adults showed similar plasma levels, 14.4 vs 17.5 µM average for CLA9,11. For CLA10,12 the average was reported as 4.3 and 6.3 µM in plasma, respectively. Another study measured total CLA level (i.e. CLA9,11 + CLA10,12) in 750 middle age European adults, reporting circulating plasma phospholipid levels, with an average of 11.2 ± 1.8 µM (Matejcic et al, 2018).

Studies that measure plasma and tissue levels of TFAs as absolute concentrations in healthy and non-healthy subjects are needed to establish reference values that allow to determine when reported levels are normal/safe or not, and to be able to associate TFA levels with their beneficial or detrimental effects in a better way.

Decreased reported intakes of TFAs together with public and private efforts to reduce partially hydrogenated oils in food products in different countries, as described earlier,

have led to decreased total and individual levels of TFAs measured in plasma over time in healthy subjects (Hulshof et al, 1999; Ratnayake et al, 2009; Vadiveloo et al, 2014; Vesper et al, 2017). Despite this, there are still groups in the population with very high intakes of TFAs, which are reflected in the highest plasma TFA concentrations reported by Abdelmagid et al. (2015): 88 µM for EA, 74 µM for TVA, 42 µM for CLA9,11 and 18 µM for CLA10,12. As shown in Figure 1.2, changes in plasma 18-carbon TFA concentrations over time are also isomer specific. This cross-sectional cohort study shows a consistent decrease in plasma concentrations of most of the common 18-carbon TFAs from 2004 to 2009, although EA increased back to the initial levels in 2010, suggesting that this group of young Canadian adults might be vulnerable to iTFA exposure. Monitoring food iTFA levels in vulnerable populations is still needed (Abdelmagid et al, 2017).

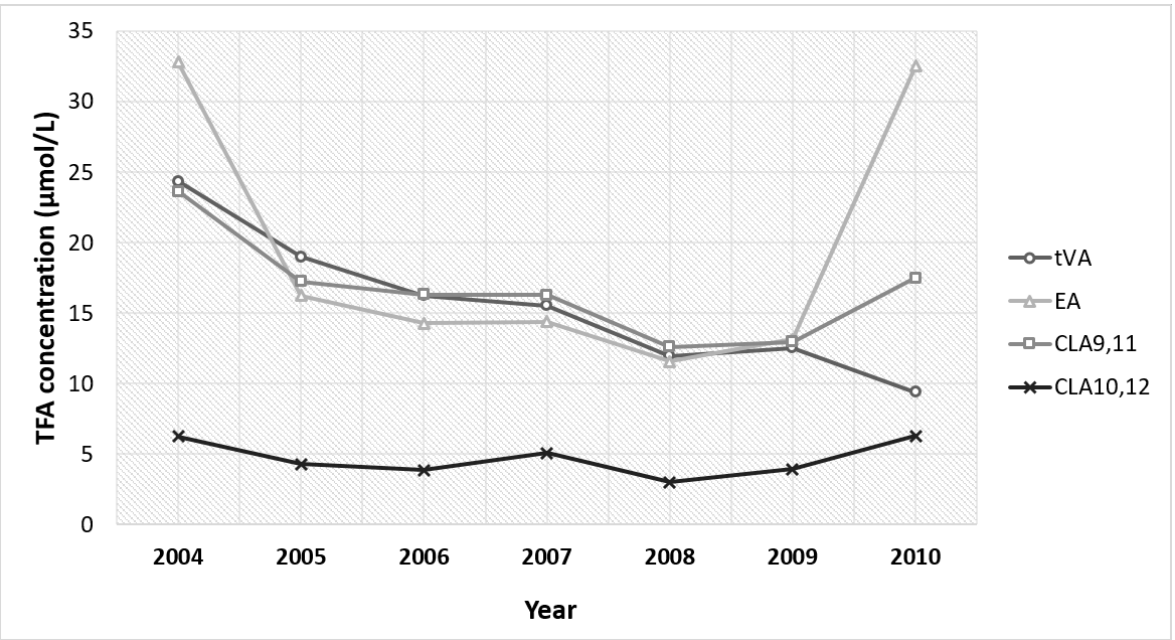



Figure 1.2: Concentrations (µmol/L) of selected 18-carbon TFAs (TVA, EA, CLA9,11 and CLA10,12) in plasma by year in young Canadian adults. Data taken from (Abdelmagid et al, 2017) with permission.

## 1.2 Inflammation and atherosclerosis

### 1.2.1 Inflammatory response in CVD

Inflammation is a component of innate immunity, and it is part of the body's response to injury or infection. The response includes an increase in blood flow, capillary dilatation, leukocyte infiltration and the localised production of chemical mediators. Crucial early steps in the inflammatory response are an increased supply of blood to the site of inflammation and an increase in vascular wall permeability that allows large molecules and cells (leukocytes, or white blood cells) to cross the endothelium. These newly arrived and activated leukocytes then express chemical mediators at the site of inflammation. Increased mediators include adhesion molecules (intercellular adhesion molecule 1, ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin on the surface of endothelial cells (ECs), causing the binding and diapedesis of leukocytes (granulocytes, monocytes and lymphocytes). Different cytokines are also produced (TNF-α, IL-1, IL-6 and IL-8), as are nitric oxide (NO), matrix metalloproteinases (MMPs), eicosanoids (prostaglandins, thromboxanes, leukotrienes, endocannabinoids, lipoxins, eoxines and others), and other products, for example reactive oxygen species (ROS, e.g. superoxide anion, hydrogen peroxide), conditional to the cell type involved, type of inflammatory stimulus, the anatomical site involved, and the phase during the inflammatory response (Calder et al, 2013; Calder et al, 2009). The inflammatory mediators are responsible for local tissue damage, systemic effects on the central nervous system, stimulation of proteolysis in skeletal muscle and lipolysis in adipose tissue, synthesis of acute phase proteins in the liver, and destruction/elimination of pathogens and toxic agents. Following the acute phase of the inflammatory process, it undergoes resolution and recovery. In fact, inflammation is usually self-limiting and is often resolved quickly due to the stimulation of negative feedback mechanisms that oppose the inflammatory signal (secretion of anti-inflammatory cytokines or pro-resolving lipid mediators, inhibition of pro-inflammatory signalling cascades, activation of regulatory cells, etc). Loss of these regulatory processes can result in excessive, inappropriate or on-going inflammation that can cause irreparable damage to host tissues (Calder, 2015b; Galli & Calder, 2009; Minihane et al, 2015). This is the case in different chronic conditions, including inflammatory diseases like arthritis, but also "lifestyle" diseases such as obesity, CHD,

type 2 diabetes mellitus, and non-alcoholic fatty liver disease (NAFLD), that are characterized by a chronic low-grade inflammatory state (Gregor & Hotamisligil, 2011; Hotamisligil, 2006).

CVDs are the leading cause of death globally. Many risk factors have been described to drive the development of CVDs, including dyslipidaemia, arterial hypertension, smoking, age, male gender, diabetes mellitus, sedentary lifestyle and stress. The most common CVDs are cardiac ischemia and cerebrovascular disease, and atherosclerosis has a key role in the pathogenesis of both of them. Hypercholesterolemia and high blood pressure were considered the main promoters of atherosclerosis for many years. Now there is increasing evidence showing that chronic inflammation is also a key factor in the aetiology of atherosclerosis, which has been described as an unresolved inflammatory condition, missing the shift from pro-inflammatory to anti-inflammatory mediators that features the resolution phase of inflammation (Viola & Soehnlein, 2015).

In atherosclerosis development, leukocyte recruitment to the sub-endothelial compartment of impaired arteries starts a sequence of events mediated by leukocyte-derived chemokines and cytokines that propagate atherosclerosis through increased inflammatory mediator production and expression of endothelial adhesion molecules, perpetuating leukocyte recruitment; promoting lipid-laden foam-cell formation; stimulating the proliferation of smooth muscle cells resulting in plaque formation and accumulation and ultimately inducing plaque instability and eventual rupture (Glass & Witztum, 2001; Hallenbeck et al, 2005; Hansson, 2005; Ross, 1999). The subsequent thrombosis also depends on the inflammatory status of the ruptured plaque (Hallenbeck et al, 2005; Minihane et al, 2015).

### 1.2.2 Atherosclerosis

Atherosclerosis is a lipoprotein-driven disease that leads to plaque formation at focal areas in the arterial blood vessels through intimal inflammation, necrosis, fibrosis, and calcification (Usman et al, 2015).

The arterial wall is composed of three layers: intima (inner layer), media (intermediate layer), and adventitia (external layer). Intima comprises a single layer of ECs, thin basal membrane, and a subendothelial layer of collagen fibres. Media is formed by smooth

muscle cells (SMC) and a network of elastin and collagen fibres. Adventitia entails mostly loose connective tissue. Atherosclerosis is characterized by the formation of atherosclerotic plaques in the subendothelial layer, SMC proliferation, accumulation of activated immune cells, and thickening of adventitia at the site of plaque formation (Fatkhullina et al, 2016). Figure 1.3 shows the immune cells, events and features of the atherosclerotic plaque.

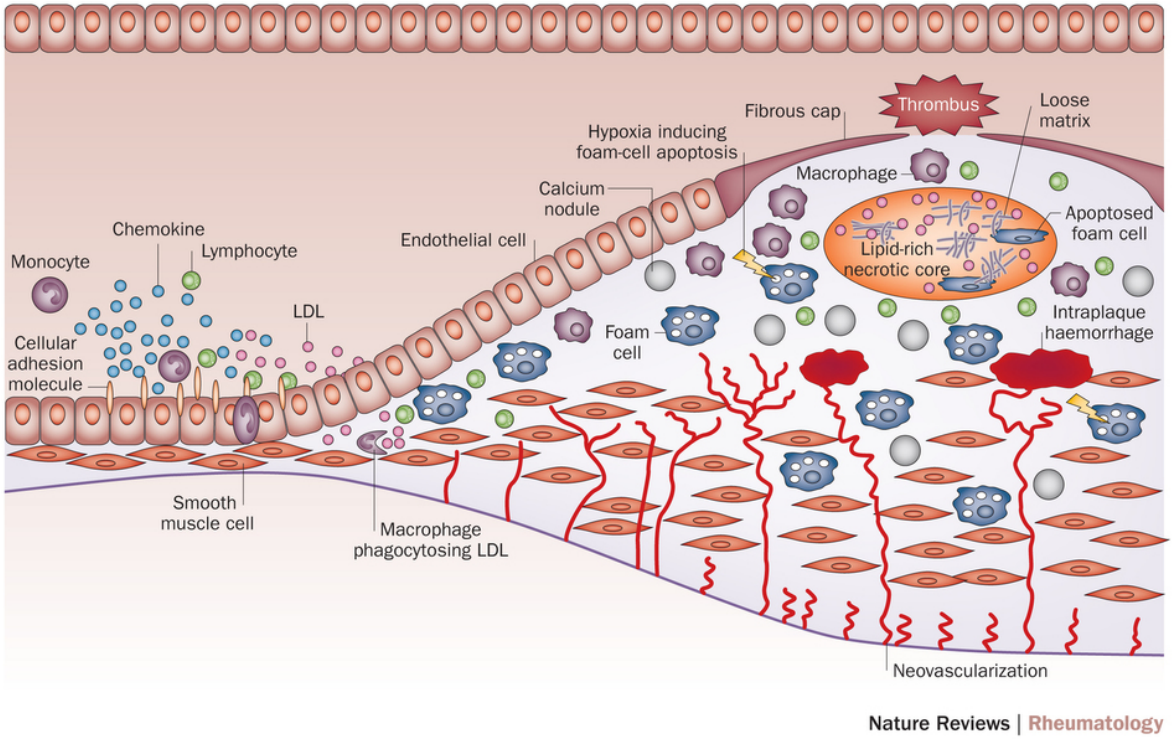



Figure 1.3: Development of the atherosclerotic plaque (Skeoch & Bruce, 2015).

Several immune cells are normally present in the arterial wall; however, their number increases significantly during atherosclerosis progression. Under normal conditions, immune cells migrate into the arterial wall and return to the circulation. In the early stages of atherosclerosis, a high concentration of low-density lipoproteins (LDL) in the plasma promotes LDL accumulation in the arterial wall and their oxidation into oxLDL, which stimulate both the innate and adaptive immune responses (Hansson, 2005; Tedgui & Mallat, 2006). Together with high blood pressure this activates ECs, promoting the expression of adhesion molecules that stimulate the migration of monocytes into the arterial wall. Monocytes roll on the ECs overlying retained LDL and adhere firmly through the interaction of integrins with EC ligands (Mestas & Ley, 2008; Usman et al, 2015). Platelet aggregation on the endothelium overlying the atherosclerotic plaque also activates the expression of adhesion molecules through the deposition of platelet-derived

chemokines on activated endothelium (Koenen et al, 2009). These leads to the entry of monocytes into the subendothelial space, where they secrete lipoprotein binding proteoglycans (such as heparin sulfate, chondroitin sulfate, dermatan sulfate and keratin sulfate) resulting in more accumulation of modified LDL, sustaining inflammation (Tabas, 2010). In the intima, monocytes can differentiate into macrophages or denditric cells under the influence of macrophage colony stimulating factor. Then, the macrophages engulf oxLDL through scavenger receptors and when the uptake exceeds efflux, lipids accumulate resulting eventually in the formation of lipid-laden foam cells. The accumulation of LDLs by macrophages promotes further growth and destabilization of the plaque by the secretion of cytokines and chemokines, generation of ROS, presentation of immune activation markers to lymphocytes or macrophage scavenger receptor, production of matrix degrading proteases and secretion of inflammatory debris into the plaque core following necrosis (Usman et al, 2015).

As inflammation progresses, macrophages and ECs secrete several peptide growth factors which act as fibrogenic mediators, promoting SMC to proliferate and migrate to the site of early lesion, generating the formation of a collagenous fibrous cap. Macrophages congregate in the central core of the atherosclerotic plaque, where they eventually die by apoptosis forming a necrotic core. With the progression of the lesion, the underlying inflammatory processes act to simultaneously decrease collagen production and increase the activity of MMPs, which degrade the dense extra-cellular matrix of the lesion cap. This can result in plaque erosion or rupture, leading to thrombus formation, vessel blockage and cardiovascular complications, such as myocardial infarction and stroke (Hansson et al, 2015).

### 1.2.3 Inflammatory signalling pathways in atherosclerosis

#### 1.2.3.1 Cytokines

Cytokines are a diverse group of protein mediators, involved in many physiological processes and playing a key role in acute and chronic inflammation. Cytokines are a varied group of molecules that include over 100 secreted factors that can be classified into several classes: interleukins, tumour necrosis factors, interferons, transforming growth factors, colony-stimulating factors, and chemokines. Cytokines are secreted by different types of cell (i.e. T cells, monocytes, macrophages, platelets, ECs, SMCs, and

adipocytes) in response to inflammation and other stimuli. The regulation of their production depends on many factors and is tightly controlled. Many cytokines are expressed in atherosclerotic plaques and all cells involved in the disease are capable of producing cytokines and responding to them. The biological effects of pro-inflammatory cytokines that may account for their pro-atherogenic activity are multiple (see table 1.3). In the early stages of atherosclerosis, cytokines can alter endothelial functions. TNF-α and IFN-γ have been shown to alter the distribution of vascular endothelial-cadherin-catenin complexes and prevent the formation of F-actin stress fibers (Kleinbongard et al, 2010), resulting in restructuring of the intercellular junctions, leading to loss of barrier function and facilitating leukocyte transmigration. Cytokines also induce the expression of chemokines and adhesion molecules on the vascular endothelium, thus favouring the recruitment, adherence, and migration of lymphocytes and monocytes into the inflamed vessel wall (Weber et al, 2008). In the intima, leukocytes can be permanently activated by locally produced cytokines, which accelerate the conversion of macrophages into foam cells by increasing the expression of scavenger receptors and promoting cell-mediated oxidation. IFN-γ can induce foam cell formation through upregulation of the scavenger receptor for phosphatidylserine and oxLDL, which has been involved in oxLDL uptake and subsequent foam cell transformation of macrophages (Shashkin et al, 2005). Cytokines also affect the function of SMC by promoting their growth, proliferation, and migration (Hansson et al, 2015). At later stages of atherosclerosis, pro-inflammatory cytokines promote destabilization of atherosclerotic plaques, apoptosis of different cells, and matrix degradation, thus accelerating plaque rupture and thrombus formation (Hansson et al, 2015; Tabas et al, 2015). Several pro-inflammatory cytokines have been shown to induce SMC and macrophage apoptosis, particularly IL-1, TNF-α, and IFN-γ and promote Fas–Fas ligand killing (Stoneman & Bennett, 2009). Pro- and anti-inflammatory cytokines significantly affect the expression of MMPs and their inhibitors, acting together with other cytokines, growth factors and oxidized lipids, inducing a significant remodeling of many components of the extracellular matrix (Newby, 2005).

Lastly, cytokines can extensively alter the antithrombotic properties of EC. IL-1 and TNF-α can increase the tissue pro-coagulant activity and suppress the anticoagulant activity mediated by the thrombomodulin-protein C system by decreasing the transcription of thrombomodulin and protein C receptor genes (Esmon, 2004). Downregulation of

anticoagulant mediators may in turn affect inflammation. Pro-inflammatory cytokines can also modify the fibrinolytic properties of ECs, increasing the production of type I plasminogen activator inhibitor and reducing the production of tissue plasminogen activator (Kruithof, 2008), which could precipitate in thrombus formation and the development of acute coronary syndromes.

Table 1.3: Summary of key inflammatory mediators involved in atherosclerosis

| Mediator | Abbreviation | Source | Target cells and Effects Relevant to Atherosclerosis |
|---|---|---|---|
| **Interleukin 6** | IL-6 | T cells, B cells, macrophages, fibroblasts, SMCs, ECs | Proliferation and migration of smooth muscle cells via mediating VEGF and TNF-α effects |
| **Interleukin 8** | IL-8 | Macrophages, SMCs, ECs, monocytes, T cells | Induces chemotaxis in target cells and is a promoter of angiogenesis |
| **Interleukin 1** | IL-1 | Macrophages, lymphocytes, SMCs, ECs | Stimulates endothelial and smooth muscle cells through NFκB and MAPK pathways (Tedgui & Mallat, 2006) |
| **Interferon γ** | IFN-γ | Th1 cells, natural killer cells, SMCs | Promotes Th1 immune response/secretion of Th1-associated cytokines, inhibits extracellular matrix synthesis by smooth muscle cells (Tedgui & Mallat, 2006) |
| **Tumour necrosis factor α** | TNFα | T cells, B cells, monocytes, macrophages, SMCs, natural killer cells | Stimulates expression of ICAM-1, VCAM-1, E and P-selectin and MCP-1 through NFκB pathway (Ohta et al, 2005) and thus promotes chemotaxis and adhesion |
| **TNF related apoptosis-inducing ligand** | TRAIL | T cells, B cells, monocytes, macrophages, SMCs, natural killer cells | Promotes smooth muscle cell proliferation and neointima formation after arterial injury (Chan et al, 2010) |
| **Vascular endothelial growth factor** | VEGF | ECs, macrophages, platelets | Stimulates proliferation and survival of endothelial cells and promotes angiogenesis and vascular permeability (Duffy AM) |
| **Monocyte chemoattractant protein-1** | MCP-1 | ECs, fibroblasts, SMCs | Chemoattractant and activates monocytes and T cells. Responsible for the transmigration of monocytes into the intima at sites of lesion formation (Szmitko et al, 2003). |

| Mediator | Abbreviation | Source | Target cells and Effects Relevant to Atherosclerosis |
|---|---|---|---|
| **Regulated on activation, normal T expressed and secreted** | RANTES/CCL5 | Expressed in atherosclerotic plaque, adipose tissue | Chemoattractant towards monocytes and memory T cells. Adhesion of monocytes to the luminal surface of the endothelium (Moore et al, 2013). |
| **Intercellular adhesion molecule 1** | ICAM-1 | ECs, leukocytes | Adhesion of monocytes to the luminal surface of the endothelium (Moore et al, 2013). |
| **Vascular cell adhesion molecule 1** | VCAM-1 | ECs | Mediation of leukocyte-endothelial cell adhesion and signal transduction as well as augmenting the production of ICAM-1. It may also have a role in the monocyte transmigration into atherosclerotic plaques (Moore et al, 2013). |
| **E-selectin** | E-selectin | ECs | Rolling and recruitment of leukocytes (Moore et al, 2013). |
| **P-selectin** | P-selectin | ECs Weibel-Palade bodies, platelets | Rolling and recruitment of leukocytes (Moore et al, 2013). |
| **L-selectin** | L-selectin | Leukocytes (neutrophils, monocytes, subsets of T cells) | Tethering, rolling of leucocytes (Pant et al, 2014) |
| **Platelet and endothelial cell adhesion molecule** | PECAM-1 | Leukocytes, ECs | Stimulates diapedesis of leucocytes (Pant et al, 2014) |

Abbreviations: SMCs, smooth muscle cells; ECs, endothelial cells; NFκB, nuclear factor kappa B; MAPK, mitogen-activated protein kinase.

#### 1.2.3.2 Chemokines

Chemokines are chemotactic cytokines that are divided into subgroups based on the position of the amino terminal cysteine residues (CC, CXC, CX3C, XC) (Koenen et al, 2009; Weber et al, 2008; Zernecke & Weber, 2014). In physiological circumstances, ECs in the intima layer of the vasculature are both anti-thrombogenic and anti-adhesive, allowing the free circulation of blood and its components. Nevertheless, in an inflammatory state the endothelium is rendered adhesive for circulating leukocytes, initiating the process of leukocyte migration (Yadav et al, 2003). Chemokines play a crucial role in the trafficking of leukocytes by the activation of heterotrimeric G proteins and associated intracellular signalling pathways (Koenen et al, 2009; Weber et al, 2008). Monocyte recruitment

occurs in the following main phases: capture and rolling; arrest; and extravasation (see figure 1.4). The capture and rolling phase for Lys6Chigh monocytes implicates immobilization of CCL5 and CXCL1 on proteoglycans and P-selectins on ECs. These chemokines interact with receptors expressed on the surface of invading monocytes. For monocyte adhesion to ECs, the binding of adhesion molecules on ECs to integrins on monocytes is required: VCAM-1 to integrin a4b1 and ICAM-1 to αLβ2 (lymphocyte function-associated antigen 1 or LFA1). Monocyte transmigration through the endothelium is mediated by chemokines produced by ECs, SMCs in the plaque and emigrated leukocytes along with VCAM-1 and PECAM-1 (Ley et al, 2007; Moore et al, 2013; Soehnlein et al, 2013).

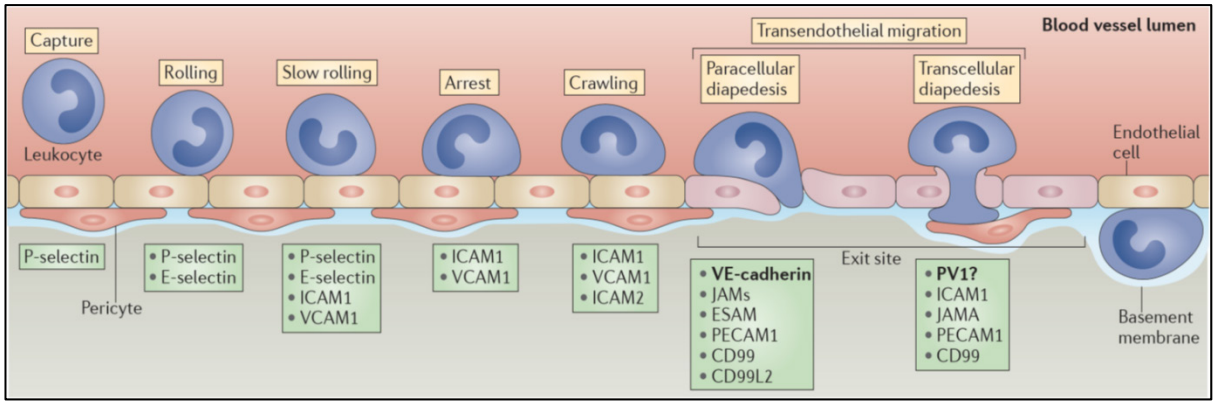



Figure 1.4: Leukocyte vascular migration cascade (Vestweber, 2015).

Abbreviations: CD99L2, CD99 antigen-like protein 2; ESAM, endothelial cell-selective adhesion molecule; ICAM, intercellular adhesion molecule; JAM, junctional adhesion molecule; LFA1, lymphocyte function-associated antigen 1; PECAM1, platelet endothelial cell adhesion molecule 1; PV1, plasmalemma vesicle protein 1 ; VCAM1, vascular cell adhesion molecule 1 ; VE, vascular endothelial.

#### 1.2.3.3 NFκB signalling pathway

The nuclear factor kappa B (NFκB) family of transcription factors are key regulators of inflammatory responses; innate and adaptive immunity; and cellular differentiation, proliferation, and survival in most multicellular organisms. The NFκB signalling system, defined by the interactions between NFκB dimers, inhibitor of NFκB (IκB) regulators, and IKK complexes, is activated by diverse stimuli, including physical, physiological and oxidative stresses (O'Dea & Hoffmann, 2009; 2010).

In mammals, the NFκB network consists of five family member protein monomers (p65/RelA, RelB, cRel, p50, and p52) and its activation is involved in inflammatory responses and regulation of genes encoding pro-inflammatory proteins including cytokines, chemokines, and adhesion molecules (Mitchell et al, 2016).

Inactive NFκB is a trimer localised within the cytosol, where NFκB dimers are bound with an inhibitory subunit (IκBα) (see Figure 1.5). NFκB is activated as a result of a signalling cascade triggered by extracellular inflammatory stimuli, such as cytokines and microbial components, through multiple mechanisms: degradation of IκB inhibitor proteins, processing of NFκB precursor proteins, and expression of NFκB monomer proteins (Huxford et al, 2011; O'Dea & Hoffmann, 2010). In general, IκB kinases are stimulated and phosphorylate IκBα, liberating NFκB dimers to migrate to the nucleus and interact with κB promoter regions, initiating the gene transcription of pro-inflammatory proteins such as TNF-α, ICAM-1, VCAM-1 and IL-1, among others.

The NFκB system is tightly regulated, and its dysregulation has been implicated in the development of diseases ranging from cancers to inflammatory and immune disorders.

It has been described that omega-3 FAs act on the NFκB pathway to decrease expression of adhesion molecules and production of inflammatory cytokines and COX-2 metabolites (Calder, 2013b). In contrast, different studies have showed that iTFA can induce pro-inflammatory responses, such as increasing the secretion of cytokines and promoting leukocyte adhesion to the endothelium, through NFκB activation (Bryk et al, 2011; Harvey et al, 2012; Pan et al, 2017; Rao & Lokesh, 2017).

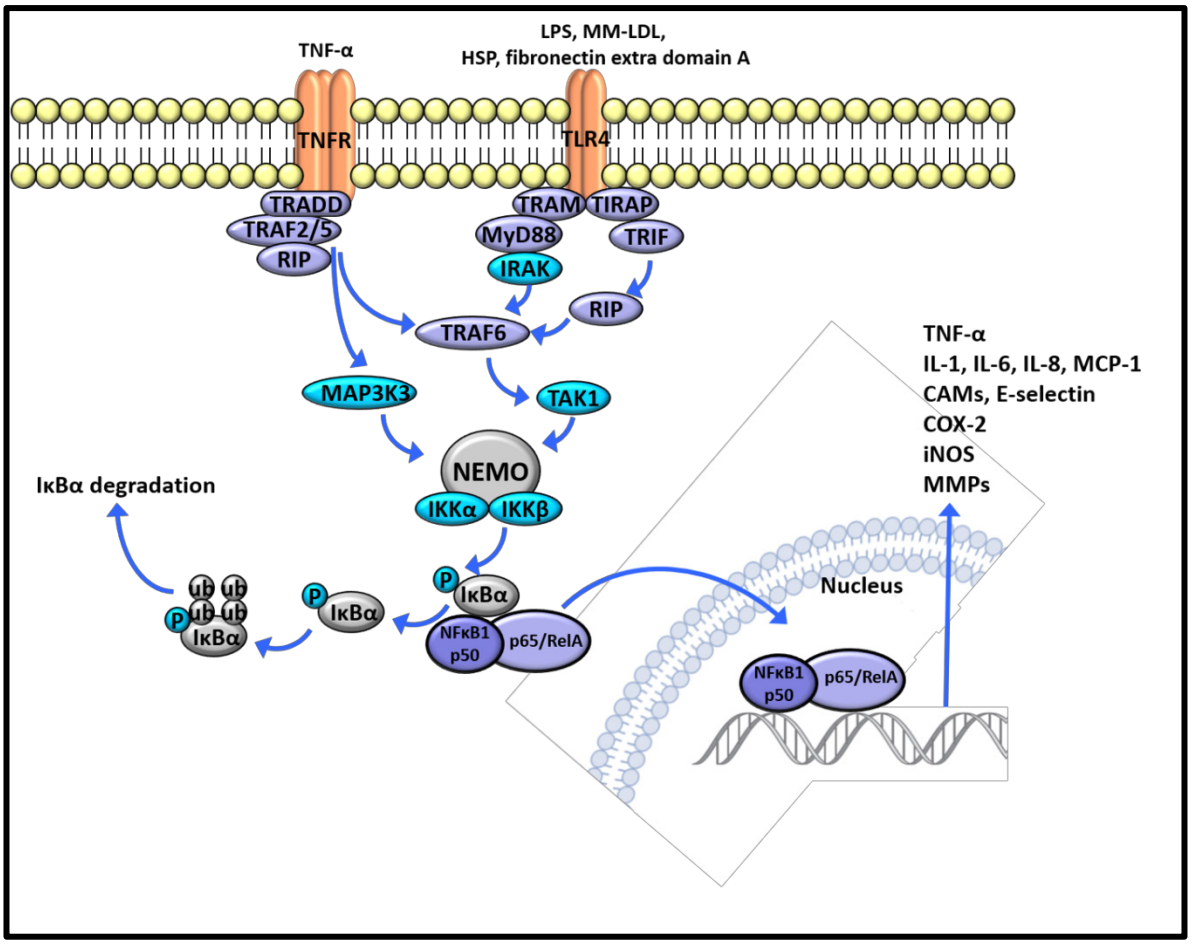



Figure 1.5: NFκB and TLR4 signalling pathways (adapted from (Calder et al, 2009)).

Abbreviations: LPS, lipopolysaccharide; MM-LDL, minimally modified low density lipoprotein; HSP, heat shock protein; TLR4, toll like receptor 4; MyD88, myeloid differentiation primary response 88; TRIF, MyD88 toll/IL-1 receptor homology domain–containing adapter inducing IFN-β; TIRAP, MyD88-adapter–like (Mal)/toll/IL-1 receptor homology domain–containing adapter protein; TRAM, TRIF-related adapter molecule; IRAK, interleukin-1 receptor-associated kinase; RIP, receptor-interacting protein kinases; TRAF, TNF receptor-associated factor; TNF-α, tumour necrosis factor alpha ; TNFR, tumour necrosis factor receptor; TRADD, tumour necrosis factor receptor type 1-associated death domain; MAP3K3, mitogen-activated protein kinase kinase 3; TAK1, transforming growth factor beta-activated kinase 1; NEMO, NFκB essential modulator; IKK, inhibitor of NFκB kinase complex; IκBα, inhibitor of NFκB alpha; NFκB, nuclear factor kappa-B; Ub, ubiquitin; P, phosphate; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; CAMs, cellular adhesion molecules; COX-2, cyclooxygenase 2; iNOS, inducible nitric oxide synthase; MMPs, matrix metalloproteinases.

#### 1.2.3.4 TLR signalling pathways

The primary defence mechanism against infections in mammals consists of a rapid innate immune response based on the recognition of molecular patterns in microbial pathogens. This immunological response is mediated by pattern recognition receptors (PRRs) in host cells that bind to microbial products and trigger a fast response. Endogenous oxidized lipids can activate the same pattern recognition receptors involved in antimicrobial

immunity in the arterial wall (Miller et al, 2011). Toll-like receptors (TLRs) are the best characterized PPRs of the innate immune system.

TLRs are type I transmembrane receptors and belong to the TLR/IL-1 receptor superfamily. The signalling outcome of TLRs can depend on the subcellular localization where the ligands engage the receptor. Each TLR can be stimulated by specific sets of pathogen-associated molecular patterns (Kawai & Akira, 2010). On ligand binding and receptor activation, the signal is transmitted via 4 different adapter molecules: MyD88, toll/IL-1 receptor homology domain–containing adapter inducing IFN-β (TRIF), MyD88-adapter–like (Mal)/toll/IL-1 receptor homology domain–containing adapter protein (TIRAP) and TRIF-related adapter molecule (TRAM).

As shown in figure 1.5, TLR4 is the only TLR that uses all 4 adaptors and activates both the MyD88- and TRIF-dependent pathways. TLR4 initially recruits TIRAP at the plasma membrane and subsequently facilitates the recruitment of MyD88 to trigger the initial activation of NFκB and MAPK45 to induce inflammatory cytokines (Akira et al, 2006; Alexopoulou et al, 2001). TLR4 subsequently undergoes dynamin-dependent endocytosis and is trafficked to the endosome, where it forms a signalling complex with TRAM and TRIF, a pathway that leads to IRF3 activation as well as the late-phase activation of NFκB and MAPK (Kagan et al, 2008; Tanimura et al, 2008).

TLRs are extensively recognized as important mediators of mammalian immune responses, capable of recognizing a wide range of exogenous and endogenous molecules, including bacterial lipopolysaccharide (LPS) and oxLDL, among others (Bae et al, 2009; Stewart et al, 2010). Once activated, these receptors initiate an acute inflammatory response through the induction of numerous pro-inflammatory mediators, such as cytokines and chemokines.

Initial studies in mice reported that the deletion of TLR4 protected the experimental animals from hypercholesterolemia-induced atherosclerosis (Michelsen et al, 2004). Consistently, single nucleotide polymorphisms in the TLR4 gene that impair TLR signalling have been related to decreased risk of atherosclerosis and ischemic events (Frantz et al, 2007).

TLR4 is expressed in human blood vessels and in atherosclerotic lesions its expression is increased (Otsui et al, 2007; Vink et al, 2002; Xu et al, 2001).

Some studies have suggested that TFAs induce inflammation in HUVECs by activation of TLR4 signalling pathway (Kondo et al, 2015; Pan et al, 2017)

## 1.3 Association of TFA and CVD

Several studies have reported that intake of TFAs correlates to higher risk of CHD. A meta-analysis of prospective cohort studies by Mozaffarian and colleagues indicated that a 2% absolute increase in energy intake from TFAs, equivalent to 4 g daily in a 2,000 kcal diet, was associated with a 23% increase in cardiovascular risk (Mozaffarian et al, 2006) (see figure 1.6).

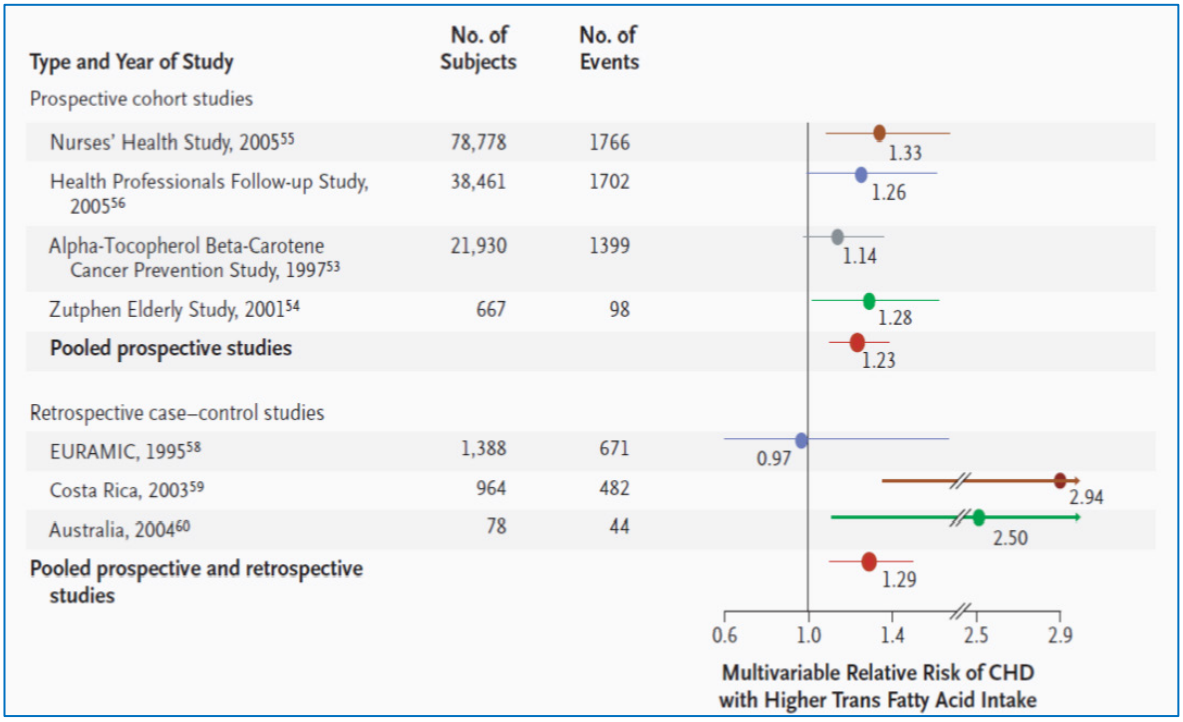



Figure 1.6: Multivariable adjusted relative risk of CHD associated with TFA intake (Mozaffarian et al, 2006).

The Nurses' Health Study showed that the highest quintile of TFA intake had a relative risk of coronary heart disease (CHD) 1.5 times greater when compared to the lowest quintile, after adjustment for age and total energy intake (Willett et al, 1993), and that higher risk was maintained over time (Hu et al, 1997; Oh et al, 2005). A nested case–control study in the same cohort was conducted using measurement of TFA levels in plasma and red blood cells, showing that higher total erythrocyte TFA content was also associated with higher CHD risk, with a relative risk 3.3 times greater in the quartile with the highest TFA

level compared to the lowest (Sun et al, 2007). Regarding the type of TFA, in the first report from the Nurses' Health Study in 1993, when total intake of TFA was partitioned into industrial and ruminant sources, the positive association with CHD risk was entirely explained by iTFAs (Willett et al, 1993).

In the Boston Health study, the intake of TFAs was directly related to the risk of myocardial infarction, with a relative risk of 2.44 in the cases compared to the controls, after adjustment for age, gender and total energy (Ascherio et al, 1994), and this risk was completely accounted for by iTFAs rather than rTFAs.

Another study in 21,930 Finnish men, showed that total TFA consumption was strongly correlated with intake of margarine but not with butter, and the estimated intake of iTFAs was positively associated with risk of CHD. In contrast, estimated intake of TFAs from ruminant sources was inversely associated to CHD, with a relative risk of 0.83 of the highest compared to the lowest quintile of intake (Pietinen et al, 1997).

In the Danish Cohort studies, where 3,686 adults were followed for 18 years, no significant associations were found between rTFA intake and risk of CHD, but among women indications of inverse associations between the absolute rTFA intake and the risk of CHD were described (Jakobsen et al, 2008).

One previous study, however, indicated a positive association between energy-adjusted rTFA intake and risk of CHD among men. Oomen and colleagues reported that for each 0.5% of energy, the fully adjusted relative risk of coronary heart disease for rTFAs, industrial 18:1 TFAs, and other industrial TFA intake was similar in the Dutch elderly men cohort (Oomen et al, 2001).

In summary, the evidence from observational studies suggests that higher CHD risk is related to consumption of industrially produced TFAs rather than rTFAs.

The cardiovascular risk associated with TFA can be explained, at least in part, by their effects on lipoproteins such as LDL and high-density lipoprotein (HDL) cholesterol, as well as inflammatory mechanisms. The effects of TFA consumption that are consistently seen in both controlled trials and observational studies comprise adverse lipid effects, including increased fasting TAGs (Mozaffarian & Clarke, 2009) and LDL cholesterol, reduced HDL cholesterol and an increased total/HDL cholesterol ratio (Mensink et al,

2003; Mozaffarian & Clarke, 2009; Sun et al, 2007); pro-inflammatory effects, including higher TNF-α system activity (increased plasma concentrations of TNF-α and higher levels of soluble TNF-α receptors), IL-6 levels and C reactive protein (CRP) concentrations (Baer et al, 2004; Han et al, 2002; Lichtenstein et al, 2003; Mozaffarian et al, 2004a; Mozaffarian et al, 2004b); and endothelial dysfunction, assessed by both circulating markers and functional measures (Baer et al, 2004; de Roos et al, 2001; Lopez-Garcia et al, 2005). These effects were significant in comparison with *cis* unsaturated fats; the adverse effects of TFAs on the total/HDL cholesterol ratio and endothelial function have also been reported when comparing with saturated fatty acids.

## 1.4 Role of fatty acids on inflammatory processes

Dietary FAs may affect inflammatory processes through effects on body weight and adipose tissue mass (since excessive adipose tissue is an inflammatory focus that releases inflammatory mediators into the bloodstream) and through changes in the membrane composition of cells involved in inflammation. Changes in the composition of cell membranes can modify membrane fluidity, lipid raft formation, cell signalling leading to altered gene expression, and the pattern of lipid and peptide mediator production (Calder, 2011). Within the cell, membrane-derived FAs and their products can influence inflammation by serving as modulators of the NFκB and PPAR-α/γ (peroxisome proliferator activated receptor alpha-gamma), transcription factor pathways related to the expression of genes encoding cytokines and chemokines, some acting in pro-inflammatory and others in anti-inflammatory ways (Calder, 2013a), and as precursors of eicosanoid and docosanoid oxidation products formed by the action of enzymes like epoxygenases, lipoxygenases and cyclooxygenases (Wall et al, 2010).

### 1.4.1 Saturated FA

Palmitic (16:0) and stearic acid (18:0) are the most common SFAs in human tissues and they are part of cell membrane phospholipids, ceramides, gangliosides and diacylglycerols (Calder, 2015a). Ceramides modulate a variety of cellular responses including cell death,

autophagy, inflammation and insulin signalling (Grosch et al, 2012) and can induce insulin resistance through different mechanisms (Fox et al, 2007; Penkov et al, 2013; Teruel et al, 2001).

SFAs are also frequently found in phospholipids and sphingolipids of lipid rafts, a collection of lipid membrane microdomains that serve as a platform for the initiation of receptor-mediated signal transduction (Simons & Toomre, 2000).

Additionally, SFAs can acylate different proteins and lipids, modulating their function. In lipid rafts, unlike phospholipids in plasma membrane, polar lipids are predominantly acylated with SFA, which facilitate the formation of liquid-ordered lipid rafts (Calder & Yaqoob, 2007; Wong et al, 2009).

Evidence of animal and *in vitro* models suggest that SFAs, particularly lauric (12:0) and palmitic acids, induce inflammation. For instance, a study showed that lauric acid induced the dimerization and recruitment of TLR4 into lipid rafts together with the association of TLR4 with downstream adaptor molecules, leading to activation of NFκB and target pro-inflammatory gene expression (Wong et al, 2009).

Epidemiological studies have also reported that exposure to SFA is related to circulating markers of inflammation in humans (Fernandez-Real et al, 2003; Klein-Platat et al, 2005), which has also being supported by intervention studies (Baer et al, 2004; Dumas et al, 2016; Lopez-Moreno et al, 2017), suggesting that dietary SFA may increase inflammation.

There is evidence, though not always consistent (Chowdhury et al, 2014; Dehghan et al, 2017; Siri-Tarino et al, 2010), of the pro-inflammatory role of some SFAs, with an effect on the production of atherogenic lipids, oxLDL and phospholipids, contributing to the development of CVD (Calder, 2015a; Ruiz-Nunez et al, 2016).

### 1.4.2 Monounsaturated FA

Monounsaturated fatty acid (MUFA) have a less evident effect on inflammation modulation. OA (18:1 ω-9) is the common cis-MUFA on the human diet and various cell membrane phospholipids contain high proportions of OA (Calder, 2015a).

When the intake of OA is increased at the expense of SFA, beneficial effects on cholesterol and lipoprotein levels (DiNicolantonio & O'Keefe, 2018; Mensink et al, 2003)

and a decrease in the postprandial levels of the adhesion molecules ICAM-1 and VCAM-1 have been described (Pacheco et al, 2008). Additionally, OA confers LDL resistance to oxidation in comparison to PUFAs (Bonanome et al, 1992; Tsimikas et al, 1999).

### 1.4.3 Polyunsaturated FA

Polyunsaturated fatty acids (PUFAs) are the most studied in relation to the effects of FA on inflammation. Omega-6 (n-6) and omega-3 (n-3) are the two major families of PUFAs and both are important constituents of membrane phospholipids (Burdge & Calder, 2015). The relative contribution of each n-6 and n-3 PUFAs in cells and tissues depends on availability from the diet, metabolic processes, the particular tissue or cell and the type of the phospholipids, given that different types of phospholipids have different FA compositions. In most cell types, arachidonic acid (ARA) is the most common PUFA present (Calder, 2017). ARA from cell membranes phospholipids can be the substrate for enzymes such as cyclooxygenase, lipoxygenase and cytochrome P450 for the production of eicosanoid mediators. Eicosanoids, including prostaglandins, thromboxanes and leukotrienes, are key regulators and mediators of inflammatory processes (Kalinski, 2012; Lewis et al, 1990; Tilley et al, 2001).

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the n-3 PUFAs most studied due to their anti-inflammatory effects. Their incorporation into cell membrane phospholipids have shown to increase the cell membrane fluidity (Calder et al, 1994), also modulating the function of lipid rafts (Schaefer et al, 2016; Wassall et al, 2018; Wong et al, 2009), where many proteins and receptors involved in cell signalling are co-localized. This can in turn modify intracellular signalling pathways, the activity of transcription factors (NFκB, PPARs and SREBPs) (Jump, 2008; Krey et al, 1997; Novak et al, 2003) and eventually the expression of different proteins related to the inflammatory response (Baker et al, 2018; Calder, 2017).

Additionally, when EPA and DHA are incorporated into cell membrane phospholipids, it is partly at the expense of ARA (Healy et al, 2000; Rees et al, 2006; Walker et al, 2015), which modifies the lipid mediator production profile from pro-inflammatory eicosanoid to

anti-inflammatory compounds, such as resolvins, protectins and maresins, from EPA and DHA (Bannenberg & Serhan, 2010; Serhan & Chiang, 2013; Serhan et al, 2008).

### 1.4.4 TFA

In relation to TFAs, the intake of iTFAs is clearly associated with CHD and associated pathologies (see earlier). The involvement of systemic inflammation and endothelial dysfunction in the pathogenesis of atherosclerosis and CVD in general, and the evidence from both observational and experimental studies that iTFAs are pro-inflammatory may provide the explanation for this. Nevertheless, more studies are required to explain the effects of iTFAs, the possible underlying mechanisms and the implications of such effects on inflammation and cardiovascular health. Regarding rTFAs, some evidence about favourable properties of these compounds *in vitro* and in animal models of disease (obesity, cancer, diabetes) has been published (Viladomiu et al, 2015), but the effects in humans are still unclear (Gebauer et al, 2015). Their involvement in the modulation of inflammatory processes is not fully understood, but some studies suggest that TVA and some CLA isomers may be hypocholesterolemic and antiatherogenic (Gebauer et al, 2011; Viladomiu et al, 2015).

#### 1.4.4.1 Evidence from epidemiological studies

Observational studies (Table 1.4) have correlated intake or levels of TFAs with clinical outcomes related to inflammation; some have looked for the differences between iTFAs and rTFAs, although the majority use total TFAs without differentiating between the sources. In 2004 Mozaffarian et al. reported that TFA intake is positively associated with markers of systemic inflammation (soluble tumour necrosis factor α receptors (sTNF-R1 and 2)) in generally healthy women, although most of the TFA intake in the participants of these cohorts came from processed foods (fried foods (18%); cookies, donuts, or sweet rolls (17%); margarine (10%); beef (9%); and crackers (4%)), suggesting that they were mainly iTFAs (Mozaffarian et al, 2004a). The same authors also showed that, in patients with chronic heart failure, TFA levels in red blood cell membranes were strongly associated with levels of IL-1, IL-1 receptor antagonist, IL-10, TNF-α, sTNF-R1 and 2, MCP-1 and brain natriuretic peptide, particularly *trans* isomers of OA and linoleic acids (LA) but

not of palmitoleic acid (Mozaffarian et al, 2004b). These findings are consistent with those of Lopez-Garcia et al. in a cross sectional study of 730 healthy women from the Nurses' Health Study I cohort. They showed that TFA intake (more strongly EA) was positively related to plasma concentrations of CRP, sTNF-R2, sE-selectin, sICAM-1, and sVCAM-1 (Lopez-Garcia et al, 2005).

In contrast, in Danish middle-aged men with a broad BMI range, intake of TFAs was not associated with levels of IL-6 or CRP, blood pressure, insulin sensitivity or blood lipids (Nielsen et al, 2011), which could be explained by the fact that reported TFA intakes were relatively low and their source was mainly ruminant fat, given Danish legislation to eliminate food products containing iTFAs since the 1990s and the high intake of milk/dairy products in Denmark. Actually, an 18-year follow-up study of 3,686 Danes, aged 30–71 years and healthy at baseline concluded there was no association between rTFA intake and risk of CHD over a wide range of intake (Jakobsen et al, 2008).

Da Silva et al. (2015) compared iTFAs and rTFAs in plasma PLs and their correlations with metabolic risk factors, including lipid profile, glycaemic profile, adiposity and blood pressure, in a cohort composed of 100 healthy non-obese and 100 obese Canadian participants. They found that plasma rTFAs (TVA and also *trans*-palmitoleic acid) levels were associated with lower insulin levels and blood pressure and higher adiponectin levels, unlike the industrial counterpart (EA) which was associated with higher total cholesterol, TAGs and glycaemia, strongly indicating that different sources of TFAs may have different impacts on metabolic markers of cardiac health (Da Silva et al, 2015). Given that adiponectin is anti-inflammatory, these findings suggest that rTFAs reduce inflammation.

A recent cross-sectional study with 5,546 adult participants, using data from the 1999-2000 cycles of the US National Health and Nutrition Examination Surveys (NHANES), showed that all of the serum TFAs measured (palmitelaidic acid, EA, TVA and linoelaidic acid) were independent predictors of plasma CRP and fibrinogen levels (Mazidi et al, 2017), and the authors suggested that all sources of TFAs enhance inflammation and should be avoided.

Table 1.4: Epidemiological studies of TFAs and inflammatory markers in humans.

| Subjects | Measurement of TFA exposure | Risk factors association | Outcomes assessed | Reference |
|---|---|---|---|---|
| 823 healthy women from the Nurses' Health Study I and II | Dietary intake assessed from 2 semi-quantitative FFQs averaged for each subject | Blood markers of systemic inflammation | TFA intake was positively associated with sTNF-R1 and sTNF-R2. TFA intake was not associated with IL-6 or CRP overall but was positively associated with IL-6 and CRP in women with higher body mass index. | (Mozaffarian et al, 2004a) |
| 86 ambulatory patients with established heart failure (cross-sectional from prospective cohort study) | Red blood cell membrane TFAs | Blood markers of systemic inflammation | TFA levels were positively associated with IL-1, IL-1 receptor antagonist, IL-10, TNF-α, sTNF-R1, sTNF-R2, MCP-1 and brain natriuretic peptide. | (Mozaffarian et al, 2004b) |
| 730 women from the Nurses' Health Study I cohort, aged 43–69 y | Dietary intake assessed from a validated FFQ in 1986 and 1990 | Blood biomarkers of Inflammation and endothelial dysfunction | CRP levels were 73% higher among those in the highest quintile of total TFA intake compared with the lowest quintile. IL-6 levels were 17% higher, sTNF-R2 5% higher, sE-selectin 20% higher, sICAM-1 10% higher, and sVCAM-1 10% higher. TFA intake was positively related to plasma concentration of CRP, sTNFR-2, sE-selectin, sICAM-1, and sVCAM-1 | (Lopez-Garcia et al, 2005) |
| 393 middle-aged men representing a broad range of BMI | Dietary intake estimated based on 7 d dietary records | Blood biomarkers of CHD risk | Intake of total TFAs 1·3 g/d, covering a daily iTFA intake of 0·4 g (0·0–1·0) and rTFA intake of 0·9 g (0·4–1·8), showed no significant associations with abdominal fatness, inflammatory markers (IL-6 and CRP), blood lipids, blood pressure and insulin homeostasis. | (Nielsen et al, 2011) |
| 200 individuals aged from 18 to 55 y, including 100 obese and 100 non-obese | Plasma phospholipid TFAs | Blood biomarkers of CHD risk | Higher rTFAs were associated with lower insulin levels and blood pressure and with higher adiponectin levels, unlike their industrial counterparts which were associated with higher total cholesterol, TAGs (obese subjects) and glycemia (non-obese subjects). | (Da Silva et al, 2015) |
| 5446 participants from the 1999-2000 cycles of the US National Health and Nutrition Examination Surveys (NHANES) | Serum TFAs (free and esterified): mostly iTFAs | Blood biomarkers of systemic inflammation | After adjustment, mean serum TFAs (palmitelaidic acid, EA, TVA and linoelaidic acid) rose with the increasing quartiles of CRP and fibrinogen. In adjusted linear regression models, serum TFAs were an independent predictor of plasma CRP and fibrinogen levels. | (Mazidi et al, 2017) |

#### 1.4.4.2 Evidence from intervention studies

There are very few intervention studies comparing the effects of iTFAs and rTFAs on biomarkers of inflammation or on inflammatory responses. Additionally, when they are tested individually, doses used, delivery method, intervention duration and washout periods, gender differences, health conditions and age variations of the subjects under study can contribute to the inability to draw clear comparisons between them (Table 1.5).

In relation to the effects of iTFAs, the double blind crossover study by Han et al. (2002), where 19 subjects with moderately elevated LDL cholesterol levels were exposed randomly to 3 diets with the same amount of fat (30%) but different proportion of iTFAs or saturated fats, reported that a soybean margarine diet (6.7% of energy from iTFAs) increased TNF-α and IL-6 production by cultured mononuclear cells in comparison with a soybean oil diet (0.6% of energy from iTFAs) (Han et al, 2002). Similarly, Baer et al. (2004) showed that an intake of 8% of energy (28.8 g/d) for 5 weeks as TFAs (spectrum of *trans* 18:1 isomers representative of the US food supply) increased blood levels of CRP, IL-6 and sE-selectin in 50 healthy adult males. When half the amount of TFAs (4% TFAs, 4% stearic acid) was consumed daily, CRP level did not increase, although fibrinogen levels did, which was suggested to be caused by stearic acid (Baer et al, 2004). Another study, conducted in overweight postmenopausal women, reported that an increased intake of iTFAs (7% of daily energy) for 16 weeks produced an increase in the circulating levels of TNF-α and its soluble receptors TNF-R1 and TNF-R2 (Bendsen et al, 2011), with no changes on CRP, IL-6 or adiponectin levels.

In contrast, in the case of rTFAs, Tricon et al. (2006) showed that a daily amount of 1.5 g CLA9,11 and 4.7 g TVA consumed through modified dairy products for six weeks had no effects on inflammatory biomarkers, insulin, glucose, total cholesterol, and TAGs in serum (Tricon et al, 2006). Another study used sheep cheese naturally rich in TVA and CLA9,11 for 10 weeks in a small sample of adults reporting a significant reduction in IL-6, IL-8, and TNF-α levels, compared to placebo (Sofi et al, 2010). Similarly, a study where 29 healthy adult volunteers underwent a CLA depletion followed by an 8 week period consuming 20 g of CLA9,11 enriched butter daily (1020 ± 167 mg CLA/day) showed decreased protein expression of NFκB in blood mononuclear cells, reduced serum levels of TNF-α, IL-2, and

IL-8 and increased levels of the anti-inflammatory cytokine IL-10 after CLA repletion compared to the levels in the depletion phase (Penedo et al, 2013).

When comparing the effect of the two most common CLA isomers, some studies suggest that CLA10,12 may have pro-inflammatory effects. Tholstrup et al. (2008) compared the effects of a CLA mixture, an oil rich in CLA9,11 or olive oil for 16 weeks in healthy postmenopausal women, showing that the oil containing CLA10,12 caused higher plasma levels of CRP, fibrinogen, and plasminogen activator inhibitor-1 (PAI-1) and of a urinary marker of lipid peroxidation, compared to the oil rich in natural CLA9,11 and the olive oil (Tholstrup et al, 2008). Other authors have also reported increased CRP after CLA10,12 or CLA mix supplementation, whether in obese men with metabolic syndrome (Riserus et al, 2002), obese adults (Steck et al, 2007) or healthy adults (Smedman et al, 2005). Nevertheless, the evidence is not consistent. Ramakers et al. (2005) found no effects on *ex vivo* cytokine production by isolated peripheral blood mononuclear cells (PBMCs) or by PBMCs present in whole blood when stimulated with LPS from a small sample of moderately overweight subjects at increased risk for CHD, after daily consumption of 3 g of CLA9,11 or CLA10,12 in an enriched dairy product for 13 weeks (Ramakers et al, 2005). A study testing an enriched butter with CLA9,11 or CLA10,12 for 5 weeks showed increases in lipid peroxidation but no effects on plasma total, LDL, and HDL cholesterol and TAGs, or inflammatory and haemostatic risk markers, nor in fasting insulin and glucose concentrations, in healthy young men (Raff et al, 2008).

One of the few published RCTs comparing the effects of iTFAs and rTFAs in healthy adults, showed that the intake of enriched dairy products with iTFAs (7% of energy) or a mix of CLAs for 3 weeks did not affect low-grade inflammation to a large degree (lower sTNF-R1 and higher sE-selectin), while both produced an increased excretion of 8-iso-$PGF_{2\alpha}$, a marker of oxidative stress (Smit et al, 2011). In the same way, Radtke et al. (2017) reported that 2% of daily energy intake as TFAs (alpine butter or margarine) or no TFA as control, during 4 weeks, did not have any adverse effect on coagulation, inflammation markers and adhesion molecules in healthy subjects. Nevertheless, the rTFA diet resulted in increased levels of total cholesterol and LDL-cholesterol compared with the other two diets, which may have been caused by some important differences in the nutritional composition between alpine butter and margarine (i.e. the SFA, MUFA and PUFA

contents). Additionally, the study did not reach the estimated total sample size and the diet of the participants was not controlled (Radtke et al, 2017).

Table 1.5: Intervention studies with TFAs in humans reporting markers of inflammation.

| Subjects | FA doses and form | Duration and design | Outcomes | Reference |
|---|---|---|---|---|
| 19 subjects (8 males, 11 females) over age 50 y with moderately elevated LDL cholesterol | 3 diets, 30% of calories as fat, of which two thirds were provided as soybean oil, soybean oil-based stick margarine, or butter (randomized order). | 32 days for each diet (washout of 2 wk. minimum). Double blind crossover design | iTFA diet ↑ PBMCs production of TNF-α (58%) and IL-6 (36%).<br>No differences in delayed-type hypersensitivity skin test response, lymphocyte proliferation, or levels of IL-2 and $PGE_2$ produced between diets. | (Han et al, 2002) |
| 50 healthy adult males with mean age 42 y | 8% of fat (total 39% of calories) or fatty acids was replaced across diets with: cholesterol, oleic acid, TFAs (*trans* 18:1 positional isomers similar to that in the US food supply), stearic acid (STE), TFAs+STE (4% of energy each), and 12:0–16:0 saturated fatty acids | 6 diets for 5 wk. each for a total controlled feeding period of 30 wk. in a randomized crossover design | TFA diet ↑ plasma CRP, IL-6 and sE-selectin (5.6%) concentrations.<br>Fibrinogen (4.4%) concentration increased after consumption of the TFA+STE diet, but not after TFA diet alone. | (Baer et al, 2004) |
| 49 healthy adult males mean age 31 y | Supplementation with 3 doses of CLA9,11 (0.59, 1.19 and 2.38 g/day) or CLA10,12 (0.63, 1.26, 2.52 g/d) | 8 wk. periods (6 months each isomer), followed by a 6 wk. washout and crossover. Randomized, double blind, crossover design. | CLA isomers did not affect lymphocyte subpopulations. Monocytes expressing ICAM-1 were ↓ after supplementation with the highest dose of both isomers than at baseline. CLA isomers ↓ mitogen-induced T lymphocyte activation in a dose-dependent manner. No effects on serum concentrations of CRP and on *ex vivo* cytokine production by PBMCs in response to LPS (TNF-α, IL-10, IL-6, IL-1β and IL-8) or in response to concanavalin A (IFN-γ, TNF-α, IL-10, IL-5, IL-4, IL-2). Supplementation had no significant effect on body composition, plasma insulin concentration, homeostasis model for insulin resistance, or revised quantitative insulin sensitivity check index. CLA10,12 ↑ LDL:HDL cholesterol and total:HDL cholesterol, whereas CLA9,11 ↓ them. CLA10,12 ↑ plasma TAGs compared to CLA9,11. | (Tricon et al, 2004a; Tricon et al, 2004b) |
| 42 overweight middle-aged adults | Yogurt-like dairy product enriched with 3 g of high oleic acid oil (control group) or 3 g of CLA9,11 or 3g of CLA10,12 instead of the oleic acid. | 13 wk. Placebo-controlled, double-blind parallel design | CLA isomers had no effect on *ex vivo* LPS-stimulated cytokine production by PBMCs and by whole blood.<br>Plasma CRP concentrations did not change. | (Ramakers et al, 2005) |
| 32 middle-aged men. | Milk, butter, and cheese providing 0.151 g/d (control) or 1.421 g/d (modified) CLA9,11 and 4.7 g/d of TVA. Incorporation of both FAs in plasma phospholipids and peripheral blood mononuclear cells was assessed (Burdge et al, 2005). | 6 wk. of intervention, 7 wk. of washout and crossover in random order | Consumption of CLA enriched dairy products did not significantly affect body weight, inflammatory markers (IL-6, sVCAM-1, sICAM-1, sE-selectin, or CRP), insulin, glucose, TAGs, or total, LDL, and HDL cholesterol but resulted in a small increase in the ratio of LDL to HDL cholesterol. | (Tricon et al, 2006) |

| Subjects | FA doses and form | Duration and design | Outcomes | Reference |
|---|---|---|---|---|
| 75 healthy postmenopausal women | Daily supplement of 5.5 g of oil rich in either CLA mixture (41.17% CLA9,11; 39.9% CLA10,12), an oil rich in CLA9,11, or olive oil (control). Total daily intake of CLA (all isomers) was 4.6 g with the mixture and 5.1 g with the CLA9,11 oil. | 16 wk. double-blind, randomized, parallel intervention study | The CLA mixture resulted in higher plasma CRP, fibrinogen and PAI-1 than in the CLA9,11 isomer group. The CLA mixture also caused higher urine 8-Iso-$PGF_{2\alpha}$ compared to CLA9,11 and olive oil.<br>Plasma IL-6, sVCAM-1, sICAM-1, MCP-1, and TNF-α were not different between the groups. | (Tholstrup et al, 2008) |
| 38 healthy young men | Butter enriched with 5.5 g/d of a CLA oil (39.4% CLA 9,11; 38.5% CLA10,12) was incorporated into bread rolls, cake, and chocolate milk. The CLA group received 4.6 g/d of total CLA (2.5 g CLA9,11 and 2.1 g CLA10,12) and the control group received 0.3 g/d of CLA9,11. | 5 wk. double-blind, randomized, parallel intervention study | CLA group had 83% higher concentration of urinary 8-Iso-$PGF_{2\alpha}$ compared to control group.<br>CLAs had no effect on plasma lipids (total, LDL, HDL, TG), CRP, plasma factor VII activity, PAI-1, insulin and glucose levels. | (Raff et al, 2008) |
| 10 adults (4 male, 6 female) | 200 g/wk. of pecorino cheese<br>(3.28% TVA, 1.56% CLA9,11) | 10 wk. (intervention, wash out and placebo), crossover design | Cheese ↓ plasma IL-6, IL-8 and TNF-α (IL-10, IL-12 and VEGF unchanged) | (Sofi et al, 2010) |
| 49 healthy overweight postmenopausal women | Partially hydrogenated soybean oil incorporated into two bread rolls (15.7 g/day iTFAs) or control oil without added TFAs. | 16 wk. | iTFA intake ↑ baseline-adjusted serum TNF-α (by 12%), plasma sTNF-R1 and 2.<br>Serum CRP, IL-6 and adiponectin and subcutaneous abdominal adipose tissue mRNA expression of IL-6, IL-8, TNF-α and adiponectin as well as ceramide content were not affected by iTFAs. | (Bendsen et al, 2011) |
| 61 healthy adults (25 male, 36 female) aged between 18 and 65 y | 7% of energy provided by oleic acid (control diet), iTFAs (mainly 18:1 *trans*), or CLA (mixture of 80% CLA9,11 and 20% CLA10,12); FAs were incorporated into margarines and yogurt drinks by enriching fat-free yogurt with 50 g high oleic sunflower oil, 50 g partially hydrogenated vegetable fat, or 50 g CLA-rich oil per L. | 3 diets for 3 wk., in random order (double blind, RCT) | iTFA diet caused 4% decrease in sTNF-R1 concentrations and 6% higher sE-selectin concentrations compared to control, with no significant effect on other inflammatory markers (IL-6, CRP, TNF-R2, MCP-1). CLA mixture did not significantly affect inflammatory markers. The urine concentration of 8-iso- $PGF_{2\alpha}$ was greater after the iTFA and the CLA diet periods than after the control period. | (Smit et al, 2011) |
| 29 healthy adults aged 22 to 36 y | CLA depletion period of restricted dairy fat intake (depletion phase; CLA intake was 5.2 ± 5.8 mg/day), followed by consuming 20 g/day of butter naturally enriched with CLA9,11 (repletion phase; CLA intake of 1020 ± 167 mg/day). | Depletion and CLA repletion period of 8-wk each | CLA-enriched butter increased serum levels of IL-10, reduced NFκB in blood and serum levels of TNFα, IL-2, IL-8 and inactive metalloproteinase-9. Reduced activity of metalloproteinases 2 and 9 in serum was observed during the CLA-repletion period. CLA-enriched butter had no effects on body composition, serum levels of adiponectin, C-reactive protein, and IL-4. | (Penedo et al, 2013) |

#### 1.4.4.3 Evidence from experimental animal studies (Table 1.6)

In an obesity model in rats (JCR:LA-cp), the animals fed a diet with TVA at 1% (w/w) during 8 weeks showed a reduction in body fat, increased insulin sensitivity, lower NAFLD activity scores, together with other metabolic benefits (Jacome-Sosa et al, 2014). Another report from the same group compared the effects of TVA and EA in the same animal model showing that both TFAs corrected the impaired IL-2 and TNF-α response to T-cell mitogen stimulation seen in obese rats, but only TVA normalized T-cell stimulated IL-1β and IFN-γ production and haptoglobin levels. Rats fed with EA produced more IL-6 compared to controls and to TVA fed groups, which was associated with a greater incorporation of EA into splenocyte PLs. Additionally, the animals fed with either TFA had higher levels of IL-6 and IL-10, suggesting that both natural and industrial TFAs can facilitate LPS-stimulated immune response in this animal model (Ruth et al, 2010). Another model in male mice fed for 4 weeks with diets containing 0.5% (w/w) of TVA, EA or CLA9,11 showed that all TFAs used caused a reduction in body weight and adipose tissue, TVA induced a reduction in LDL cholesterol and TNF-α levels, while CLA9,11 induced higher levels of TNF-α (Wang et al, 2015).

Other authors have used diets with different amounts of partially hydrogenated oils (rich in EA and/or linoelaidic acid) and reported the effect on different parameters related to inflammation. Longhi et al. (2017), using diets with low and high content of lipids in the form of lard or partially hydrogenated soybean oil (PHSO), showed that PHSO diets (high and low) increased oxLDL levels in serum and cerebrospinal fluid (CSF). Additionally, animals fed with PHSO diets had higher levels of IL-1, IL-6 and TNF-α in CSF, showed impairment of insulin sensitivity and alteration in the antioxidant enzyme activities in hepatic tissue (Longhi et al, 2017). Another study, using diets rich in ALA, partially hydrogenated vegetable fat (PHVF) or both mixed in different proportions, reported higher levels of $PGE_2$, $TXB_2$, $LTB_4$ and $LTC_4$, upregulation of COX-2, phospholipase A2 and NFκB p65 and downregulation of PPARγ in macrophages of rats fed with PHVF. These effects were to different extents normalised by including ALA in the PHVF diet (Rao & Lokesh, 2017). In agreement, when longer chain n-3 FAs (EPA and DHA) were compared with iTFAs in a model of myocardial infarction, Siddiqui et al. (2009) reported that iTFAs

adversely affected survival, while n-3 FAs had beneficial effects on survival. In addition, animals fed with TFAs had variable degrees of aortic atherosclerotic lesions, lacked the ability to develop collaterals around the site of occlusion and showed increased circulating levels of sICAM-1, the opposite to what was observed in the animals fed with n-3 FA-enriched diet (Siddiqui et al, 2009).

Studies using CLAs show inconsistent results. Poirier et al. (2006) reported that the administration of CLA10,12 by gavage at a dose of 20 mg/day for 7 days in mice led to the upregulation of TNF-α, MCP-1 and IL-6 gene expression in white adipose tissue (WAT) without affecting their serum levels, together with macrophage infiltration in WAT, reduction in body weight and adipose tissue mass, lower serum levels of leptin, adiponectin and higher levels of insulin and resistin (Poirier et al, 2006). Similarly, another study, using enriched diets with 0.06%, 0.2%, and 0.6% (w/w) of mixed CLA10,12 with LA (50/50), mixed CLA10,12 with CLA9,11 (50/50) or LA alone as a control in young male mice for 6 weeks, showed that the intermediate and higher intakes of CLA10,12 reduced adiposity, increased serum levels of MCP-1 and IL-6 and increased liver steatosis (Shen et al, 2013).

In two murine models of arthritis, collagen antibody-induced arthritis and collagen-induced arthritis, where the mice were exposed to CLAs prior to the induction of joint swelling or after the onset of disease, respectively, both CLA isomers showed anti-inflammatory effects (Butz et al, 2007; Huebner et al, 2010). In contrast, in another model where the mice were exposed to the CLA isomers prior to the onset of arthritis, the diet enriched with CLA10,12 (0.25% w/w) resulted in a more severe initial inflammatory response at the onset of disease. Instead, CLA9,11 (0.5% w/w) reduced the incidence of the disease by 39% compared to control mice fed with corn oil, also increasing IL-4 and IL-10 levels in the animals paws. The authors suggested that CLA10,12 may have acted by driving Th1 type responses during the adaptive immune response, whereas CLA9,11 may have induced a Th2 dominant adaptive immune response (Muhlenbeck et al, 2017).

Studies in animal models show more consistent outcomes relating the exposure to CLA9,11 and anti-inflammatory responses. Using a murine asthma model, colorectal cancer in mice and a model of LPS-induced inflammation in mice, CLA9,11 induced a reduction in the allergic airway inflammation, decreased percentages of macrophages in the mesenteric lymph nodes and produced downregulation of colonic TNF-α mRNA

expression, and reduced the serum levels of the pro-inflammatory cytokines IFN-γ, IL-12, and IL-1β, in response to LPS-induced septic shock (Evans et al, 2010; Jaudszus et al, 2008; Reynolds et al, 2009).

Table 1.6: Studies of 18-carbon TFAs in animal models reporting markers of inflammation

| Animal model | FA used, doses and duration | Measurement of TFAs | Outcome | Reference |
|---|---|---|---|---|
| Wild-type C57BL/6J female mice | Daily gavage was performed with 0.1 g sunflower oil or 0.1 g of a mixture with 0.08 g sunflower oil and 0.02 g TAGs containing 79% CLA10,12 (total CLA isomers 90.4% [79% t10,c12; 7% c9,t11; and 3.1% t/t]), oleic acid 2.5%, linoleic acid 0.2%, and saturated fatty acids 6.6%. For 1, 3 or 7 days. | --- | Oral administration of CLA10,12 led to ↓ in body weight and adipose tissue mass accompanied by an insulin-resistant state characterized by hyperinsulinemia, ↑ resistin levels and ↓ leptin and adiponectin levels triggered after seven gavages. The gene expression of MCP-1, IL-6 and TNF-α ↑ in white adipose tissue, while their serum levels of these inflammatory mediators did not change. CLA supplementation also induced SOCS3 and produced a reduction in PPARγ gene expression in WAT. Additionally, immunohistochemistry revealed marked macrophage infiltration into adipose tissue. | (Poirier et al, 2006) |
| Wistar rats: coronary ligation model C57/BL6 mice: femoral artery ligation model | n-3 PUFA diet (mixture of maize and fish oil), with 21% of fats as n-3 PUFAs. TFA diet (mixture of maize and primex shortening, rich in EA and linoelaidic acid) with 17% of fats as TFAs. Experimental diets for 6 months in the coronary artery model, and 3 weeks in the femoral artery ligation model. | Plasma (only %) | Animals on the TFA diet had a lower survival rate due to sudden cardiac death and exhibited variable degrees of aortic atherosclerotic lesions. | (Siddiqui et al, 2009) |
| JCR:LA-cp rats | EA and TVA (1% w/w), 16 weeks | Plasma PL. Splenocyte phosphatidylethanolamine and phosphatidylcholine. | Obese rats had ↑ serum haptoglobin and impaired T-cell stimulated cytokine responses. TVA and EA improved T-cell stimulated cytokine production, but only TVA normalized serum haptoglobin. TVA also normalized T-cell stimulated IL-1β and IFN-γ production | (Ruth et al, 2010) |
| 129Sv male mice | Linoleic acid (LA) alone, CLA10,12 mixed with LA (50/50) or CLA10,12 mixed with CLA9,11 (50/50) in three doses in the diet (w/w): 0.06% (low), 0.2% (intermediate), and 0.6% (high), equivalent to 70, 240, and 700 mg/kg body weight. For 7 weeks. | WAT, liver and muscle tissue | The intermediate and high doses of both CLA treatments ↓ total body weight gain and total WAT depot weights, compared to control. Higher dose of CLA caused hepatic steatosis, hepatomegaly and ↑ serum levels of MCP-1. IL-6 serum levels were ↑ with the intermediate dose of the CLA isomer mixture. Intermediate or low doses of CLA10,12 or mixed CLAs ↑ mRNA markers or activators of browning in WAT, together with ↑ their protein or activity levels. Gene expression of MCP-1, IL-6, F4/80, and TNF-α was ↑ in the intermediate and high dose of CLA10,12 + LA or CLA isomer mixture in WAT. | (Shen et al, 2013) |

| Animal model | FA used, doses and duration | Measurement of TFAs | Outcome | Reference |
|---|---|---|---|---|
| JCR:LA-cp rats (obesity model) | TVA (1% w/w), 8 weeks | --- | ↓ total body fat (−6%), stimulated adipose tissue redistribution [reduced mesenteric fat (−17%) while increasing inguinal fat (29%)] ↓ adipocyte size (−44%), ↑ metabolic rate (7%), ↑ insulin sensitivity [lower HOMA-IR (−59%)], ↓ non-alcoholic fatty liver disease activity scores (−34%), ↓ hepatic (−27%) and intestinal (−39%) TAG secretion relative to control diet. Upregulation of hepatic ApoA1, SRP1, DGAT2 genes. Downregulation of intestinal SREBP1, FAS, UCP2 genes. | (Jacome-Sosa et al, 2014) |
| Male mice | 0.5 g/100 g of diet of TVA, EA or CLA9,11 for 4 weeks | --- | After 10 days, all FAs used induced a reduction in the body weight.<br>TVA produced a reduction in LDL cholesterol levels.<br>CLA9,11 and TVA tended to increase the expression of CYP7α1<br>CLA9,11 induced higher levels of TNF-α, while TVA reduce them, compared to control group. | (Wang et al, 2015) |
| Wistar rats | Partially hydrogenated soybean oil, 90 days. TFA content in diets used (g/kg): Low Lard (0.28), High Lard (1.44), Low PHSO (34.38) and High PHSO (223.4). | --- | Cerebrospinal parameters: PHSO diets increased LDL antibody, IL-1, IL-6 and TNF-α. Animals treated with PHSO diets also showed lower levels of IL-10.<br>Serum parameters: animals fed with PHSO diets had higher fasting glucose and insulin levels (both higher in the low concentration of PHSO). Additionally, PHSO diets led to higher levels of oxLDL<br>Hepatic parameters: PHSO diet induced alterations in TAG levels (lower), SOD activities and ratio of SOD/CAT + GPx | (Longhi et al, 2017) |
| Wistar rats | Groundnut oil (10 wt%), partially hydrogenated vegetable fat (PHVF, mainly EA) (10 wt%), ALA-rich linseed oil (LSO, 10 wt%), PHVF blended with LSO at 2.5, 5.0 and 7.5 wt% levels. For 60 days. | Serum lipids | Macrophages from rats fed PHVF showed a down-regulated expression of PPARγ and up-regulated expressions of cytosolic phospholipase A2, cyclooxygenase-2, 5-lipoxygenase and NFκB p65. Macrophages from rats fed PHVF secreted higher levels of pro-inflammatory eicosanoids and cytokines. Macrophages from rats fed PHVF blended with LSO at incremental amounts showed a significant reduction in the protein expressions of pro-inflammatory markers in dose-dependent manner. | (Rao & Lokesh, 2017) |
| DBA/1 mice | Corn oil (CO, 6 wt%), 5.75% CO plus 0.25% CLA10,12, or 5.5% CO plus 0.5% CLA9,11, prior to arthritis development (3 weeks before 1ary immunization and for 70 days; or starting at the 2ary immunization, for 35 days) | Liver | CLA10,12: mice fed with CLA10,12 isomer at the 2ry immunization showed a more severe initial inflammatory onset of disease, with a trend toward increased plasma anti-CII IgG1 antibodies levels<br>CLA9,11: This CLA isomer decreased arthritis incidence by 39% compared to control mice. In mice that did not develop arthritis, there was a significant 49% reduction in anti-CII IgG2a antibody levels in CLA9,11 fed mice compared to control mice fed corn oil.<br>CLA9,11 exposure produced increased levels of IL-4 and IL-10 compared to control mice, independent of arthritis induction. | (Muhlenbeck et al, 2017) |

#### 1.4.4.4 Evidence from *in vitro* studies

In unstimulated human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs), linoelaidic acid and/or EA have been shown to induce gene expression, surface expression or protein levels of ICAM-1, VCAM-1, IL-6 and TLR4, the phosphorylation and nuclear translocation of NFκB p65 and IκBα, the adhesion of monocytes and lymphocytes, and to impair insulin-mediated NO production together with increased ROS production (Bryk et al, 2011; Harvey et al, 2012; Iwata et al, 2011; Pan et al, 2017) as depicted in table 1.7. In contrast, studies using TVA in HUVECs or human microvascular endothelial cells (HMECs) found no effects on, or downregulation of, inflammatory gene expression (Da Silva et al, 2017a; Iwata et al, 2011; Livingstone et al, 2014). When HepG2 cells were exposed to a pro-inflammatory stimulus, TVA was shown to downregulate TNF-α and IL-8 genes (Da Silva et al, 2017b).

However, there are reports describing some similar effects of iTFAs and rTFAs in different cell models (both anti- and pro-inflammatory). For example, Da Silva et al. (2017) showed that both TVA (25, 50 and 150 μM) and EA (5 to 150 μM) downregulated gene expression of TNF-α, VCAM-1 and SOD-2 together with reducing the secretion of prostaglandin $E_2$ in HUVECs stimulated with TNF-α (Da Silva et al, 2017b). In another model of human coronary arterial smooth muscle cells (HCASMCs), a high concentration of TVA and EA (200 μM for 20 h) increased the levels of IL-6 and MCP-1, respectively (Soto-Vaca et al, 2013).

In relation to the effects of CLAs, some studies have reported anti-inflammatory effects in HUVECs. Sneddon et al. (2006) reported that CLA10,12 was able to suppress adhesion of THP-1 cells and isolated human PBMCs dose-dependently in HUVECs treated with TNF-α, which was related to the suppression of platelet-activating factor production . Similarly, Goua et al. (2008) showed that CLA10,12 and a CLA mix reduced ICAM-1 and VCAM-1 expression, while the CLA mix used at 25 μM was able to decrease NFκB activity by 30% in both HUVECs and smooth muscle cells treated with TNF-α . Another study by Stachowska et al. (2012) showed that the incubation of monocytes from healthy donors with CLA9,11 and CLA10,12 at 100 μM for 7 days reduced the expression of the integrins VLA-4 and Mac-1. When HUVECs were exposed to the same concentrations of CLA isomers, both caused a reduction in the surface expression of VCAM-1, but only CLA9,11 reduced ICAM-

1 compared to control. Additionally, both CLA isomers showed a strong tendency to reduce the binding of monocytes to HUVECs (Stachowska et al, 2012).

Table 1.7: *In vitro* studies of 18-carbon TFAs and inflammation

| Cell type | FA used / Inflammatory stimulus | Outcome | Reference |
|---|---|---|---|
| 3T3-L1 adipocytes | CLA10,12 (50 μM for 0, 8, 16, 24, 48, and 168 h) | In 3T3-L1 cells, CLA10,12 induced IL-6 gene expression and secretion (NFκB dependent) and decreased gene expression of PPARγ, whereas SOCS3 mRNA levels were ↑, with lower uptake of glucose. Nuclear localization of the p65 subunit of NFκB was markedly ↑ in the CLA10,12-treated cells. PPARγ protein was ↓ in the presence of CLA 10,12. | (Poirier et al, 2006) |
| HUVECs<br>THP-1 cells<br>Isolated human peripheral blood monocytes | Linoleic acid, CLA9,11, CLA10,12<br>CLA mix (50:50), 6, 12.5, 25 and 50 μM FA for 24 h<br>TNF-α (5 ng/ml) or IL-1β (10 U/ml) for 2.5 and 6 h | CLA ↓ binding of both THP-1 and isolated human peripheral blood monocytes to HUVECs (40%), with the CLA10,12 isomer suppressing adhesion dose-dependently.<br>CLA was shown to inhibit cytokine-induced binding by suppressing PAF production (IL-1β, VCAM-1 and ICAM-1 levels and pro-inflammatory cytokine expression were unaffected by CLA) | (Sneddon et al, 2006) |
| HUVECs<br>Primary aortic vascular smooth muscle cells (vSMCs) | EPA, DHA and palmitic acid<br>(PA), Linoleic acid, CLA9,11 , CLA10, 12, CLA mix (50:50)<br>25 μM for 24 h<br>TNF-α (5 ng/ml for either 6 h for ADM or NF-kB activity or 5 min, for phosphorylation of IkBα) | HUVECs: CLA10,12 and CLA mix, EPA and DHA ↓ ICAM-1 expression (15–35%) at 12.5, 25 and/or 50 μM.<br>↓ VCAM-1 expression by 25 μM CLA10,12 and mix; omega-3 PUFA and other concentrations of CLA and TNF-α induced E-selectin expression were unaffected.<br>↓ NFκB activity (29% and 30%, HUVECs and vSMCs) by 25 μM CLA mix<br>CLA isomers alone induced IκBα phosphorylation (activation NFκB pathway) | (Goua et al, 2008) |
| HAECs | *Trans*-18:2, DHA<br>5 μM, 48 h | HAECs treated with *trans*-18:2 showed significantly increased expression of ICAM-1 and nitrosylation of cellular proteins than those treated with DHA | (Siddiqui et al, 2009) |
| Human microvascular endothelial cells (HMECs) | EA, Linoelaidic acid, TVA Palmitic acid, Linoleic acid<br>(100 μM, 3h) | EA and Linoelaidic acid ↑ IL-6 levels and phosphorylation of IκBα, impaired insulin signalling (↓ Akt and eNOS phosphorylation) and NO production and ↑ superoxide production. | (Iwata et al, 2011) |
| HAECs | EA, Linoelaidic acid at 50, 100 and 200 μM; 45 min, 6 and 16 h. | Both TFAs ↑ the surface expression of ICAM-1 (100 μM, 3 fold increase) and VCAM-1 (100 μM, 8 fold increase) in a dose-dependent manner. The expression of both was increased after 6 h FA exposure measured by RT-PCR.<br>Adhesion of monocytes and lymphocytes was significantly increased when HAECs were treated with TFAs.<br>Treatment of HAECs with both of the TFAs for 45 min induced ↑ phosphorylation of NFκB p65.<br>Both TFAs significantly increased the intracellular ROS production in a dose-dependent manner (100 μM inducing over a 2-fold increase). | (Bryk et al, 2011) |

| Cell type | FA used / Inflammatory stimulus | Outcome | Reference |
|---|---|---|---|
| Isolated human peripheral blood monocytes<br>HUVECs | CLA9,11; CLA10,12; LA<br>Monocytes: 100 µM for 7 days<br>HUVECs: 30 min, 12 h or 24 h without or with 100 µM, stimulation with ox-LDL (50 mg/ml) for 8 h before the adhesion assay | Both CLAs ↓ the expression of CD49d/CD29 (VLA-4) and CD11b/CD18 (Mac-1) on macrophages.<br>Both CLAs ↓ the surface expression of VCAM in HUVECs, CLA9,11 by 31.5% and CLA10,12 by 23%, compared to control cells.<br>CLA9,11 ↓ the surface expression of ICAM-1 in HUVECs by 52% compared to control cells.<br>Trend to reduce adhesion of macrophages to HUVECs in cells cultured with CLAs | (Stachowska et al, 2012) |
| HAECs | OA, LA, EA, Linoelaidic, Stearic: 5, 25, 50 µM, 24 and 48h | EA and linoleaidic ↑ expression ICAM-1<br>Both iTFAs and stearic acid ↑ phosphorylation of NFκB. | (Harvey et al, 2012) |
| HCASMCs and human coronary endothelial cells | Butyric, lauric, myristic, palmitic, stearic, oleic, linoleic, linolenic, arachidonic, EPA, DHA, EA, TVA and CLA (200 µM, HCAECs 8h; HCASMCs 20h) | ↑ IL-6 and MCP-1 levels were observed only in HCASMCs for TVA and EA, respectively.<br>Linoleic acid was less pro-inflammatory in both cell lines when compared to CLA. | (Soto-Vaca et al, 2013) |
| HUVECs<br>HepG2 cells (hepatocellular carcinoma) | TVA, EA, *trans*-palmitoleic (5-150 µM)<br>Mixture of TVA and *trans*-palmitoleic (150/50 µM) TNF-α (2 ng/ml, 24h) | HUVECs: rTFAs and EA ↓ inflammatory gene expression (TNF-α, VCAM-1 and SOD-2<br>HepG2 cells: rTFAs ↓ inflammatory gene expression (TNF, IL-8) | (Da Silva et al, 2017b) |
| HUVECs | OA, EA (100 µM) | EA ↑ nuclear translocation of NFκB p65 and phosphorylated ERK1/2; ↑ expression of TLR-4<br>Lipid rafts may be involved in TFA induced-inflammation in HUVECs. | (Pan et al, 2017) |
| HUVECs | TVA, EA, *trans*-palmitoleic (50 µM)<br>Mixture of TVA and *trans*-palmitoleic (150/50 µM) | rTFAs ↓ PG excretion and inflammatory gene expression (TNF and VCAM-1).<br>rTFAs and iTFAs ↑ different classes of F2-isoP (EA ↑ class VI F2-isoP isomers, TVA ↑ mostly class III isomers) | (Da Silva et al, 2017a) |

## 1.5 Objective and Aims

In recent decades, many countries worldwide have experienced changes in lifestyles that impact on human food consumption patterns (Naska et al, 2006; Naughton et al, 2015; Singh et al, 2015). One of the most important trends is the increased consumption of "ready to eat" or ultra-processed foods (Adams & White, 2015; Monteiro et al, 2013; Moubarac et al, 2013). Additionally, even meals eaten in the home environment are often purchased as pre-made processed meals from "take away" restaurants or supermarkets (Adams et al, 2015; Kant & Graubard, 2004; Solberg et al, 2015).

In the United Kingdom, between one-fifth and one-quarter of individuals eat meals prepared out-of-home weekly (Adams et al, 2015). Similarly, in Australia it has been reported that 28% of people consume take away meals at least twice a week (Smith et al, 2009). Meanwhile in United States (US), a study showed that more than three-quarters of dietary energy in purchases by US households comes from moderately (15.9%) and highly processed (61.0%) foods and beverages (Poti et al, 2015). These trends have also been described for children and adolescents. For example, a study of a representative sample of US children estimated that from 66% to 84% of total daily energy, saturated fat, cholesterol, total sugar, added sugars, and sodium intake were contributed by processed foods (Eicher-Miller et al, 2015).

Given that food and nutrition play a key role in the prevention and development of diseases, the effects of these changes in dietary patterns on the current global epidemiological profile should be considered. Regarding the quality of processed foods, the evidence suggests that their role in the burden of global disease is related to the high energy density, and contribution to intake of salt, sugar, total fat, saturated fat, cholesterol and TFAs (Astrup et al, 2008; Ludwig, 2011; Moubarac et al, 2013). Given the increasing consumption of industrialized food, understanding the mechanisms by which their constituents are associated with the increasing prevalence of cardiovascular disease is required. One such component requiring investigation is the family of TFA.

CHD and stroke have remained the leading causes of death globally in the last 15 years (WHO, 2018); therefore, decreasing the risk for these CVDs by reducing the intake of harmful TFAs continues to be a major public health objective worldwide. Even before the WHO announcement in 2018 recommending the elimination of industrially produced TFAs from the food supply (Ghebreyesus & Frieden, 2018), many countries had adopted TFA regulations, including the European Union (Astrup, 2006; EFSA, 2018; WHO, 2015). Nevertheless, more than 110 countries have not yet established regulations against TFAs, meaning that 5 billion people are still at risk from iTFA exposure (WHO, 2019). However, it is not clear how specific TFA isomers differ in their biological activity and mechanisms of action with regard to inflammation.

As shown previously, there are not many studies comparing the inflammatory effects of TFAs of industrial and ruminant origin. Considering the need for greater knowledge about the possible differential effects of TFAs on inflammation, the objective of this project is to study the inflammatory response in cultured EA.hy926 endothelial cells exposed to different 18-carbon *trans* and *cis* fatty acids, with the TFAs being of both ruminant and industrial origin. The FAs used are shown in table 1.8.

Table 1.8: *Cis* and *trans* fatty acids used in this project.

| **Fatty Acid** | **Formal Name** | **Origin** | **Shorthand nomenclature** |
|---|---|---|---|
| Linoleic acid | *Cis*-9*, cis*-12-octadecadienoic acid | Natural, vegetable FA | cis,cis-Δ9,Δ12 18:2n−6 |
| Oleic acid | *Cis*-9- octadecenoic acid | Natural, vegetable FA | 18:1n-9 |
| Elaidic acid | *Trans*-9- octadecenoic acid | Hydrogenated vegetable oils, industrialized FA | trans-Δ9 18:1n−9t |
| *Trans* vaccenic acid | *Trans* 11-octadecenoic acid | Natural, ruminant FA | trans-Δ11 18:1n-7t |
| *Cis* vaccenic acid | *Cis*-11-octadecenoic acid | Natural, de novo synthesis. | 18:1n-7 |
| Conjugated *cis*-9, *trans*-11 linoleic acid | 9Z,11E-octadeca-9,11-dienoic acid | Natural, ruminant FA | *cis, trans*-Δ9,Δ11 18:2n-7 |
| Conjugated *trans*-10, *cis*-12 linoleic acid | 10E,12Z-octadeca-9,11-dienoic acid | Natural, ruminant FA | *trans, cis*-Δ10,Δ12 18:2n-6 |

The aims of this project were to compare the effects of the *trans* isomers *trans* vaccenic acid and elaidic acid, from ruminant and industrial origin respectively, and also comparing the effects of the two main conjugated linoleic acid isomers of ruminant origin, in ECs cultured both under basal conditions and after TNF-α stimulation, to simulate situations of both normal functioning and of low-medium level inflammation (Damas et al, 1992; Dentener et al, 2006; Mozes et al, 2011).

It is anticipated that this research will provide novel insights into the mechanisms through which TFAs in general and 18-carbon iTFAs and rTFAs in particular affect inflammation and the functioning of human endothelial cells, which is important in relation to development of atherosclerosis and the consequent risk for CVD.

# Chapter 2 General Methods

## 2.1 Endothelial cell model

EA.hy926 cells (ATCC®, CRL2922, Manassas, USA) were grown in Dulbeccos's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum (10%), L-glutamine (0.4 mM), penicillin (50 U/mL), streptomycin (50 µg/mL) and HAT (100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine) at 37ºC in a humidified 95% air/5% $CO_2$ incubator. Before their use in experiments, cells were grown in T-175 flasks until confluence. ECs were used between passages 6 and 25.

## 2.2 Fatty acid treatment

*Trans* vaccenic acid, elaidic acid, *cis* vaccenic acid, conjugated *cis*-9,*trans*-11 and *trans*-10, *cis*-12 linoleic acids, oleic acid and linoleic acid were prepared as 1, 10 and 50 mM stock solutions in 100% ethanol. Before each experiment, the stock solutions were diluted in warm complete culture medium to yield final concentrations of 1, 10 and 50 µM. The corresponding control was a 0.1% ethanol solution diluted in complete medium.

For the experiments, cells were seeded in 96 well plates (MTT assay and ELISA), 6 well plates (RT-PCR, adhesion assay, flow cytometry) or T25 flasks (gas chromatography), cultured in complete medium and exposed to different fatty acids for 48 h.

## 2.3 TNF-α treatment

Based on the conditions optimized for studying inflammatory responses of cultured EA.hy926 cells performed previously (Baker, 2018), after the fatty acid incubation period, cells were incubated with or without TNF-α (1 ng/mL; 20 units/ml) for 6 or 24 h, depending on the assay to be performed, as described in Figure 2.1.

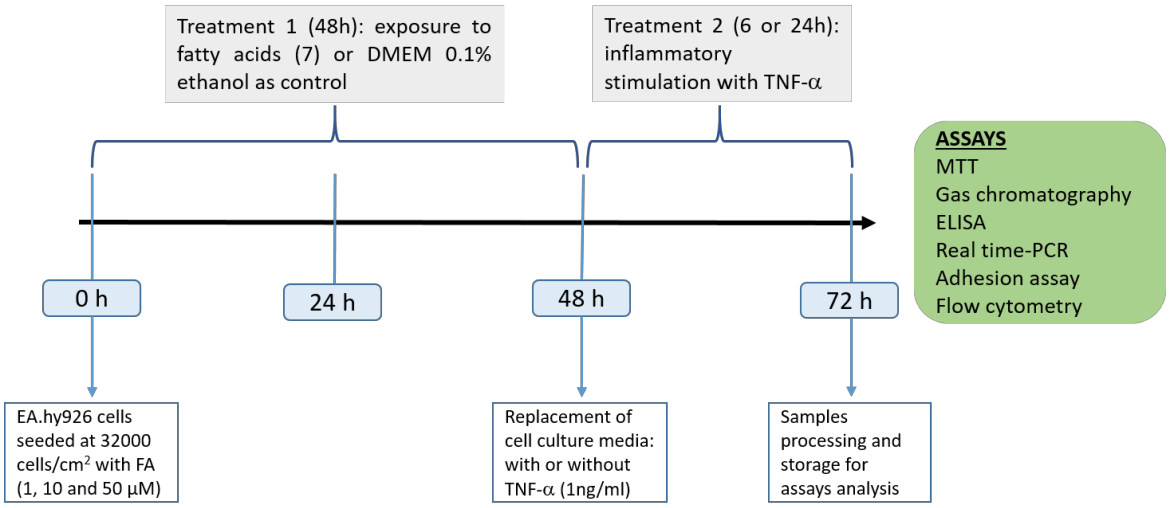



Figure 2.1: Experimental design. Treatment of EA.hy926 cells with fatty acids and TNFα

## 2.4 MTT assay for cell viability

The MTT assay was used to test the cytotoxic effect of the fatty acids at different concentrations.

### 2.4.1 MTT assay principle

The MTT assay is based on the measurement of a marker activity associated with viable cell number. MTT assay is a tetrazolium reduction assay, where MTT substrate (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is positively charged and readily penetrates viable eukaryotic cells. Viable cells with active metabolism convert MTT into a purple coloured formazan product with an absorbance maximum near 570 nm. Dead cells lose the ability to convert MTT into formazan, thus colour formation works as a suitable marker of only the viable cells. The exact cellular mechanism of MTT reduction into formazan is not fully understood, but likely involves the action of dehydrogenase enzymes to produce NADH or similar reducing molecules that transfer electrons to MTT. The reduced product of the MTT tetrazolium accumulates as an insoluble precipitate inside cells, near the cell surface and in the culture medium. Therefore, formazan has to be solubilized, in this case with dimethyl sulfoxide (DMSO), prior to recording absorbance readings. Acidification of the solubilizing solution has the benefit of changing the colour of phenol red to yellow colour that may have less interference with absorbance readings.

### 2.4.2 MTT assay procedure

For the assessment of cell viability, EA.hy926 cells were incubated (96 well plate, $1x10^4$ cell/100 µL per well) with different concentrations of TVA, EA, CVA, CLA9,11, CLA10,12, OA or LA (1, 10 and 50 µM) for 48 h and exposed to full medium with or without TNF-α (1 ng/mL) for 24 h.

Thereafter, the cells were checked under the microscope and the culture medium was replaced by supplemented DMEM also containing 0.05 mg/mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Solon, OH, USA) and cultivated for another 4 h in the incubator (37°C). Then, supernatant was removed and 50 µL of DMSO was added to each well and the plate was incubated for 10 minutes at 37°C. Finally, the absorbance was measured at 550 nm. Figure 2.2 shows the MTT assay procedure steps.

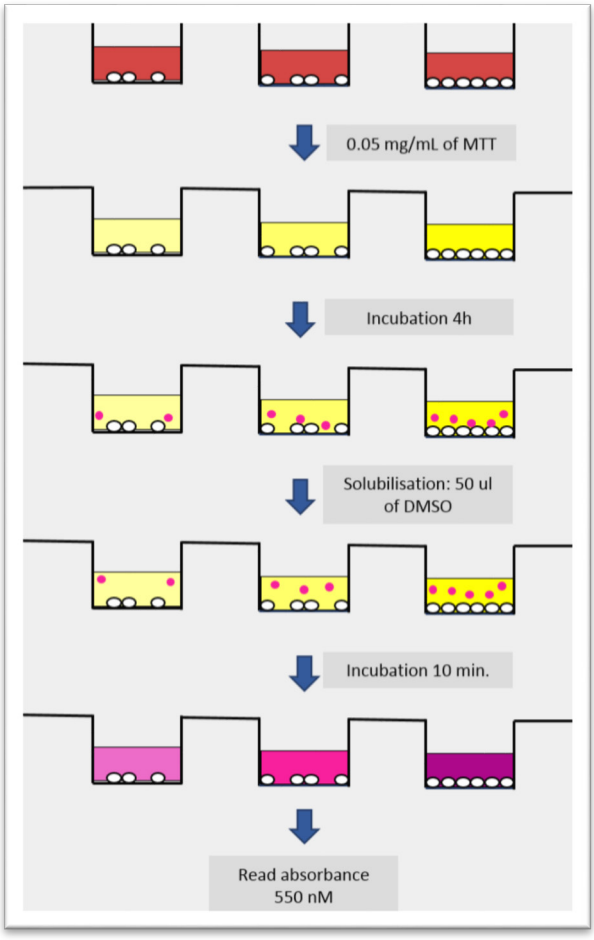



Figure 2.2: Schematic of MTT assay procedure.

Effects of fatty acids and TNF-α on cell viability were normalized according to the control group value (set to 100%).

## 2.5 Gas chromatography

Gas chromatography (GC) was used to test the FAs concentrations in the culture medium and to confirm the incorporation of the FAs into the cells.

For FA concentration testing, each FA was diluted in full warm medium from the respective stock in 100% ethanol.

For FA incorporation, cells were seeded in T25 $cm^2$ flaks (5 x $10^5$ cells/mL) for 48 h with each fatty acid in different concentrations (initially 0.1, 1, 5, 10, 50 and 100 µM). Afterwards, the cells were checked under the microscope, scraped and counted with a Beckman Coulter cell counter. EA.hy926 cells were resuspended to have 1 x $10^6$ cells/800 µL of 0.9% NaCl solution.

### 2.5.1 Gas chromatography principles

GC is a method employed to separate FAs (in this case fatty acid methyl esters) based on the temperature at which they become volatile and their affinity to a silica capillary column. This depends on the carbon chain length, the number and position of double bonds. The longer the chain length of the fatty acid the higher the temperature at which it enters the vapour phase and the opposite occurs with the number of double bonds. A methyl ester group is added to the carboxylic end of the fatty acid to form a fatty acid methyl ester (FAME), this lowers the boiling point, allowing separation of a wide range of fatty acids at moderate temperatures; in practice the methylation is combined with the release of fatty acids from phospholipids, triglycerides and cholesteryl esters. The gas chromatograph has a heated injection port, a silica capillary column situated in an oven and a flame ionization detector. FAMEs are injected into the gas chromatograph port and heated at high temperatures to become volatile. Then, FAMES are carried by a helium flow through the capillary column, which has a lower temperature than the injection port letting FAMEs condense on the column lining. Subsequently, the column is heated and FAMEs are dissociated from the column lining as they reach their boiling point. The end of the column is located within the flame ionization detector, held at 250ºC. The hydrogen flame causes FAME combustion, generating an ion current proportional to the amount of FAME in the sample. This process results in a chromatogram containing peaks, each corresponding to a different FAME.

### 2.5.2 Gas chromatography procedure

#### 2.5.2.1 Lipid extraction and formation of FAMEs

The total lipid fraction was extracted from cell pellets and culture medium, after adding the internal standard, into 5 mL of chloroform:methanol (2:1, vol:vol) and 1 mL of NaCl (1M). Consequently, after vortexing, centrifuging (845 g for 10 minutes) and colleting the lower phase, lipids were dried under nitrogen at 40°C and then resuspended in toluene. To obtain the FAMEs, the lipid extracts were incubated at 50°C for 2 h with 1 mL of methanol containing 2% (vol:vol) $H_2SO_4$. After methylation, the samples were left at room temperature for approximately 5 minutes to cool and then neutralized with a solution containing $KHCO_3$ and $K_2CO_3$ (1 mL). Then, FAMEs were dissolved in 1 mL of hexane and dried under nitrogen at 40°C. Finally, the samples were resuspended in 50 µL (FA incorporation to cells) or 150 µL (FA concentration checking in DMEM) of hexane and transferred into GC autosampler vials for GC analysis.

### 2.5.3 GC analysis

GC analysis was performed in a Hewlett-Packard 6890 gas chromatograph fitted with a SGE BPX-70 capillary column (30 m x 0.2 mm x 0.25 µm) and a flame ionising detector. Split ratio was programmed to 2:1 to allow a higher sample uptake in the column. Injector port was set at 300ºC and helium was used as the carrier gas for FAME transport. To condense FAMEs, the oven was kept at 115ºC for 2 minutes increasing 10ºC/minute; a maximum temperature of 200ºC was held for 18 minutes. After this cycle, the column reached 245°C. FAME histograms produced were analysed with Agilent ChemStation software. 37 FAMEs were used as standard to identify fatty acids according to retention time and for software calibration. Figure 2.3 shows the GC trace of a sample of EA.hy926 cells cultured in complete medium.

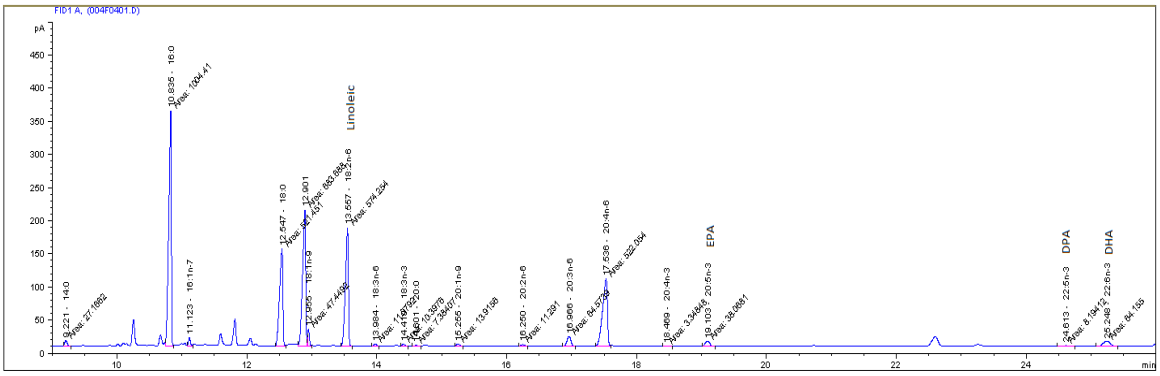



Figure 2.3: Chromatogram of EA.hy926 cells incubated in culture medium for 48 h.

## 2.6 ELISA

Cell culture supernatants were assayed by Human Magnetic Luminex Screening Assay ELISA (R&D Systems, Minneapolis, MN, USA) to measure the appearance of inflammatory factors MCP-1, IL-6, IL-8, VEGF, RANTES and ICAM-1. The cells were seeded with the FAs in 96 well plates (1 x $10^4$ cells/100 µL per well) for 48 h and then stimulated with TNF-α for 24 h. Before the supernatants of each well were collected and stored at -80°C until analysis, the cells were checked under the microscope.

### 2.6.1 Principle of Luminex assay

Luminex is an immunoassay based on multi-analyte technology that enables simultaneous detection and quantification of different secreted proteins, in this case cytokines and chemokines. This assay uses colour-coded beads, pre-coated with analyte-specific antibodies, which bind to the analyte of interest (see figure 2.4). Unbound substances are washed away, and biotinylated detection antibodies specific to the analytes of interest are added to form an antibody-antigen sandwich. Samples are washed again to remove any unbound substances, and incubated with phycoerythrin (PE)-conjugate streptavidin, which binds to the biotinylated antibodies. Unbound substances are again washed away and microparticles are resuspended in wash buffer and read using the Luminex MAGPIX Analyzer. This analyzer uses a magnet that captures and holds the magnetic beads in a

monolayer, while two spectrally different light-emitting diodes (LEDs) illuminate the beads. One LED identifies the analyte that is being detected and, the second LED determines the magnitude of the PE-derived signal.

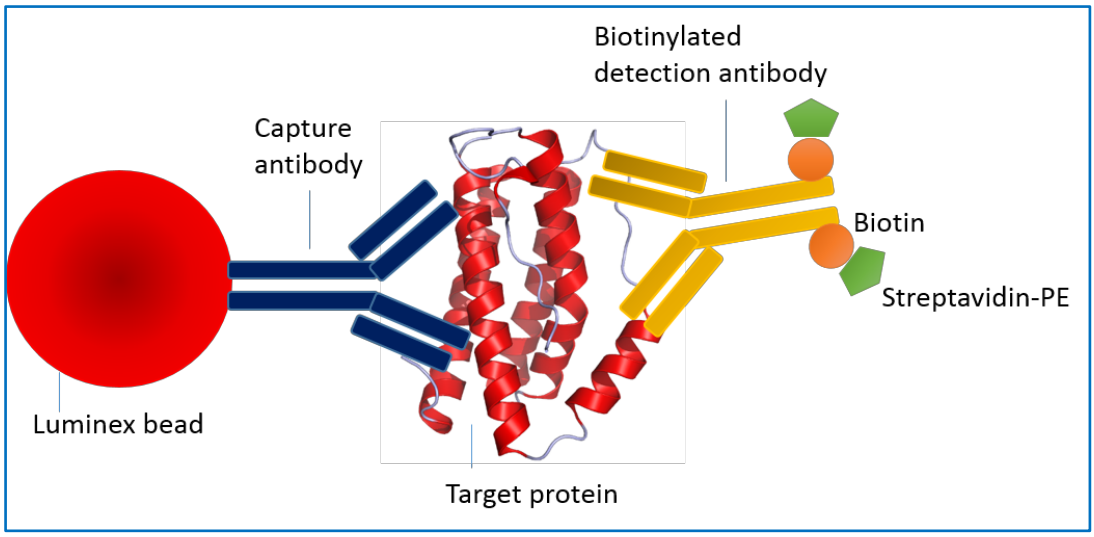



Figure 2.4: Schematic of antibody-antigen sandwich principle of luminex assay

### 2.6.2 Luminex assay procedure

All reagents and standards were prepared according to the manufacturer's instructions; for IL-6, VEGF, RANTES and ICAM-1 undiluted samples of culture medium were analysed, for MCP-1 and IL-8, samples were diluted 1:5 in diluent provided by the manufacturer (Calibrator Diluent RD6-52). First, 50 µL of microparticle cocktail and 50 µL of sample or standard were added into a 96 well clear bottomed black plate which was then securely covered with a foil plate sealer to be incubated for 2 h at room temperature on a horizontal orbital microplate shaker at 800 rpm. Then, the plate was attached to a magnetic microplate device and washed 3 times with 100 µL wash buffer. 50 µL of Biotin Antibody Cocktail was added to each well and plate covered again with a foil sealer and incubated for 1 h on a horizontal plate shaker at 800 rpm. The plate was washed again 3 times with 100 µL wash buffer. 50 µL of Streptavidin-PE was added to each well and the plate was sealed for a 30 minutes incubation on a plate shaker at 800 rpm. The washing step was repeated and each well was resuspended in 100 µL wash buffer. The plate was incubated for 2 minutes on a plate shaker at 800 rpm. Finally, the plate was analysed on the Bi-Plex 200 (Bio-Rad) using the Bio-Plex Manager 6.1 software.

## 2.7 Real Time PCR

The effect of each fatty acid and TNF-α on gene expression was studied by RT-PCR. The cells were seeded in six well plates (density of 6 x $10^5$ cells/mL) with for 48 h and then stimulated with TNF-α. Changes in inflammatory gene expression after TNF-α stimulation for 6 h were analysed by RT-PCR. The expression of NFκB subunit 1 (NFκB1) (Hs00765730_m1), PPAR-α (Hs00947536_m1), PPAR-γ (Hs00234592_m1), TLR-4 (Hs00152939_m1), IL-6 (Hs00985639_m1), IκBα (Hs00355671_g1) IKK-β (Hs00233287_m1), COX-2 (Hs00153133_m1) and MCP-1 (Hs00234140_m1) were assessed by qRT-PCR with TaqMan® Gene Expression primers (Thermo Fisher Scientific, UK).

First, the total RNA for each time point sample was extracted with ReliaPrep™ RNA Cell Miniprep System (Promega, UK). RNA quality, concentration and integrity were measured by Nanodrop Spectrophotometer and Bioanalyzer, respectively. cDNA was synthesized from the total RNA using GoScript™ Reverse Transcriptase (Promega, UK). Quantification of gene expression was carried out initially (time-course) with B2M (Hs00187842_m1, TaqMan® Gene Expression, Thermo Fisher Scientific, UK) and β-actin (Hs01060665_g1) as internal control genes.

### 2.7.1 Principle of RT-PCR

The fatty acids that have been used as treatment are signals for the cells to increase or decrease the expression of genes, in this case, of the inflammation pathway. Therefore, the transcription of those genes to mRNA changes, in order that higher or lower levels of the proteins the genes codes for may be translated. This means the expression of genes in cells exposed to different treatments can be compared to control samples, by comparing the levels of mRNA for those genes. To do this, total RNA is extracted from the cell samples and levels of the specific mRNA transcribed from the gene of interest measured. The enzyme, reverse transcriptase, then uses the RNA as a template to form complementary strands of DNA (cDNA), resulting in cDNA for every RNA extracted, in the same proportions that are present in the samples. The use of specific primers for the sequence of the messenger RNA (mRNA) of the gene of interest, allows to precisely assess the level of expression of that particular gene. This level is then compared to the level of expression measured in a control

group, for example, to determine whether there is a significant increase or decrease in expression of the gene between groups.

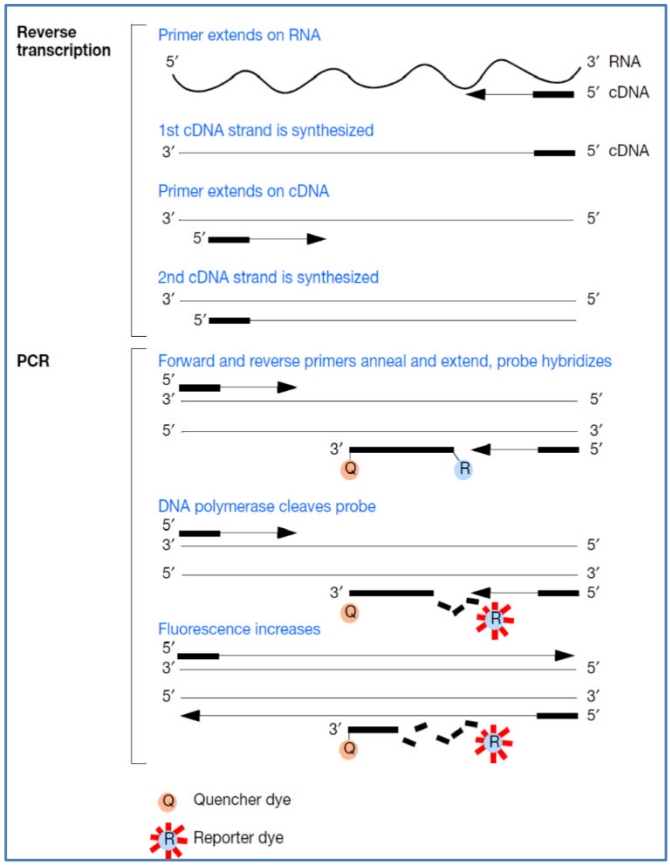



Figure 2.5: Schematic of Real Time PCR with TaqMan probes.

Real-time quantitative PCR is a measurement to detect the products generated during each cycle of the PCR process, which are directly proportional to the amount of template prior to the start of the PCR process. This is achieved by using specific primers, which only anneal to the cDNA specific to the gene of interest, and measuring the point at which the amount of amplified DNA reaches a threshold level (Cq value). The earlier the amount of DNA within a sample reaches the 'threshold level', compared to other samples, the more cDNA template was present; and therefore the gene was more highly expressed in the cell sample. In this case, levels of DNA can be measured by the use of a probe (TaqMan), where a fluorescence emitting dye (e.g. FAM) is bound to one end of a short DNA sequence with a molecule of quencher (e.g. TAMRA) at the other (Figure 2.5). While these molecules are bound in close proximity to each other, no fluorescence is emitted. However, once the probe binds to its specific site and the extension step begins, Taq DNA polymerase uses its

exonuclease activity to 'chew up' the oligonucleotide sequence holding the dye and quencher molecule close together. As the dye and quencher start to move apart, the dye emits a fluorescent signal. One unit of fluorescence is emitted per copy of the gene to which it specifically binds; therefore, the amount of fluorescence detected is proportional to the amount of mRNA for the gene of interest present in the sample. This signal is recorded by the qPCR machine and converted into Cq values. The details of each step in the thermocycler are described in the following table.

Table 2.1: Details of the real time PCR cycles

| Step | Description of the cycle | Event |
|---|---|---|
| 1 | 50°C for 2 minutes | |
| 2 | 95°C for 10 minutes | To activate the Taq polymerase enzyme |
| 3 | 95°C for 15 seconds per cycle | Denaturation of 1x double stranded (ds) DNA to 2x single stranded (ss) DNA |
| 4 | 60°C for 60 seconds per cycle | Probe anneals between binding sites for forward (F) and reverse (R) primers. The TaqMan probe hybridizes to the sequence. The 5' to 3' nucleolytic activity of the DNA polymerase cleaves the hybridized fluorogenic probe between the reporter dye and the quencher dye. The fragments of reporter dye are displaced from the target, resulting in an increase in fluorescence. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. Data is collected at this stage. |
| Steps 2 and 3 are repeated for 40 cycles. The process is repeated with each round of PCR, resulting in a proportional increase in fluorescent signal detected as the amount of DNA within the sample doubles. | | |

### 2.7.2 RT-PCR procedures

#### 2.7.2.1 Isolation of RNA

Total RNA was extracted from each cells sample using ReliaPrep™ RNA Cell Miniprep System following the manufacturer's instructions. After discarding the cell supernatant and washing with PBS, the reagent BL+ 1-thioglycerol was added to each well (250 µL). Cells were scraped and cells lysates were stored at -80°C until processing.

Cell lysates were thawed, resuspended and mixed with 85 µL 100 % isopropanol. Then, the total volume of each sample was transferred to a ReliaPrep™ Minicolumn and collection

tube and centrifuged at 13,000 g for 30 seconds. 500 µL RNA wash solution was added and the samples were again centrifuged at 13,000 g for 30 seconds. 30 µl of DNase 1 mix (24 µl of Yellow Core Buffer, 3 µl 0.09 M $MnCl_2$, 3 µl of DNase I enzyme) was added directly to Minicolumn membrane and samples were incubated at room temperature for 15 minutes. Following incubation, 200 µL column wash solution was added and samples were centrifuged at 13,000 g for 15 seconds. 500 µL RNA wash solution was added and samples centrifuged at 13,000 g for 30 seconds. The Minicolumns were then transferred to new collection tubes and 300 µL RNA wash solution added and samples centrifuged at 13,000 g for 2 minutes. Minicolumns were then transferred to elution tubes, 15 µL of nuclease free water added and samples were centrifuged again at 13,000 g for 1 minute. Minicolumns were then discarded and the RNA quantity and quality was assessed by Nanodrop. The elution tube containing the purified RNA was stored at -80°C.

#### 2.7.2.2 NanoDrop analysis

The quantity and purity of the RNA was measured using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). The analysis is based on the principle that nucleic acids absorb ultraviolet light in a specific pattern. The RNA sample is exposed to ultraviolet light at a wavelength of 260 nanometres (nm) and a photo-detector measures the light that passes through the sample. Some of the ultraviolet light will pass through and some will be absorbed by the RNA sample. The more light absorbed by the sample, the higher is the nucleic acid concentration, resulting in less light striking the photodetector and producing a higher optical density (OD).

The Beer-Lambert Law equation is used to relate the amount of light absorbed to the concentration of the absorbing molecule in ng/µL, with an extinction coefficient of 0.025 $(\mu g/ml)^{-1}$ $cm^{-1}$ for RNA at a wavelength of 260 nm.

The ratio of the absorbance at 260 and 280 nm ($A_{260/280}$) is used to assess the purity of nucleic acids, generally utilized to assess the amount of protein contamination from the nucleic acid isolation process since proteins absorb at 280 nm and phenol at 270 nm. The $A_{260/280}$ ratio for pure RNA is ~2.0. The ratio of the absorbance at 260 and 230 nm ($A_{260/230}$) is used to assess contamination by guanidine thiocyanate or other organic compounds. The $A_{260/230}$ ratio for pure RNA is ~1.8-2.2.

#### 2.7.2.3 Bioanalyzer analysis

The integrity of the RNA starting material was determined using the Agilent 2100 Bioanalyzer System. By electrophoretic separation on microfabricated chips, RNA samples are separated and consequently detected through laser induced fluorescence detection. The Bioanalyzer software produces an electropherogram and gel-like image and displays results such as sample concentration and the ratio of the 18S and 28S ribosomal subunits. The electropherogram provides a detailed visual assessment of the integrity of an RNA sample by showing a detailed picture of the size and distribution of RNA fragments.

To standardize the process of RNA integrity interpretation, the Bioanalyzer software generates the RNA Integrity Number (RIN), removing individual interpretation in RNA quality control and considering the entire electrophoretic trace. The RIN software algorithm classifies eukaryotic total RNA based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact.

The procedure includes loading 1 µL of each RNA sample into a chip and reading it on the Bioanalyzer, which was carried out by Melissa Doherty.

#### 2.7.2.4 cDNA synthesis

GoScript™ Reverse Transcriptase was used to synthesise cDNA from total RNA according to the manufacturer's specifications. 1 µg of purified RNA mixed with nuclease free water was heated to 70°C for 5 minutes with Oligo$(dT)_{15}$ primers and immediately cooled on ice for at least 5 minutes. Then, 9 µL of reverse transcription reaction mix (4 µL of GoScript™ 5x reaction buffer, 2 µL of $MgCl_2$ (25mM), 1 µL of PCR nucleotide mix, 1 µL of Recombinant RNasin® Ribonuclease inhibitor, 1 µL of GoScript™ reverse transcriptase, per reaction) was added. Samples (20 µL in total) were then loaded into a Thermal Reactor and programmed to anneal at 25°C for 5 minutes, followed by extension at 42°C for an hour and inactivation at 70°C for 15 minutes.

#### 2.7.2.5 qRT-PCR

The expression of NFκB1, IKKβ, IκBα, PPAR-α, PPAR-γ, TLR4, COX-2, MCP-1 and IL-6 were assessed by qRT-PCR with TaqMan® Gene Expression primers. cDNA was diluted to 5 ng/µL.

Using a 96 well qPCR plate, 4 µL of sample were added to 16 µL of PCR mix (10 µL of master mix, 1 µL of gene primer/probe and 5 µL of nuclease free water, per reaction) per well. The plate was sealed and centrifuged at a low speed for 1 minute. The plate was then loaded into a thermocycler (Applied Biosystems 7500) with a cycle of 50°C for 2 minutes, followed by a cycle of 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and finally a cycle of 60°C for 1 minute. Each sample was analysed in duplicate.

#### 2.7.2.6 Relative quantification of gene expression

mRNA levels were calculated using the Cq values by double delta Cq (ΔΔCq) values expressed as fold change of the target gene in experimental samples relative to control samples (Livak & Schmittgen, 2001).

A normalization factor based on the expression levels of the best-performing housekeeping genes was calculated using the geometric mean. Then, the average Cq values for the duplicates of target genes in experimental and control conditions were calculated. Delta Cq (ΔCq) values were calculated from the differences between target gene and reference gene for experimental and control samples. The difference between the ΔCq of experimental samples and the ΔCq of control samples results in the double delta Cq value (ΔΔCq). Finally, the 2^ΔΔCq is calculated to express the fold change.

## 2.8 Adhesion assay

The effect of each fatty acid and TNF-α on adhesion of monocytes to cultured EA.hy926 cells was studied by a static cell adhesion assay. The EA.hy926 cells were seeded in 96-well flat bottom plates (density of 2 x $10^5$ cells/mL, 1 x $10^5$ cell per well) with FA for 48 hrs and then stimulated with TNF-α (1 ng/mL) for 6 hrs.

The monocyte cell line THP-1 (ECACC - 88081201) was obtained from ECACC. The cells were thawed and subcultured following the supplier's instructions. Cells were cultured at 5-7 x $10^5$ cells/mL in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS, L-glutamine (0.4 mM), penicillin (50 U/mL) and streptomycin (50 µg/mL). Cells were maintained at 37°C in 5% $CO_2$. Preceding the experiments, cells were grown in T-75 flasks until confluent. THP-1 cells were used between passages 7 and 17.

The static adhesion of THP-1 monocytes to EA.hy926 cells was determined using the Vybrant™ Cell Adhesion Assay Kit (Thermo Fisher Scientific).

### 2.8.1 Principle of the static adhesion assay

The Vybrant™ Cell Adhesion Assay Kit (Thermo Fisher Scientific) utilizes the fluorogenic dye calcein acetoxymethyl ester (calcein AM) for measuring cell adhesion. Calcein AM is non-fluorescent but, once loaded into cells, it is cleaved by endogenous esterases to produce highly fluorescent calcein. Calcein provides a brightly fluorescent, pH-independent, cytoplasmic cell marker, which interferes minimally with the cell adhesion process. Calcein's absorbance maximum of 494 nm and emission maximum of 517 nm are suited for detection by instruments equipped with standard fluorescein filters.

As shown in figure 2.6, THP-1 monocytes are labelled with calcein AM by incubating them for 30 minutes. These cells are then incubated with the EA.hy926 cells for 1 h to allow the cell-to-cell adhesion to occur. After removal of non-adherent cells, calcein fluorescence is used to calculate the proportion of adherent cells.

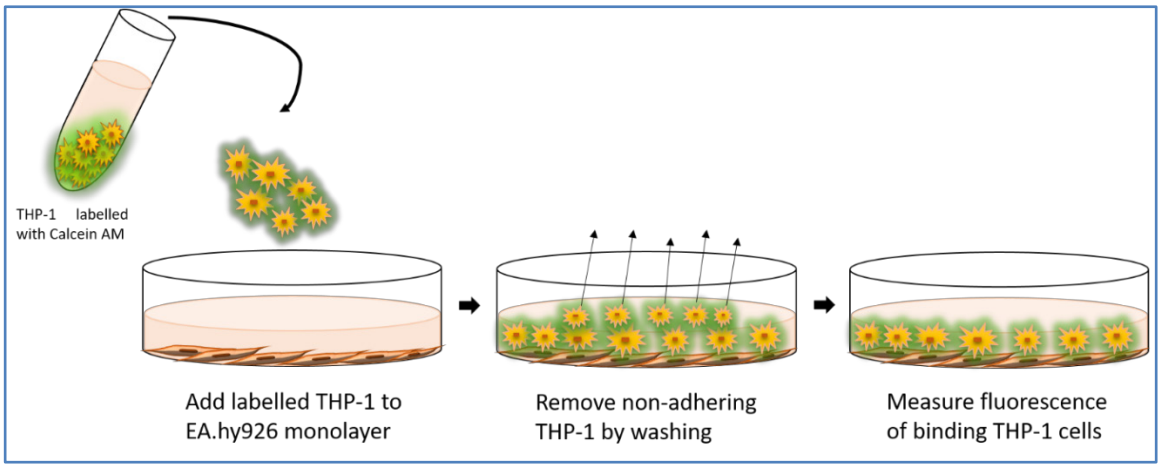



Figure 2.6: Schematic of Vybrant Cell Adhesion Assay principle.

### 2.8.2 Adhesion Assay Procedure

#### 2.8.2.1 Vybrant™ Cell Adhesion Assay Kit

Vybrant cell adhesion kit assay protocol instructions were followed. THP-1 cells were counted and washed twice with RPMI (without supplements). Then, the cells were incubated with calcein (5 µL/mL) at a density of 5 x $10^6$/mL for 30 minutes.

After TNF-α stimulation and the observation of the cells under the microscope, supernatant was removed from the EA.hy926 cell cultures and the cells were washed twice with RPMI (non-supplemented). Calcein labelled THP-1 cell suspension (5 x $10^4$ cells in 100 µL) was washed twice with RPMI and added to each well containing 3 x $10^4$ ECs. Cells were co-incubated for 1 h at 37°C. Non adherent THP-1 cells were removed by gentle washing using a pipette, and adherent cells were washed a further 3 times with RPMI (non-supplemented). Finally, 200 µL of PBS was added to each well and fluorescence was read on the Glomax Discover System (Promega) at 475 nm of absorbance and 520 nm of emission.

### 2.8.3 Imaging of THP-1 binding

Images of fluorescence-labelled THP-1 monocytes adhered to EA.hy926 cells were taken with fluorescence microscope Nikon Elipse Ti using NIS elements software (version 4.30). The images were captured at a magnification of 100x under transmitted light and fluorescence filter for green fluorescence protein.

## 2.9 Flow cytometry

The effect of each fatty acid and TNF-α on the expression of ICAM-1 on the surface of cultured EA.hy926 cells was studied through flow cytometry. Endothelial cells were seeded in six well plates (density of 6 x $10^5$ cells/mL) with FA for 48 h and then stimulated with TNF-α.

### 2.9.1 Principle of flow cytometry

The basic principle of flow cytometry is the passage of cells in single file in front of a beam of a laser so they can be detected and counted. Cell components are fluorescently labelled and then excited by the laser to emit light at varying wavelengths and this fluorescence can then be measured to determine the amount and type of cells present in a sample.

Each sample can be analysed as the cells pass through the liquid stream and the beam of laser light is directed at it in a hydrodynamically-focused way. Several detectors are placed around the stream, at the point where the fluid passes through the light beam, one of them

is in line with the light beam and is used to measure Forward Scatter (FSC). Another is placed perpendicular to the stream and is used to measure Side Scatter (SSC). A third detector measures fluorescence.

As the cells pass through the beam of light and scatter the light beams, the fluorescently labelled cell components are excited by the laser and emit light at a longer wavelength than the light source. Consequently, the detectors collect a combination of scattered and fluorescent light, data which are then analyzed by a computer that is attached to the flow cytometer using CellQuest Pro software.

The brightness of each detector, according to the fluorescent emission peak, is adjusted. Using the light measurements, different information can be collected about the physical and chemical structure of the cells. In general, FSC detects cell volume whereas the SSC reflects the inner complexity of the particle (granularity or nuclear structure), as depicted in Figure 2.7.

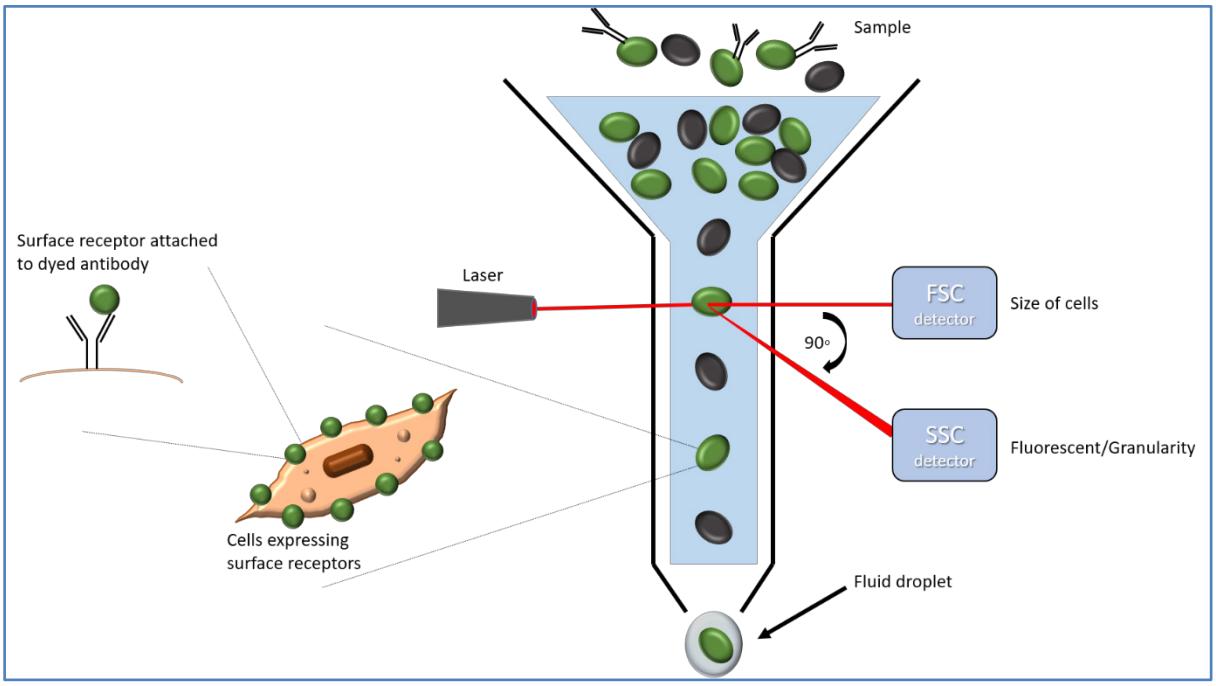



Figure 2.7: Schematic of flow cytometry principle.

### 2.9.2 Procedure

Confluent EA.hy926 cells were scraped from T175 flasks and resuspended in DMEM at a density of 5.5 x $10^5$ cells per mL. The cells were seeded in 6-well plates for 48 h with FAs followed by 6 h incubation with either DMEM or TNF-α at 1 ng/mL.

After incubation, the cells were checked under the microscope, supernatant was removed and 1 mL cold PBS added to each well. Cells were detached by scraping on ice and transferred to FACS tubes. Consequently, the cells were centrifuged at 304 g for 15 minutes, 4ºC. Supernatant was discarded and cell pellets were then resuspended in 100 µL of PBS with 2% FBS. After vortexing, cells were stained with (PE-Cy$^{TM}$ 5)-conjugated anti-human CD54 (BD Biosciences) at 10 µg/mL for 30 minutes at 4ºC in darkness. Mouse IgG1 κ (PE-Cy$^{TM}$ 5) isotype (BD Biosciences) was used as a negative control. Unstained negative controls were also prepared; these were incubated with PBS 2% FBS alone.

Following the incubation with the antibody, samples were washed twice with PBS containing 2% FBS and centrifuged at 304 g for 5 minutes. Supernatant was discarded and pellets resuspended in 200 µL BD CellFIX (diluted 1/10 in PBS with 2% FBS). FACS tubes were then stored at 4ºC overnight and were analysed the following morning in a FACS Calibur flow cytometer (Becton Dickinson). Data were collected using CellQuest Pro software and analysed using FlowJo.

For each sample 10,000 events were collected. Forward and side scatter were used to determine the cell size and granularity and a gate was drawn around the cell population. Cells within this gate were analysed to determine PE-Cy$^{TM}$ 5 fluorescent staining.

## 2.10 Statistical analysis

Data distribution and homoscedasticity were analysed through Kolmogorov-Smirnov and Levene tests, respectively. Data are presented as mean ± SEM and analysed by one-way analysis of variance or two-way analysis of variance (two-way ANOVA), after log transformation if needed (according to sample distribution), followed by a post hoc test (Tukey's or Dunnet). Analyses were performed using GraphPad Prism 6.0. Differences were considered significant when $p<0.05$.

# Chapter 3 Toxicity and incorporation of 18-carbon *trans* fatty acids when incubated with endothelial cells

## 3.1 Introduction

Prior to determining the effects of 18-carbon *cis* and *trans* FAs of different origin on the inflammatory responses of ECs, assessing cell viability and FA composition changes was considered important. The viability of the cells was evaluated using the MTT assay, which assesses metabolic activity, and the incorporation of FAs of interest into the cells was determined using gas chromatography (GC).

Cytotoxic effects of FAs have been observed in HUVECs and other cell lines. Long-chain saturated FAs have been shown to induce human aortic endothelial cell apoptosis (Harvey et al, 2010). EA has also been reported to induce apoptosis of HUVECs in a dose-dependent manner, significant from the lowest concentration used (50 to 400 μM) and measured using flow cytometry (Qiu et al, 2012). In contrast, other authors reported that OA did not induce cell death in different EC models, while LA triggered programmed cell death only at the highest concentration used (300 μM) in human aortic ECs and in endothelial progenitor cells (Artwohl et al, 2008).

To be able to compare the effect on the inflammatory response of the FAs of interest, it is necessary to check that the FA treatments do not produce cytotoxicity, and that each FA is incorporated into the cells to be able to exert its biological effects.

The research described in this chapter compares the effects of different FAs on cell viability and FA composition in EA.hy926 cells, with those in control conditions.

## 3.2 Objectives and Aims

The research objectives of this chapter were to evaluate the toxicity of the FAs of interest, in different concentrations, on EA.hy926 cells and to measure their incorporation into the cells.

The specific aims were to:

- Evaluate EA.hy926 cell viability after culture with each FA and TNF-α, after initial testing with *trans* vaccenic acid and elaidic acid.

- Assess FA incorporation into EA.hy926 cells after culture with each FA, after initial testing with *trans* vaccenic and elaidic acid.

## 3.3 Methods

### 3.3.1 Reagents

Reagents and materials used for experiments are described in Appendix A.

### 3.3.2 Cell culture and treatment

For the cell viability experiments, confluent EA.hy926 cell cultures were scraped from flasks and resuspended in culture medium at a density of 5.5 x $10^5$ cells per mL. Cells were seeded at 5.5 x $10^4$ cells per well in 96-well flat bottom plates, incubated for 48 h at 37$^o$C with FAs at different concentrations, followed by 24 h incubation with TNFα at 1 ng/mL.

For the FA incorporation experiments, confluent EA.hy926 cultures were scraped from flasks and resuspended in culture medium at a density of 5.5 x $10^5$ cells per mL. Cells were seeded at 5.5 x $10^4$ cells per well in T25 flasks, incubated for 48 h at 37$^o$C with FAs at different concentrations. Afterwards, cells were counted and adjusted to have 1 x $10^6$ cells/800 µL of 0.9% NaCl solution.

### 3.3.3 Determination of cell viability using the MTT procedure

The MTT assay procedures are described in Chapter 2.

### 3.3.4 Determination of cell fatty acid composition using gas chromatography (GC)

GC assay procedures are described in Chapter 2.

### 3.3.5 Statistical analysis

Viability of ECs was expressed as a % of control, control being unstimulated cells. Data are expressed as mean ± SEM; data analysis was performed in PRISM and Excel. Multiple group differences were compared using two-way ANOVA, and, where the ANOVA was significant, Dunnett's multiple comparison test was used.

## 3.4 Results

### 3.4.1 Viability of EA.hy926 cells incubated with TNF-α, TVA and EA

Using the cell viability assay (MTT assay), it was observed that TNF-α (1 ng/mL) for 24 h was not toxic for the EA.hy926 cells. However, 100 µM of both FAs tested initially (TVA and EA) decreased cell viability by 20% (Figure 3.1). Consequently, it was decided to work with FA concentrations up to 50 µM in the next, and all later, experiments. Cells remained viable at this FA concentration.

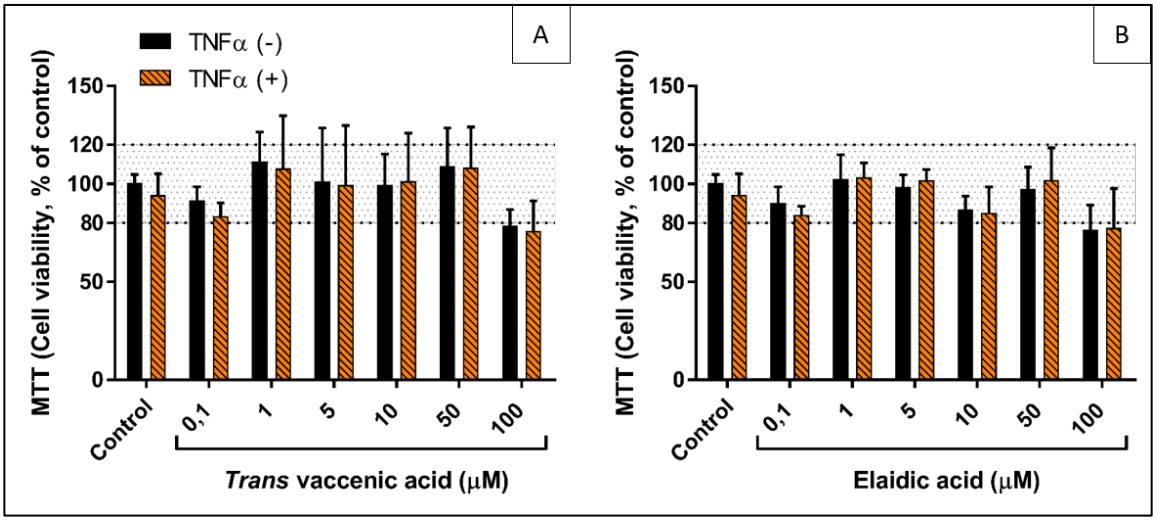



Figure 3.1: Viability test of EA.hy926 incubated with TVA and EA.
EA.hy926 cells were incubated for 48 h with supplemented DMEM containing 0.1% of ethanol (CTL) or different concentrations (0.1, 1, 5, 10, 50 and 100 µM) of TVA and EA, followed by incubation with (+) or without (-) TNF-α (1 ng/mL) for 24 h. Bars are mean ± SEM of 6 samples performed in 2 experiments.

When cell viability was tested using all FAs at concentrations of 1, 10 and 50 µM, it was observed they were not toxic for EA.hy926 cells, with the exception of CLA10,12 at 50 µM, which decreased cell viability by 50-60% (figure 3.2). Therefore, for the experiment comparing the effects of CLAs and LA, it was decided to work only with concentrations 1 and 10 µM.

To support the results from the MTT assay, photographs of EA.hy926 cells pre-incubated with the FAs at the highest concentration (50 µM), followed by the incubation with TNF-α (1 ng/mL) for 24 h, were taken. Figure 3.3 shows that TNF-α and all FAs, with the exception of CLA10,12, allowed the cells to grow healthy and reach confluence, similar to control cells.

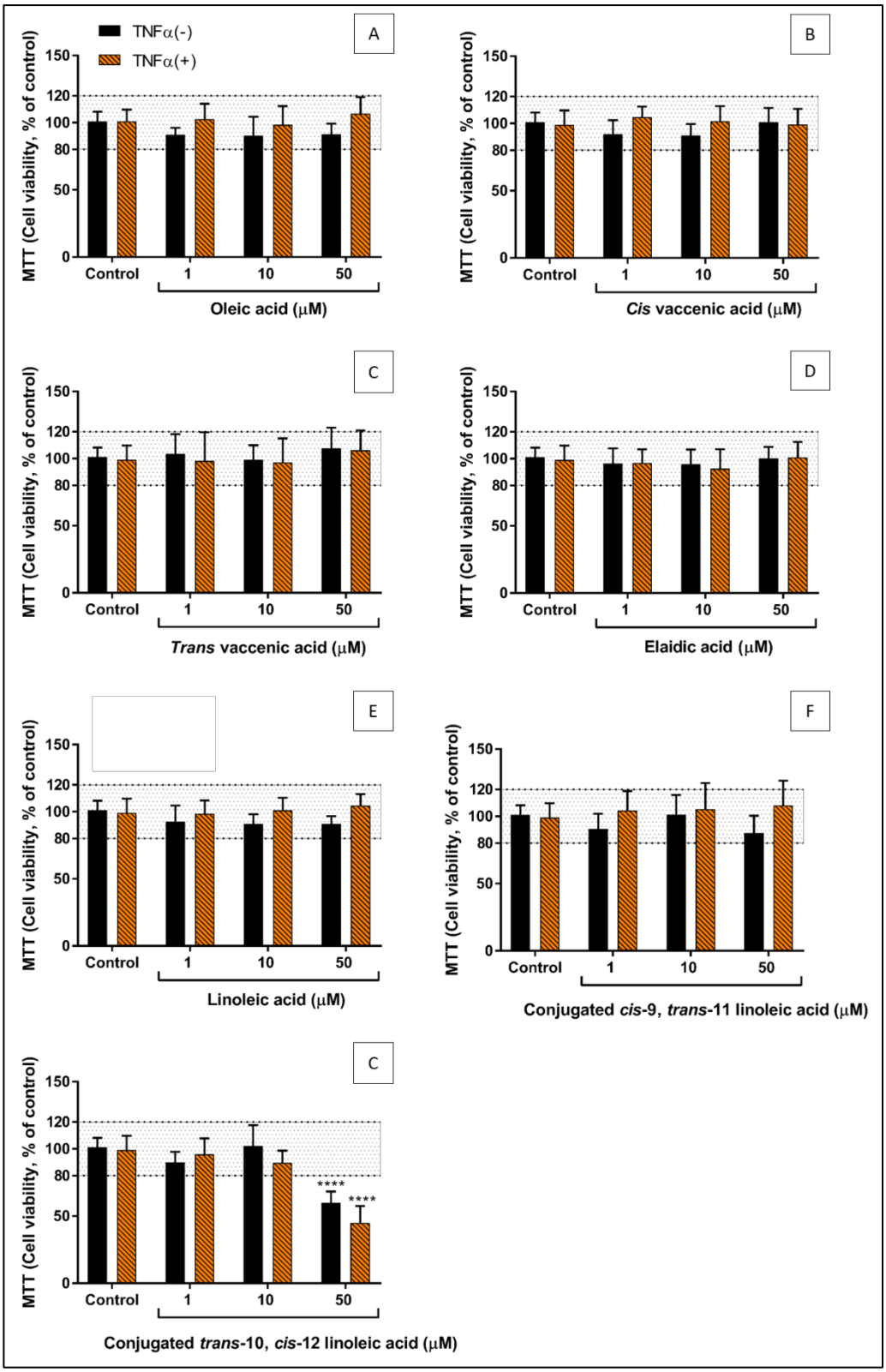



Figure 3.2: Viability of EA.hy926 cells after incubation with FAs.
EA.hy926 cells were incubated for 48 h with supplemented DMEM containing 0.1% of ethanol (CTL) or different concentrations (1, 10, and 50 µM) of different FAs, followed by incubation with (+) or without (-) TNF-α (1 ng/mL) for 24 h. Bars are mean ± SEM of 9 samples performed in 3 experiments. Two-way ANOVA with Dunnett's as post-hoc test. ****p<0.0001 vs Control.

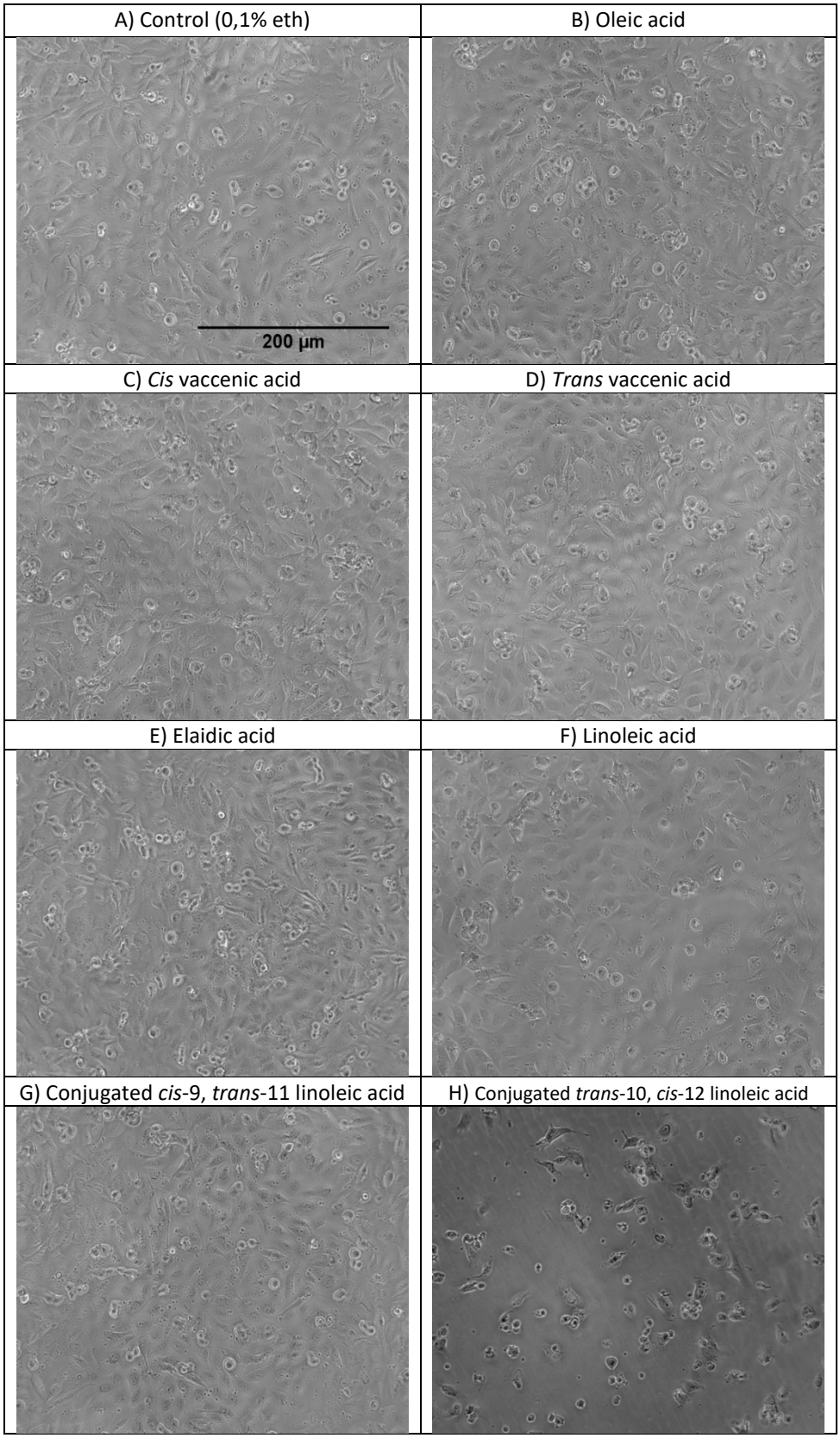



Figure 3.3: Representative visualisation of of EA.hy926 cells after FA exposure (50 µM). EA.hy926 cells after incubation for 48 h with supplemented DMEM containing 0.1% of ethanol (control; (A)) or with OA (B), CVA (C), TVA (D), EA (E), LA (F), CLA9,11 (G), CLA10,12 (H) followed by incubation with TNF-α (1 ng/mL) for 24 h. ECs were visualised by fluorescence microscope Nikon Elipse Ti at a magnification of 100x under transmitted light.

### 3.4.2 Fatty acid incorporation into EA.hy926 cells

FA incorporation into cells was confirmed by GC. Figure 3.4 shows that the administration of TVA or EA increased their incorporation into ECs in a dose-dependent manner. Additionally, cell exposure to TVA acid resulted in the appearance of two unknown products (Fig 3.5 (A and B)), while the exposure to EA generated the presence of one unknown product (Fig. 3.5 (C)).

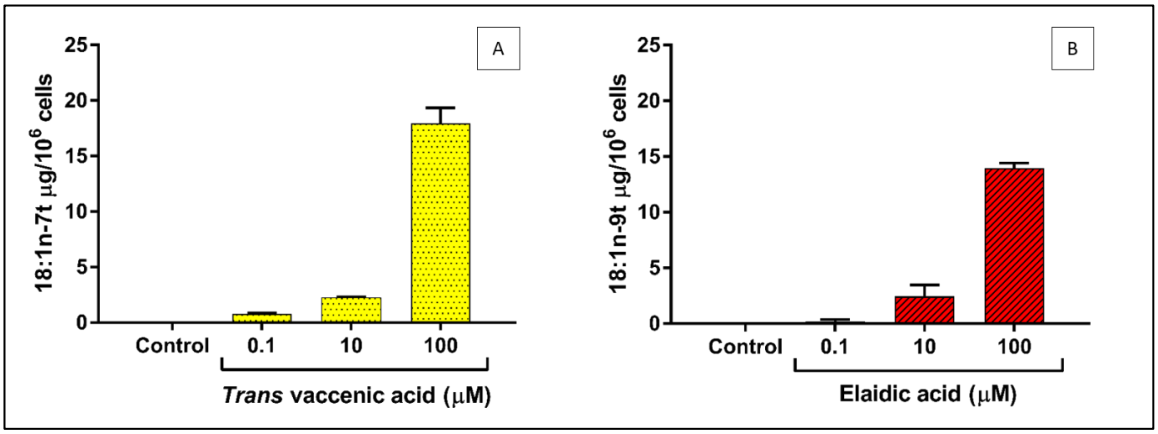



Figure 3.4: Incorporation of TVA (A) and EA (B) into ECs.
EA.hy926 cells incubated for 48 h with DMEM containing 0.1% of ethanol (Control ) or different concentrations (0.1, 10 and 100 µM) of FAs. Bars are mean ± SEM of 3 samples performed in 1 experiment.

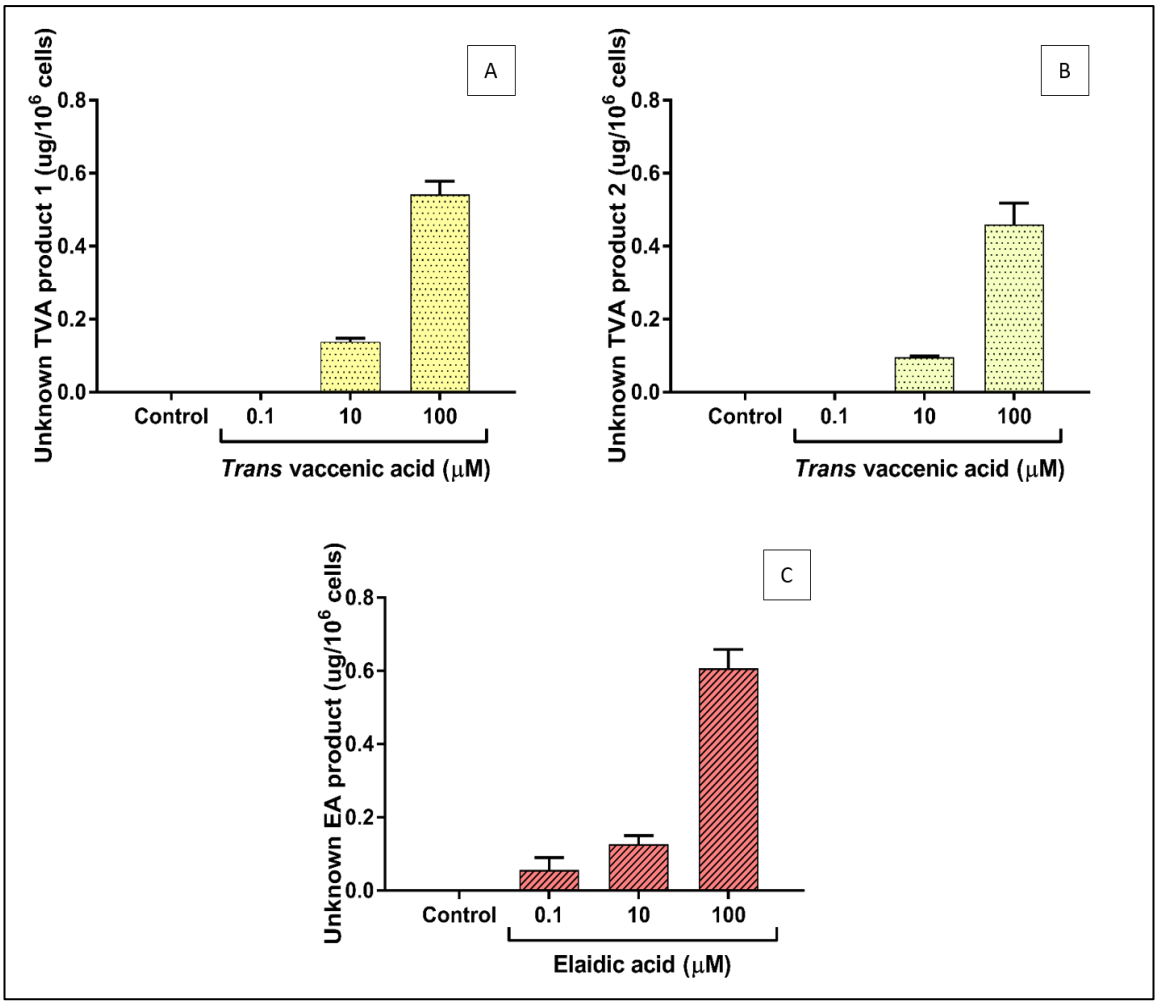



Figure 3.5: Incorporation into EA.hy926 cells of unknown metabolic product of TVA (A-B) and EA (B).
EA.Hy926 cells were incubated for 48h with DMEM containing 0,1% of ethanol (Control) or different concentrations (0.1, 10 and 100 μM) of FAs. Bars are mean ± SEM of 3 samples performed in 1 experiment.

Then, FA incorporation into EA.hy926 cells was assayed using all FAs being studied.

Figure 3.6 shows that the incorporation of each of the FAs used increased as their concentration increased from 1 to 50 μM. CVA was incorporated in a higher amount than TVA, while OA and EA were incorporated to a similar extent. When used at 50 μM, TVA was incorporated into the ECs in a slightly higher quantity than EA.

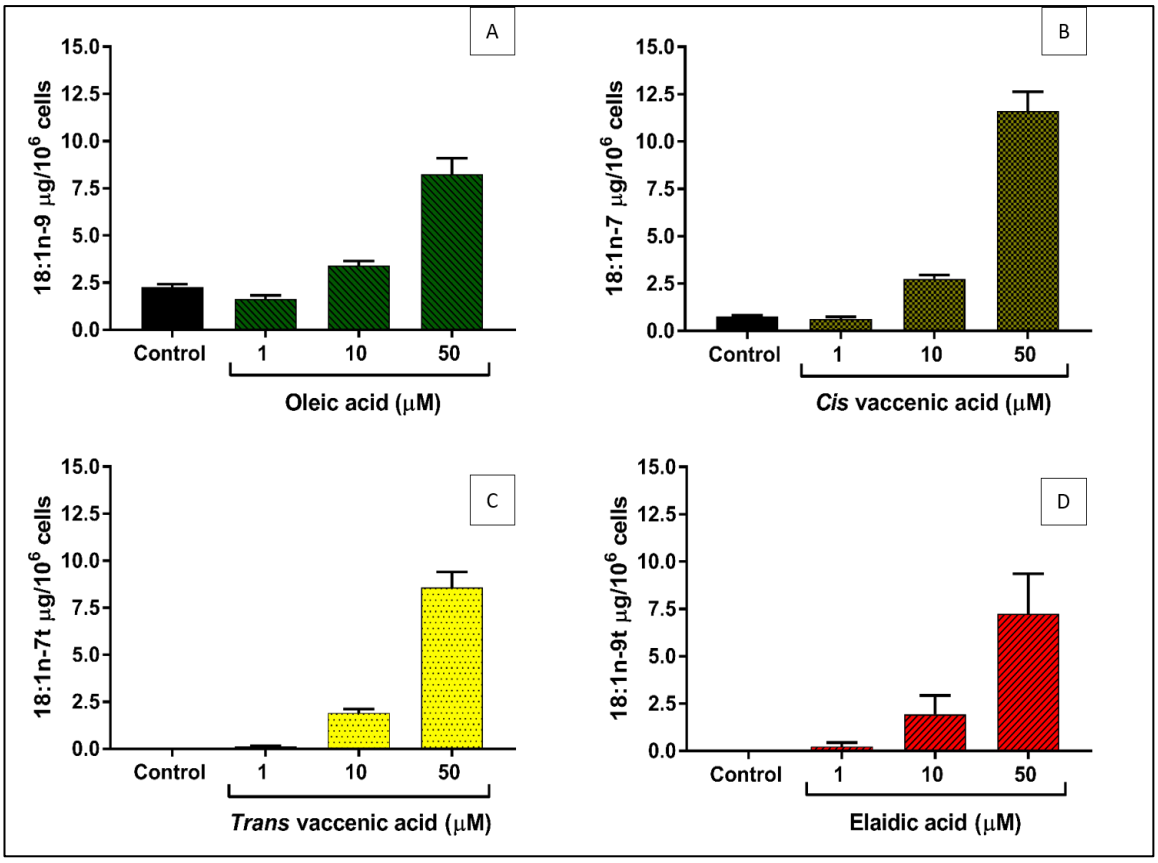



Figure 3.6: Incorporation of FAs into ECs.
EA.hy926 cells were incubated for 48 h with DMEM containing 0.1% of ethanol (Control) or different concentrations (1, 10 and 50 µM) of FA. Bars are mean ± SEM of 6 to 9 samples performed in 3 experiments.

Figure 3.7 shows that the CLA isomers and LA were also incorporated into EA.hy926 cells in a dose-dependent manner. CLA10,12 was incorporated into the ECs in a higher quantity (at least 50% more) than CLA9,11.

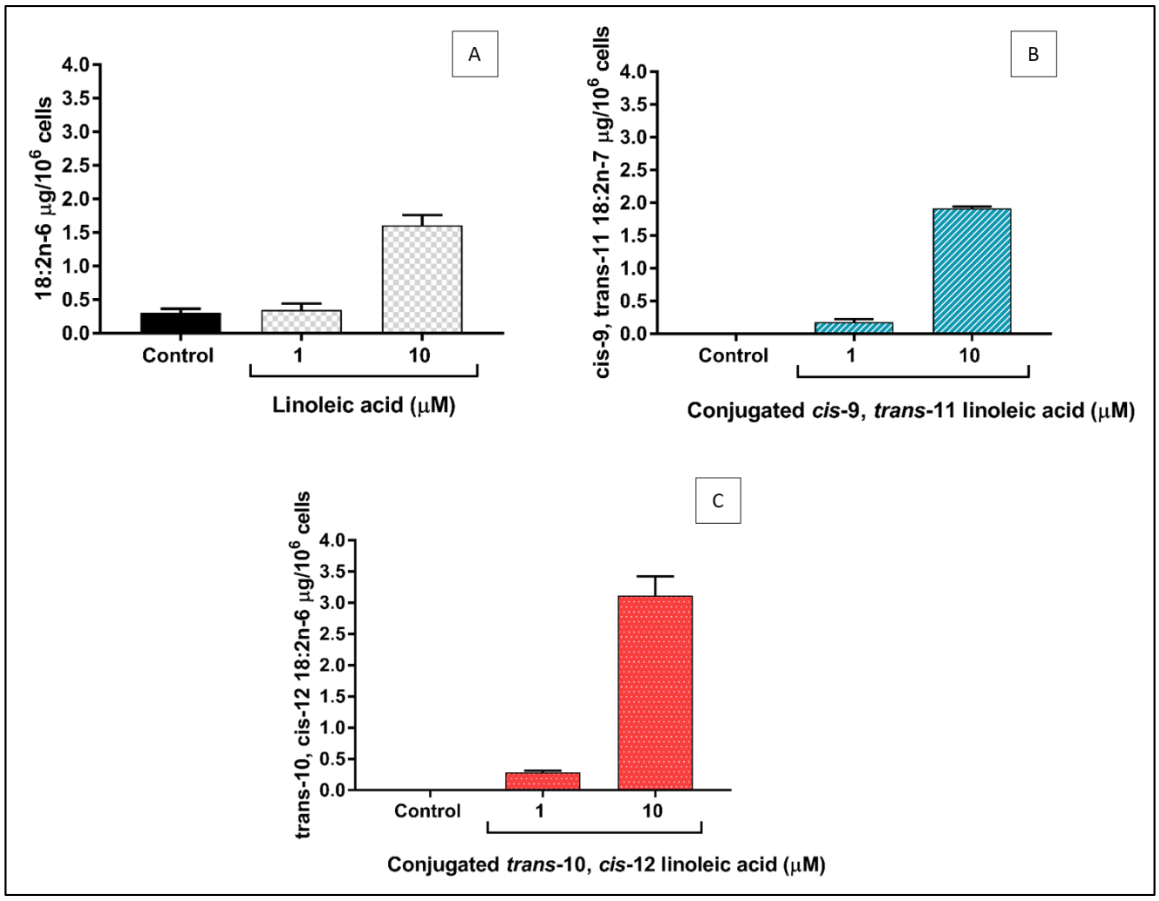



Figure 3.7: Incorporation of CLAs and LA into ECs.
EA.hy926 cells incubated for 48 h with DMEM containing 0.1% of ethanol (Control) or different concentrations (1, 10 and 50 µM) of FA. Bars are mean ± SEM of 6 to 9 samples performed in 3 experiments.

## 3.5 Discussion

The aims of the research described in this chapter were to determine the viability of EA.hy926 cells after culture with TNF-α and various FAs and to determine the incorporation of the different FAs into EA.hy926 cells. These aims were met.

FA induced cell viability alteration in ECs depends on the vascular origin of the cells, the respective FAs and the concentrations that they are used at, and the growth/proliferation status of the cells (Artwohl et al, 2008). In the current model, when TVA and EA were used at 100 µM, with or without TNF-α stimulation, cell viability was reduced by 20% (Figure 3.1). Therefore, it was decided to use a maximum concentration of 50 µM in the next, and all later, experiments. When cell viability was assayed using all of the FAs of interest, CLA10,12 at 50 µM reduced it by 50 to 60% (Figure 3.2), which was also supported by the

image of the EA.hy926 monolayer under the microscope (Figure 3.3 (H)). None of the other FAs used at 50 μM affected the viability of the ECs and they all allowed the cells to reach confluence, looking healthy and normal after 48 h incubation with the FA and 24 h incubation with TNF-α, as observed in the images (Figure 3.3).

Consequently, in the experiments comparing the effects of TVA and EA with their *cis* isomers, the concentrations of FA used were 1, 10 and 50 μM, while when comparing the CLAs and LA, the FA concentrations used were 1 and 10 μM.

Other authors have reported that the viability of human aortic ECs was not affected by treatment with 5 or 50 μM of CLA9,11 or CLA10,12 for 24 h (Eder et al, 2003; Ringseis et al, 2006). The cell model used or the time of exposure to the FA might explain these differences. In relation to the other FAs studied, there are reports of cytotoxicity (Artwohl et al, 2008; Harvey et al, 2012; Qiu et al, 2012; Zapolska-Downar et al, 2005), but in general, this was at higher concentrations than the ones used in the current research.

Before the FA incorporation into the ECs was tested, the initial concentration of each FA stock was checked through GC (see Appendix B for example), as well as the FA profile of the supplemented DMEM (see Appendix C). Solvents such as DMSO or in this case ethanol are used to dissolve the FAs and with every use a small amount of the solvent may get evaporated, changing the concentration of the FA. Based on this, it was decided also to check the concentration of the FA stocks in use before every set of experiments.

Cellular uptake of the FAs of interest was confirmed by GC analysis. All FAs used were incorporated into the cells in a dose-dependent manner (Figure 3.6 and 3.7). The non-appearance of TFAs in control EC samples indicates that when *trans* isomers were found, these originated from the respective TFA treatment.

It is assumed that most of the FA incorporation will be into the membrane of the cells, which provides an association between FA exposure and their functional effects. This is supported by the findings of Ecker et al. (2010). They showed that CLA9,11 incorporates preferably to phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and activates *de novo* glycerolphospholipid synthesis in macrophages, results that the authors suggest to be related to the anti-inflammatory effects of CLA9,11 (Ecker et al, 2010). Harvey et al. (2012) also reported that EA and linoelaidic acid (50 μM, 24 h) are incorporated into cellular phospholipids in ECs, with low incorporation into the triacylglycerol fraction, suggesting

that these TFAs resemble SFAs in the way they are incorporated into the cells (Harvey et al, 2012). It has also been suggested that EA induces inflammation in HUVECs through lipid rafts (Li et al, 2017; Pan et al, 2017), microdomains of the cellular membrane that act as regulators of different cellular events including cell-death, inflammation and various cell signalling pathways (Calder & Yaqoob, 2007; Lemaire-Ewing et al, 2012)

Additionally, the exposure of cells to TVA and EA (Figure 3.5) generated FA metabolites. There is evidence that TVA can be converted into CLA9,11 in different human, mouse and ruminant tissues (adipose tissue, mammary glands) (Griinari et al, 2000; Kuhnt et al, 2007; Mosley et al, 2006a; Mosley et al, 2006b; Santora et al, 2000; Turpeinen et al, 2002), through Δ9 desaturation. The estimated average conversion rate of TVA to CLA9,11 reported in humans is between 19-25% (Turpeinen et al, 2002).

The metabolism of EA has been related to an increase in C18:3 FAs (Krogager et al, 2015), or another unknown FAs. It was not possible to identify the metabolic products of TVA and EA through mass spectrometry; currently inquiries are being made to know if it is possible to identify these metabolites using the tools on lipidmap.org. It would be important to identify them and to determine if they contribute in any way to the effects on inflammation induced by TVA and EA.

## 3.6 Conclusions

In general, the observations made here confirmed the incorporation of the FAs provided to cultured EA.hy926 cells. Regardless of the changes in FA composition, the cells remained viable when they were exposed to 1, 10 and 50 µM of each of the FAs of interest, with the exception of CLA10,12 at 50 µM. Based on these results, in the next experiments TVA, EA, CVA and OA were used at concentrations of 1, 10 and 50 µM, while CLAs and LA were used at 1 and 10 µM. The effects of these FAs on inflammatory responses of EA.hy926 cells will be described in the next chapters. It is also noteworthy that ECs can metabolise some TFAs to FA derivatives, although these have not yet been identified. However one of the metabolic products of TVA is likely to be CLA9,11.

# Chapter 4 Effect of 18-carbon *trans* fatty acids on the production of inflammatory mediators by cultured EA.hy926 cells

## 4.1 Introduction

Endothelial cells are the barrier between the bloodstream and underlying tissues. The endothelial cell monolayer can be crossed by lipoproteins and immune cells such as monocytes and lymphocytes which can then become resident in the sub-endothelial space, playing an important role in the initiation and progression of atherosclerosis (see section 1.2.2).

Several studies, in different experimental models, have reported pro-inflammatory outcomes related to exposure of endothelial cells to TFAs, specifically those from industrial sources. In aortic endothelial cells, 100 μM elaidic acid induced the expression of ICAM-1 and VCAM-1 (Bryk et al, 2011). Dietary supplementation with partially hydrogenated soybean oil (high in elaidic acid) affected inflammatory parameters in cerebrospinal fluid and blood of Wistar rats, with elevated levels of TNF-α, IL-1 and IL-6 (Longhi et al, 2017). In 730 women, greater TFA intake was associated with higher blood levels of sE-selectin, sICAM-1 and sVCAM-1, which are circulating markers indicative of endothelial dysfunction, after adjustment for other risk factors (Lopez-Garcia et al, 2005).

Evidence in relation to the inflammatory effects of TFAs of ruminant origin is less consistent, with effects sometimes being opposite to those of iTFAs (Blewett et al, 2009; Da Silva et al, 2015; Jaudszus et al, 2012). Most prospective studies show that diets enriched in rTFAs have no significant associations with plasma CRP levels and may even be associated with lower plasma levels of cytokines like IL-6, IL-8 and TNF-α (Ramakers et al, 2005; Sofi et al, 2010; Tricon et al, 2006).

In relation to the effects of CLAs, some studies have reported anti-inflammatory effects in HUVECs. For example, Goua et al. (2008) showed that CLA10,12 and a CLA mix reduced ICAM-1 and VCAM-1 expression. Another study by Stachowska et al. (2012) showed that when HUVECs were exposed to 100 μM of CLA isomers, both CLA9,11 and CLA10,12 caused a reduction in the surface expression of VCAM-1, but only CLA9,11 reduced ICAM-1 surface expression compared to control.

Given the lack of studies comparing TFAs of different origins at physiological concentrations, the research described in this chapter compares the effects of 18-carbon *cis* and *trans* FAs of interest (*trans* vaccenic acid, elaidic acid, *cis* vaccenic acid, conjugated

*cis*-9, *trans*-11 and *trans*-10, *cis*-12 linoleic acids, oleic acid and linoleic acid) on the production of cytokines and adhesion molecules by EA.hy926 cells with those in control conditions.

## 4.2 Objectives and aims

The objective of the research described in this chapter was to compare the effects of the 18-carbon *cis* and *trans* FAs of interest on the production of cytokines and adhesion molecules in EA.hy926 cells.

The specific aims were to:

- Measure the effects of different *cis* and *trans* 18-carbon FAs on the production of inflammatory mediators (ICAM-1, MCP-1, VEGF, IL-6, IL-8, and RANTES) by cultured ECs with and without TNF-α stimulation.
- Compare the effects of 18 carbons *trans* isomers from industrial and ruminant origin on the production of inflammatory mediators by cultured ECs.
- Compare the effects of two conjugated linoleic acid isomers of ruminant origin and linoleic acid on the production of inflammatory mediators by cultured ECs.

## 4.3 Methods

### 4.3.1 Reagents

Reagents and materials used for experiments are described in Appendix A.

### 4.3.2 Production of inflammatory mediators by cultured EA.hy926 cells

For these experiments, confluent EA.hy926 cultures were scraped from flasks and resuspended in culture medium at a density of 2 x $10^5$ cells per mL. Cells were seeded at 1 x $10^4$ cells per well in 96-well flat bottom plates, incubated for 48 h at 37$^o$C with FAs at different concentrations, followed by 24 h incubation with either DMEM or TNF-α at 1

ng/mL. Then, supernatant was removed and stored at -80$^{o}$C until analysis. Concentrations of cytokines, chemokines, growth factors and adhesion molecules in EC culture supernatants were determined simultaneously using Human Magnetic Luminex Screening Assay kits (R&D Systems) using the assay described in Chapter 2.

### 4.3.3 Statistical analysis

Concentration of cytokines and inflammatory mediators in the cell media were calculated using the Bio-Plex manager 6.0 software (Bio-Rad). Data are expressed as mean ± SEM. Analyses were performed using GraphPad Prism 6.0 and Excel. Comparisons between samples were made using two or one-way ANOVA and, where the ANOVA was significant, Tukey's post hoc test was used.

## 4.4 Results

### 4.4.1 The effect of *trans* vaccenic and elaidic acid on production of inflammatory mediators by EA.hy926 cells

Incubation of EA.hy926 cells with TVA tended to produce a decrease in basal (i.e., unstimulated) concentrations of some of the mediators measured (MCP-1, RANTES, IL-6), while incubation with EA did not produce changes in cytokine levels compared to the control (Figure 4.1). TNF-α (1 ng/ml) stimulation for 24 h induced a significant increase in the levels of all the mediators measured, being very effective as an inflammatory stimulus in this cell model. Cells pre-incubated with TVA had a lower concentrations (interpreted as lower production) of ICAM-1 (0.1 μM), VEGF (1 μM), and RANTES (0.1 and 1 μM) after inflammatory stimulation, compared to the control cells (Fig 4.1 B, C and D, respectively). This anti-inflammatory effect was seen at the lower concentrations of TVA used (from 0.1 and 1 μM). On the other hand, pre-incubation with EA tended to increase levels of inflammatory mediators after TNF-α stimulation, although the level of cytokine production was not statistically different from the control. When comparing the effects between the FAs tested, TVA induced a significantly lower level of ICAM-1 (1 μM), VEGF (1 μM), and RANTES (0.1 and 1 μM) in relation to EA (Fig 4.1 B, C and D, respectively). Based

on these results, it was decided to work with 1, 10 and 50 µM concentrations for the next experiments using all the FAs of interest. Additionally, it was observed that after TNF-α stimulation, IL-8 and MCP-1 values were very high, moving out of their standard curve. Even though the luminex software was used to extrapolate those values, it was decided to buy a separate kit to measure both cytokines after diluting the supernatant samples for the next experiments, using all the FAs of interest. After testing different dilutions, a dilution of 1/5 was chosen.

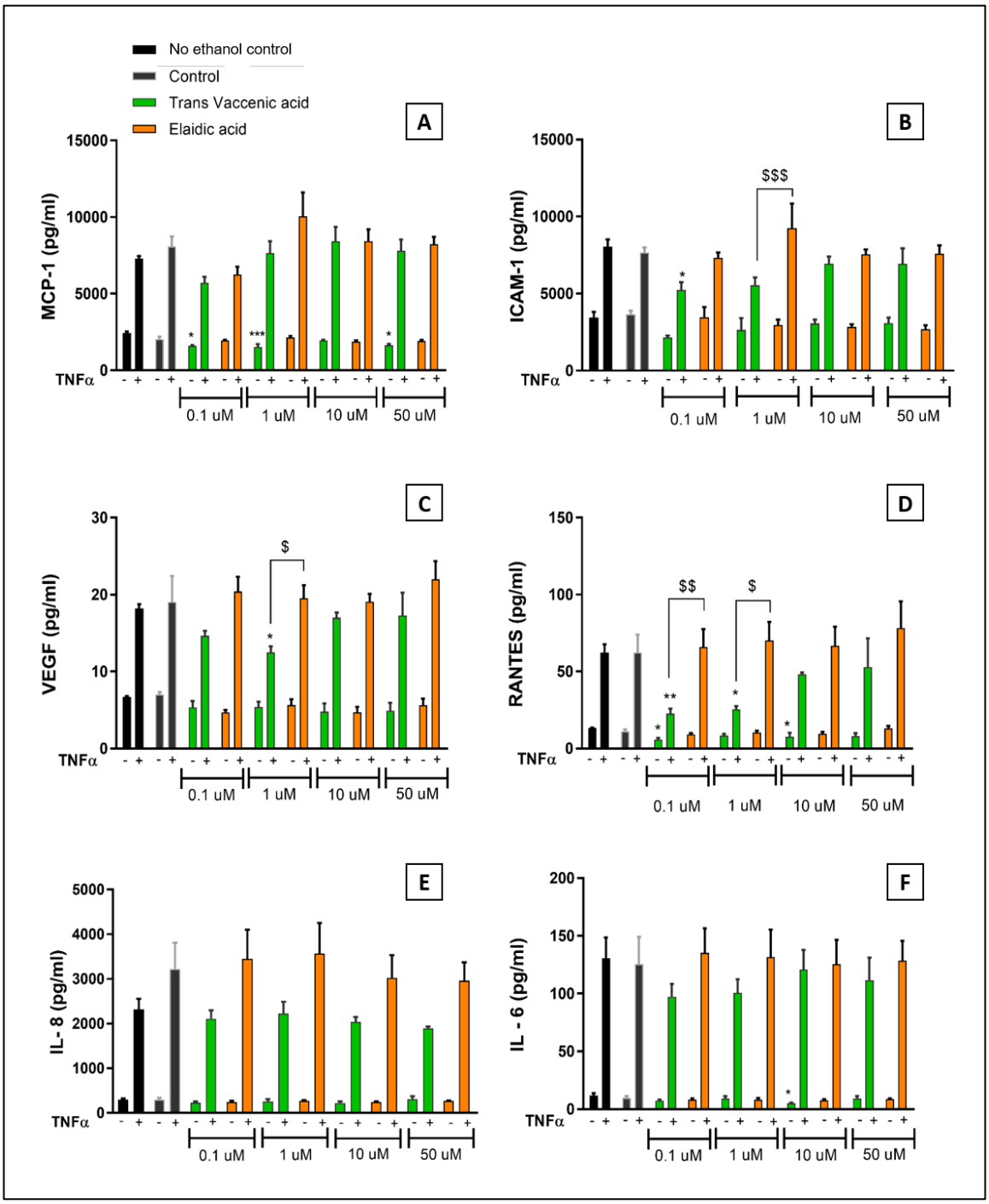



Figure 4.1: Effects of TVA and EA exposure on inflammatory mediators level in the medium of EA.hy926 cells.

Concentrations (pg/mL) of MCP-1 (A), ICAM-1 (B), VEGF (C), RANTES (D), IL-8 (E) and IL-6 (F) in the medium of EA.hy926 cells incubated for 48 h with DMEM (no ethanol control) or DMEM containing 0.1% of ethanol (Control) or several concentrations (0.1, 1, 10, and 50 µM) of *trans* vaccenic or elaidic acid, followed by incubation with (+) or without (-) TNF-α (1 ng/mL) for 24 h. Bars are mean ± SEM of 6 samples performed in 2 experiments. Two-way ANOVA with Tukey's as post-hoc test: *p<0.05; **p<0.01; ***p<0.001 vs. Control; $p<0.05, $$p<0.01 and $$$p<0.001, differences between fatty acids at the same concentration.

### 4.4.2 Effect of 18-carbon *trans* isomers from industrial and ruminant sources on the production of inflammatory mediators by EA.hy926 cells.

When all the FAs of interest were used together, each experiment could be assayed in one 96 well plate. Even though similar trends were observed amongst the different experiments performed, sometimes there were differences in the ranges of fluorescence values measured each time. Therefore, it was decided to analyse and present the values as % of control.

#### 4.4.2.1 MCP-1

Figure 4.2(A) shows that pre-incubation of ECs with TVA induced a significant reduction in the levels of MCP-1 for all the concentrations of TVA used. EA also produced a significant decrease in MCP-1 levels at 1 and 10 µM, which was lost at 50 µM. The *cis* isomers, CVA and OA, had similar effects to the *trans* isomers, reducing the levels of MCP-1, at almost all the concentrations used, with the exception of CVA at 1 µM.

In a pro-inflammatory state, after TNF-α stimulation (Figure 4.2(B)), FA pre-exposure did not induce changes in the supernatant levels of MCP-1 compared to control at the two lowest FA concentrations used. However, at 50 µM, EA produced a significant increase of MCP-1 levels, compared to control and to TVA.

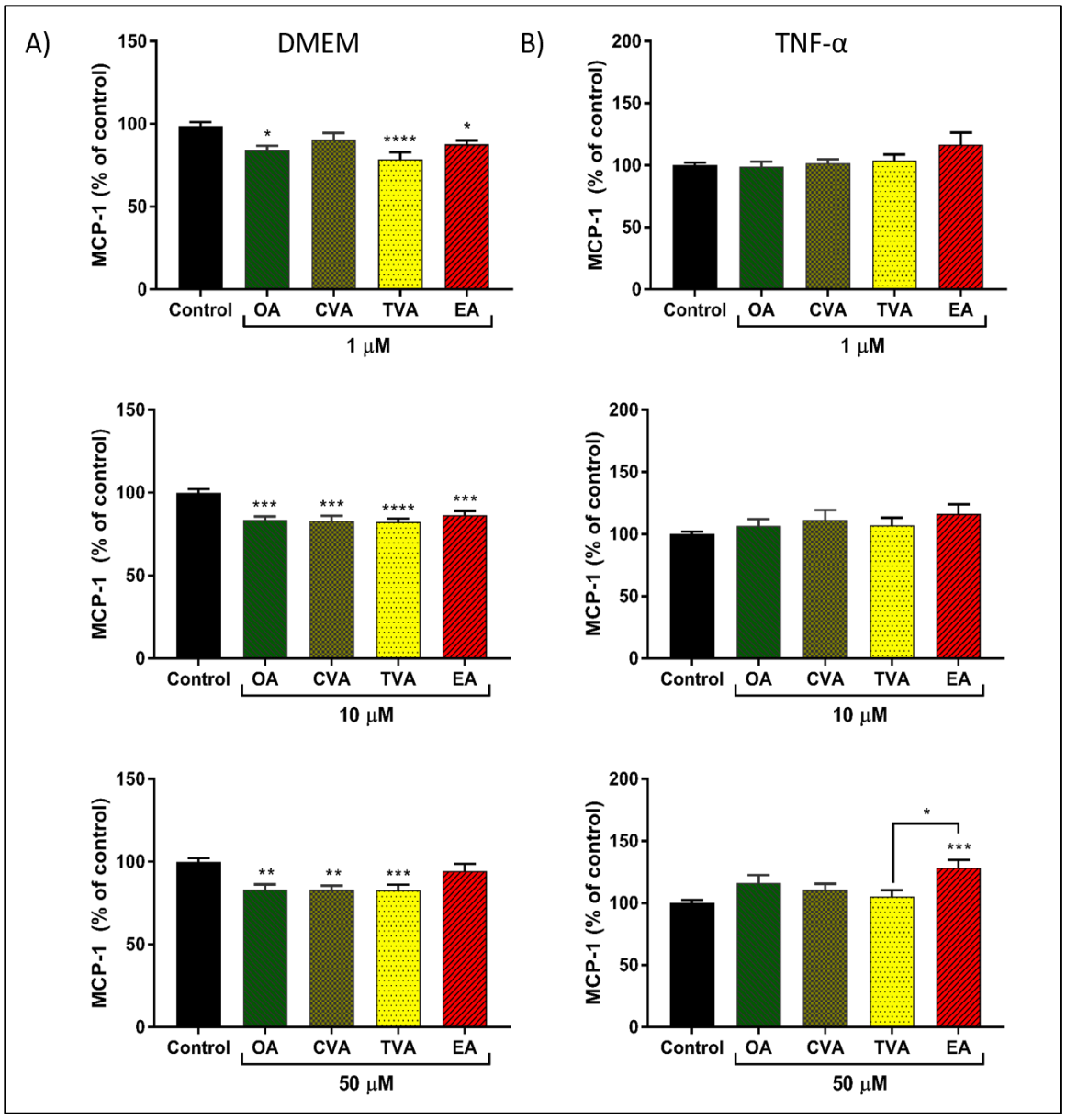



Figure 4.2: Concentration (% of control) of MCP-1 in the medium of EA.hy926 cells exposed to FAs.

ECs were incubated for 48 h with DMEM containing 0.1% of ethanol (Control) or several concentrations (1 μM; 10 μM; 50 μM) of FAs, followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 24 h. Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. OA = oleic acid, CVA = *cis* vaccenic acid, TVA = *trans* vaccenic acid, EA = elaidic acid.

#### 4.4.2.2 ICAM-1

As shown in Figure 4.3(A), none of the FAs used, at any concentration, induced changes in ICAM-1 levels in the cell supernatant under basal (i.e., unstimulated) conditions. After TNF-α stimulation (1 ng/mL) for 24 h, only the treatment with TVA at 1 μM produced a

significant reduction in the supernatant levels of ICAM-1, compared to control and to EA (Figure 4.3(B)).

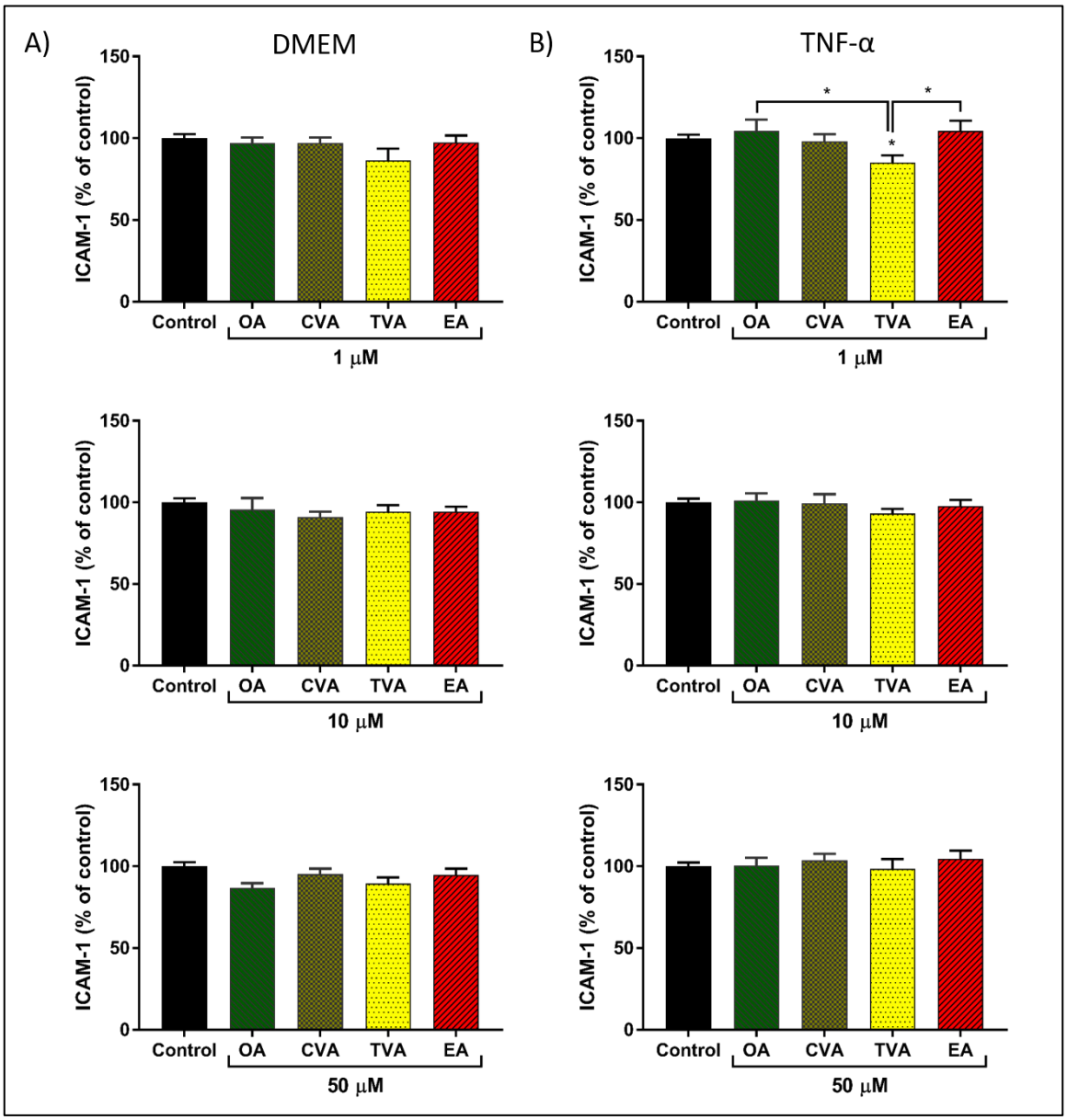



Figure 4.3: Concentration (% of control) of ICAM-1 in the medium of EA.hy926 cells exposed to FAs.

ECs were incubated for 48 h with DMEM containing 0.1% of ethanol (Control) or several concentrations (1 μM; 10 μM; 50 μM) of FAs, followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 24 h. Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *$p<0.05$. OA = oleic acid, CVA = *cis* vaccenic acid, TVA = *trans* vaccenic acid, EA = elaidic acid.

#### 4.4.2.3 VEGF

In basal conditions, only TVA at 1 µM produced decreased levels of VEGF in the supernatant. The other FAs, especially the *cis* isomers, tended to show the same effect at all the concentrations used (Figure 4.4(A)). After TNF-α stimulation (Figure 4.4(B)), pre-incubation with TVA at 1 µM produced decreased levels of VEGF compared to control and to EA. At the highest concentration used, EA tended to induce increased levels of VEGF compared to control, behaving significantly differently from OA and TVA.

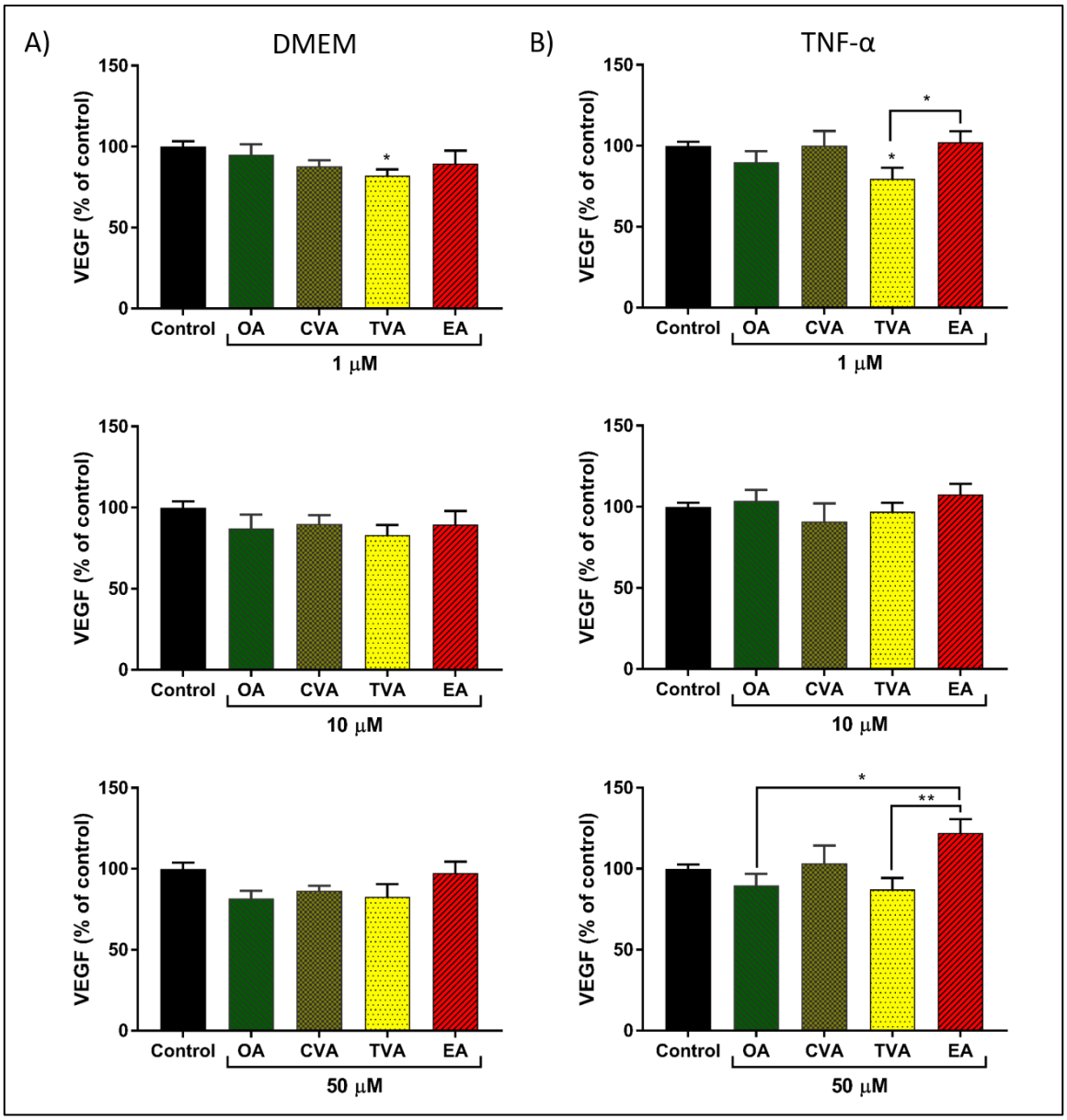



Figure 4.4: Concentration (% of control) of VEGF in the medium of EA.hy926 exposed to FAs. ECs were incubated for 48 h with DMEM containing 0.1% of ethanol (Control) or several concentrations (1 µM; 10 µM; 50 µM) of FAs, followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 24 h. Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; **p<0.01. OA = oleic acid, CVA = *cis* vaccenic acid, TVA = *trans* vaccenic acid, EA = elaidic acid.

#### 4.4.2.4 RANTES

Figure 4.5(A) shows that pre-incubation of ECs with CVA and TVA induced decreased levels of RANTES at 1 and 50 µM for CVA; and at 10 and 50 µM for TVA. Additionally, EA tended to induce increased levels of RANTES at 50 µM compared to control, behaving significantly different from the other FAs used. After TNF-α stimulation, CVA and TVA induced significant changes in RANTES levels, with decreased levels at 10 µM for CVA and at 1 µM for TVA.

Additionally, EA induced significantly increased levels of RANTES at 50 µM compared to control, acting significantly different than the other FAs used (Figure 4.5(B)).

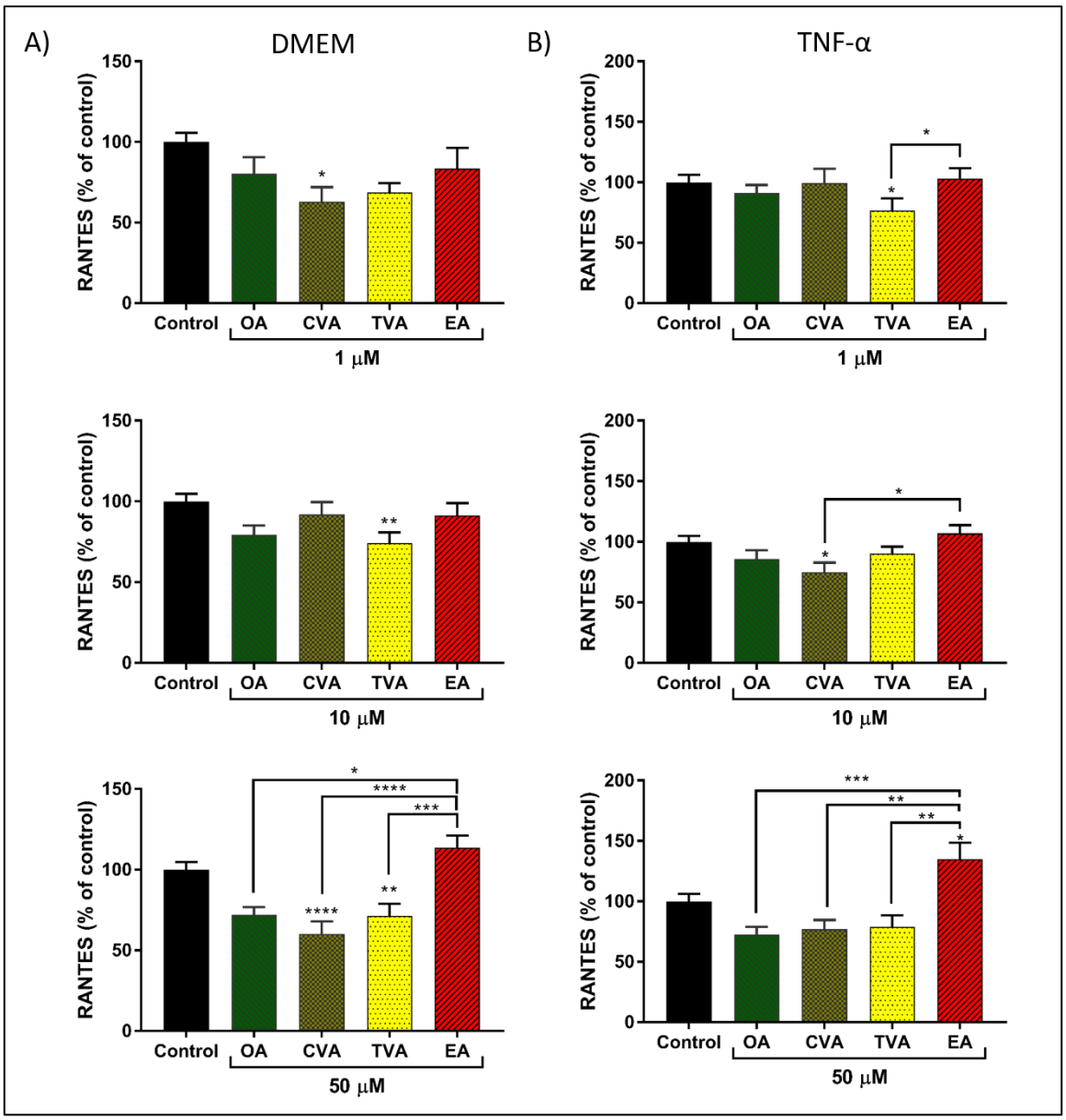



Figure 4.5: Concentration (% of control) of RANTES in the medium of EA.hy926 cells exposed to FAs.

ECs were incubated for 48 h with DMEM containing 0.1% of ethanol (Control) or several concentrations (1 µM; 10 µM; 50 µM) of FAs, followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 24 h. Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. OA = oleic acid, CVA = *cis* vaccenic acid, TVA = *trans* vaccenic acid, EA = elaidic acid.

#### 4.4.2.5 IL-8

Under basal conditions, OA and TVA induced significant changes in IL-8 levels, with decreased basal levels at all concentrations for OA; and at 1 and 10 µM for TVA. CV and EA also reduced IL-8 levels at 1 µM compared to control (Figure 4.6(A)). As shown in figure 4.6(B), exposure to EA at 1 and 50 µM, before TNF-α stimulation, produced a significant increase in IL-8 levels in the supernatant, which was also the case for OA and CVA at 50 µM.

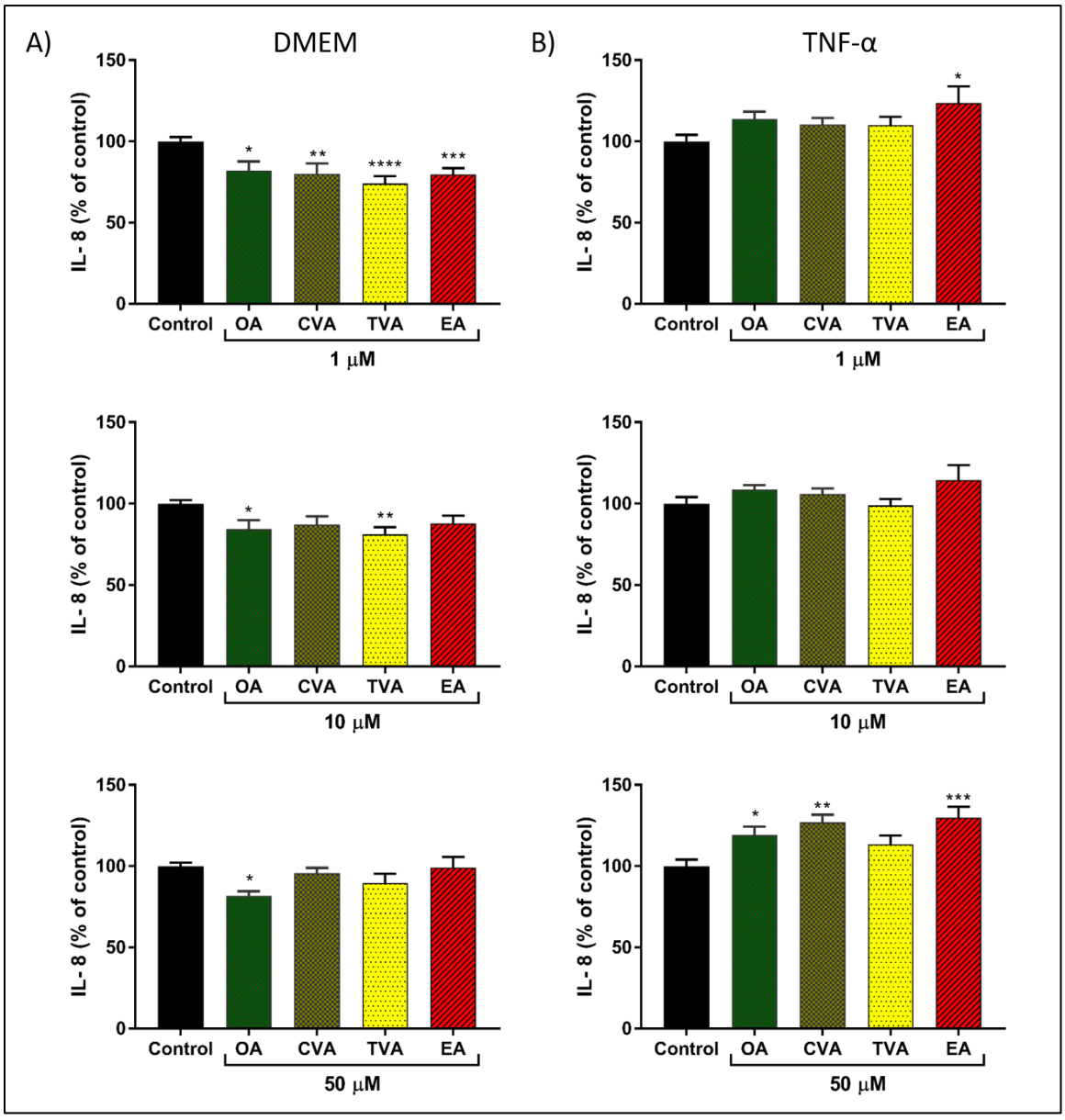



Figure 4.6: Concentration (% of control) of IL-8 in the medium of EA.hy926 cells exposed to FAs. ECs were incubated for 48 h with DMEM containing 0.1% of ethanol (Control) or several concentrations (1 µM; 10 µM; 50 µM) of FAs, followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 24 h. Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. OA = oleic acid, CVA = *cis* vaccenic acid, TVA = *trans* vaccenic acid, EA = elaidic acid.

#### 4.4.2.6 IL-6

In basal conditions, TVA induced a reduction in IL-6 levels at all the concentrations used. CVA at 1 µM, OA at 10 and 50 µM and EA at 1 and 10 µM produced the same effect (Figure 4.7(A)). For IL-6 levels after TNF-α stimulation, FA treatments did not induce significant changes compared to the control, although EA at 10 µM behaved significantly different from TVA (Figure 4.7(B)).

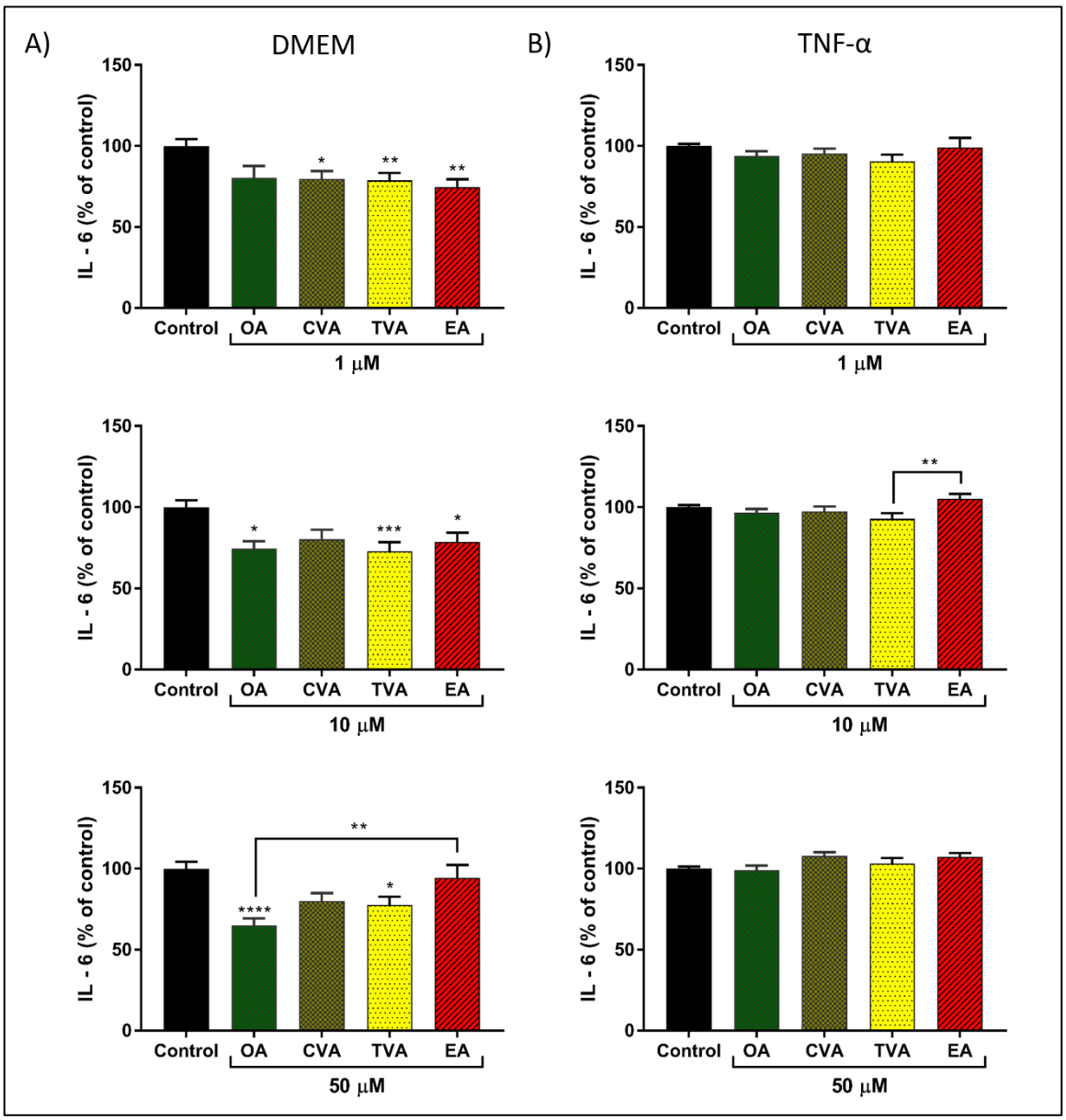



Figure 4.7: Concentration (% of control) of IL-6 in the medium of EA.hy926 cells exposed to FAs. ECs were incubated for 48 h with DMEM containing 0.1% of ethanol (Control) or several concentrations (1 µM; 10 µM; 50 µM) of FAs, followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 24 h. Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. OA = oleic acid, CVA = *cis* vaccenic acid, TVA = *trans* vaccenic acid, EA = elaidic acid.

### 4.4.3 Effect of CLAs on the production of inflammatory mediators by EA.hy926 cells.

In this experiment, where CLA isomers were used to analyse their effect on the production of inflammatory mediators in basal condition and after TNF-α stimulation,

only 1 and 10 µM FA concentrations were used due to the toxicity described in chapter 3 when CLA10,12 was tested at 50 µM.

#### 4.4.3.1 MCP-1

Figure 4.8(A) shows that CLA9,11 at 1 µM reduced MCP-1 levels, while CLA10,12 produced the same effect at 10 µM. Their *cis* isomer, linoleic acid, also decreased basal levels of MCP-1 at both concentrations used. After TNF-α stimulation (Figure 4.8(B)), pre-incubation with CLAs or LA did not induce changes in MCP-1 levels.

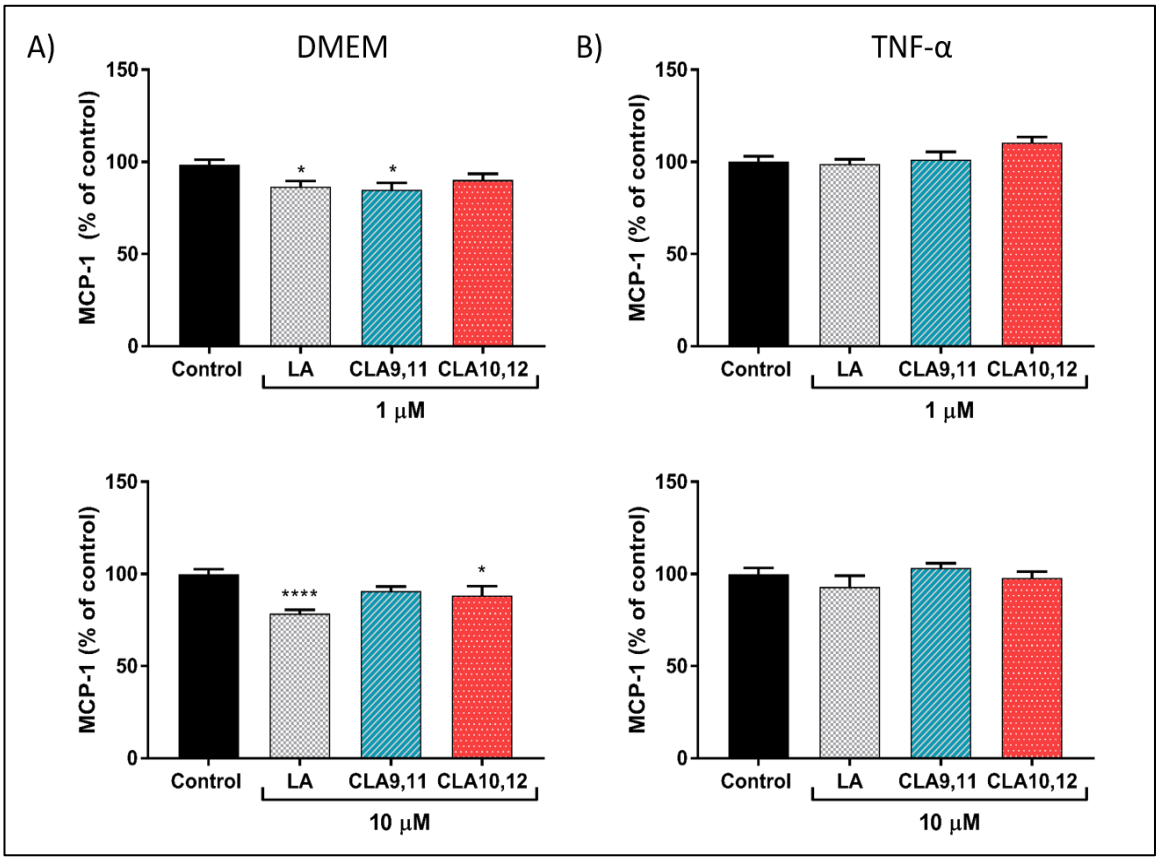



Figure 4.8: Concentration (% of control) of MCP-1 in the medium of EA.hy926 cells exposed to CLAs.
ECs were incubated for 48 h with DMEM containing 0.1% of ethanol (Control) or 1 µM and 10 µM of FAs, followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 24 h. Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; ****p<0.0001. LA = linoleic acid, CLA9,11 = conjugated *cis*-9, *trans*-11 linoleic acid, CLA10,12 = conjugated *trans*-10, *cis*-12 linoleic acid.

#### 4.4.3.2 ICAM-1

Figure 4.9(A) shows that, in basal conditions, only the treatment with LA at 10 µM induced a significant decrease in ICAM-1 levels in the cell supernatant. After TNF-α stimulation, pre-incubation with CLA10,12 at 1 µM induced a significant increase in ICAM-1 levels in the cell supernatant (Figure 4.9(B)).

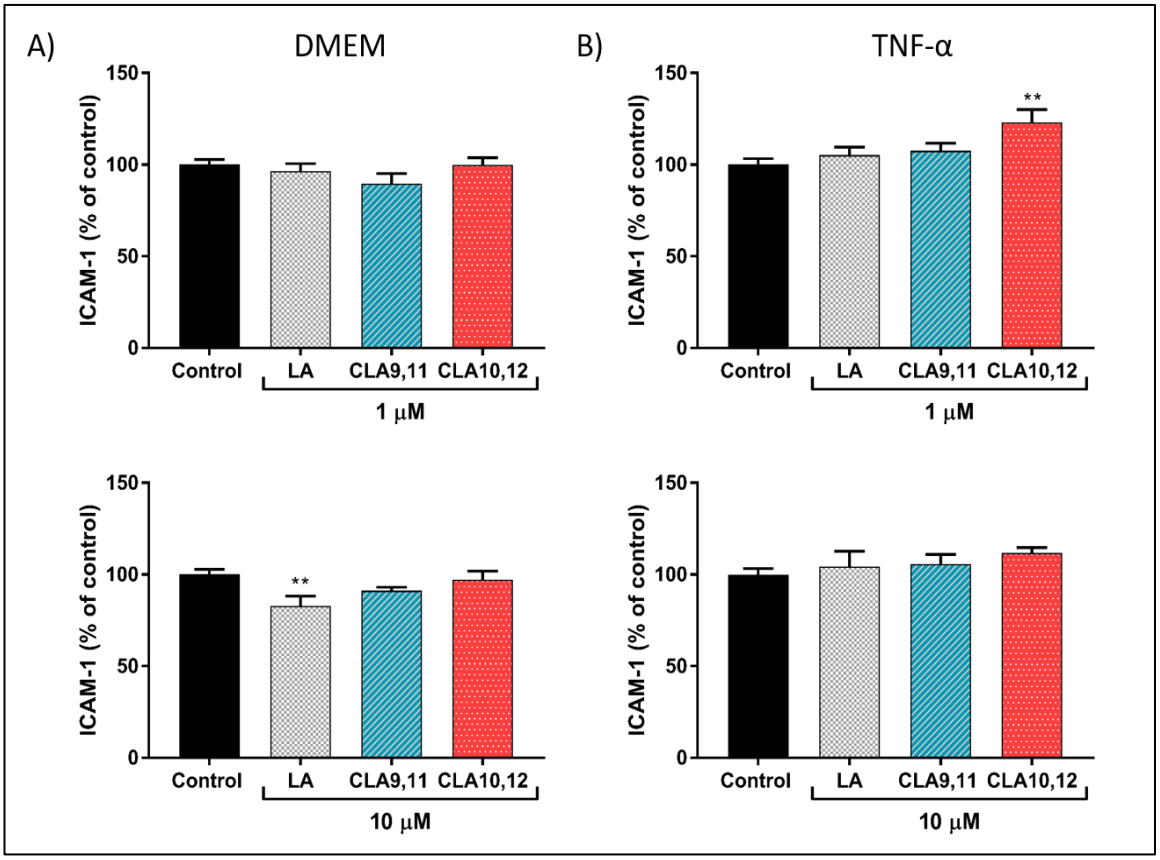



Figure 4.9: Concentration (% of control) of ICAM-1 in the medium of EA.hy926 cells exposed to CLAs.
ECs were incubated for 48 h with DMEM containing 0.1% of ethanol (Control) or 1 µM and 10 µM of FAs, followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 24 h. Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. **p<0.01. LA = linoleic acid, CLA9,11 = conjugated *cis*-9, *trans*-11 linoleic acid, CLA10,12 = conjugated *trans*-10, *cis*-12 linoleic acid.

#### 4.4.3.3 VEGF

As shown in Figure 4.10(A), none of the FAs used, at either concentration, induced changes in VEGF levels in the cell supernatant in basal conditions. After TNF-α stimulation (Figure 4.10(B)), pre-incubation with CLA10,12 at both concentrations induced a

significant increase in VEGF levels in the cell supernatant, behaving differently than LA and CLA9,11.

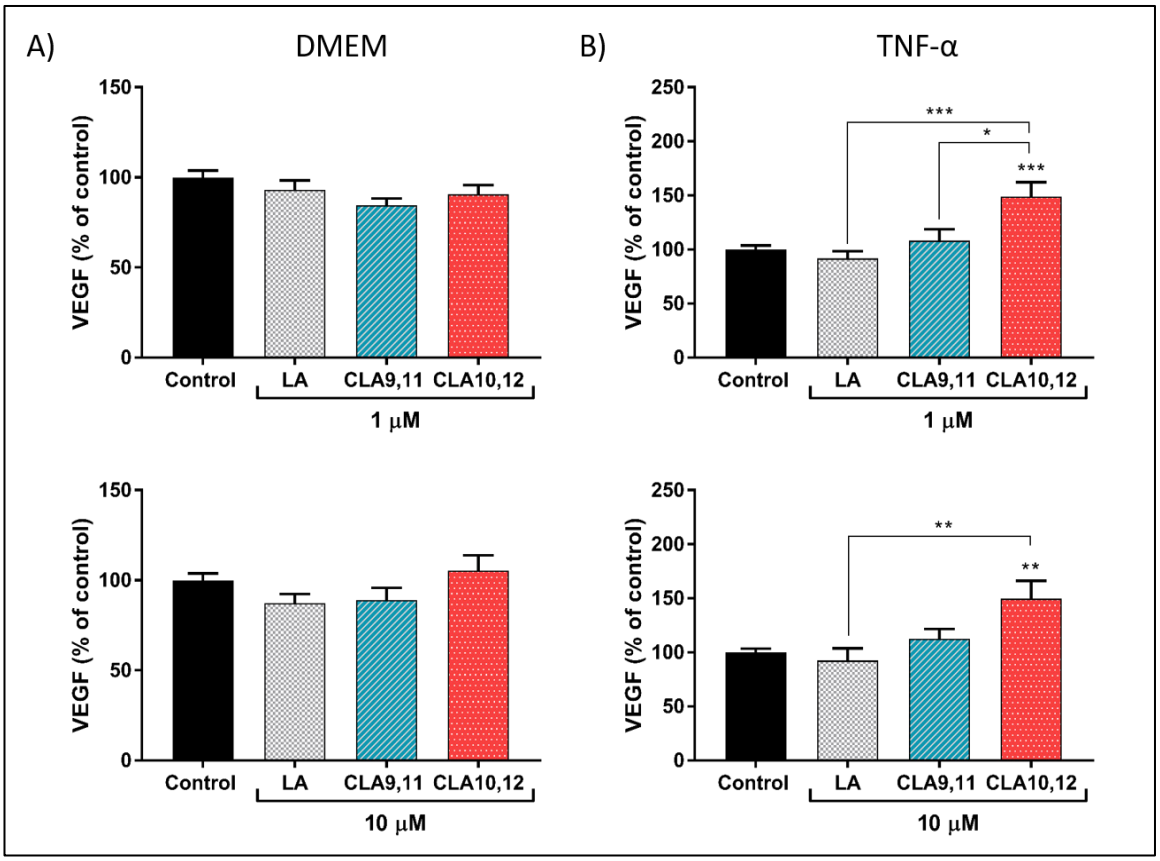



Figure 4.10: Concentration (% of control) of VEGF in the medium of EA.hy926 cells exposed to CLAs.
ECs were incubated for 48 h with DMEM containing 0.1% of ethanol (Control) or 1 μM and 10 μM of FAs, followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 24 h. Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; **p<0.01; ***p<0.001. LA = linoleic acid, CLA9,11 = conjugated *cis*-9, *trans*-11 linoleic acid, CLA10,12 = conjugated *trans*-10, *cis*-12 linoleic acid.

#### 4.4.3.4 RANTES

For RANTES levels in EA.hy926 cell supernatants (Figure 4.11(A)), only CLA9,11 induced significant changes with decreased levels at 1 μM, while both CLAs and LA induced a reduction in RANTES production when used at 10 μM in basal conditions. After TNF-α stimulation (Figure 4.11(B)), pre-incubation with CLA10,12 at both concentrations induced a significant increase in RANTES levels in the cell supernatant, behaving differently from LA and CLA9,11.

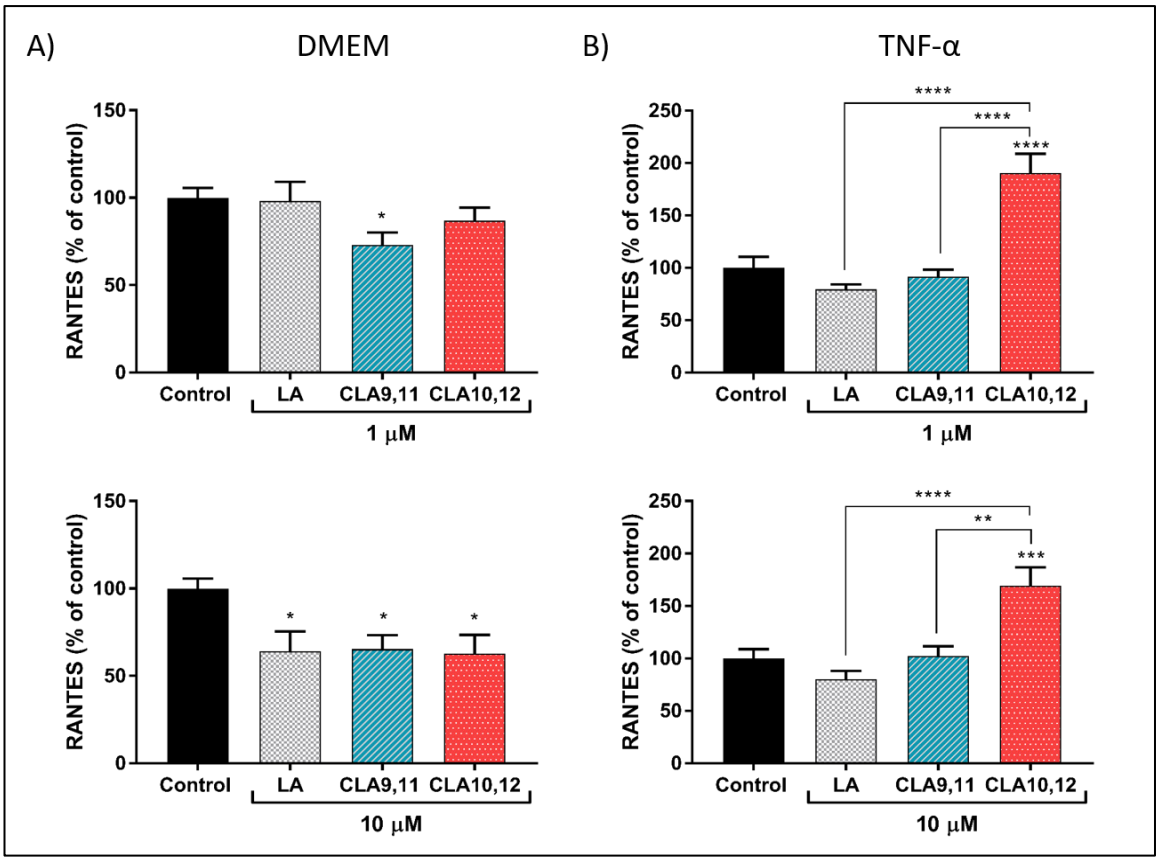



Figure 4.11: Concentration (% of control) of RANTES in the medium of EA.hy926 cells exposed to CLAs.
ECs were incubated for 48 h with DMEM containing 0.1% of ethanol (Control) or 1 µM and 10 µM of FAs, followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 24 h. Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. LA = linoleic acid, CLA9,11 = conjugated *cis*-9, *trans*-11 linoleic acid, CLA10,12 = conjugated *trans*-10, *cis*-12 linoleic acid.

#### 4.4.3.5 IL-8

In basal conditions, exposure to both CLAs at 1 µM produced a decrease in IL-8 levels in the supernatant. CLA9,11 and LA also induced decreased IL-8 production when used at 10 µM (Figure 4.12(A)). After TNF-α stimulation, LA at 1 µM induced a significant increase in IL-8 levels in the cell supernatant (Figure 4.12(B)). The CLAs were without effect.

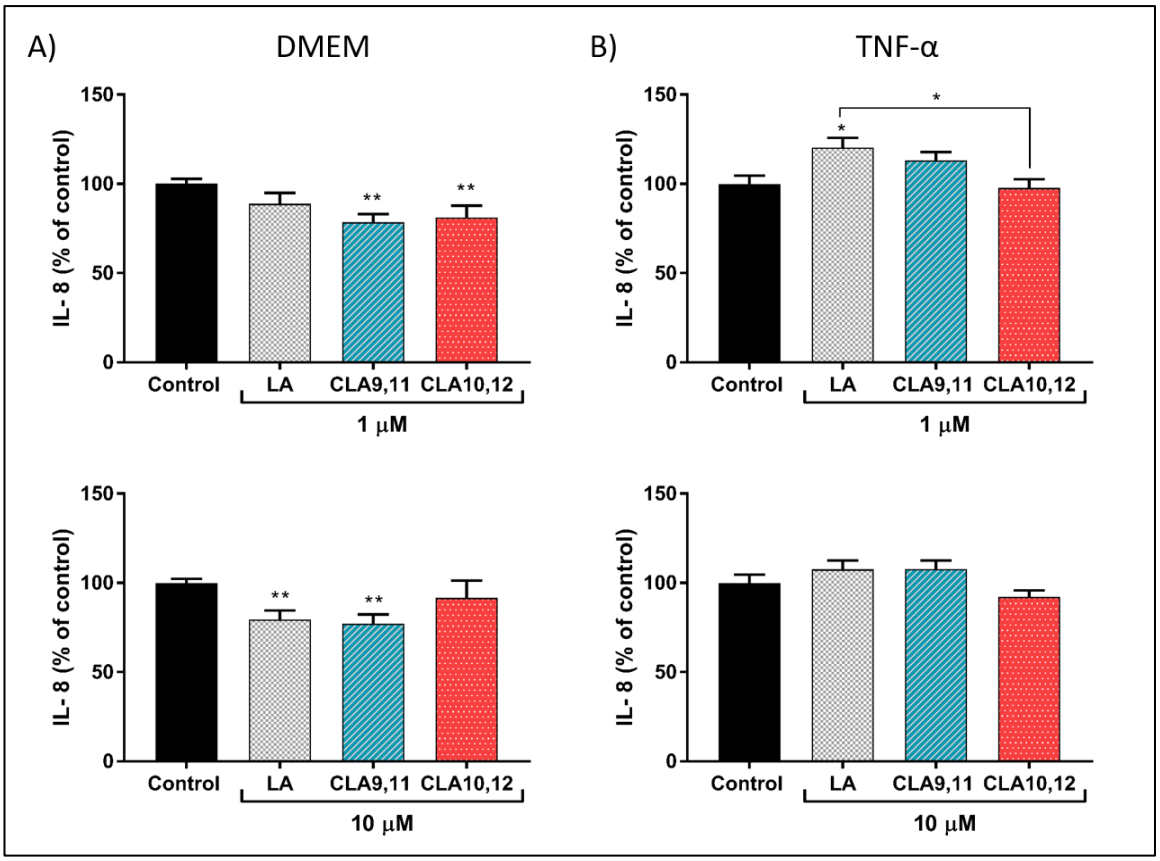



Figure 4.12: Concentration (% of control) of IL-8 in the medium of EA.hy926 cells exposed to CLAs. ECs were incubated for 48 h with DMEM containing 0.1% of ethanol (Control) or 1 μM and 10 μM of FAs, followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 24 h. Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; **p<0.01. LA = linoleic acid, CLA9,11 = conjugated *cis*-9, *trans*-11 linoleic acid, CLA10,12 = conjugated *trans*-10, *cis*-12 linoleic acid.

#### 4.4.3.6 IL-6

For basal IL-6 levels in EA.hy926 cell supernatants (Figure 4.13(A)), CLA9,11 induced significant changes, with decreased levels at 1 μM, whereas, at 10 μM CLA10,12 produced increased levels, behaving differently from LA and CLA9,11. For IL-6 after TNF-α stimulation (Figure 4.13(B)), LA at 1 μM reduced levels, behaving significantly different from CLA10,12. At 10 μM, CLA10,12 produced increased levels of IL-6, behaving differently from LA and CLA9,11.

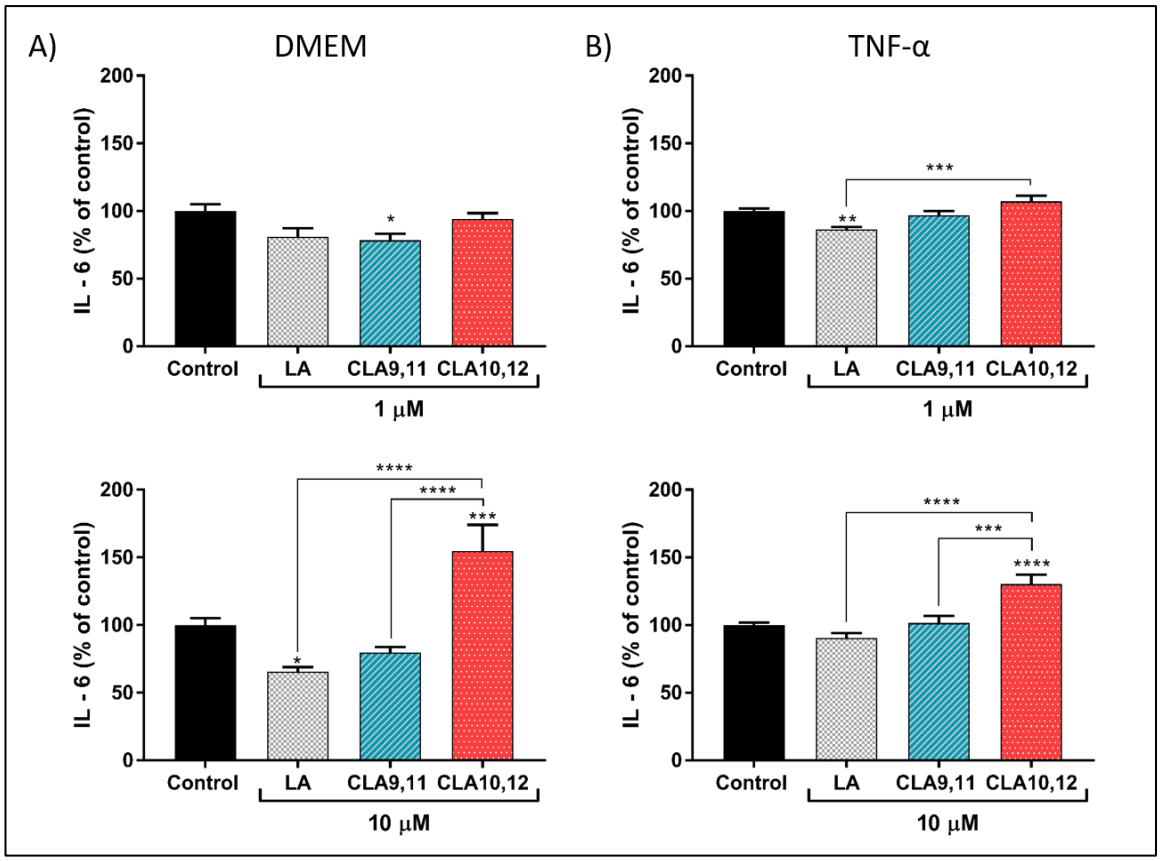



Figure 4.13: Concentration (% of control) of IL-6 in the medium of EA.hy926 cells exposed to CLAs. ECs were incubated for 48 h with DMEM containing 0.1% of ethanol (Control) or 1 µM and 10 µM of FAs, followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 24 h. Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; ***p<0.001; ****p<0.0001. LA = linoleic acid, CLA9,11 = conjugated *cis*-9, *trans*-11 linoleic acid, CLA10,12 = conjugated *trans*-10, *cis*-12 linoleic acid.

## 4.5 Discussion

The aims of the research described in this chapter were to compare the effects of different *cis* and *trans* 18-carbon FAs on production of various inflammatory mediators (ICAM-1, MCP-1, VEGF, IL-6, IL-8, and RANTES) by cultured ECs, in basal conditions and after stimulation with TNF-α. These aims were met.

### 4.5.1 Comparing the effect of 18-carbon *trans* isomers from industrial and ruminant origin

In non-stimulated ECs, Figure 4.1 shows that low concentrations of TVA (0.1 to 10 µM) were enough to result in decreased levels of MCP-1, RANTES and IL-6 compared to the control (Figure 4.1), suggesting an anti-inflammatory effect of TVA in the absence of a classic inflammatory stimulus. EA did not evoke any significant changes in the cells under

the same non-stimulated conditions. In the next experiments performed, the anti-inflammatory effects induced by TVA were maintained and extended to VEGF and IL-8 (Figures 4.6 and 4.10). Wang et al. (2012) showed evidence that TVA may be a ligand of PPAR-γ and PPAR-α (Wang et al, 2012), possibly exerting anti-inflammatory effects through PPAR activation. PPARs can inhibit IKK activity in response to inflammatory stimuli causing the inhibition of IκB degradation and of NFκB nuclear translocation (Genolet et al, 2004).

Surprisingly, EA also showed some anti-inflammatory effects under basal conditions in the subsequent experiments, reducing the production of MCP-1 at 1 and 10 µM, of IL-8 at 1 µM and of IL-6 at 1 and 10 µM, as shown in figures 4.2, 4.10 and 4.12, respectively. The difference between experiments may be related to the transformation of the results to % of control, instead of using the concentration in pg/ml. In unstimulated HUVECs, HAECs and HMECs linoelaidic acid and/or EA have been shown to induce gene expression, surface expression or protein levels of ICAM-1, IL-6, VCAM-1 and toll-like receptor 4 (TLR-4) (Bryk et al, 2011; Harvey et al, 2012; Iwata et al, 2011; Pan et al, 2017). Iwata et al. (2011) showed that incubation for 3 h with 50 and 100 µM of EA induced increased basal levels of IL-6 in HMECs, measured by ELISA. Figure 4.7 shows no significant induction of this cytokine with a FA incubation period of 48 h at the same concentration. In fact, EA reduced the basal levels of IL-6 at 1 and 10 µM in ECs. This difference may be related to the type of cells used or times of exposure.

Studies in humans and in animal models have reported some indications that in certain conditions iTFAs and rTFAs may have similar effects. In an obesity model in rats (JCR:LA-cp), where TVA and EA were included in the diet at 1% (w/w) during 16 weeks, it was reported that both TFAs corrected the impaired IL-2 and TNF-α response to T-cell mitogen stimulation seen in obese rats, but only TVA normalized IL-1β and IFN-γ production by stimulated T-cells and haptoglobin levels (Ruth et al, 2010). Another model in male mice fed for 4 weeks with 0.5% (w/w) of TVA, EA or CLA9,11 enriched diets, identified that all TFAs used induced a reduction in body weight and adipose tissue; TVA also induced a reduction in LDL cholesterol and TNF-α levels, while CLA9,11 induced higher levels of TNF-α (Wang et al, 2015). Regarding the evidence in humans, one of the few published RCTs comparing the effects of iTFAs and rTFAs in healthy adults, showed that the intake of enriched dairy products with iTFAs (7% of energy) or a mix of CLAs for 3 weeks did not

affect low-grade inflammation to a large degree (lower TNF-R1 and higher sE-selectin), while both induced an increased excretion of 8-iso-$PGF_{2\alpha}$, a marker of oxidative stress (Smit et al, 2011). In the same way, another study reported that 2% of daily energy intake as TFAs (alpine butter or margarine) or no TFA as control, during 4 weeks, did not have any adverse effect on coagulation, inflammation markers and adhesion molecules in healthy subjects (Radtke et al, 2017). Concordantly, the results reported here suggest that in basal conditions, EA may also exert some anti-inflammatory effects in a similar way to TVA, especially when it was used at low concentrations.

After TNF-α stimulation, an anti-inflammatory effect of TVA was observed as a reduction of ICAM-1, VEGF and RANTES levels (Figure 4.1) at the lowest concentrations used (0.1 and 1 μM). This anti-inflammatory effect was also maintained in the later experiments performed, as shown in Figures 4.5, 4.7 and 4.9, respectively. Studies in endothelial and other cell line models have reported beneficial effects of TVA (Da Silva et al, 2017b; Iwata et al, 2011; Jaudszus et al, 2012), to some extent concordant with the current results. For example, Iwata et al. (2011) reported that TVA did not induce any inflammatory responses in comparison to EA and linoelaidic acid, specifically in relation to NF-kB activation, levels of IL-6 and superoxide production. Jaudszus et al. (2012) showed that TVA was able to significantly decrease the percentage of both IL-2 and TNF-α expressing Th cells induced by alloreactive stimulation in human PBMCs. Da Silva et al. (2017) showed recently that exposure to TVA at concentrations above 25 μM significantly reduced the TNF-α-induced gene expression of TNF-α, VCAM-1 and superoxide dismutase 2 in HUVECs, although independently of PPAR-γ activation; the effect may be due to an alteration of fatty acids in cell membranes as a result of the high incorporation of TFAs .

In accordance with other studies, the results described in this chapter also show that the exposure to EA increased cytokine production in stimulated EA.hy926 cells (MCP-1, RANTES and IL-8) when compared to ECs cultured in control conditions (0.1% ethanol), tending to also increase the production of VEGF and IL-6. Harvey et al. (2012) showed that the exposure of human-derived aortic endothelial cells to EA (5–50 μM) induced enhanced ICAM-1 surface expression, indicative of the inflammatory status of ECs. The same study showed that neither OA nor LA evoked a pro-inflammatory phenotype in ECs at the maximal 50 μM treatments, which also agrees with the current results where, in

general, the *cis* isomers used did not induce important pro-inflammatory changes in cytokines and adhesion molecules.

It has also been described that linoelaidic acid and EA promote leukocyte adhesion to the endothelium through increasing oxidative stress and NFκB activation (Bryk et al, 2011). Other authors postulate that lipid rafts are involved in the inflammatory effects of TFAs. Pan et al. (2017) showed that levels of ICAM-1, VCAM-1 and IL-6 were significantly increased in HUVECs after exposure to EA but these effects were annulled when lipid rafts were destroyed. The same study showed that lipid raft formation might increase NFκB activation in HUVECs treated with TFA. Therefore, the expression of NFκB pathway genes was analysed (see chapter 5) to determine if the increased cytokine production observed in this model is related to an effect of the FAs on this pathway.

### 4.5.2 Comparing the effect of two CLA isomers of ruminant origin and LA on the production of inflammatory mediators by cultured ECs

In non-stimulated ECs, CLA9,11 had neutral or anti-inflammatory effects, reducing the production of MCP-1 (1 μM), RANTES (1 and 10 μM), IL-8 (1 and 10 μM) and IL-6 (1 μM). CLA10,12 exposure reduced the same cytokines in basal conditions.

Conversely, after TNF-α stimulation, CLA10,12 showed a consistent pro-inflammatory effect, increasing levels of ICAM-1, VEGF, RANTES and IL-6. It has been described that CLA, particularly the *cis*-9, *trans*-11 isomer, is able to reduce pro-inflammatory mediator production, like VCAM-1 and ICAM-1, in HUVECs (Stachowska et al, 2012). Another study showed that HUVECs pre-incubated for 24 h with 25 μM of the different CLA isomers or a 50/50 mix of both isomers had reduced protein expression of ICAM-1 and VCAM-1, after stimulation with TNF-α (5 ng/ml) for 6 h (Goua et al, 2008). In contrast, in an animal model of $ApoE^{-/-}$ mice, the development of atherosclerotic lesions was impaired by a diet supplemented with CLA9,11 (12 weeks), while pro-atherogenic effects were observed in mice fed with the CLA10,12 diet, demonstrating differential atherogenic effects of CLA isomers (Arbones-Mainar et al, 2006). The results shown here also indicate that CLA isomers may elicit differential actions in TNF-α stimulated ECs. A proposed mechanism through which CLAs produce pleiotropic effects is via activation of PPARs (Brown et al,

2003; Moya-Camarena et al, 1999; Yu et al, 2002). Moreover, the NFκB pathway can be inhibited by activation of PPARs (Daynes & Jones, 2002). Therefore the expression of PPARα and γ was also analysed (see chapter 5) to determine if the effect of CLA isomers on cytokine production is related to this pathway.

### 4.5.3 Conclusions

Overall, these results show differential effects of the FAs tested on inflammatory responses of ECs *in vitro* (summary in table 4.1). The exposure of EA.hy926 cells to EA and CLA10,12 increased the levels of most of the cytokines and adhesion molecules measured after inflammatory stimulation, having neutral or some anti-inflammatory effects in basal conditions. TVA caused reduced production of MCP-1, VEGF, RANTES, IL-8 and IL-6 in non-stimulated cells, which was also seen after inflammatory stimulation for ICAM-1, VEGF and RANTES at 1 μM, having otherwise neutral effects. Similarly, CLA9,11 showed some anti-inflammatory effects in non-stimulated cells (MCP-1, RANTES, IL-8 and IL-6), with null effects after TNF-α stimulation. The *cis*-isomers usually showed neutral or mild anti-inflammatory effects, with the exception of OA and CVA increasing the levels of IL-8 when used at 50 μM, and LA at 1 μM.

Table 4.1: Summary of effects of FAs on inflammatory mediator concentrations in the medium of EA.hy926 cells.

| | **Fatty acid (unstimulated ECs)** | | | | | | |
|---|---|---|---|---|---|---|---|
| **Mediator** | OA | CVA | TVA | EA | LA | CLA9,11 | CLA10,12 |
| MCP-1 | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ |
| ICAM-1 | ↔ | ↔ | ↔ | ↔ | ↓ | ↔ | ↔ |
| VEGF | ↔ | ↔ | ↓ | ↔ | ↔ | ↔ | ↔ |
| RANTES | ↔ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ |
| IL-8 | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ |
| IL-6 | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↑ |
| | **Fatty acid (TNF-α stimulated ECs)** | | | | | | |
| MCP-1 | ↔ | ↔ | ↔ | ↑ | ↔ | ↔ | ↔ |
| ICAM-1 | ↔ | ↔ | ↓ | ↔ | ↔ | ↔ | ↑ |
| VEGF | ↔ | ↔ | ↓ | ↔ | ↔ | ↔ | ↑ |
| RANTES | ↔ | ↓ | ↓ | ↑ | ↔ | ↔ | ↑ |
| IL-8 | ↑ | ↑ | ↔ | ↑ | ↑ | ↔ | ↔ |
| IL-6 | ↔ | ↔ | ↔ | ↔ | ↓ | ↔ | ↑ |

Based in these results, it is suggested that EA (an iTFA) and TVA (a rTFA) have opposite effects on the production of cytokines in the EC model used here, particularly when a pro-inflammatory state is induced. In a similar way, the CLA isomers compared here also seem to have differential effects, both reducing the production of cytokines under basal conditions, although, in the case of CLA10,12, increasing their levels after TNF-α stimulation. These findings generally support the contention that iTFAs can be pro-inflammatory while rTFAs may be without effect or anti-inflammatory. Later chapters will explore some of the possible mechanisms involved and other actions of 18-carbon TFAs related to atherosclerosis.

# Chapter 5 Effect of 18-carbon *trans* fatty acids on inflammatory pathway gene expression in cultured EA.hy926 cells

## 5.1 Introduction

The research described in Chapter 4 showed that some 18-carbon TFAs can directly affect inflammation and can modify the response to a well-recognised inflammatory stimulant, TNF-α. The inflammatory response of cultured endothelial cells (ECs) was assessed as the production of known inflammatory mediators including cytokines and chemokines, determined by measuring the concentrations of those mediators in the cell culture medium. TNF-α triggers intracellular signalling processes that activate transcription factors such as NFκB. The activated NFκB dimers translocates to the EC nucleus where it upregulates the expression of genes encoding inflammatory mediators including several of those reported to be affected by 18-carbon TFAs as well as the genes encoding cyclooxygenase-2 (COX-2) and the inhibitory subunit of κB (IκBα) (see Figure 1.4, Chapter 1). In order to better understand the effects of 18-carbon TFAs on inflammation and their possible mechanism of action, their impact on transcription factor activation and inflammatory gene expression in cultured ECs was studied. Prior to assessing the effects of the FAs on inflammatory gene expression, a time course of the effect of TNF-α stimulation on genes of interest was performed.

TNF-α increases the level of inflammatory mediators (e.g. cytokines and chemokines) produced by EA.hy926 cells in a time dependent manner (Chapter 4). If this involves alterations in gene expression these should occur before the secretion of cytokines into the cell supernatant; consequently a time course using time points at 30 minutes, 1, 3, 6 and 9 h post-stimulation was chosen. The relative expression of genes encoding transcription factors and inflammatory cytokines was assessed through real-time PCR.

The effects of some TFAs on inflammatory pathway gene expression has been studied previously, but because these previous studies have used different cell and animal models, and different TFAs and FA concentrations it is difficult to identify and compare the precise molecular pathways by which TFAs of different sources contribute to inflammation relevant to the development of atherosclerosis.

The research described in this chapter compares the effects of seven 18-carbon *cis* and *trans* FAs of different origins on gene expression in EA.hy926 cells with those in control conditions.

## 5.2 Objective and Aim

The research objectives developed in this chapter were to measure the effects of 18-carbon *cis* and *trans* fatty acids on expression of selected genes in the inflammatory pathway of EA.hy926 cells, after optimizing the experimental conditions.

The specific aims were to:

- Perform a time course experiment to measure the appearance of mRNA for PPAR-α, PPAR-γ, NFκB1 and MCP-1 following TNF-α stimulation of cultured ECs.
- Evaluate changes in gene expression of NFκB1, IKKβ, IKβα, PPAR-α, PPAR-γ, TLR4, COX-2, MCP-1 and IL- 6 after exposure to the fatty acids, with and without TNF-α stimulation.
- Compare the effect of 18-carbon *trans* isomers from industrial and ruminant origin on the expression of selected genes in the inflammatory pathway of EA.hy926 cells.
- Compare the effect of two conjugated linoleic acid isomers of ruminant origin and linoleic acid on the expression of selected genes in the inflammatory pathway of EA.hy926 cells.

## 5.3 Methods

### 5.3.1 Reagents

Reagents and materials used for experiments are described in Appendix A.

### 5.3.2 EA.hy926 cell culture

For the time course experiment, confluent EA.hy926 cultures were scraped from flasks and resuspended in culture medium at a density of 5.5 x $10^5$ cells per mL. Cells (1 mL) were seeded in 6-well flat bottom plates, incubated for 48 h at 37$^o$C with DMEM, followed by incubation with either DMEM or TNF-α at 1 ng/mL for different durations (30 minutes, 1, 2, 6 and 9 h). At each time point, cells were removed, resuspended in BL-TG

buffer following ReliaPrep™ RNA Cell Miniprep System instructions and stored at -80$^{o}$C until analysis.

For the subsequent experiments, confluent EA.hy926 cultures were scraped from flasks and resuspended in culture medium at a density of 5.5 x $10^5$ cells per mL. Cells were seeded at 5.5 x $10^5$ cells per well in 6-well flat bottom plates, incubated for 48 h at 37$^{o}$C with FAs at a concentration of 1, 10 and 50 µM, followed by 24 h incubation with DMEM or TNF-α at 1 ng/mL. Then, cells were removed, resuspended in BL-TG buffer following ReliaPrep™ RNA Cell Miniprep System instructions and stored at -80$^{o}$C until analysis.

### 5.3.3 PCR Procedure

PCR assay procedures were as described in Chapter 2.

### 5.3.4 geNorm analysis

For accurate gene quantification, it is essential to normalise RT-PCR data to the most stable reference ("house-keeping") genes which are the ones that are not affected by the experimental conditions (i.e., exposure to FAs or TNF-α). A geNorm kit (Primerdesign Ltd.) was used for selecting the best candidate reference genes. To perform geNorm analysis, the expression of 8 reference genes in a representative set of samples was measured. Duplicate samples of each condition were used, of cells exposed to the different FAs, at 1, 10 and 50 µM, with or without TNF-α stimulation. The geNorm software qbase+ provided with the kit was used to rank the reference genes in order of stability of expression.

### 5.3.5 Statistical analysis

Relative gene expression was calculated using double delta Cq (ΔΔCq) values expressed as fold change of the target gene in experimental samples relative to control samples (see section 2.7.2.6). Data are expressed as mean ± SEM. Analyses were performed using GraphPad Prism 6.0 and Excel. Comparisons between samples were made using One-way ANOVA and post hoc test.

## 5.4 Results

### 5.4.1 Time course of changes in inflammatory pathway gene expression in EA.hy926 cells

To determine when to measure changes in the expression of inflammatory genes in cells exposed to different FAs and stimulated with TNF-α, a time course was performed. For the NFκB1 gene, the biggest change in mRNA expression was observed at 3 h of stimulation (Fig. 5.1A). In the case of the PPAR-α gene, the inflammatory stimulus did not produce any significant change in the expression, although it is possible to see a trend of decreased expression over time (Fig. 5.1B). For the MCP-1 gene, there were differences from the first time point (3 h), but the largest change in mRNA expression was detected at 9 h (Fig. 6C). PPAR-γ mRNA was not detected (data not shown).

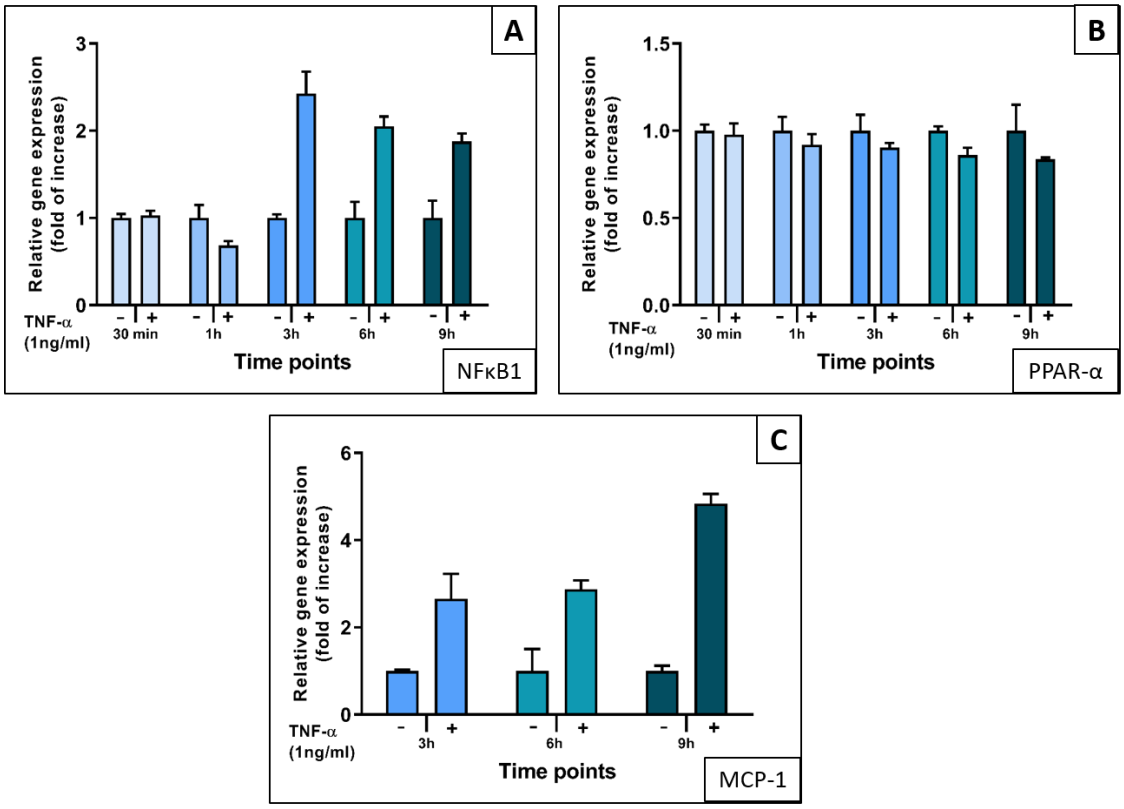



Figure 5.1: Time-course for the expression of genes for NFκB1 (A), PPAR-α (B), and MCP-1 (C). EA.hy926 cells were incubated for 48 h with DMEM containing 0.1% ethanol (Control) followed by incubation with (+) or without (-) TNF-α (1 ng/mL) in different time points. All Cq values were normalized to Beta-2-Microglobulin (B2M). Bars are mean ± SEM of 3 samples performed in 1 experiment.

Based on these results, a time point of 6 h for TNF-α stimulation was chosen to evaluate the effects of FA treatment on the expression of genes of the inflammatory signalling pathway in EA.hy926 cells.

### 5.4.2 RNA analysis

RNA quantity, purity and integrity were measured. The first two were carried out through NanoDrop analysis. Table 5.1 shows the results of this analysis in a representative set of RNA samples, indicating enough quantity and good purity, judged by $A_{260/280}$ (~2.0.) and $A_{260/230}$ (~1.8-2.2.) ratios.

Table 5.1: NanoDrop analysis of RNA quantity and purity.

| Samples | ng/µl | 260/280 | 260/230 |
|---|---|---|---|
| Control (-) | 640.63 | 2.14 | 2.16 |
| OA 1 µM (-) | 654.3 | 2.14 | 2.15 |
| CVA 1 µM (-) | 654.16 | 2.13 | 2.15 |
| TVA 1 µM (-) | 718.06 | 2.13 | 2.07 |
| EA 1 µM (-) | 583.6 | 2.13 | 2.16 |
| LA 1 µM (-) | 682.21 | 2.13 | 2.03 |
| CLA9,11 1 µM (-) | 639.18 | 2.12 | 2.16 |
| CLA10,12 1 µM (-) | 695.81 | 2.12 | 2.08 |
| OA 10 µM (-) | 786.84 | 2.10 | 2.08 |
| CVA 10 µM (-) | 809.46 | 2.06 | 2.14 |
| TVA 10 µM (-) | 844.88 | 2.10 | 2.08 |
| EA 10 µM (-) | 665.55 | 2.10 | 2.19 |
| LA 10 µM (-) | 737.5 | 2.12 | 2.19 |
| CLA9,11 10 µM (-) | 794.09 | 2.07 | 2.16 |
| CLA10,12 10 µM (-) | 742.34 | 2.11 | 2.08 |
| OA 50 µM (-) | 701.63 | 2.14 | 2.19 |
| CVA 50 µM (-) | 1053.1 | 2.12 | 2.17 |
| TVA 50 µM (-) | 988.4 | 2.14 | 2.19 |
| EA 50 µM (-) | 812.06 | 2.16 | 2.17 |
| Control (+) | 599.49 | 2.07 | 1.93 |
| OA 1 µM (+) | 716.64 | 2.11 | 2.15 |
| CVA 1 µM (+) | 572.17 | 2.12 | 2.03 |
| TVA 1 µM (+) | 665.95 | 2.10 | 2.10 |
| EA 1 µM (+) | 732.96 | 2.11 | 2.11 |
| LA 1 µM (+) | 742.17 | 2.12 | 2.18 |
| CLA9,11 1 µM (+) | 641.85 | 2.16 | 2.13 |
| CLA10,12 1 µM (+) | 654.13 | 2.14 | 2.13 |
| OA 10 µM (+) | 590.45 | 2.11 | 2.00 |
| CVA 10 µM (+) | 630.04 | 2.13 | 1.95 |
| TVA 10 µM (+) | 580.65 | 2.12 | 1.96 |
| EA 10 µM (+) | 605.45 | 2.08 | 2.14 |
| LA 10 µM (+) | 550.69 | 2.11 | 2.00 |
| CLA9,11 10 µM (+) | 658.25 | 2.07 | 2.13 |
| CLA10,12 10 µM (+) | 668.29 | 2.07 | 2.05 |
| OA 50 µM (+) | 749.56 | 2.15 | 2.13 |
| CVA 50 µM (+) | 633.63 | 2.14 | 2.18 |
| TVA 50 µM (+) | 713.65 | 2.09 | 2.07 |
| EA 50 µM (+) | 699.03 | 2.14 | 2.18 |

The analysis depicted in figures 5.2, 5.3 and 5.4 shows high RNA Integrity Number (RIN) scores, indicating good RNA integrity.

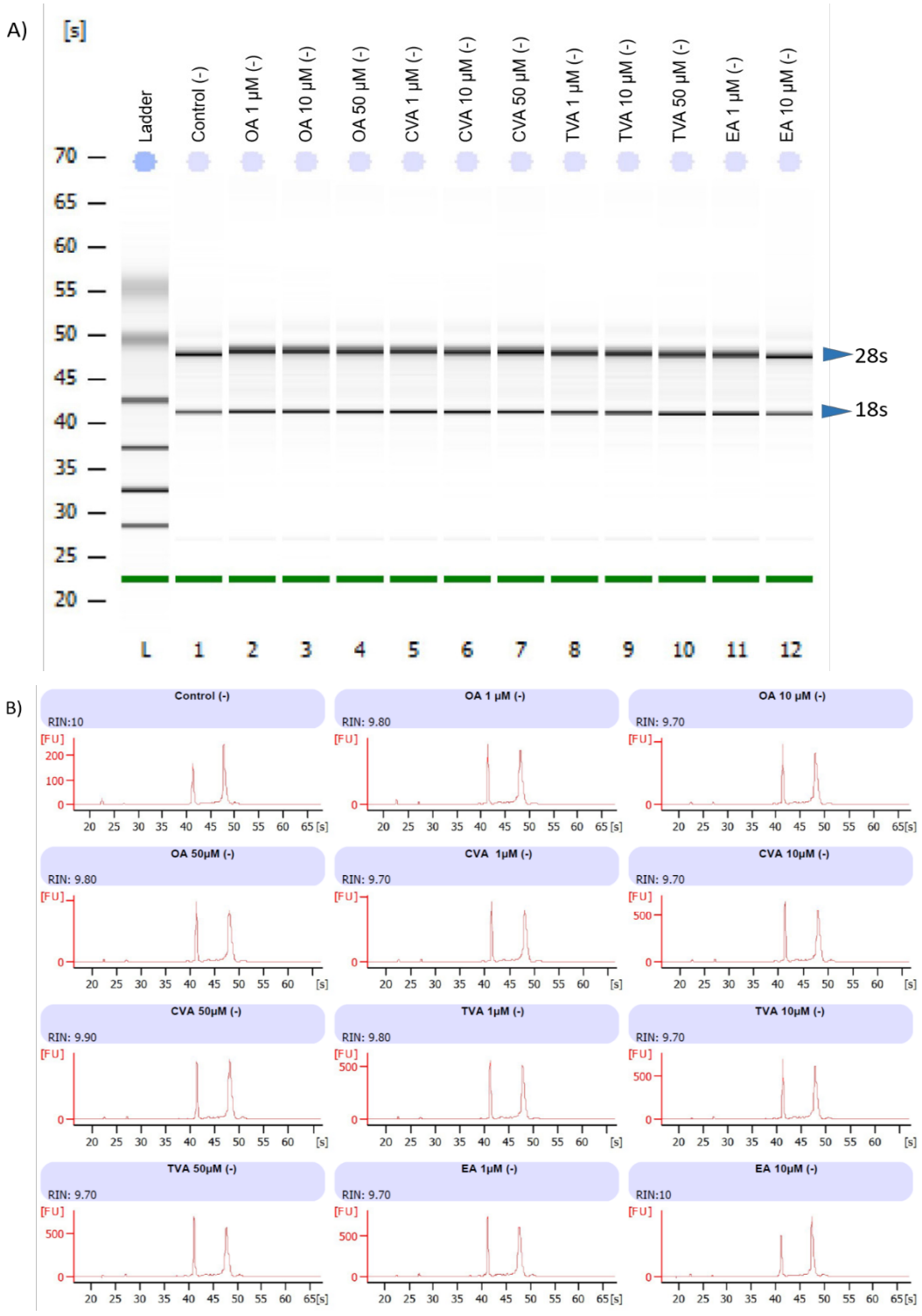



Figure 5.2: Bioanalizer profiles of RNA extracted from EA.hy926 cells exposed to FAs. ECs were incubated for 48 h with 1, 10 and 50 µM of FA in DMEM containing 0.1% of ethanol (Control) followed by incubation with DMEM for 6 h (-). A) Gel-like image, bands represent 18s and 28s ribosomal RNA subunits. B) Electropherogram with 18s and 28s peaks. RIN scores were measured by Agilent bioanalizer.

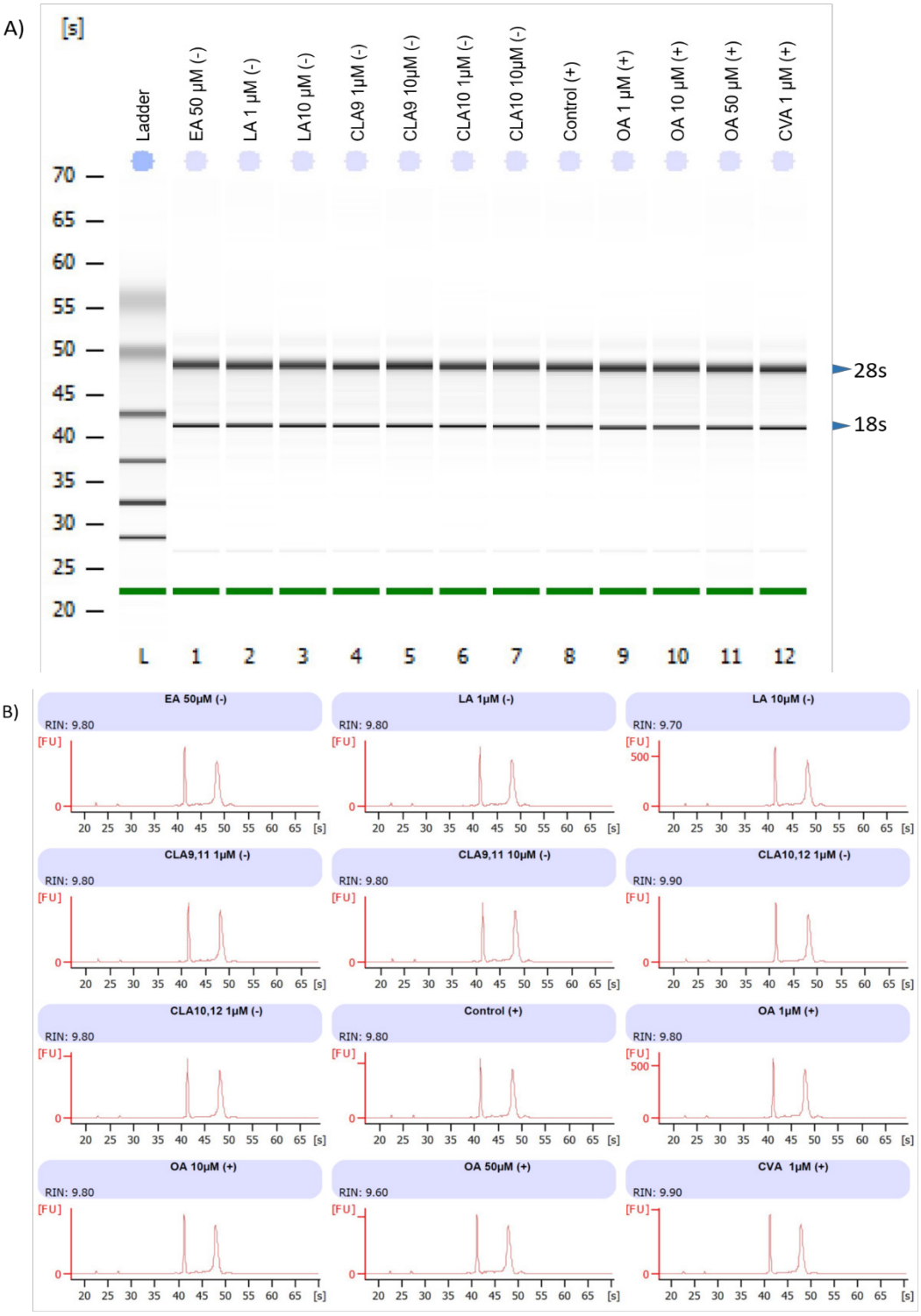



Figure 5.3: Bioanalizer profiles of RNA extracted from EA.hy926 cells exposed to FAs.
ECs were incubated for 48 h with 1, 10 and 50 µM of FA in DMEM containing 0.1% of ethanol (Control) followed by incubation with DMEM (-) or TNF-α (1 ng/mL, (+)) for 6 h. A) Gel-like image, bands represent 18s and 28s ribosomal RNA subunits. B) Electropherogram with 18s and 28s peaks. RIN scores were measured by Agilent bioanalizer.

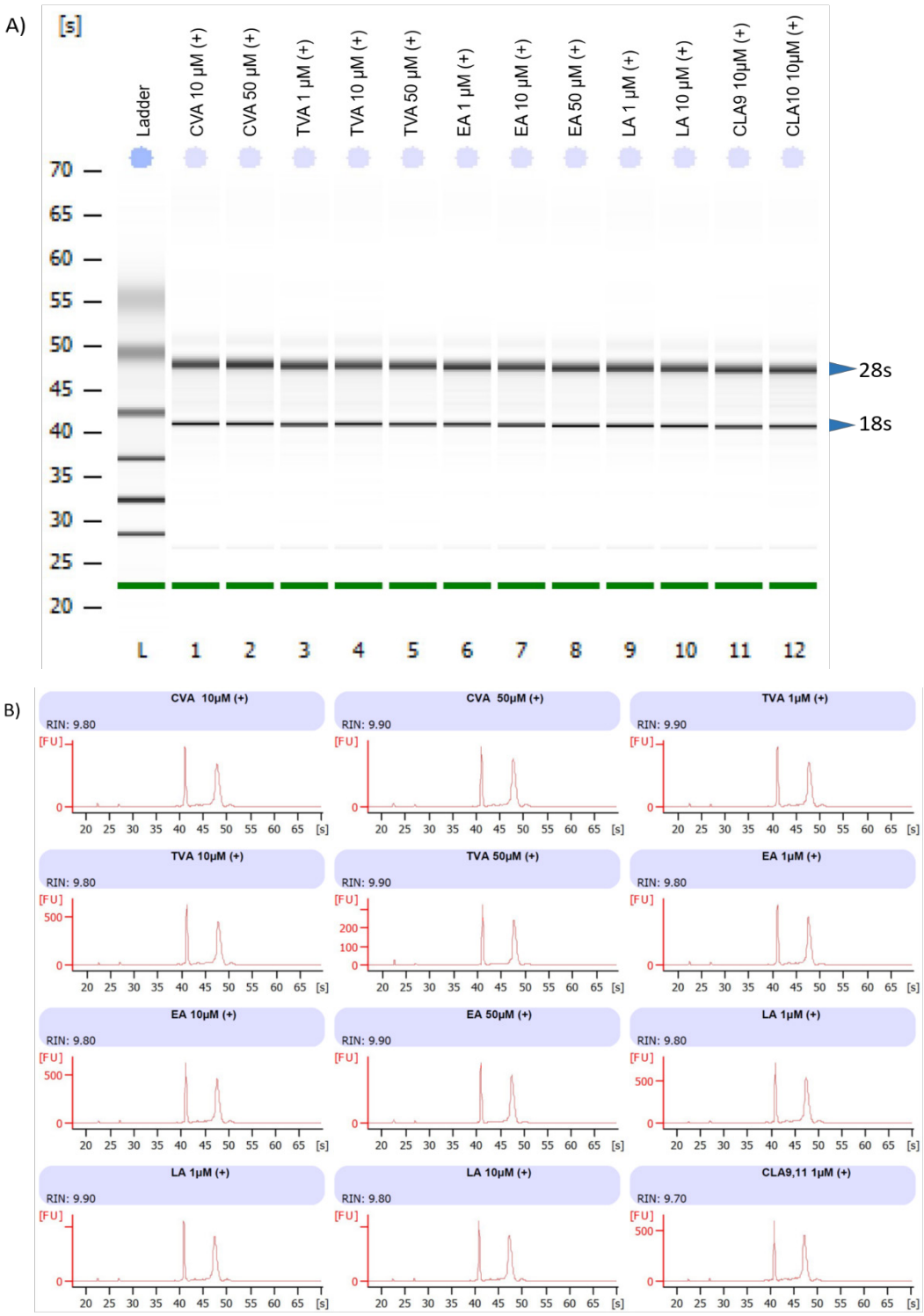



Figure 5.4: Bioanalizer profiles of RNA extracted from EA.hy926 cells exposed to FAs.

ECs were incubated for 48 h with 1, 10 and 50 µM of FA in DMEM containing 0.1% of ethanol (Control) followed by incubation with DMEM (-) or TNF-α (1 ng/mL, (+)) for 6 h. A) Gel-like image, bands represent 18s and 28s ribosomal RNA subunits. B) Electropherogram with 18s and 28s peaks. RIN scores were measured by Agilent bioanalizer.

### 5.4.3 Reference gene identification

A representative set of cDNA samples for all the experimental conditions was analysed using the geNorm kit. The optimal number of reference genes both for unstimulated and TNF-$\alpha$ stimulated cells was 2 (geNorm V < 0.15 when comparing a normalization factor based on the 2 or 3 most stable targets). As shown in Figure 5.5, for unstimulated cells, these genes were YWHAZ and RPL13A. For stimulated cells, the most stable reference genes were RPL13A and CYC1 (Figure 5.6). Consequently, the optimal normalization factor can be calculated as the geometric mean of the reference targets selected for each experimental condition.

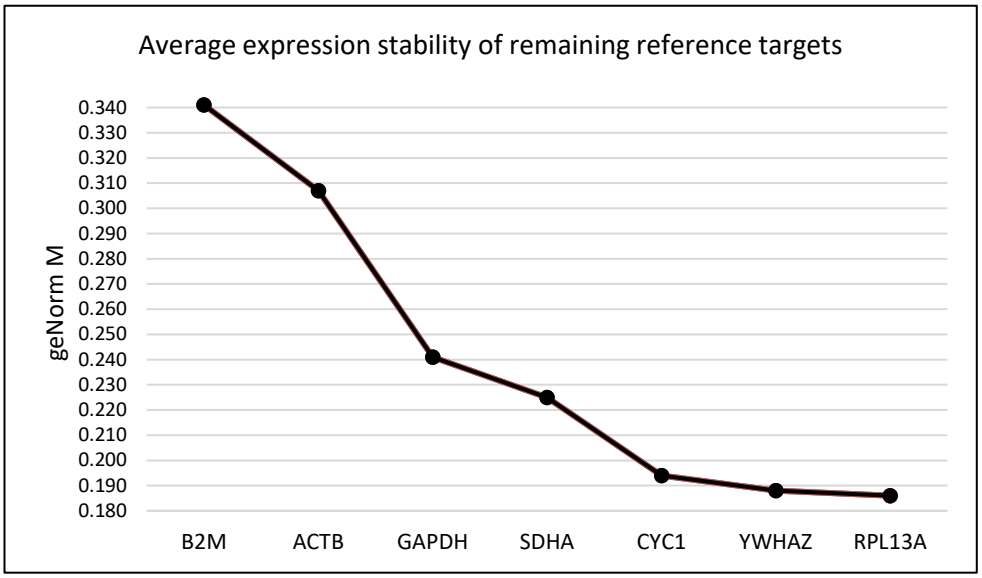



Figure 5.5: geNorm qbase+ analysis for reference genes suitable for unstimulated EA.hy926 cells.

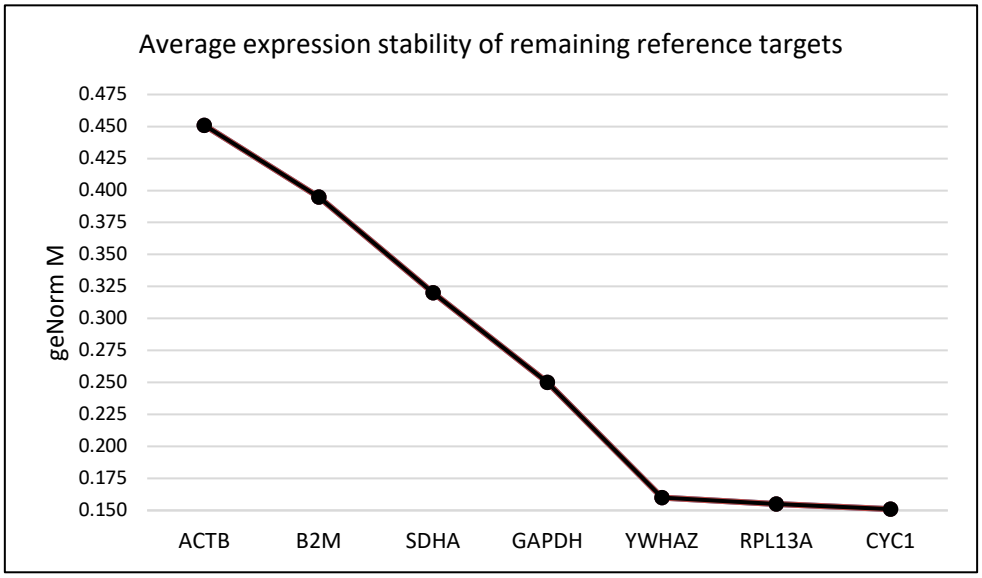



Figure 5.6: geNorm qbase+ analysis for reference genes suitable for the TNF-α (1 ng/mL) stimulated EA.hy926 cells.

### 5.4.4 Effect of 18-carbon *trans* isomers from industrial and ruminant sources on gene expression in EA.hy926 cells.

#### 5.4.4.1 NFκB1 relative gene expression

As shown in Figure 5.7(A), none of the FAs used, at any concentration, induced significant changes in NFκB1 mRNA levels in EA.hy926 cells under basal conditions (i.e. unstimulated). After TNF-α stimulation, TVA at 1 µM induced a significant reduction in the relative expression of NFκB1 compared to the control. The other FAs used did not did induce any changes in NFκB1 relative expression (Figure 5.7(B)).

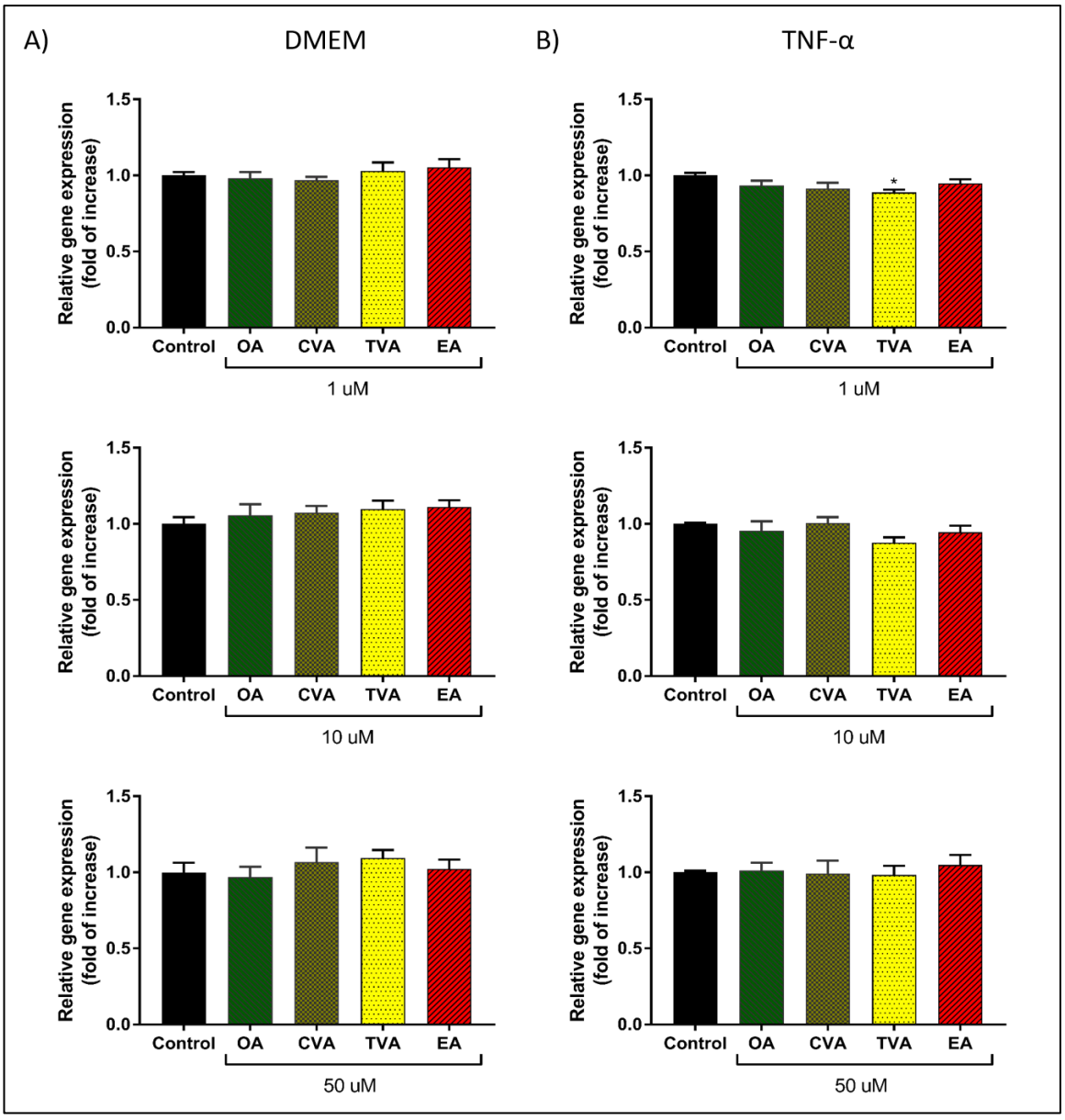



Figure 5.7: Gene expression of NFκB1 in EA.hy926 cells exposed to FAs.
ECs were incubated for 48 h with 1, 10 and 50 µM of FAs in DMEM containing 0.1% of ethanol (Control) followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 6 h. Cq values were normalized by the geometric mean of reference targets YWHAZ and RPL13A genes (A) or RPL13A and CYC1 genes (B). Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05. OA = oleic acid, CVA = *cis* vaccenic acid, TVA = *trans* vaccenic acid, EA = elaidic acid.

#### 5.4.4.2 IKBKB (IKKβ) for relative gene expression

Similar to NFκB1, none of the FAs used, at any concentration, induced significant changes in IKKβ mRNA levels in EA.hy926 cells under basal conditions. Nevertheless, TVA showed a trend to downregulate the relative expression of this gene at 10 µM (p=0.1) (Figure 5.8(A)).

In a pro-inflammatory state, after TNF-α stimulation (Figure 5.8(B)), FA exposure did not induce significant changes in the relative expression of IKKβ. However, at 10 µM OA tended to downregulate IKKβ mRNA levels (p=0.08).

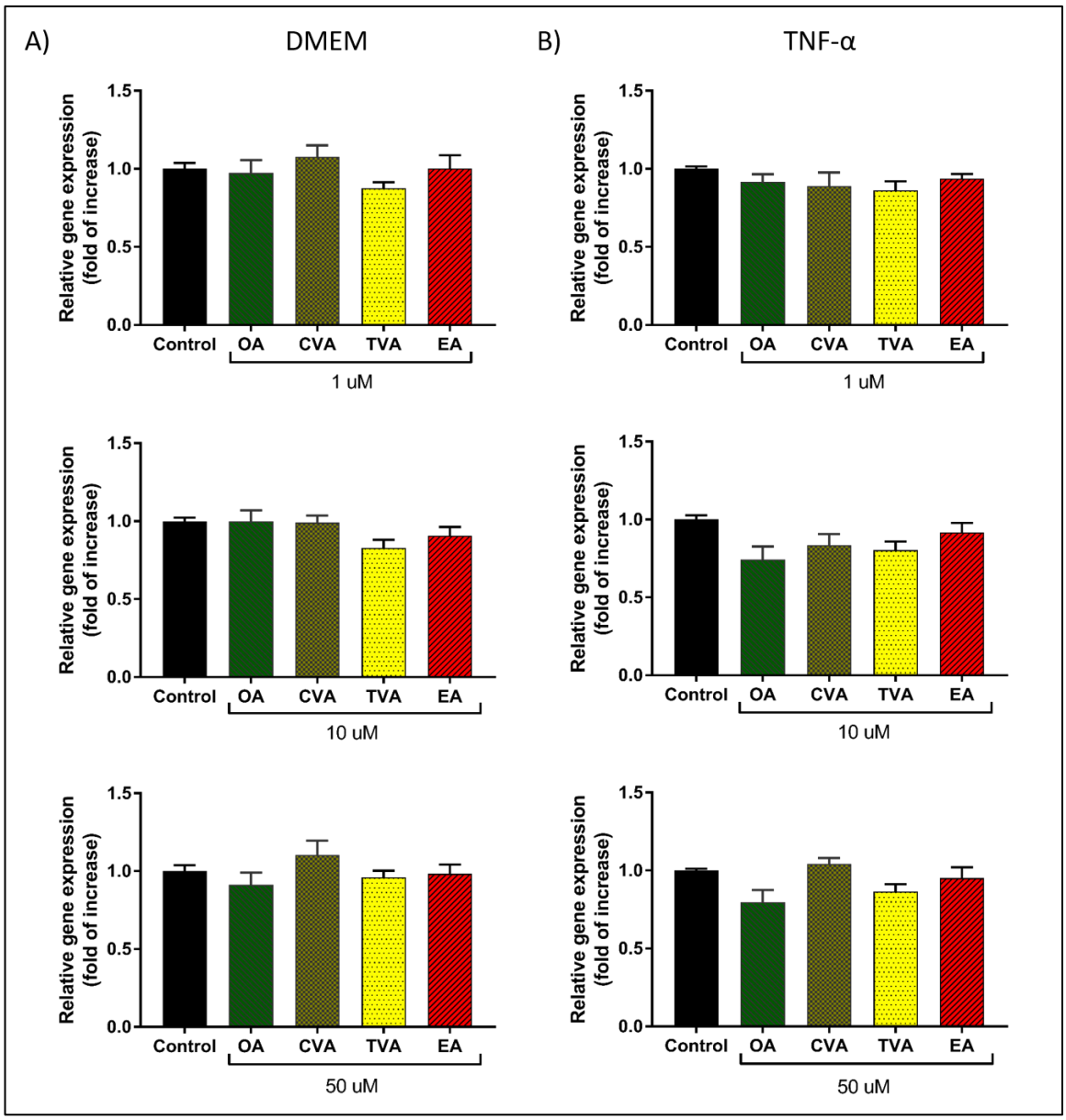



Figure 5.8: Relative gene expression of IKBKB (for IKK-β) in EA.hy926 cells exposed to FAs. ECs were incubated for 48 h with 1, 10 and 50 µM of FAs in DMEM containing 0.1% of ethanol (Control) followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 6 h. Cq values were normalized by the geometric mean of reference targets YWHAZ and RPL13A genes (A) or RPL13A and CYC1 genes (B). Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. OA = oleic acid, CVA = *cis* vaccenic acid, TVA = *trans* vaccenic acid, EA = elaidic acid.

#### 5.4.4.3 NFKBIA (IκBα) relative gene expression

Figure 5.9(A) shows that pre-incubation of ECs with EA behaved significantly different from TVA at 10 µM. The other FAs used, did not induce significant changes in IκBα mRNA levels in EA.hy926 cells at basal conditions. As shown in Figure 5.9(B), none of the FAs used, at any concentration, induced significant changes in the relative gene expression of IκBα after TNF-α stimulation.

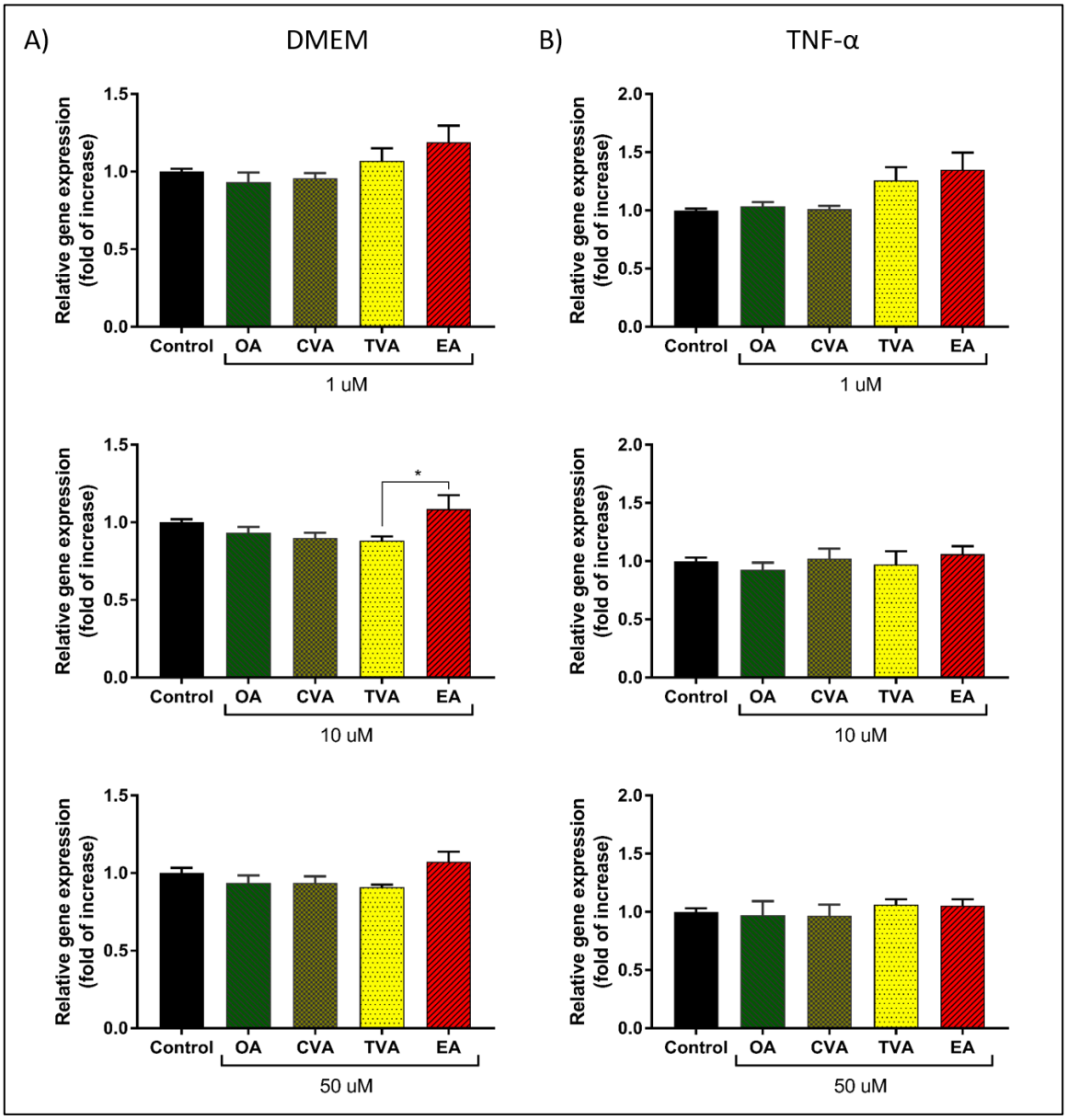



Figure 5.9: Relative gene expression of NFKBIA (for IκBα) in EA.hy926 cells exposed to FAs. ECs were incubated for 48 h with 1, 10 and 50 µM of FAs in DMEM containing 0.1% of ethanol (Control) followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 6 h. Cq values were normalized by the geometric mean of reference targets YWHAZ and RPL13A genes (A) or RPL13A and CYC1 genes (B). Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05. OA = oleic acid, CVA = *cis* vaccenic acid, TVA = *trans* vaccenic acid, EA = elaidic acid.

#### 5.4.4.4 PPAR-α relative gene expression

For the peroxisome proliferator-activated receptor alpha (PPAR-α) gene, all the FA used at 10 µM downregulated its relative expression under basal conditions, compared to control (Figure 5.10(A)). After TNF-α stimulation, as shown in Figure 5.10(B), both TFAs and their cis isomers were similar to control. Only TVA showed a trend to reduce mRNA levels of PPAR-α at 1 µM (p=0.1), which was significantly different from mRNA expression of EA treated cells.

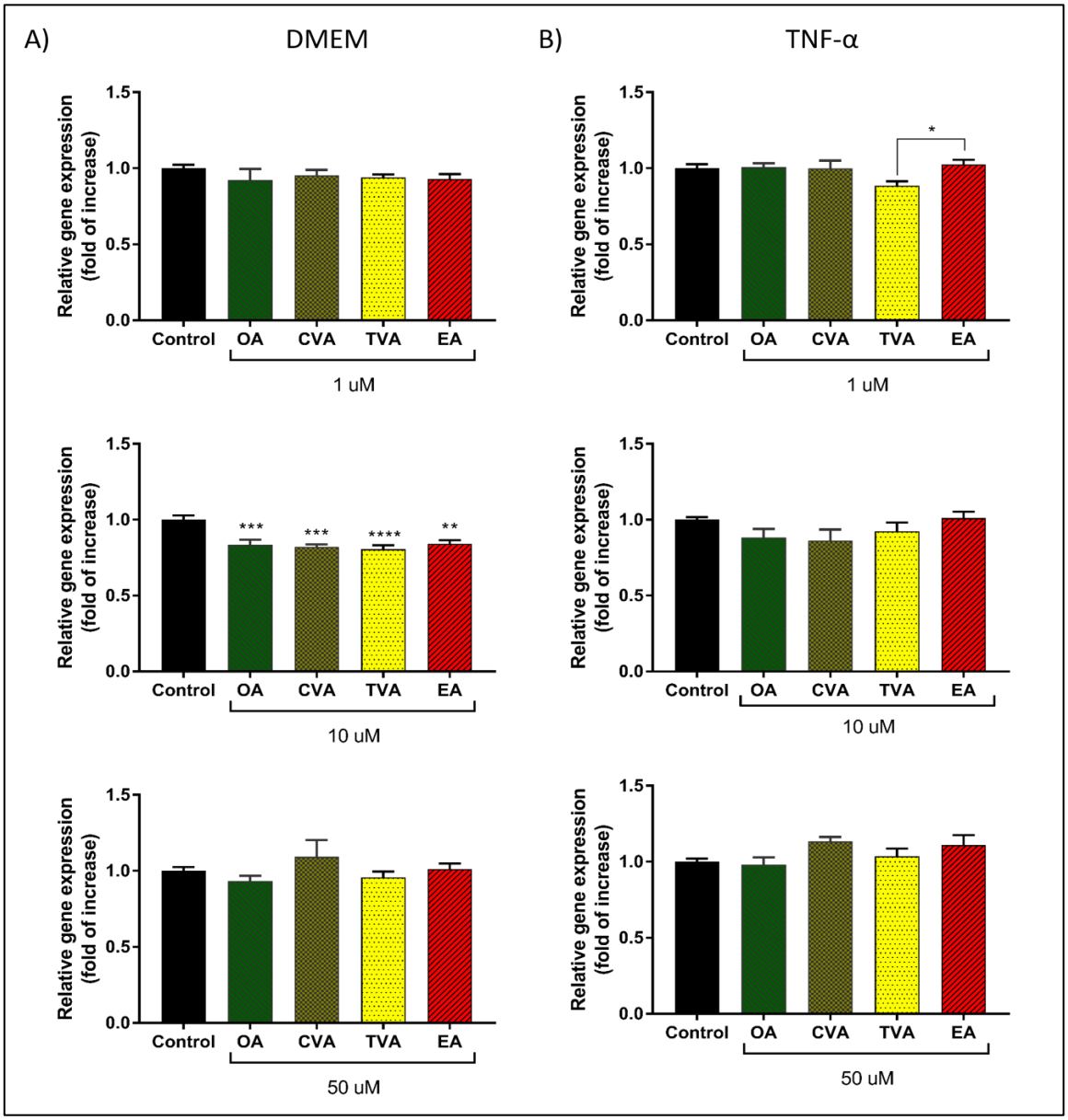



Figure 5.10: Relative gene expression of PPAR-α in EA.hy926 cells exposed to FAs.
ECs were incubated for 48 h with 1, 10 and 50 µM of FAs in DMEM containing 0.1% of ethanol (Control) followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 6 h. Cq values were normalized by the geometric mean of reference targets YWHAZ and RPL13A genes (A) or RPL13A and CYC1 genes (B). Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. OA = oleic acid, CVA = *cis* vaccenic acid, TVA = *trans* vaccenic acid, EA = elaidic acid.

#### 5.4.4.5 Toll like receptor 4 (TLR4) relative gene expression

Figure 5.11(A) shows that pre-incubation of ECs with 10 µM CVA significantly downregulated TLR4 relative gene expression, acting significantly different from its *trans*-isomer, TVA. At 50 µM, both cis isomers modulated TLR4 gene expression producing downregulation.

In a pro-inflammatory state, after TNF-α stimulation (Figure 5.11(B)), EA at 1 µM behaved significantly different from its *cis*-isomer OA. When EA was used at 50 µM, caused a significant upregulation of the relative TLR4 gene expression.

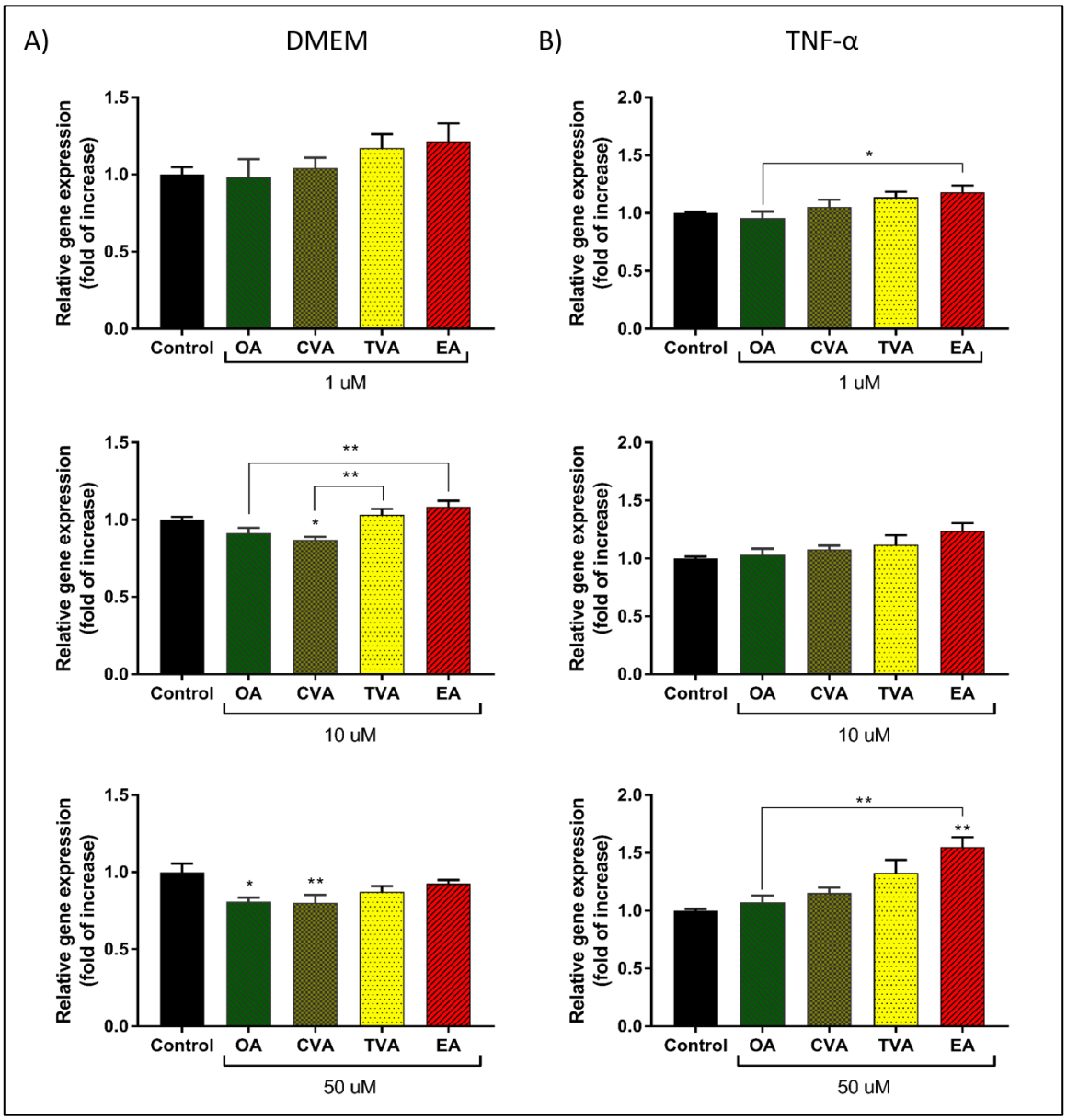



Figure 5.11: Relative gene expression of TLR4 in EA.hy926 cells exposed to FAs.
ECs were incubated for 48 h with 1, 10 and 50 µM of FAs in DMEM containing 0.1% of ethanol (Control) followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 6 h. Cq values were normalized by the geometric mean of reference targets YWHAZ and RPL13A genes (A) or RPL13A and CYC1 genes (B). Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; **p<0.01. OA = oleic acid, CVA = *cis* vaccenic acid, TVA = *trans* vaccenic acid, EA = elaidic acid.

#### 5.4.4.6 Prostaglandin-endoperoxide synthase 2 (PTGS2) relative gene expression

For the gene encoding COX-2, EA at 1 µM behaved significantly different from TVA. When used at 10 µM, TVA induced a significant reduction in COX-2 mRNA levels. When used at 50 µM, EA induced downregulation (Figure 5.12(A)).

After TNF-α stimulation (1 ng/mL) for 6 h, only the highest FA concentration produced significant modulation of COX-2 gene expression: both CVA and EA induced an increment in the levels of gene expression compared to control (Figure 5.12(B)).

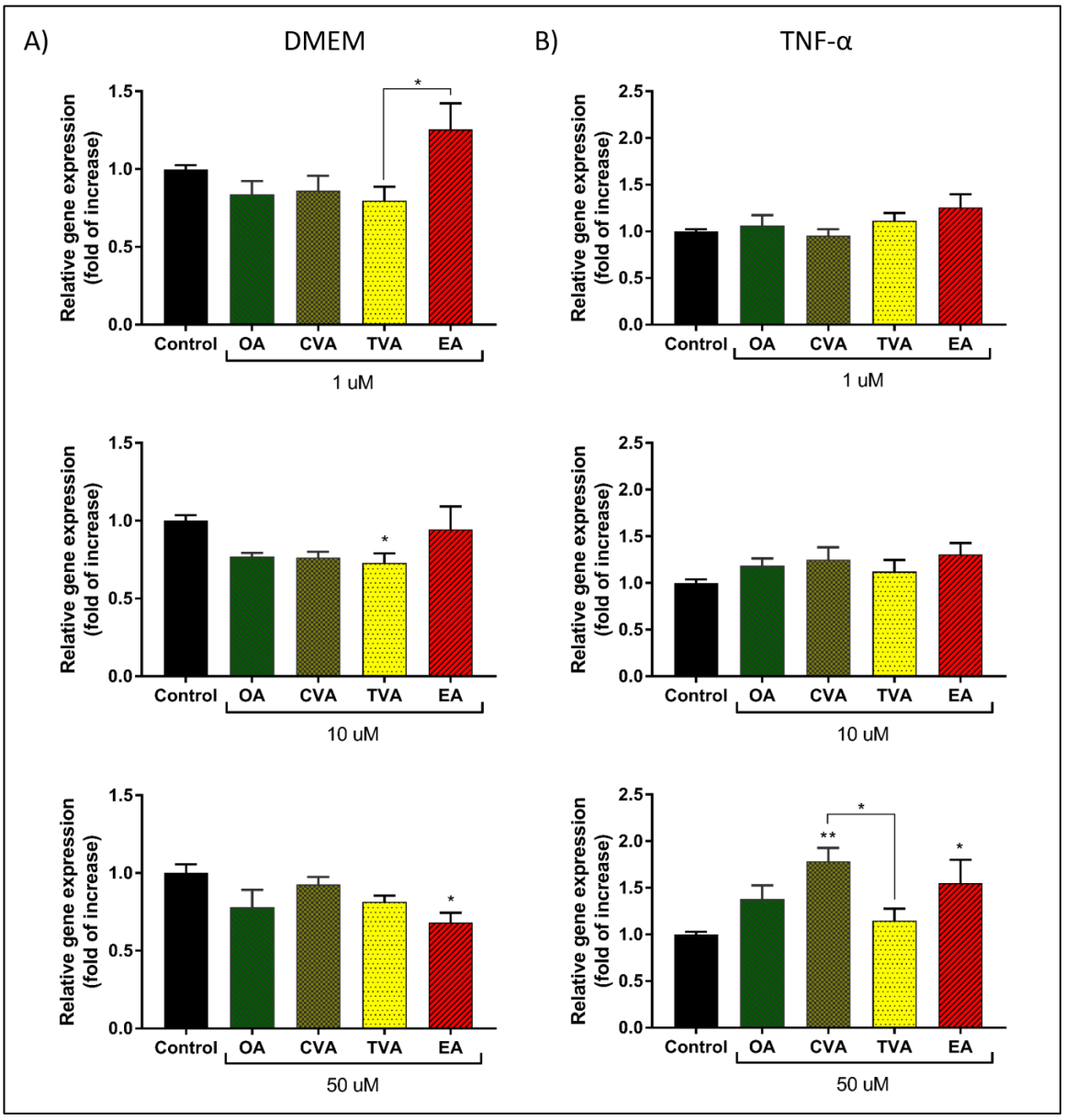



Figure 5.12: Relative gene expression of PTGS2 (for COX-2) in EA.hy926 cells exposed to FAs. ECs were incubated for 48 h with 1, 10 and 50 µM of FAs in DMEM containing 0.1% of ethanol (Control) followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 6 h. Cq values were normalized by the geometric mean of reference targets YWHAZ and RPL13A genes (A) or RPL13A and CYC1 genes (B). Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; **p<0.01. OA = oleic acid, CVA = *cis* vaccenic acid, TVA = *trans* vaccenic acid, EA = elaidic acid.

### 5.4.4.7 Chemokine (C-C motif) ligand 2 (CCL2) relative gene expression

For the gene encoding monocyte chemoattractant protein 1 (MCP-1), pre-incubation with EA at 1 µM produced upregulation, acting significantly different from OA. OA at 50 µM induced a reduced gene expression compared to control (Figure 5.13(A)). After TNF-α stimulation, as shown in Figure 5.13(B), both TFAs and their *cis* isomers were similar to control when used at 1 and 10 µM.

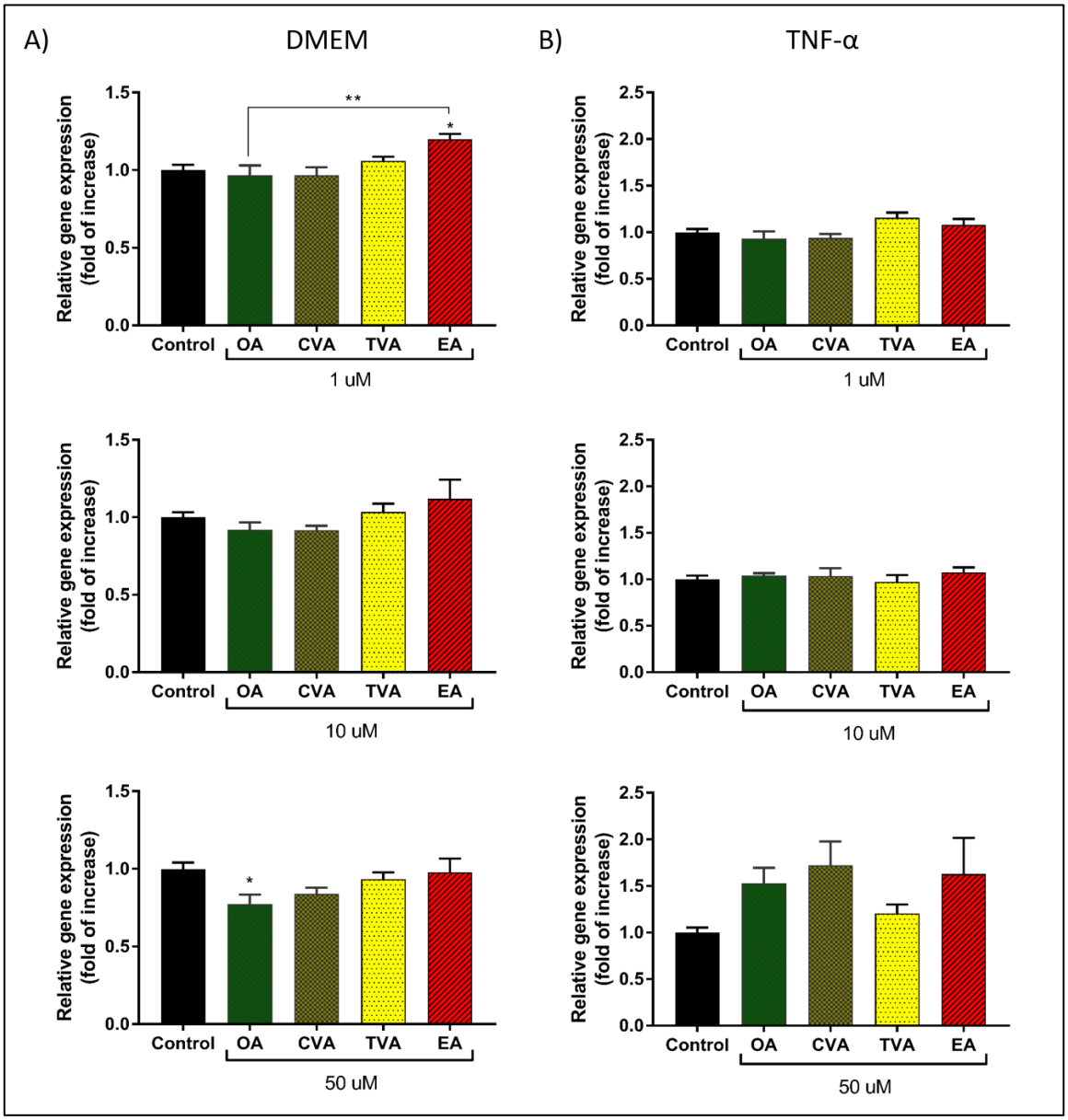



Figure 5.13: Relative gene expression of CCL2 (for MCP-1) in EA.hy926 cells exposed to FAs. ECs were incubated for 48 h with 1, 10 and 50 µM of FAs in DMEM containing 0.1% of ethanol (Control) followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 6 h. Cq values were normalized by the geometric mean of reference targets YWHAZ and RPL13A genes (A) or RPL13A and CYC1 genes (B). Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; **p<0.01. OA = oleic acid, CVA = *cis* vaccenic acid, TVA = *trans* vaccenic acid, EA = elaidic acid.

#### 5.4.4.8 Interleukin-6 (IL-6) relative gene expression

For IL-6 gene, pre-incubation with TVA, CVA and OA at 10 µM downregulated expression under basal conditions compared to control. At the same concentration EA behaved significantly different from OA. When used at 50 µM, all the FAs induced a reduction in the mRNA levels of this gene (Figure 5.14(A)). In the pro-inflammatory state (Figure 5.14(B)), both TFAs and their *cis* isomers behaved similar to control when used at 1 and 10 µM. When used at 50 µM, EA also behaved significantly different from OA.

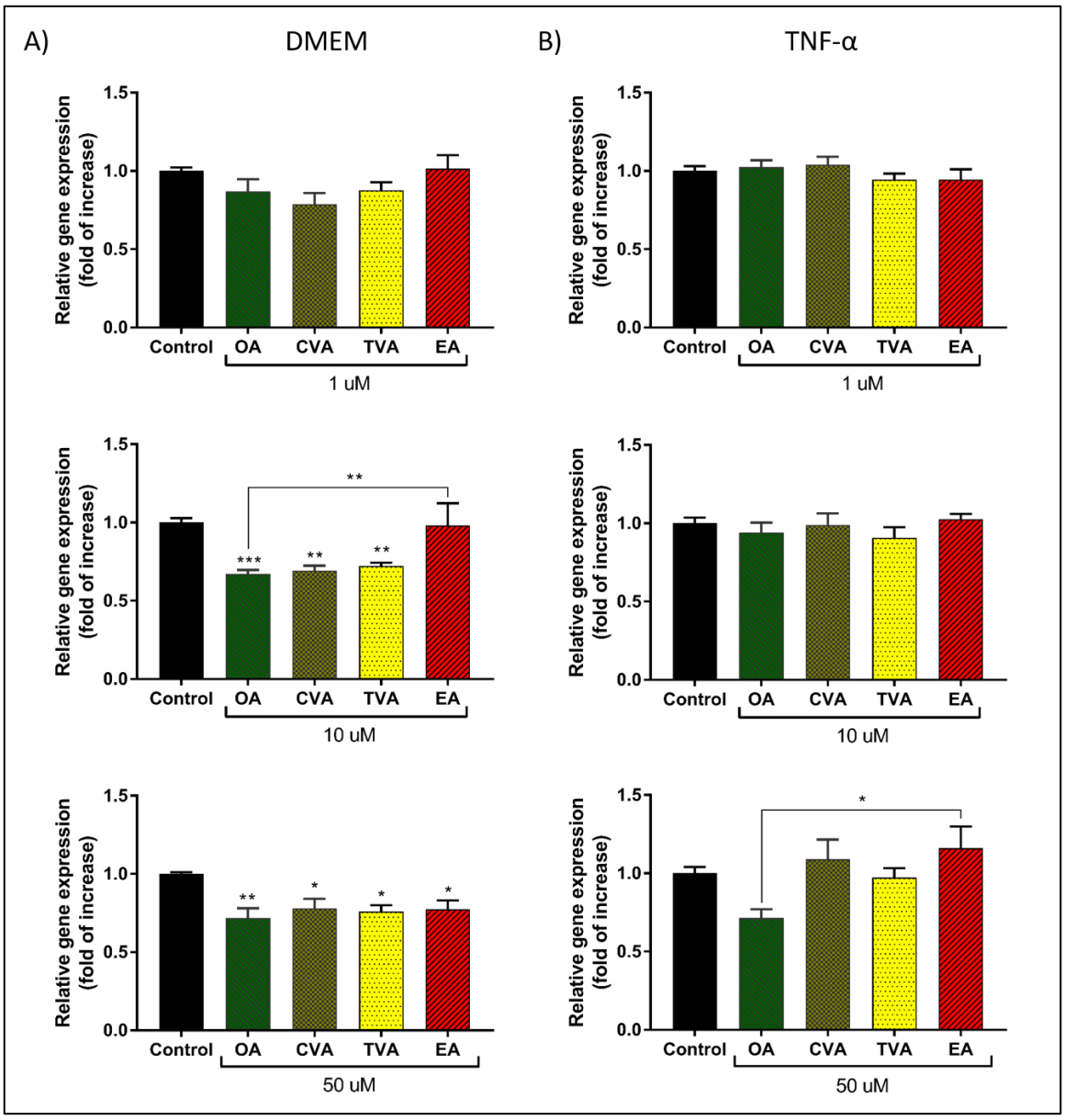



Figure 5.14: Relative gene expression of IL-6 in EA.hy926 cells incubated exposed to FAs. ECs were for 48 h with 1, 10 and 50 µM of FAs in DMEM containing 0.1% of ethanol (Control) followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 6 h. Cq values were normalized by the geometric mean of reference targets YWHAZ and RPL13A genes (A) or RPL13A and CYC1 genes (B). Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; **p<0.01; ***p<0.001. OA = oleic acid, = *cis* vaccenic acid, TVA = *trans* vaccenic acid, EA = elaidic acid.

### 5.4.5 Effect of CLA9,11 and CLA10,12 on gene expression in EA.hy926 cells.

#### 5.4.5.1 NFκB1 relative gene expression

As shown in Figure 5.15(A), both CLAs increased NFκB1 gene expression under basal conditions (10 µM). After TNF-α stimulation, both CLAs at 1 µM induced a significant reduction in the relative expression of NFκB1 gene compared to control, acting differently from LA. When used at 10 µM only CLA9,11 sustained this effect (Figure 5.15(B)).

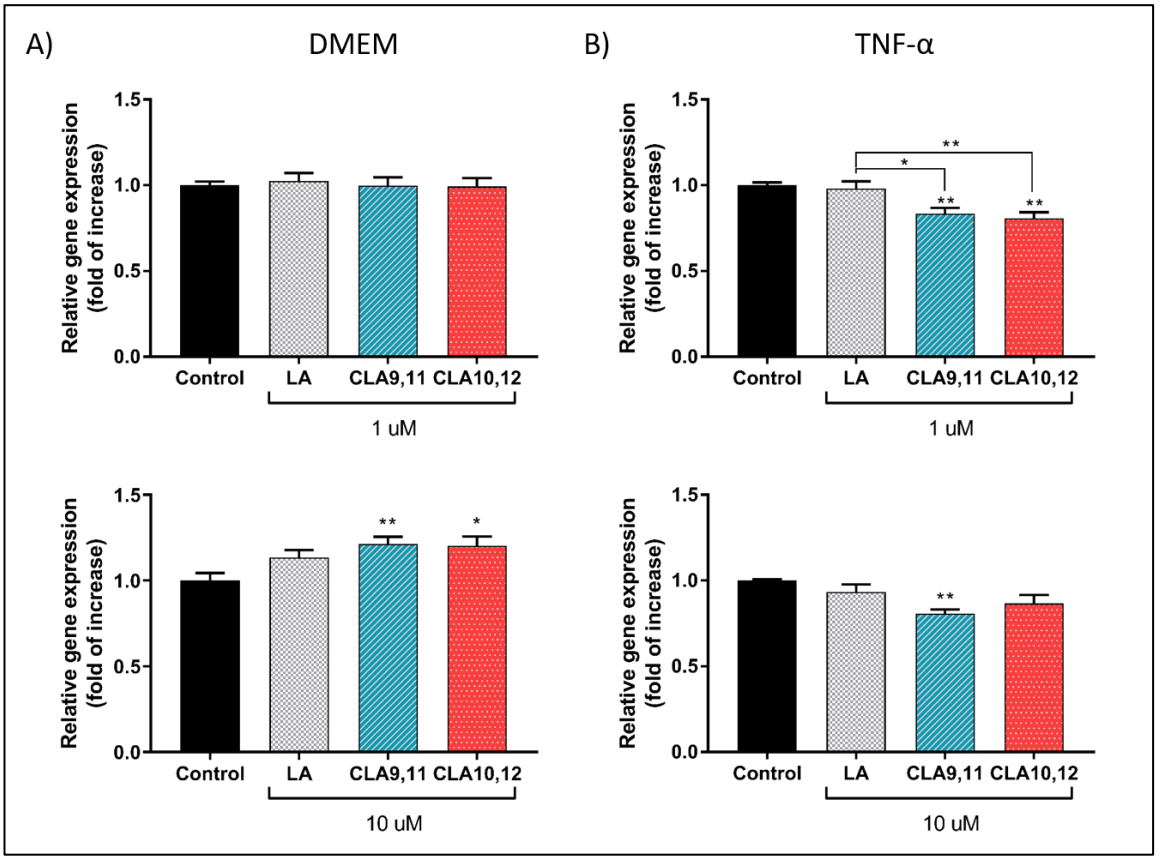



Figure 5.15: Gene expression of NFκB1 in EA.hy926 cells exposed to CLAs.
ECs were incubated for 48 h with 1 and 10 µM of FAs in DMEM containing 0.1% of ethanol (Control) followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 6 h. Cq values were normalized by the geometric mean of reference targets YWHAZ and RPL13A genes (A) or RPL13A and CYC1 genes (B). Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; **p<0.01. LA = linoleic acid, CLA9,11 = conjugated *cis*-9, *trans*-11 linoleic acid, CLA10,12 = conjugated *trans*-10, *cis*-12 linoleic acid.

#### 5.4.5.2 IKBKB relative gene expression

For the gene encoding IKK-β, pre-incubation with both CLAs did not induce significant changes in its expression under basal conditions. Nevertheless, CLA10,12 behaved significantly different than CLA9,11 at 10 µM (Figure 5.16(A)). After TNF-α stimulation

(Figure 5.16(B)), the FAs used behaved in different ways. CLA10,12 increased the mRNA levels of IKK-β when used at 1 µM. Instead, LA had the opposite effect at both concentrations used downregulating the expression of IKK-β gene. CLA9,11 had no effect at either concentration used.

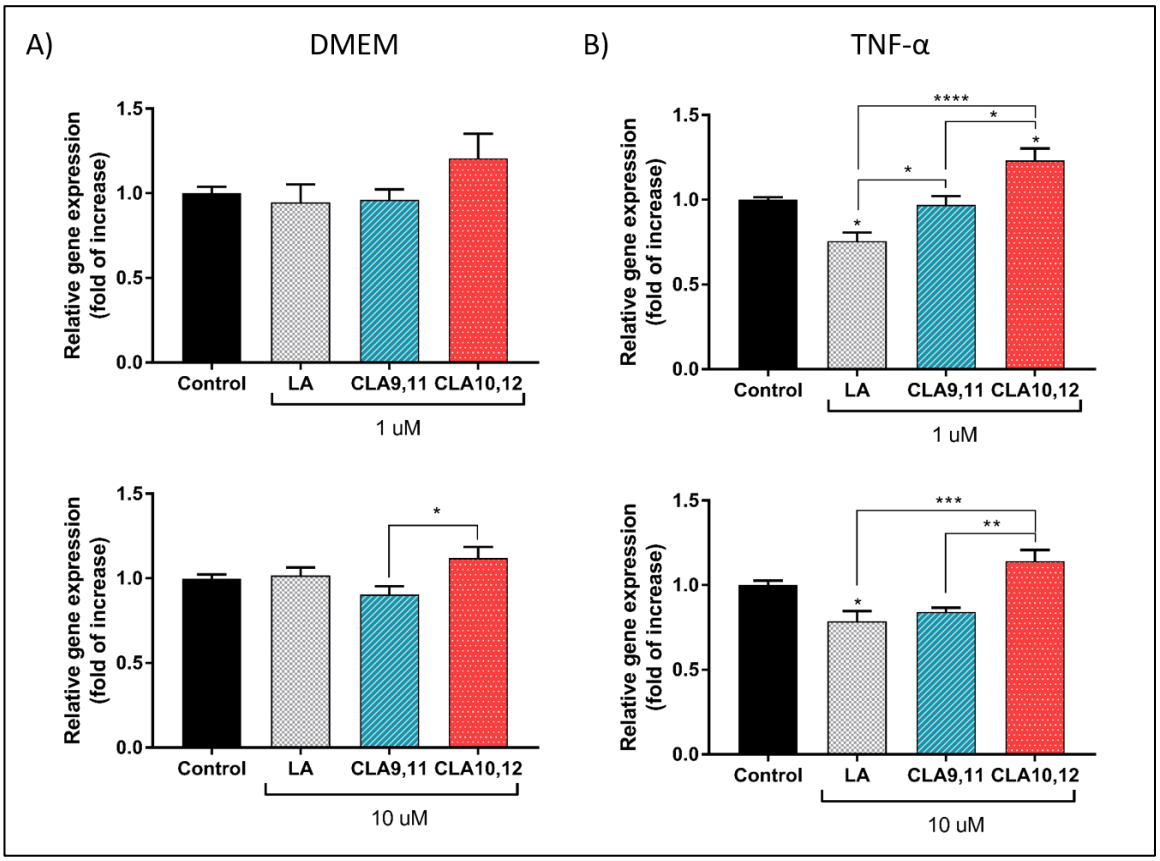



Figure 5.16: Relative gene expression of IKBKB (for IKK-β) in EA.hy926 cells exposed to CLAs. ECs were incubated for 48 h with 1 and 10 µM of FAs in DMEM containing 0.1% of ethanol (Control) followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 6 h. Cq values were normalized by the geometric mean of reference targets YWHAZ and RPL13A genes (A) or RPL13A and CYC1 genes (B). Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. LA = linoleic acid, CLA9,11 = conjugated *cis*-9, *trans*-11 linoleic acid, CLA10,12 = conjugated *trans*-10, *cis*-12 linoleic acid.

#### 5.4.5.3 NFKBIA (IκBα) relative gene expression

As shown in Figure 5.17(A), pre-incubation of ECs with CLA10,12 seemed to upregulate the relative gene expression of IκBα at both concentrations used, behaving significantly different from LA. CLA9,11 and LA did not induce changes in IκBα mRNA levels under basal conditions. After TNF-α stimulation (Figure 5.17(B)) none of the FAs used, at either

concentration, induced significant changes in the relative gene expression of IκBα. However, when used at 1 µM, LA behaved significantly different from CLA10,12.

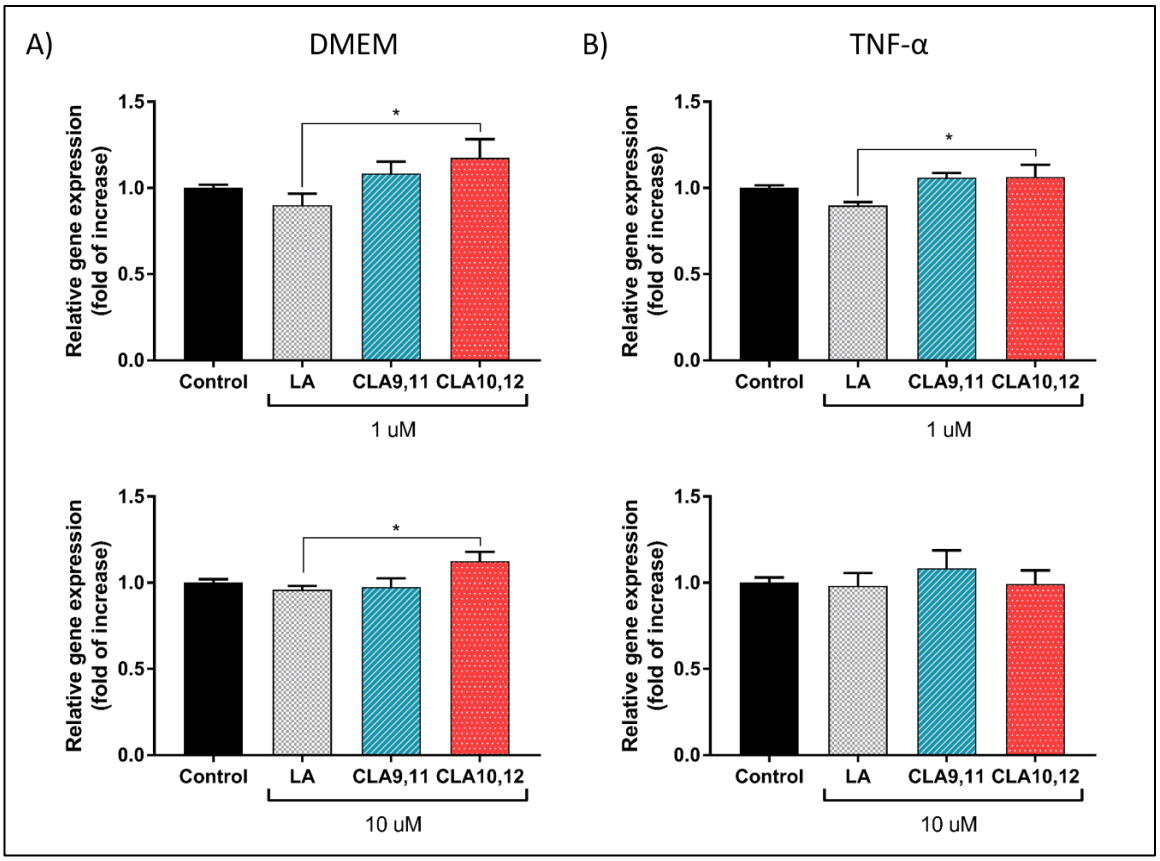



Figure 5.17: Relative gene expression of NFKBIA (for IκBα) in EA.hy926 cells exposed to CLAs. ECs were incubated for 48 h with 1 and 10 µM of FAs in DMEM containing 0.1% of ethanol (Control) followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 6 h. Cq values were normalized by the geometric mean of reference targets YWHAZ and RPL13A genes (A) or RPL13A and CYC1 genes (B). Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05. LA = linoleic acid, CLA9,11 = conjugated *cis*-9, *trans*-11 linoleic acid, CLA10,12 = conjugated *trans*-10, *cis*-12 linoleic acid.

#### 5.4.5.4 PPAR-α relative gene expression

For PPAR-α gene, both CLAs and LA used at 10 µM downregulated its relative gene expression under basal conditions, compared to control (Figure 5.18(A)). After TNF-α stimulation, as shown in Figure 5.18(B), CLA10,12 behaved significantly differently than CLA9,11 and LA only at 10 µM.

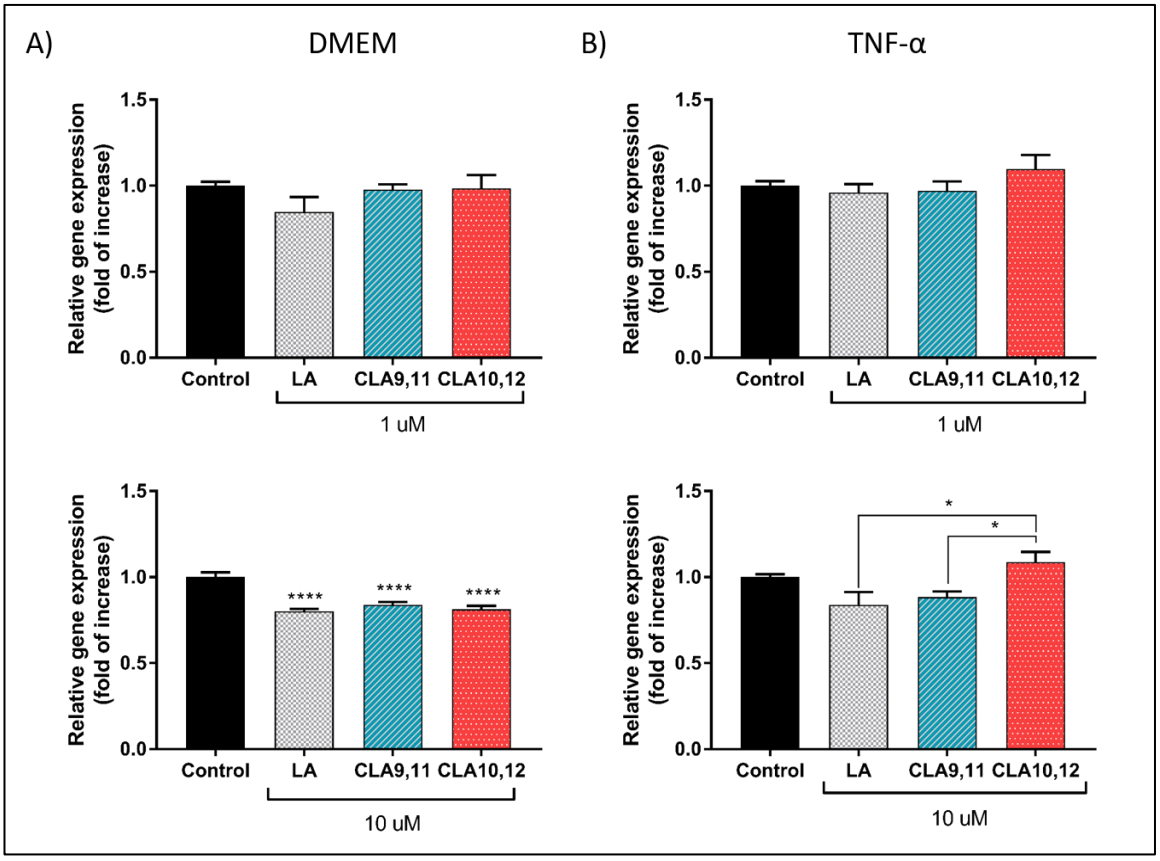



Figure 5.18: Relative gene expression of PPAR-α in EA.hy926 cells exposed to CLAs.
ECs were incubated for 48 h with 1 and 10 µM of FAs in DMEM containing 0.1% of ethanol (Control) followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 6 h. Cq values were normalized by the geometric mean of reference targets YWHAZ and RPL13A genes (A) or RPL13A and CYC1 genes (B). Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; ****p<0.0001. LA = linoleic acid, CLA9,11 = conjugated *cis*-9, *trans*-11 linoleic acid, CLA10,12 = conjugated *trans*-10, *cis*-12 linoleic acid.

#### 5.4.5.5 Toll like receptor 4 (TLR4) relative gene expression

For the gene encoding TLR4, pre-incubation with both CLAs did not induce significant changes in expression under basal conditions (Figure 5.19(A)). This was maintained after TNF-α stimulation (Figure 5.19(B)).

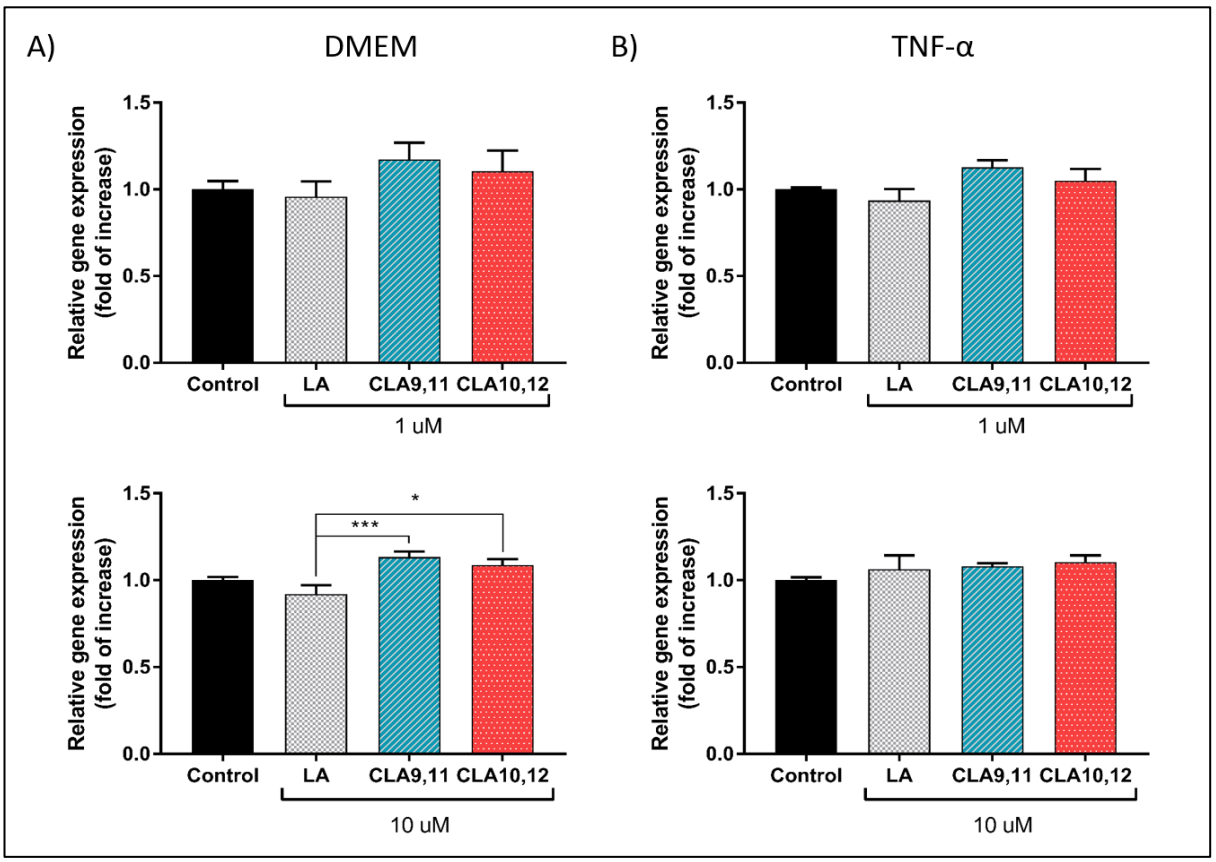



Figure 5.19: Relative gene expression of TLR4 in EA.hy926 cells exposed to CLAs.
ECs were incubated for 48 h with 1 and 10 µM of FAs in DMEM containing 0.1% of ethanol (Control) followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 6 h. Cq values were normalized by the geometric mean of reference targets YWHAZ and RPL13A genes (A) or RPL13A and CYC1 genes (B). Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; ***p<0.001. LA = linoleic acid, CLA9,11 = conjugated *cis*-9, *trans*-11 linoleic acid, CLA10,12 = conjugated *trans*-10, *cis*-12 linoleic acid.

#### 5.4.5.6 Prostaglandin-endoperoxide synthase 2 (PTGS2) relative gene expression

Figure 5.20(A) shows that pre-incubation of ECs with either CLA or LA at 10 µM downregulated the expression of the gene encoding COX-2 under basal conditions, compared to control. This effect was also observed as a trend when the CLAs were used at 1 µM (p=0.052 and 0.08, respectively). In contrast, after TNF-α stimulation (1 ng/mL)

for 24 h, both CLAs, but particularly CLA9,11, seemed to increase the mRNA levels of the gene encoding COX-2, although with high variability (Figure 5.20(B))

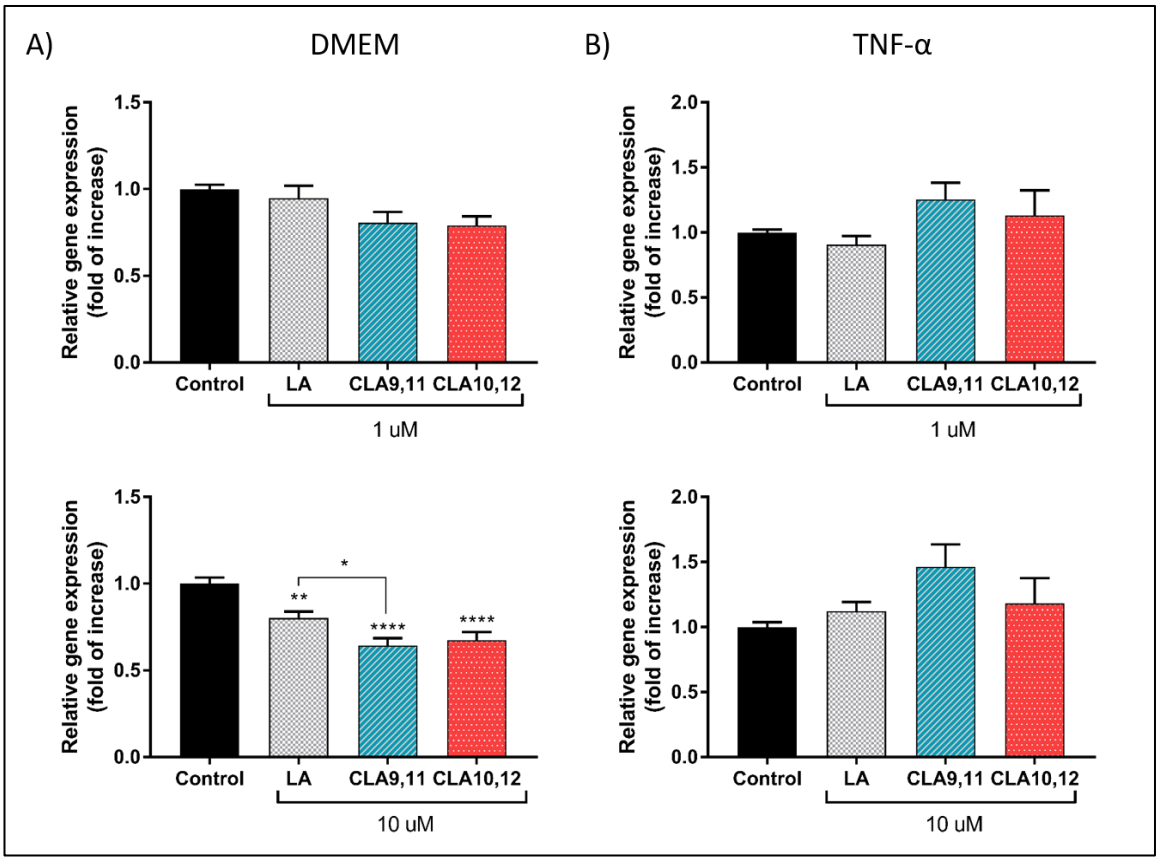



Figure 5.20: Relative gene expression of PTGS2 (for COX-2) in EA.hy926 cells exposed to CLAs. ECs were incubated for 48 h with 1 and 10 µM of FAs in DMEM containing 0.1% of ethanol (Control) followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 6 h. Cq values were normalized by the geometric mean of reference targets YWHAZ and RPL13A genes (A) or RPL13A and CYC1 genes (B). Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; **p<0.01; ****p<0.0001. LA = linoleic acid, CLA9,11 = conjugated *cis*-9, *trans*-11 linoleic acid, CLA10,12 = conjugated *trans*-10, *cis*-12 linoleic acid.

#### 5.4.5.7 Chemokine (C-C motif) ligand 2 (CCL2) relative gene expression

For the gene encoding MCP-1, pre-incubation with either CLA did not induce significant changes in its expression under basal conditions (Figure 5.21(A)). This was maintained after TNF-α stimulation (Figure 5.21(B)).

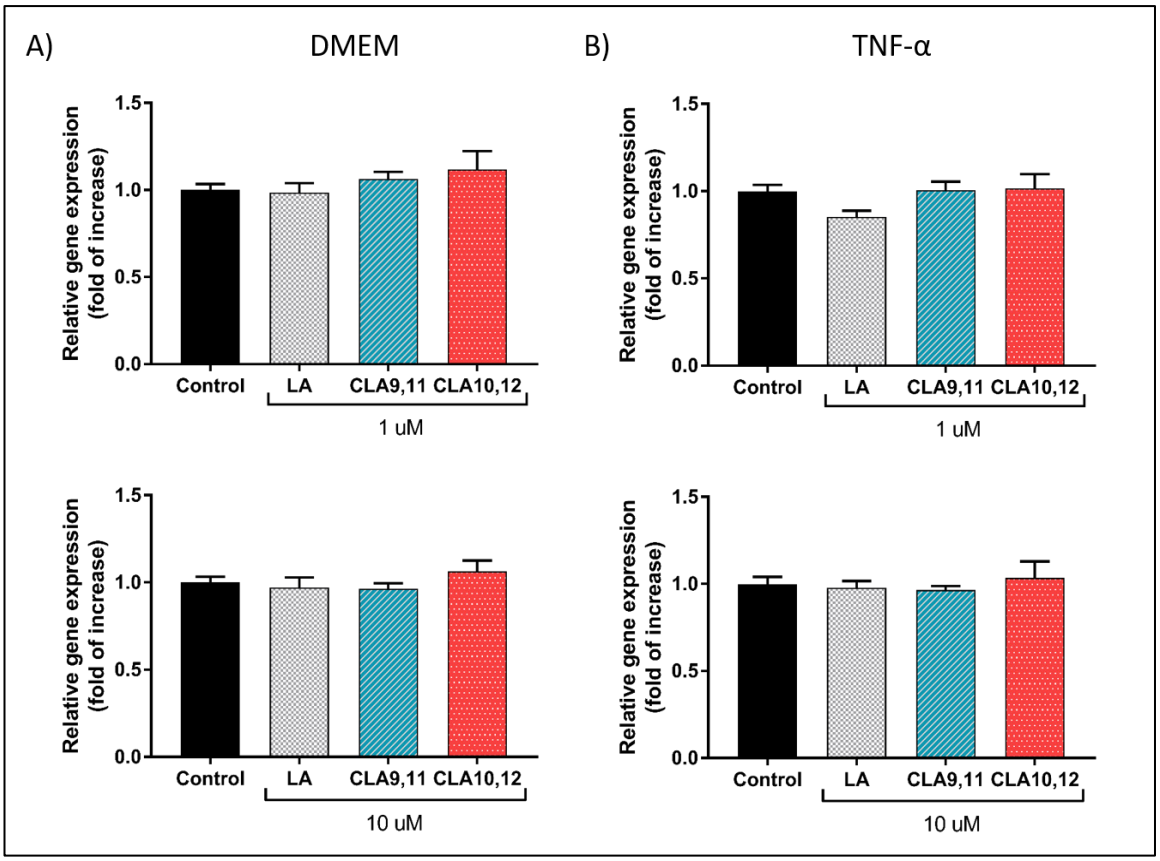



Figure 5.21: Relative gene expression of CCL2 (for MCP-1) in EA.hy926 cells exposed to CLAs. ECs were incubated for 48 h with 1 and 10 µM of FAs in DMEM containing 0.1% of ethanol (Control) followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 6 h. Cq values were normalized by the geometric mean of reference targets YWHAZ and RPL13A genes (A) or RPL13A and CYC1 genes (B). Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. *p<0.05; ***p<0.001. LA = linoleic acid, CLA9,11 = conjugated *cis*-9, *trans*-11 linoleic acid, CLA10,12 = conjugated *trans*-10, *cis*-12 linoleic acid.

#### 5.4.5.8 Interleukin-6 (IL-6) relative gene expression

As shown in Figure 5.22(A), pre-incubation of ECs with either CLA at 10 µM downregulated the expression of the gene encoding IL-6 under basal conditions, acting significantly differently from LA. Instead, after TNF-α stimulation, pre-incubation with the CLAs did not induce changes in IL-6 gene expression (Figure 5.22(B)).

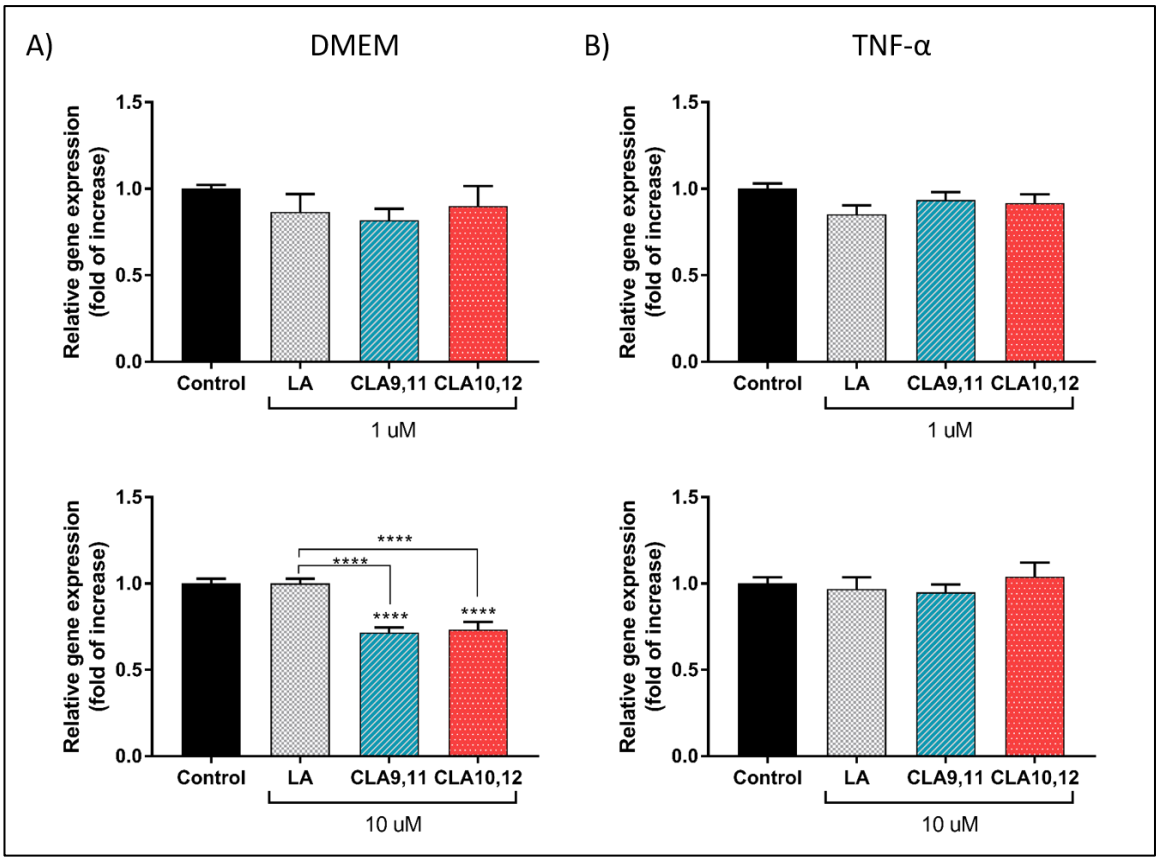



Figure 5.22: Relative gene expression of IL-6 in EA.hy926 cells exposed to CLAs.
ECs were incubated for 48 h with 1 and 10 µM of FAs in DMEM containing 0.1% of ethanol (Control) followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 6 h. Cq values were normalized by the geometric mean of reference targets YWHAZ and RPL13A genes (A) or RPL13A and CYC1 genes (B). Bars are mean ± SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test. ****p<0.0001. LA = linoleic acid, CLA9,11 = conjugated *cis*-9, *trans*-11 linoleic acid, CLA10,12 = conjugated *trans*-10, *cis*-12 linoleic acid.

## 5.5 Discussion

The aims of the research described in this chapter were to determine the effects of various 18 carbon *cis* and *trans* FAs on the expression of genes involved in the inflammatory pathway of EA.hy926 cells, after optimizing the experimental conditions. These aims were met.

The time course experiments allowed identification of an adequate time of stimulation with TNF-α for the different array of genes of interest (i.e. transcription factors, transcription factor modulators, enzymes, cytokines, chemokines). Stimulation with TNF-

α for 6 h produced changes in the expression of 2 of the genes tested initially (Figure 5.1), and this time was selected for use in the later experiments.

RNA analysis through NanoDrop and Bioanalyzer showed high quantities, quality and integrity of the RNA samples generated from cultured ECs (Table 5.1, Figures 5.2, 5.3 and 5.4). The geNorm kit was used to identify the reference genes for the RT-PCR analysis; this is superior to the usual selection of a single gene such as β-actin or GAPDH. For unstimulated cells the identified reference genes were YWHAZ and RPL13A (Figure 5.5), while for the stimulated cells they were RPL13A and CYC1 (Figure 5.6). This approach complies with the MIQE guidelines (Bustin et al, 2009), allowing reliable and robust analysis of relative gene expression data within the experimental conditions used. An optimal normalization factor was calculated as the geometric mean of these reference genes in order to quantify the relative gene expression of the genes of interest (NFκB, IKKβ, Iκβα, PPAR-α, PPAR-γ, TLR4, COX-2, MCP-1 and IL- 6).

### 5.5.1 Comparing the effect of 18 carbon *trans* isomers from industrial and ruminant origin

Activation of NFκB is a critical process for the regulation of a variety of genes participating in the inflammatory response (see Figure 1.5, Chapter 1), including those encoding for inflammatory cytokines, COX-2 and adhesion molecules (de Winther et al, 2005; Lu et al, 2012). Therefore, a reduced expression of the NFκB gene could be considered anti-inflammatory.

EA and its *cis* isomer OA did not show any significant effect on the relative gene expression of NFκB1, either in basal conditions or after TNF-α stimulation (Figures 5.7 and 5.8). This effect is consistent with the almost null changes induced by EA on the expression of both IKKβ and IκBα (Figures 5.9 to 5.12). Kinase IKKβ function is to phosphorylate the inhibitory subunit of NFκB, IκBα, causing NFκB activation and translocation to the nucleus. Consequently, increased expression of the gene encoding IKKβ, leading to higher levels of its protein, could cause increased NFκB activity. In contrast, increased expression of the gene encoding IκBα may be anti-inflammatory, given that an increase in this protein would lead to higher levels of the inactive trimeric form of NFκB.

On the other hand, in a pro-inflammatory state, pre-incubation with the lowest concentration (1 μM) of TVA seemed to slightly decrease the expression of NFκB1 (Figure 5.8), while a trend to do the same was observed at 10 μM. This might suggest an anti-inflammatory action of TVA. In basal conditions, TVA had no apparent effect on NFκB1 gene expression (Figure 5.7). TVA, and also CVA, did not induce any significant changes in IKKβ or Iκβα mRNA appearance (Figures 5.9 to 5.12).

To some extent concordant with the results shown here, studies using TVA in HUVECs or human microvascular endothelial cells (HMECs) found no or only mild effects on inflammatory gene expression (Iwata et al. 2011, Livingstone et al. 2014, Da Silva et al. 2017), although these studies reported genes other than the ones investigated here. Iwata et al. (2011) reported on TNF, ICAM-1 and iNOS gene expression, showing no effects when HMECs were exposed to TVA and EA at increasing concentrations (25 to 100 μM) for 3 h. Livingstone et al. (2014) used HAECs from healthy and diabetic donors and showed no effect of TVA (20 μM) on ICAM-1, insulin receptor, VCAM, eNOS or E-selectin gene expression. However, Da Silva et al. (2017) showed that when cells are exposed to a pro-inflammatory stimulus, TVA and EA induced downregulation of TNF, VCAM-1 and SOD2 genes in HUVECs, although only TVA downregulated TNF and IL-8 genes in HepG2 cells.

In HAECs it has been described that EA increases the levels of activated NFκB protein, measured by western blot and flow cytometry, while OA did not appear to alter the phosphorylation status of this transcription factor (Bryk et al, 2011; Harvey et al, 2012). Others have reported that EA, at a concentration of 100 μM, increased nuclear translocation of NFκB p65 in HUVECs (Pan et al, 2017). It would be interesting to test in this model if the TFAs of interest have a differential effect on NFκB phosphorylation or nuclear translocation.

PPARs play multiple regulatory functions, including the control of macrophage activity and inflammation (Crisafulli & Cuzzocrea, 2009) and several FAs exert their anti-inflammatory effects, at least partly, through their agonism of PPARs (Deckelbaum et al, 2006). When activated, PPAR-α binds to response elements on DNA, increasing the transcription of anti-inflammatory proteins, such as IκBα (Delerive et al, 2000), interfering with the nuclear translocation of NFκB. Therefore, PPARα negatively regulates gene

expression of pro-inflammatory proteins by antagonizing the activities of other transcription factors, including NFκB (Pontis et al, 2016).

The FAs used in this study did not show major effects in terms of PPAR-α gene expression. While all the FAs induced a reduced PPAR-α mRNA expression when used at 10 μM in unstimulated ECs, which could be regarded as pro-inflammatory, no changes were induced at the other concentrations or when a pro-inflammatory state was achieved with TNF-α stimulation (Figures 5.12 and 5.13).

TLRs act as sensors to detect a wide variety of microbial components and produce innate immune responses including inflammation. Toll-like receptor-4 signalling pathway stimulation culminates with the activation of NFκB, which controls the expression of inflammatory cytokines genes. Therefore, the upregulation of TLR4 gene expression could be considered pro-inflammatory.

Under basal conditions, EA and TV did not induce any significant changes in the gene expression of TLR4, although their *cis* isomers did (Figure 5.15). CVA reduced TLR4 mRNA expression when used at 10 and 50 μM, while OA did the same at 50 μM. After TNF-α stimulation, EA induced a significant upregulation of TLR4 gene expression (Figure 5.16), which could be considered as pro-inflammatory.

In agreement with the observations made here, Pan et al. (2017) proposed that TFAs induced inflammation in HUVECs through their effects on TLR4 protein expression within lipid rafts. Another study reported that EA induced the phosphorylation of TLR-mediated signals in the mouse aorta and in HUVECs, and TLR inhibition attenuated the TFA-induced pro-thrombogenic phenotypes of ECs implying that EA impairs endothelial anti-thrombogenic functions through a TLR-mediated pathway and increases the thrombogenic tendency in mice (Kondo et al, 2015). The upregulation of TLR4 gene expression caused by EA shown here may result in enhanced TLR4 activity. Nevertheless, given that TLR4 protein expression was not evaluated, whether EA might act as a ligand of TLR4 or if EA altered cell membrane components (like lipid rafts) that could modulate its expression or activity remains unclear.

Cyclooxygenase-2 (COX-2) is the enzyme responsible for catalysing the rate-limiting step of prostaglandin (PG) synthesis from FAs. The type of FA substrates available in the cellular membrane influences the profile of the PGs synthesized (Calder, 2011). Usually

the major substrate is arachidonic acid which gives rise to the mainly pro-inflammatory 2-series PGs.

Under basal conditions, all of the FAs tended to reduce COX-2 mRNA expression when used at 50 µM. Only TVA induced a significant decrease in the expression of COX-2 when used at 10 µM, while EA tended to do the opposite when used at 1 µM (Figures 5.17). Instead, after TNF-α stimulation, EA and CVA induced a significant increase in the expression of COX-2 gene when used at 50 µM (Figures 5.18). In agreement with these results, a study showed that peritoneal macrophages from Wistar rats fed for two months with a diet supplemented with partially hydrogenated vegetable fat, and therefore high in EA, showed an upregulation of COX-2 and NFκB p65 proteins together with downregulation of PPAR-γ, measured by western blot (Rao & Lokesh, 2017). In contrast, another study reported that TVA and EA (50 µM) were able to reduce PG secretion in HUVECs after stimulation with TNF-α (24 h, 2 ng/mL), which could be caused by a reduction in COX-2 activity (Da Silva et al, 2017a).

The expression of COX-2 is regulated by numerous transcription factors including NFκB, the cyclic-AMP response element binding protein (CREB) and the CCAAT-enhancer binding protein (C/EBP). COX-2 expression can also be regulated post-transcriptionally, at the level of mRNA stability (Tsatsanis et al, 2006). The current study did not observe a positive relationship between the effects of the different FAs on the expression of NFκB and PTGS2 genes.

MCP-1 is one of the key chemokines that regulate migration and infiltration of monocytes/macrophages into the subendothelial space, being considered an early indicator of endothelial dysfunction (Martynowicz et al, 2014). On the other hand, IL-6 has different roles in activation of ECs, promotion of lymphocyte proliferation and differentiation, coordinating the influx of inflammatory cells into the atherosclerotic lesion (Hartman & Frishman, 2014).

EA at the lowest concentration used induced a significant increase in the expression of MCP-1 and OA had the opposite effect when used at 50 µM under basal conditions (Figure 5.19). In the induced pro-inflammatory state, EA, OA and CV tended to increase the mRNA of the gene encoding MCP-1 when used at 50 µM (Figure 5.20). In the case of IL-6 gene expression, all the FAs induced its downregulation at 50 µM under basal

conditions, which was also observed for TV, CV and OA at 10 µM (Figure 5.21). No significant changes in IL-6 gene expression were observed after TNF-α stimulation (Figure 5.22), although EA tended to produce upregulation.

In a similar model, of human microvascular endothelial cells, EA in increasing concentrations (25 to 100 µM) was able to increase the levels of IL-6 and NFκB activation measured by phosphorylation of IκBα , and reduced endothelial insulin signalling and NO production, whereas TVA was not associated with any of these responses (Iwata et al, 2011). Although these results are not concordant, in that study IL-6 and phosphorylated Iκβα were measured through ELISA and gene expression by real time-PCR was not assayed. A study in 3T3-L1 adipocytes and THP-1 macrophages showed that 24 h incubation with OA and EA (500 µM) had no detectable effect on MCP-1 expression and secretion (Cullberg et al, 2014). However the high concentration of FAs used suggests that cell viability may have been affected although this was not tested.

There is no previous research on the effects of these TFAs of different origin on the expression of the genes of interest in ECs.

### 5.5.2 Comparing the effect of two CLA isomers of ruminant origin and LA on the inflammatory gene expression pathway

Both CLAs were shown to induce downregulation of NFκB1 gene expression after TNF-α stimulation (Figure 5.24). Paradoxically, at 1 µM CLA10,12 also induced increased expression of the gene encoding IKKβ (Figure 5.26), which may be considered as contradictory effects.

A study in HUVECs reported that a mix (50% each) of the same CLAs used in this project reduced NFκB activity by 25-30%, measured by transfection with a NFκB-luciferase reporter gene (Goua et al, 2008). In contrast, CLA10,12 was shown to increase the nuclear localization of p65 subunit of NFκB in 3T3-L1 adipocytes (Poirier et al, 2006). In the case of CLA9,11, a study in dendritic cells showed that this FA (50 µM, 48 h) decreased NFκB activity in basal conditions and also after LPS stimulation by preventing the degradation of Iκβα (Loscher et al, 2005). This evidence and the new results shown here suggest that the effects of CLAs on NFκB gene expression or activity might be cell type and isomer specific.

At 10 µM, the CLAs and LA induced the downregulation of PPAR-α gene expression, but only under basal conditions (Figure 5.29), while in the pro-inflammatory state CLA10,12 tended to cause upregulation (Figure 5.30).

There is no previous research on the effects of CLAs on PPAR-α gene expression in ECs. Some studies in mice and *in vitro* have reported effects of CLAs on PPAR-γ. For example, a study in mice reported that CLA10,12 supplementation induced a reduction in PPAR-γ gene expression in white adipose tissue, which was associated with reduced adipose tissue mass, increased expression of pro-inflammatory genes (TNF-α, MCP-1, IL-6) and insulin resistance (Poirier et al, 2006). This effect on PPAR-γ gene expression was also observed in 3T3-L1 adipocytes in the same study. Other authors proposed that supplementation with a mixture of CLAs ameliorated colitis through a PPAR-γ-dependent mechanism in a mice model (Bassaganya-Riera et al, 2004). In the current study PPARγ gene expression was not detectable.

The CLA isomers and LA did not induce significant changes in the expression of TLR4 (Figure 5.31 and 5.32). Also in the case of TLR4, there is not previous research relating its actions or expression in ECs with CLA exposure.

The CLAs and LA induced the downregulation of COX-2 gene expression, but only under basal conditions (Figure 5.33), with a trend to increase it after TNF-α stimulation for the CLAs (Figure 5.34).

In contrast to these results, a study performed in ECs isolated from porcine pulmonary arteries showed that pre-incubation for 24 h with linoleic acid (20 µM) amplified the TNF-α mediated endothelial inflammatory response, inducing oxidative stress, p38 MAPK, NFκB, COX-2, and $PGE_2$ (Wang et al, 2008a). Others have reported that the exposure of mouse macrophages to various CLA isomers reduced the COX-2 mRNA expression, COX-2 promoter activity and the production of $PGE_2$ in response to IFN-γ (Yu et al, 2002), although the study used CLA concentrations (100 and 200 µM) that according to results reported previously (Chapter 3) are cytotoxic, at least in the EC model used here.

The CLAs and LA did not induce any change in the expression of the MCP-1 encoding gene (Figure 5.35 and 5.36), which was similar to the results for the expression of IL-6 after TNF-α stimulation (Figure 5.38). Instead, both CLAs at 10 µM induced downregulation of IL-6 gene expression under basal conditions, which could be considered anti-

inflammatory if it is translated into lower cytokine levels. This was the case for CLA9,11, as shown in chapter 4.

Studies using CLAs in animal models show inconsistent results. Poirier et al. (2006) reported that the administration of CLA10,12 by gavage at a dose of 20 mg/day for 7 days in mice led to the upregulation of TNF-α, MCP-1 and IL-6 gene expression in white adipose tissue without affecting their serum levels (Poirier et al, 2006). Similarly, another study, using enriched diets with 0.06%, 0.2%, and 0.6% (w/w) of mixed CLA10,12 with linoleic acid (50/50), mixed CLA10,12 with CLA9,11 (50/50) or linoleic acid alone as control diet in young male mice for 6 weeks, showed that the intermediate and higher intakes of CLA10,12 reduced adiposity, increased serum levels of MCP-1 and IL-6 and enhanced liver steatosis (Shen et al, 2013).

On the other hand, some *in vitro* studies have reported anti-inflammatory effects of CLAs in HUVECs, but these effects were related to lower adhesion of THP-1 cells and isolated human PBMCs dose-dependently in HUVECs treated with TNF-α by CLA10,12 (Sneddon et al, 2006), or reduced ICAM-1 and VCAM-1 expression in HUVECs exposed to CLA10,12 and a CLA mix (Goua et al, 2008).

### 5.5.3 Conclusions

Overall, differential effects of the TFAs used were observed on the expression of genes involved in the inflammatory pathway of EA.hy926 cells.

Table 5.2: Summary of effects of FA treatment on the expression of genes involved in the inflammatory pathway of EA.hy926 cells.

| | **Fatty acid (unstimulated cells)** | | | | | | |
|---|---|---|---|---|---|---|---|
| **Gene** | OA | CVA | TVA | EA | LA | CLA9,11 | CLA10,12 |
| NFκB1 | ↔ | ↔ | ↔ | ↔ | ↔ | ↔/↑ | ↔/↑ |
| IKBKB (for IKKB) | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ |
| NFKBIA (for IκBα) | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ |
| PPARα | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ |
| TLR4 | ↓ | ↓ | ↔ | ↔ | ↔ | ↔ | ↔ |
| PTGS2 (for COX-2) | ↔ | ↔ | ↓ | ↓ | ↓ | ↓ | ↓ |
| CCL2 (for MCP-1) | ↓ | ↔ | ↔ | ↑ | ↔ | ↔ | ↔ |
| IL-6 | ↓ | ↓ | ↓ | ↓ | ↔ | ↓ | ↓ |
| **Gene** | **Fatty acid (TNF-α stimulated cells)** | | | | | | |
| NFκB1 | ↔ | ↔ | ↓ | ↔ | ↔ | ↓ | ↓ |
| IKBKB (for IKKB) | ↔ | ↔ | ↔ | ↔ | ↓ | ↔ | ↑ |
| NFKBIA (for IκBα) | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ |
| PPARα | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ |
| TLR4 | ↔ | ↔ | ↔ | ↑ | ↔ | ↔ | ↔ |
| PTGS2 (for COX-2) | ↔ | ↑ | ↔ | ↑ | ↔ | ↔ | ↔ |
| CCL2 (for MCP-1) | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ |
| IL-6 | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ |

The exposure of EA.hy926 cells to EA increased the mRNA levels of some of the inflammatory pathway genes measured after TNF-α stimulation, having neutral or lowering gene expression effects under basal conditions, which is consistent with the results shown in chapter 4. EA decreased gene expression of COX-2 and IL-6 under basal conditions, although it upregulated MCP-1. In the induced pro-inflammatory state, EA upregulated TLR4 and COX-2, tending to do the same for MCP-1 and IL-6 gene expression. In contrast, TVA had very few effects on the inflammatory pathway gene expression, decreasing gene expression of COX-2 and IL-6 at basal conditions and doing the same after TNF-α stimulation for NFκB1 mRNA expression. The results suggest that TFAs from different sources do behave differently in terms of their effect on the gene expression of the inflammatory pathway in this EC experimental model, especially after TNF-α stimulation.

For the CLA isomers, their effects were similar. Both of them upregulated NFκB1 and caused downregulation of COX-2 and IL-6 gene expression under basal conditions, and lowered gene expression of NFκB1 after TNF-α stimulation. The effects of both CLA isomers on gene expression can be regarded as anti-inflammatory, which differs from the findings shown in chapter 4 where CLA10,12 generated an increase of pro-inflammatory cytokines levels after TNF-α stimulation. These findings suggest a complex modulatory role of the CLA10,12 isomer on inflammation.

# Chapter 6 Effect of 18-carbon *trans* fatty acids on binding of monocytes to cultured EA.hy926 cells

## 6.1 Introduction

Previously it was shown that TVA has neutral or anti-inflammatory effects on ECs under basal conditions and after inflammatory stimulation (Chapter 4 and 5), and that CLA9,11 also has some anti-inflammatory actions in basal conditions. In contrast, EA and CLA10,12 showed pro-inflammatory effects in the EC model used, particularly after TNF-α stimulation. These effects were identified by measuring inflammatory cytokines and chemokines being secreted from ECs to the cell culture media (Chapter 4) and through differences in expression of genes involved in the inflammatory pathway (Chapter 5). These effects could affect the binding of monocytes to the ECs, which is considered a key early step in the progression of atherosclerosis (section 1.2.2).

The activation of the NFκB signalling pathway leads to EC activation, which initially comprises the stimulation of the expression and subsequent production of chemoattractants and cell-surface adhesion molecules, such as VCAM-1, ICAM-1, P-selectin and E-selectin, which mediate initial capture, tethering and rolling along the endothelium of circulating leukocytes, including monocytes. The activation of ECs can be induced by numerous mediators, including pro-inflammatory cytokines, such as TNF-α and IL-6, as well as by oxidative stress, hypercholesterolemia, and hypertension among others (see section 1.2.1, chapter 1).

The levels of sICAM-1 in the supernatant of ECs were shown to be reduced by TVA and increased by CLA10,12 when used at 1 μM (Chapter 4). It has been reported that sICAM-1 reflects ICAM-1 expression on cells (Leeuwenberg et al, 1992), which is constitutively expressed or inducible on the cell surface of different cell lines such as ECs (Fonsatti et al, 1997; Myers et al, 1992).

The effects of the TFAs of interest on the adhesion of monocytes to ECs have not been explored extensively. There are only a few studies reporting effects of CLAs and EA on monocyte adhesion, using different *in vitro* models and often high FAs concentrations (Bryk et al, 2011; Sneddon et al, 2006; Stachowska et al, 2012).To my knowledge, the effects of TVA on monocyte adhesion has not been reported.

The study by Bryk et al. (2011) reported that iTFAs such as EA or linoelaidic acid can induce an increased adhesion of monocytes and lymphocytes to HAECs together with a

dose-dependent increase of cell surface expression of ICAM-1 and VCAM-1 (Bryk et al, 2011). The study by Stachowska et al. (2012) showed that CLAs induced only a trend to reduce macrophage adhesion to HUVECs even though CLA9,11 decreased ICAM-1 cell surface expression by 52% in comparison to control cells (Stachowska et al, 2012).

In order to identify if the effects of 18-carbon TFAs on the levels of pro-inflammatory mediators in the ECs supernatant and on the inflammatory pathway gene expression have a functional effect on the activation of ECs and subsequent monocytes adhesion, the monocytic THP-1 cell line was used to perform static adhesion assays with EA.hy926 cells pre-incubated with the FAs of interest. Additionally, EC surface expression of ICAM-1 was measured using anti-human CD54 antibodies and flow cytometry technique.

## 6.2 Objective and aims

The objectives of the research described in this chapter were to compare the effects of the 18-carbon *cis* and *trans* FAs of interest on the adhesion of monocytes to cultured EA.hy926 cells and on the endothelial cell surface expression of ICAM-1.

The specific aims were to:

- Measure the effects of different *cis* and *trans* 18-carbon FAs on the adhesion of monocytes to cultured ECs with and without TNF-α stimulation.
- Measure the effects of different *cis* and *trans* 18-carbon FAs on the expression of ICAM-1 on the surface of cultured ECs.
- Compare the effects of 18 carbon *trans* isomers from industrial and ruminant origin on the adhesion of monocytes to cultured ECs and on the expression of ICAM-1 on the surface cultured ECs.
- Compare the effects of two conjugated linoleic acid isomers of ruminant origin and linoleic acid on the adhesion of monocytes to cultured ECs and on the expression of ICAM-1 on the surface of cultured ECs.

## 6.3 Methods

### 6.3.1 Reagents

The reagents and materials used for these experiments are described in Appendix A.

### 6.3.2 Adhesion assay procedures

For these experiments, confluent EA.hy926 cell cultures were scraped from flasks and resuspended at a density of 2 x $10^5$ cells per mL. Cells were seeded at 1 x $10^4$ cells per well in 96-well flat bottom plates, incubated for 48 h at 37$^o$C with various FAs at a concentration of 1, 10 and 50 µM followed by 6 h incubation with DMEM or TNF-α at 1 ng/mL. Plates with ECs were washed twice with RPMI without supplements before adding the THP-1 cells.

The monocyte THP-1 cell line (ECACC - 88081201) was obtained from ECACC. The cells were thawed and subcultured following the supplier's instructions. Cells were cultured at 5-7 x $10^5$ cells/mL in culture medium which consisted of Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS, L-glutamine (0.4 mM), penicillin, streptomycin and HAT (100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine). Cells were maintained at 37$^o$C in 5% $CO_2$. Prior to use in experiments cells were grown in T-75 flasks until confluent, using them for experiments between passages 5 and 15.

Adhesion of THP-1 monocytes to EA.hy926 cells was determined using the Vybrant™ Cell Adhesion Assay Kit (Thermo Fisher) as described in Chapter 2.

### 6.3.3 Flow cytometry procedures

For these experiments, confluent EA.hy926 cell cultures were scraped from T-175 flasks and resuspended in DMEM at a density of 5.5 x $10^5$ cells per mL. Cells were seeded at that density per well in 6-well plates, incubated for 48 h at 37$^o$C with various TFAs at a concentration of 1, 10 and 50 µM followed by 6 h incubation with TNF-α at 1 ng/mL.

To determine the cell surface expression of ICAM-1, (PE-Cy$^{TM}$ 5)-conjugated anti-human CD54 (BD Biosciences) was used as described in Chapter 2. Flow cytometry was conducted as described in Chapter2.

### 6.3.4 Statistical analysis

THP-1 monocyte adhesion to EA.hy926 cells pre-incubated with FAs is expressed as a % of control, control being non-FA treated.

Cell surface ICAM-1 expression data are expressed as Δ change (subtraction of the % of gated CD54 positive cells of the EA.hy926 cells incubated with FA to the % of gated CD54 positive cells of the EA.hy926 cells incubated with DMEM containing 0.1% of ethanol (Control)), % of control of gated CD54 positive cells and % of control of median fluorescence intensity (MFI), which relates to the levels of ICAM-1 expression per cell.

Data are expressed as mean ± SEM; data analysis was performed in PRISM and Excel. Multiple group differences were compared using One-way ANOVA, and, where the ANOVA was significant, Tukey's as post hoc test was used.

## 6.4 Results

### 6.4.1 Effect of 18-carbon TFAs on the adhesion of monocytes to cultured ECs

To investigate the effect of different *cis* and *trans* 18-carbon FAs on the adhesion of monocytes to the endothelial monolayer, EA.hy926 cells were exposed to FAs at concentrations of 1, 10 and 50 μM for 48 h followed by 6 h incubation with DMEM or TNF-α stimulation at 1 ng/mL. Monolayers were then co-incubated with calcein labelled THP-1 cells for 1 h, which was determined by previous work in our research group as being optimal for binding to ECs that had been subject to TNF-α stimulation at 1 ng/mL.

#### 6.4.1.1 Effect of 18-carbon *trans* isomers from industrial and ruminant sources on the adhesion of monocytes to cultured ECs

Under basal conditions, pre-incubation of EA.hy926 cells with TVA at 1 µM and OA at 50 µM induced decreased adhesion of THP-1 monocytes (Figure 6.1(A)). In contrast, EA used at 10 µM tended to increase monocyte adhesion, which became a statistically significant effect at 50 µM, with EA acting differently from TVA and OA at both higher concentrations used (Figure 6.1(A)).

After TNF-α stimulation, pre-incubation of EA.hy926 cells with TVA, OA and CVA at 1 µM decreased adhesion of THP-1 monocytes. When the FAs were used at 10 µM, none of them induced changes in monocyte adhesion compared to control. Once again EA at 50 µM increased monocyte adhesion (Figure 6.1(B)).

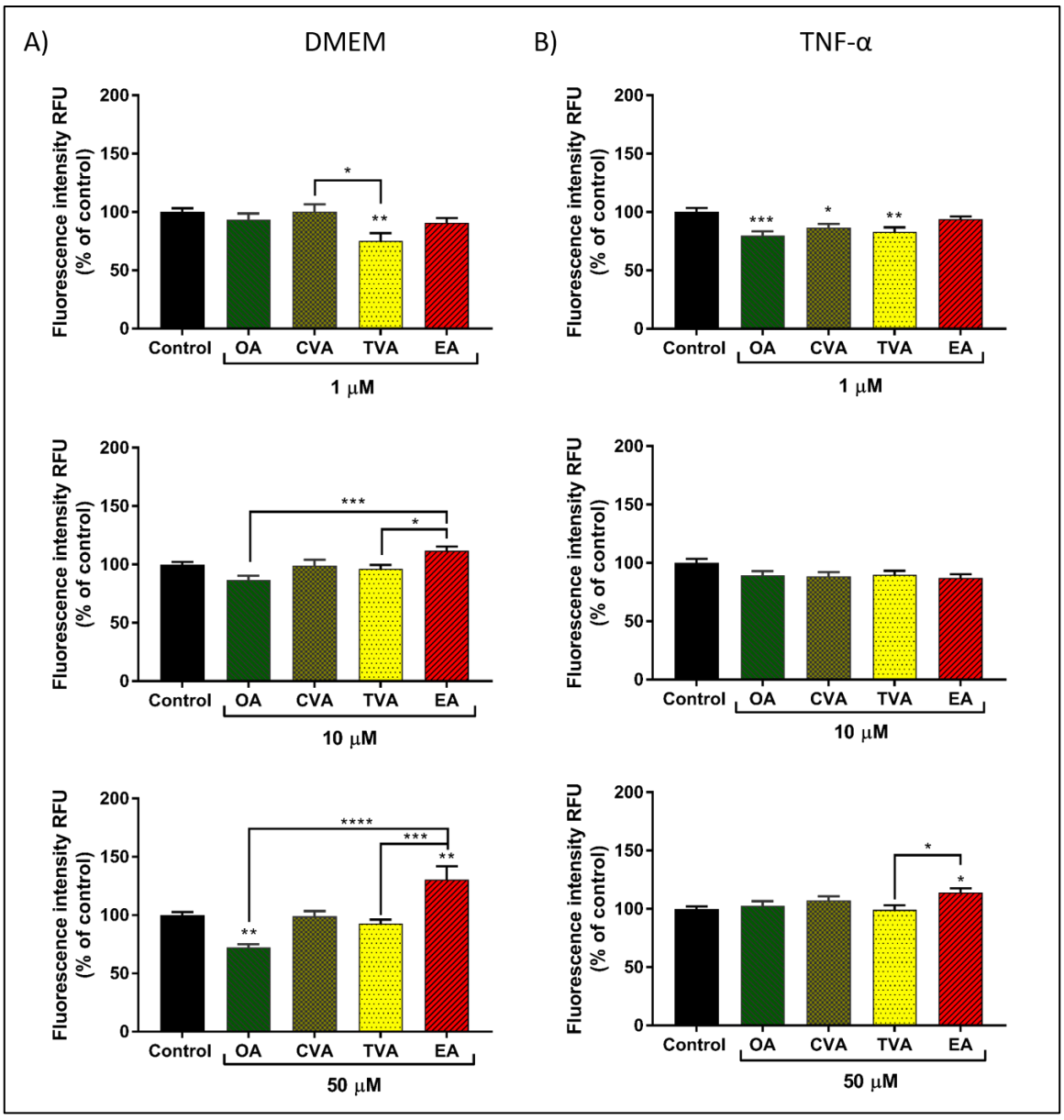



Figure 6.1: Adhesion of THP-1 cells (% of control) to EA.hy926 cells exposed to FAs. ECs were incubated for 48 h with DMEM containing 0.1% of ethanol (Control) or several concentrations (1 µM; 10 µM; 50 µM) of FAs, followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 24 h and 1 h co-incubation with THP-1 cells. Bars are mean ±SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. OA = oleic acid, CVA = *cis* vaccenic acid, TVA = *trans* vaccenic acid, EA = elaidic acid.

Images taken under the fluorescence microscope agree with these quantitative results, showing a higher number of THP-1 monocytes (green spots) when ECs were pre-incubated with EA both with and without TNF-α stimulation (Figures 6.2 and 6.3).

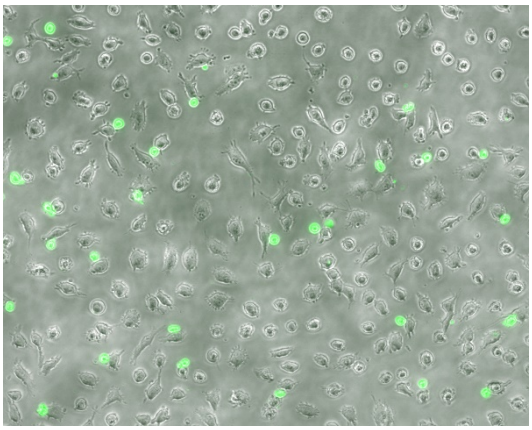
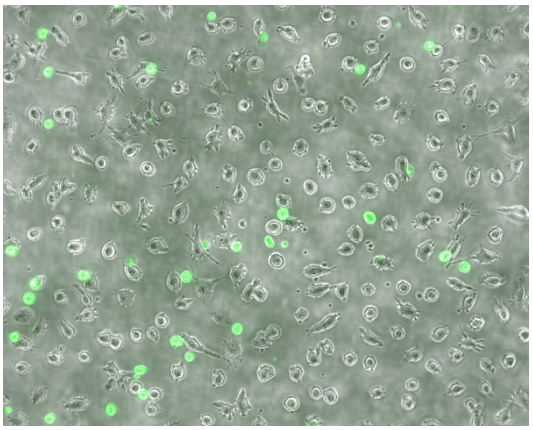
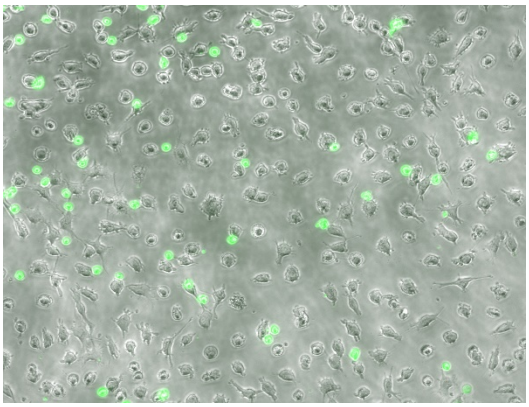
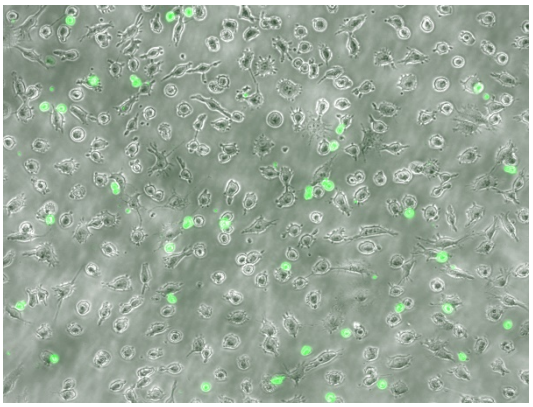
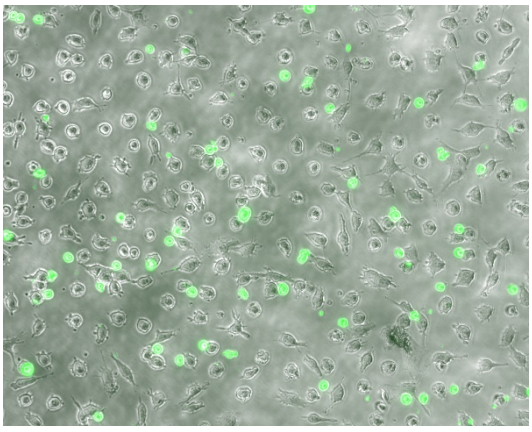
| A) Control (0,1% eth) | B) Oleic acid |
|---|---|
|  |  |
| C) *Cis* vaccenic acid | D) *Trans* vaccenic acid |
|  |  |
| E) Elaidic acid | |
|  | |

Figure 6.2: Representative visualisation of adhesion of calcein-labelled THP-1 cells to EA.hy926 after exposure to FAs (50 µM).
Adhesion of THP-1 cells to EA.hy926 cells without pre-incubation with FA (control (A)) or with 48 h prior exposure to oleic acid (B), *cis* vaccenic acid (C), *trans* vaccenic acid (D), elaidic acid (E) followed by incubation with DMEM for 6 h and 1 h co-incubation with calcein-labelled THP-1 cells. Attached THP-1 cells were visualised by fluorescence microscope Nikon Elipse Ti at a magnification of 100x under transmitted light.

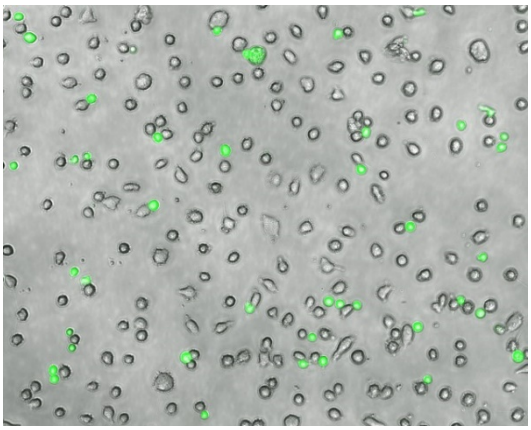
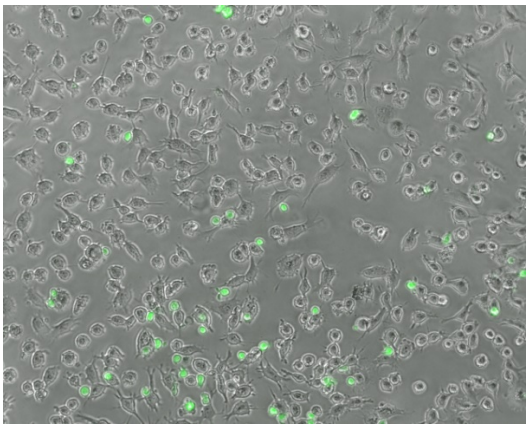
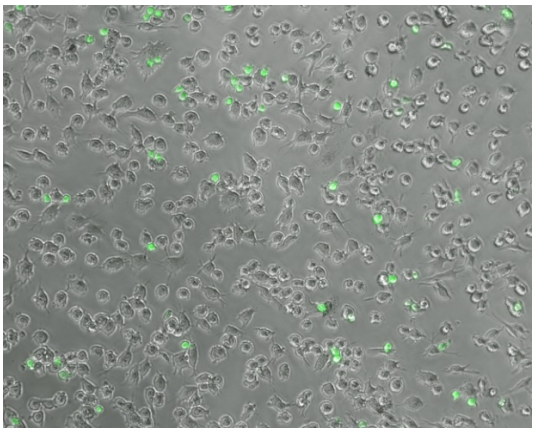
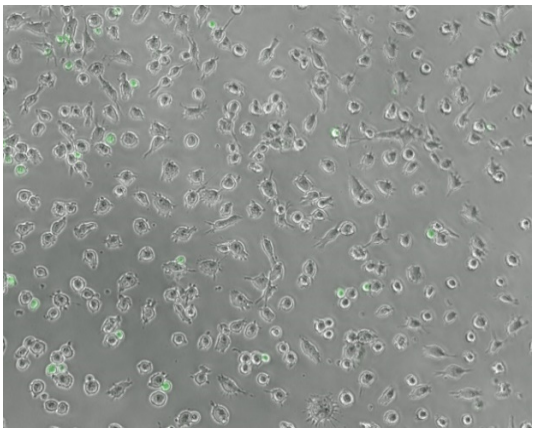
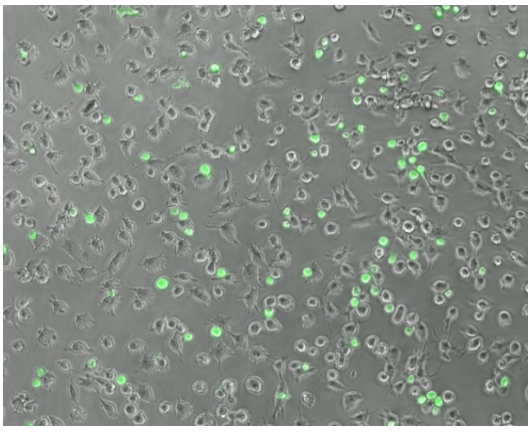
| A) Control (0,1% eth) | B) Oleic acid |
|---|---|
|  |  |
| C) *Cis* vaccenic acid | D) *Trans* vaccenic acid |
|  |  |
| E) Elaidic acid | |
|  | |

Figure 6.3: Visualisation of adhesion of calcein-labelled THP-1 cells to EA.hy926 after exposure to FAs (50 µM) and TNF-α (1 ng/mL).
Adhesion of THP-1 cells to activated EA.hy926 cells without prior incubation with FA (control; CTL (A)) or with 48 h prior exposure to oleic acid (B), *cis* vaccenic acid (C), *trans* vaccenic acid (D), elaidic acid (E) followed by incubation with TNF-α (1 ng/mL) for 6 h and 1 h co-incubation with calcein-labelled THP-1 cells. Attached THP-1 cells were visualised by fluorescence microscope Nikon Elipse Ti at a magnification of 100x under transmitted light.

### 6.4.1.2 Comparing the effect of two CLA isomers of ruminant origin and LA on the adhesion of monocytes to cultured ECs

Figure 6.4(A) shows that treatment of EA.hy926 cells with any of the three FAs at 1 µM did not significantly affect adhesion of THP-1 cells to the ECs under basal conditions. When used at 10 µM, pre-incubation with LA induced a reduction in THP-1 adhesion, acting significantly different from the CLA isomers.

In a pro-inflammatory state, both LA and CLA9,11 reduced adhesion of THP-1 cells compared to stimulated control cells, while CLA10,12 had no effect on THP-1 adhesion (Figure 6.4(B)).

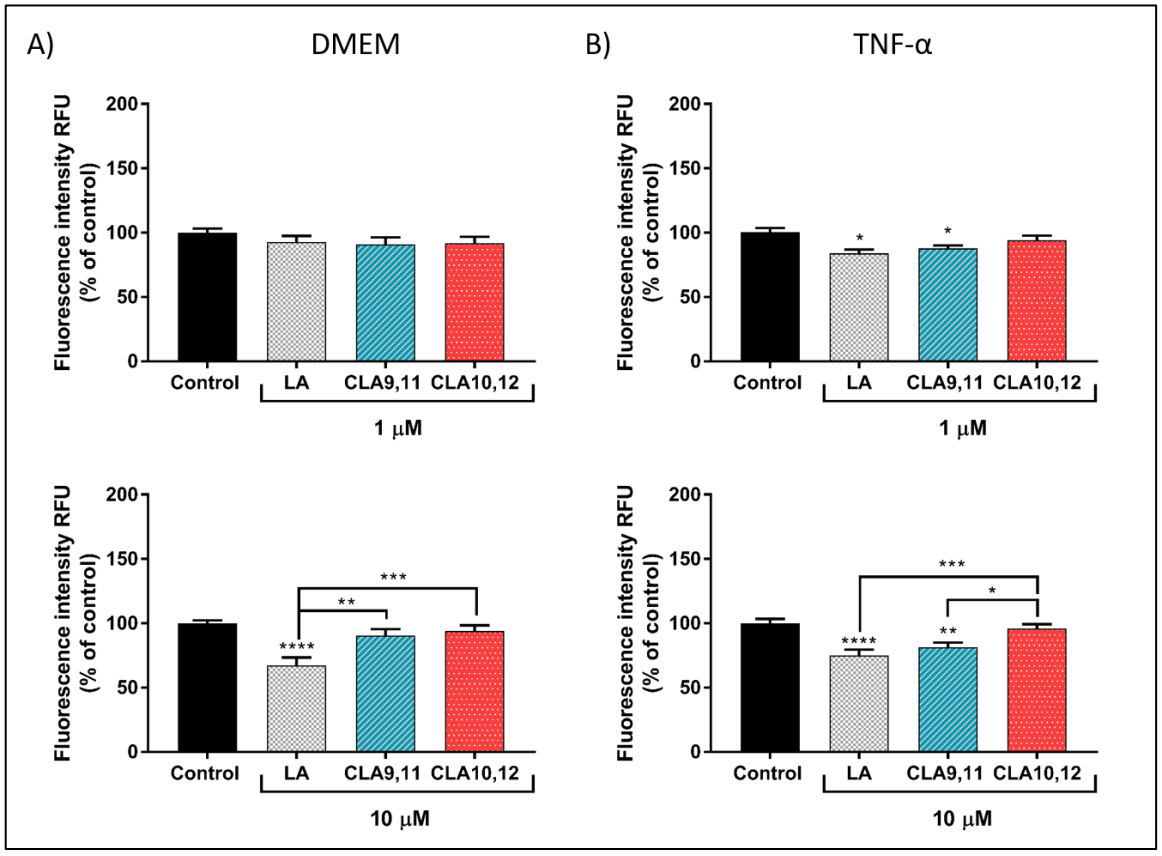



Figure 6.4: Adhesion of THP-1 cells (% of control) to EA.hy926 cells exposed to FAs.
ECs were incubated for 48 h with DMEM containing 0.1% of ethanol (Control) or 1 µM and 10 µM of FAs, followed by incubation with A) DMEM or B) TNF-α (1 ng/mL) for 24 h and 1 h co-incubation with THP-1 cells. Bars are mean ±SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. LA = linoleic acid, CLA9,11 = conjugated *cis*-9, *trans*-11 linoleic acid, CLA10,12 = conjugated *trans*-10, *cis*-12 linoleic acid.

Images taken under the fluorescence microscope agree with the quantitative results, showing lower amounts of THP-1 monocytes (green spots) when ECs were pre-incubated

with CLA9,11 in TNF-α stimulated cells and LA both with and without TNF-α stimulation (Figures 6.5 and 6.6).

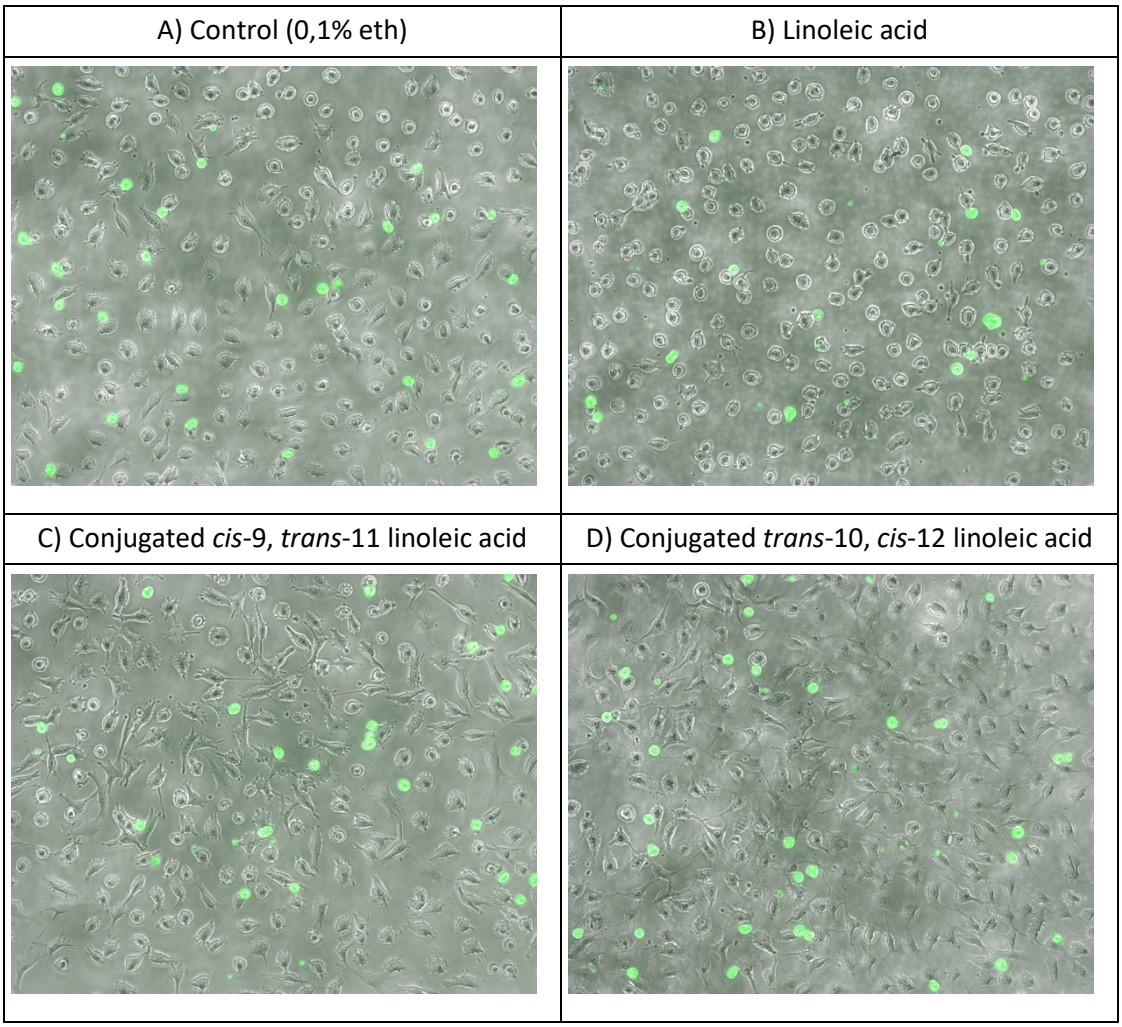



Figure 6.5: Visualisation of adhesion of calcein-labelled THP-1 cells to EA.hy926 after exposure to CLAs (10 µM).
Adhesion of THP-1 cells to activated EA.hy926 cells without prior incubation with FA (control; CTL (A)) or with 48 h prior exposure to linoleic acid (B), conjugated *cis*-9, *trans*-11 linoleic acid (C), conjugated *trans*-10, *cis*-12 linoleic acid (D) followed by incubation with DMEM for 6 h and 1 h co-incubation with calcein-labelled THP-1 cells. Attached THP-1 cells were visualised by fluorescence microscope Nikon Elipse Ti at a magnification of 100x under transmitted light.

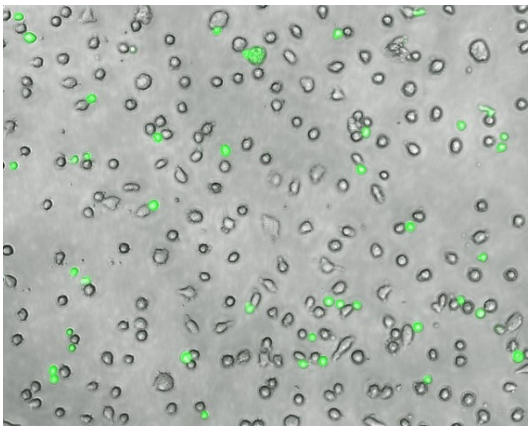
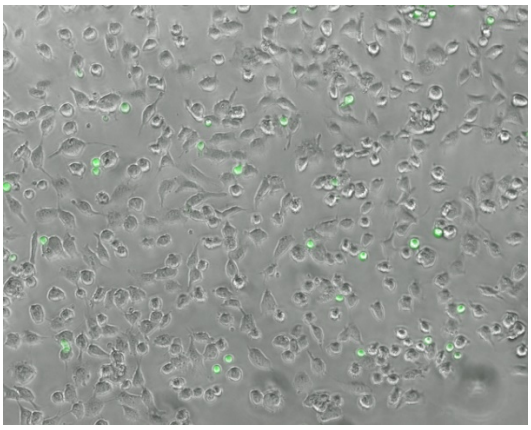
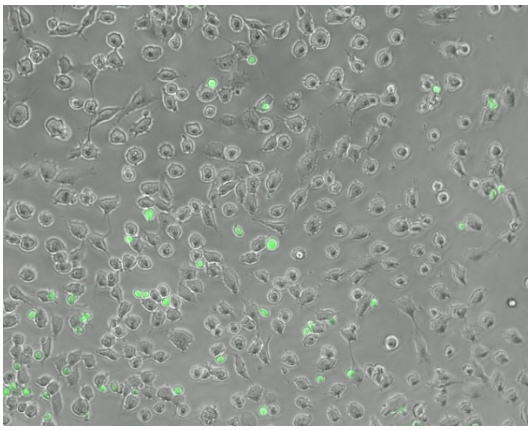
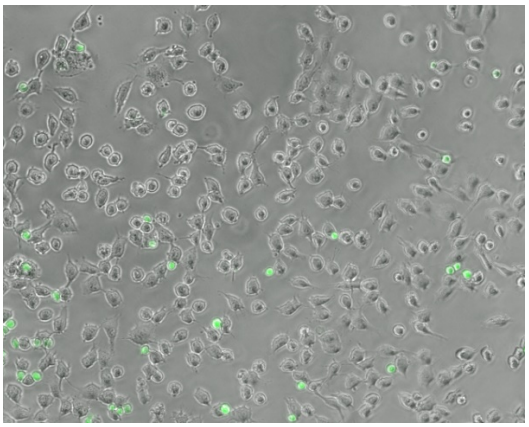
| A) Control (0,1% eth) | B) Linoleic acid |
|---|---|
|  |  |
| C) Conjugated *cis*-9, *trans*-11 linoleic acid | D) Conjugated *trans*-10, *cis*-12 linoleic acid |
|  |  |

Figure 6.6: Visualisation of adhesion of calcein-labelled THP-1 cells to EA.hy926 after FA after exposure to CLAs (10 µM) and TNF-α (1 ng/mL).
Adhesion of THP-1 cells to activated EA.hy926 cells without prior incubation with FA (control; CTL (A)) or with 48 h prior exposure to linoleic acid (B), conjugated *cis*-9, *trans*-11 linoleic acid (C), conjugated *trans*-10, *cis*-12 linoleic acid (D) followed by incubation with TNF-α (1 ng/mL) for 6 h and 1 h co-incubation with calcein-labelled THP-1 cells. Attached THP-1 cells were visualised by fluorescence microscope Nikon Elipse Ti at a magnification of 100x under transmitted light.

### 6.4.2 Effect of 18-carbon TFAs on the surface expression of ICAM-1 on cultured ECs.

The experiments described in this section were performed only with TNF-α stimulated ECs given that when the cells were analysed under basal conditions, only 0 to 4% of gated ECs were ICAM-1 positive.

Figure 6.7(A) shows the gating of unstained TNF-α stimulated EA.hy926 cells. Incubation of EA.hy926 cells with TNF-α significantly up-regulated cell surface ICAM-1 expression (Figure 6.7(B)).

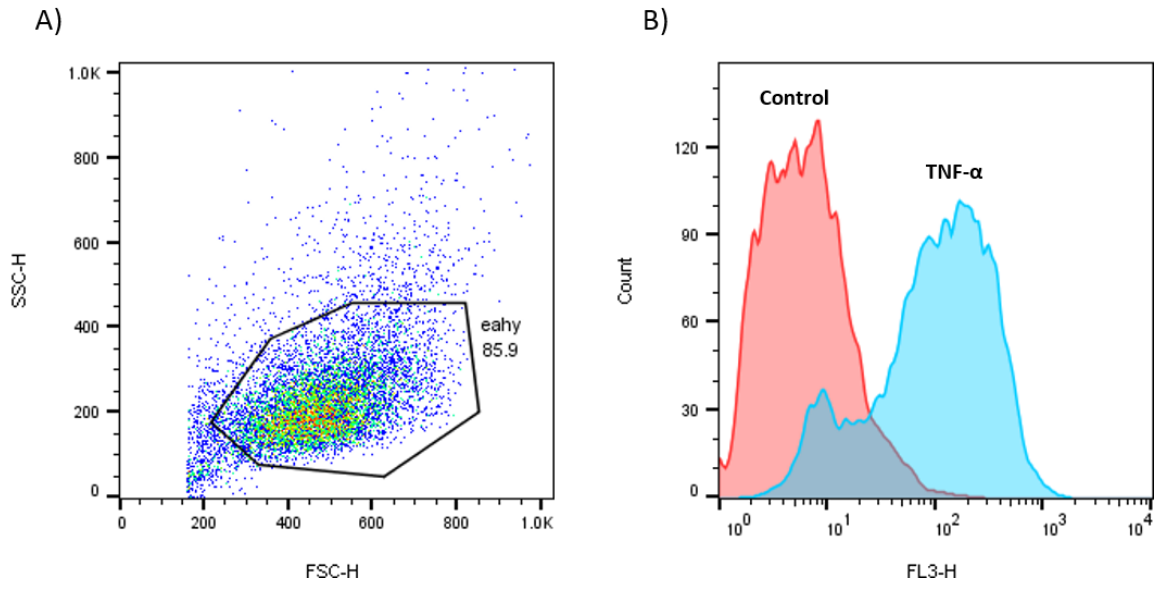



Figure 6.7: Flow cytometry plots for cell surface ICAM-1 analysis.
A) Gated unstained TNF-α stimulated EA.hy926 cells. B) Gated unstimulated (red) and TNF-α stimulated (blue) (PE-CyTM 5)-conjugated CD54 antibody stained EA.hy926 cells.

#### 6.4.2.1 Effect of 18-carbon *trans* isomers from industrial and ruminant sources on the surface expression of ICAM-1 in cultured ECs.

FA exposure was shown to have differential effects depending on individual FA and FA concentration. TVA exposure showed a trend to reduce the % of cells expressing ICAM-1 with all the concentrations used, behaving significantly differently from EA, which tended to increase ICAM-1 expressing cells (Figure 6.8(A)).

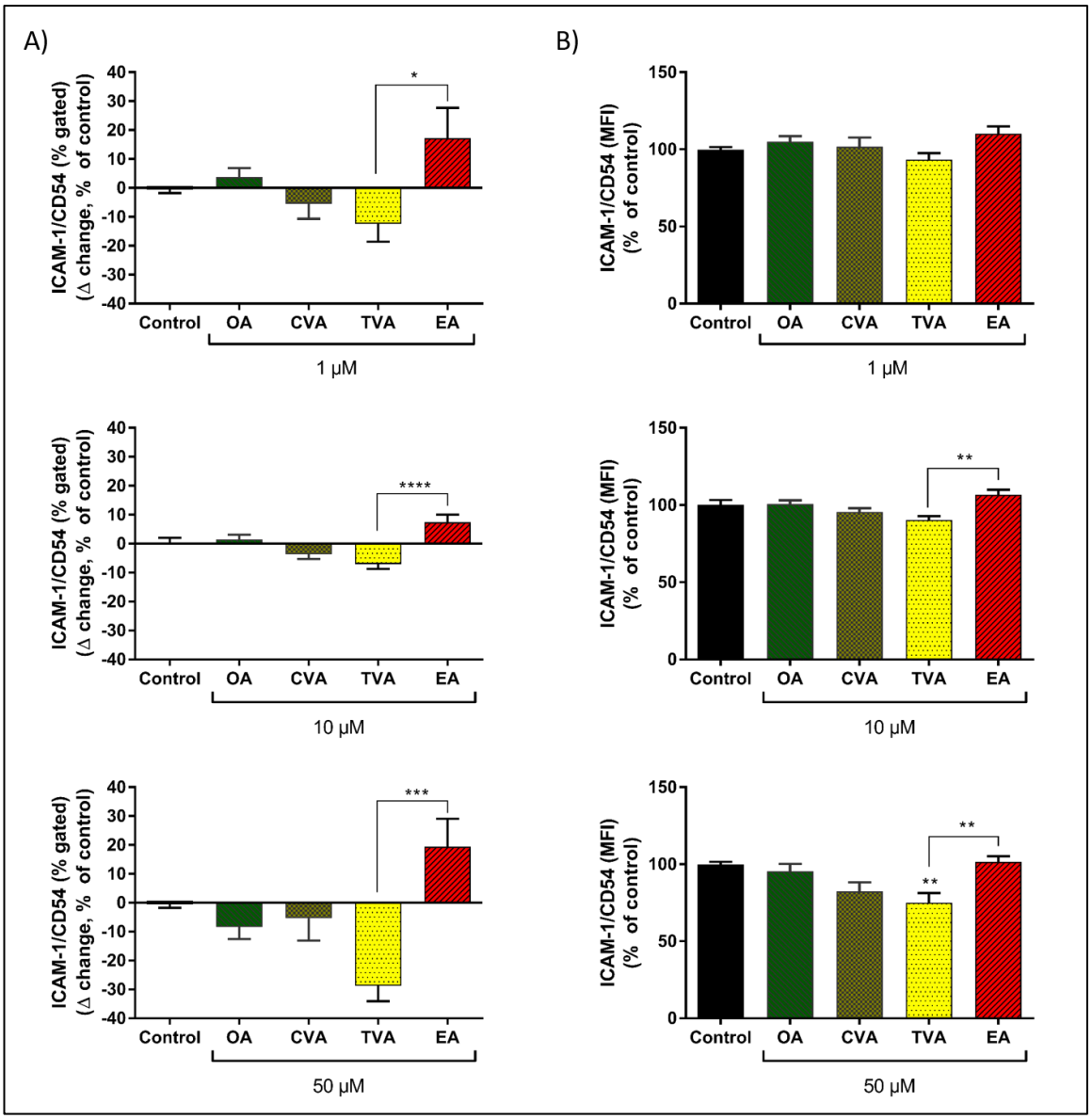



Figure 6.8: Cell surface expression of ICAM-1 after exposure to FAs.
A) % gated (Δ change, % of control) and B) median fluorescence intensity (% of control), in EA.hy926 cells incubated for 48 h with DMEM containing 0.1% of ethanol (Control) or several concentrations (1 µM; 10 µM; 50 µM) of FAs, followed by incubation with TNF-α (1 ng/mL) for 6 h. Bars are mean ±SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test: *p<0.05; **p<0.01; ****p<0.0001. OA = oleic acid, CVA = *cis* vaccenic acid, TVA = *trans* vaccenic acid, EA = elaidic acid.

The changes in the % of cells expressing ICAM-1 were consistent with the changes in the levels of ICAM-1 expression on the total of EA.hy926 cells analysed per sample (i.e. MFI). As shown in figure 6.8(B), TVA also tended to reduce ICAM-1 expression on ECs, inducing a significant change when used at 50 µM. EA tended to induce the opposite effect, behaving significantly differently than TVA when used at 10 and 50 µM.

### 6.4.2.2 Comparing the effect of two CLA isomers of ruminant origin and LA on the surface ICAM-1 expression in cultured ECs.

The cells exposed to CLA9,11 showed no change in either % of ECs expressing ICAM-1 or on the levels of ICAM-1 expression per cell (Figure 6.9), behaving similar to control. CLA10,12 induced an increase in the % of cells expressing ICAM-1 when used at 1 µM, behaving significantly differently than CLA9,11 and LA at that concentration, while inducing a significant reduction both on % of ECs expressing ICAM-1 and on the levels of ICAM-1 expression per cell when used at 10 µM (Figure 6.9. (A) and (B)).

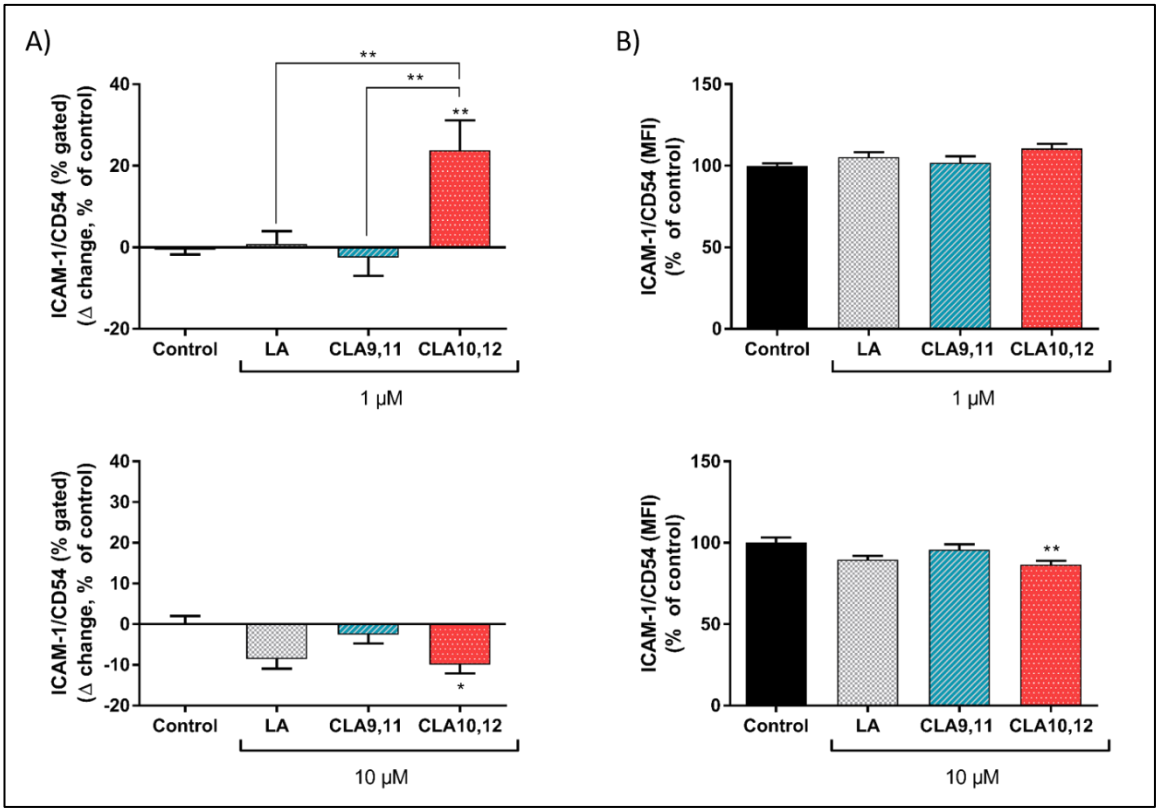



Figure 6.9: Cell surface expression of ICAM-1 after exposure to CLAs.
A) % gated (Δ change, % of control) and B) median fluorescence intensity (% of control), in EA.hy926 cells incubated for 48 h with DMEM containing 0.1% of ethanol (Control) or 1 and 10 µM of FAs, followed by incubation with TNF-α (1 ng/mL) for 6 h. Bars are mean ±SEM of 9 samples performed in 3 experiments. One-way ANOVA with Tukey's as post-hoc test: *p<0.05; **p<0.01. LA = linoleic acid, CLA9,11 = conjugated *cis*-9, *trans*-11 linoleic acid, CLA10,12 = conjugated *trans*-10, *cis*-12 linoleic acid.

## 6.5 Discussion

The aims of the research described in this chapter were to compare the effects of different *cis* and *trans* 18-carbon FAs on the adhesion of monocytes to cultured ECs, in basal conditions and after stimulation (of the ECs) with TNF-α and on the cell surface expression of ICAM-1 in cultured TNF-α stimulated ECs. These aims were met.

The recruitment of monocytes is regulated by the expression of adhesion molecules and chemokines on the activated endothelium surface. The chemokines stimulate the circulating cells, such as leukocytes, to bind to ECs in order to initiate their migration through the endothelium into sites of inflammation. Adhesion molecules expressed on ECs play a key role in the leukocyte-adhesion interactions (Baker et al, 2018).

Chemoattractants that were measured in the EC supernatants in this research include monocyte chemoattractant protein 1 (MCP-1), regulated on activation normal T cell expressed and secreted (RANTES) and interleukin 8 (IL-8) (see Chapter 4). In addition, the soluble form of the adhesion molecule intercellular adhesion molecule 1 (sICAM-1) was also measured in the cell culture media. sICAM-1 reflects ICAM-1 expression on cells (Leeuwenberg et al, 1992), which is constitutively expressed or inducible on the cell surface of different cell lines such as ECs (Fonsatti et al, 1997; Myers et al, 1992).

As explained above all of these molecules are involved in monocyte activation and trafficking across the endothelium. As reported in Chapter 4, the FAs studied within this model modulated the levels of these inflammatory mediators, after which it was decided to explore if these effects could change the functionality of the ECs in terms of affecting the adhesion of monocytes.

ICAM-1 is involved in monocyte activation and trafficking across the endothelium (Lehmann et al, 2003; Reiss & Engelhardt, 1999), which explains its presence in atherosclerotic lesions (Poston et al, 1992; van der Wal et al, 1992) and its role in their progression (Kitagawa et al, 2002). Leukocyte adhesion to the endothelium via ICAM-1 elicits increased intracellular $Ca^{2+}$, the activation of p38 and Rho, while VCAM-1 binding leads to rac1 activation. It has been suggested that the activation of these signalling molecules facilitates transmigration by triggering EC contraction and by weakening the bonds of the junctional adhesion molecules (Lawson & Wolf, 2009).

Given the role of cell surface ICAM-1 on the adhesion of monocytes to ECs, it was decided to also determine the effect of the FAs of interest on the EC surface expression of ICAM-1.

### 6.5.1 Comparing the effect of 18-carbon *trans* isomers from industrial and ruminant origin

In non-stimulated ECs, Figure 6.1(A) shows that the lowest concentration of TVA was enough to produce decreased monocyte adhesion. The other concentrations used (10 and 50 µM) did not evoke any significant changes. In contrast, EA showed a dose-response effect, inducing a significant increase of THP-1 cell adhesion to ECs only at 50 µM under the same non-stimulated conditions.

After TNF-α stimulation (Figure 6.1(B)), TVA maintained its previous effect of reducing adhesion together with OA and CVA (1 µM). EA also maintained its effect increasing the adhesion of THP-1 cells to EA.hy926 monolayer when used at 50 µM.

These results suggest an anti-inflammatory effect of TVA both in the absence of a classic inflammatory stimulus and in its presence. On the other hand, the augmented adhesion of monocytes caused by EA observed with unstimulated and TNF-α stimulated ECs suggests a change in these cells functionality, which could contribute to the development of atherosclerosis.

There are no studies describing the effect of TVA in static or flow adhesion assays, although it has been reported that the treatment with a diet supplemented with this ruminant TFA can reduce TNF-α levels in a mouse model (Wang et al, 2015) and also in humans (Sofi et al, 2010). TNF-α is a pro-inflammatory cytokine that affects a variety of cell processes. Through the activation of the NFκB signalling pathway, TNF-α stimulates expression of adhesion molecules such as E-selectin, VCAM-1 and ICAM-1 (Kułdo et al, 2005), which in turn would affect the adhesion and transmigration of monocytes.

In chapter 4, EA treatment was shown to increase the levels of MCP-1, RANTES and IL-8 after inflammatory stimulation, having neutral or some anti-inflammatory effects in basal conditions. Instead, the treatment with TVA caused a reduction of MCP-1, VEGF, RANTES, IL-8 and IL-6 in non-stimulated cells, which was maintained for ICAM-1, VEGF and RANTES

at 1 µM after inflammatory stimulation, having otherwise neutral effects. The results of both chapters can be related, particularly in the experiments where a pro-inflammatory state was induced. Under basal conditions, only a trend of higher levels of RANTES was observed when EA was used at 50 µM.

As shown in Figure 6.8, and in agreement with the findings described in the previous chapters, TVA showed opposite effects to EA, tending to decrease % of cells expressing ICAM-1 and producing a significant decrease in the levels of its expression (50 µM, $p<0.01$).

These results suggest an anti-inflammatory endothelial cell phenotype induced by TVA that is consistent with the findings shown previously.

There are no other studies describing the effect of TVA on cell surface expression of ICAM-1 or comparing the effect of ruminant versus industrialized TFAs on this outcome. In the study by Livingstone et al. (2014) aortic ECs from healthy and diabetic donors were incubated for 24 h with TVA (20 µM). They measured soluble VCAM-1 and ICAM-1 in the cell culture supernatant by ELISA; however, the levels were below the detectable range. Additionally, the same study showed that TVA did not affect the relative gene expression of ICAM-1.

In this study, EA did not induce significant changes in the surface expression of ICAM-1 in EA.hy926 cells, although it showed a trend to increase both in the % of cells expressing ICAM-1 and in the level of expression as MFI (Figure 6.8).

A few studies have reported the effects of iTFA on the surface expression of adhesion molecules in ECs. Bryk et al. (2011) showed that linoelaidic acid and EA can induce the expression of ICAM-1 and VCAM-1 on the surface of aortic endothelial cells together with overexpression of ICAM-1 and VCAM-1 mRNA, resulting in an increased adhesion of human monocytes and lymphocytes to the ECs. However, the FA concentrations used in this study were much higher than in the current study (50 to 200 µM). Harvey et al. (2012) showed the same effect for surface ICAM-1 in a similar *in vitro* model using lower concentrations (25 and 50 µM). Siddiqui et al. (2009) reported that linoelaidic acid (5 µM) significantly stimulated ICAM-1 expression on the cell surface of HAECs, while treatment with the n-3 FA DHA significantly inhibited the expression of ICAM-1. The same study reported that a diet containing 17% of iTFAs (EA and linoelaidic acid) adversely affected

survival due to sudden cardiac death in rats with coronary ligation, while n-3 FAs had beneficial effects on survival. In addition, animals fed with iTFAs had variable degrees of aortic atherosclerotic lesions, lacked the ability to develop collaterals around the site of occlusion, and showed increased circulating levels of soluble ICAM-1 (sICAM-1), the opposite to what it was observed in the animals fed with n-3 FA-enriched diet (Siddiqui et al, 2009).

The difference with the findings described here might be related to the type of cell line used, times of exposure and use of inflammatory stimulus.

Interestingly, although the surface expression of ICAM-1 on ECs was not significantly changed by the pre-incubation with EA at any of the concentrations used here, the adhesion of monocytes did increase when EA was used at 50 µM, which suggests that EA might be acting on other pro-inflammatory molecules to activate ECs.

### 6.5.2 Comparing the effect of two CLA isomers of ruminant origin and LA.

In non-stimulated ECs, CLA isomers had neutral effects, comparable to control, at both concentrations. However, LA significantly affected monocyte adhesion inducing a reduction when used at 10 µM (Figure 6.4(A)).

In TNF-α stimulated ECs, both CLA9,11 and LA significantly reduced the adhesion of THP-1 cells to the EA.hy926 cell monolayer at both concentrations used, being more potent and acting significantly different than CLA10,12 isomer when used at 10 µM (Figure 6.4(B)).

These results suggest an anti-inflammatory effect of CLA9,11 in the induced pro-inflammatory condition, which could play a protective role against the development of atherosclerosis.

CLA9,11 had neutral effects on ICAM-1 cell surface expression, comparable to control, at both concentrations used. In contrast, CLA10,12 increased significantly the % of cells expressing ICAM-1 when used at 1 µM ($p<0.01$), producing the opposite effect when used at 10 µM ($p<0.05$) (Figure 6.9(A)).

These results suggest a complex modulatory effect of CLA10,12 on ICAM-1 cell surface expression.

Sneddon et al. (2006) reported that both CLA9,11 and CLA10,12 were able to suppress adhesion of THP-1 cells and isolated human PBMCs to HUVECs treated with TNF-α, with no induced changes on VCAM-1 or ICAM-1 expression measured by western blot; nevertheless, the inflammatory stimulation in this study was higher than in the current study (TNF-α (5 ng/ml) or IL-1β (10 U/ml)). Similarly, Goua et al. (2008) showed that CLA10,12 and a CLA mix reduced ICAM-1 and VCAM-1 expression, while the CLA mix used at 25 μM was able to decrease NFκB activity by 30% in both HUVECs and smooth muscle cells treated with TNF-α. Another study by Stachowska et al. (2012) showed that the incubation of monocytes from healthy donors with CLA9,11 and CLA10,12 at 100 μM for 7 days reduced the expression of the integrins VLA-4 and Mac-1. When HUVECs were exposed to the same concentrations of CLA isomers, both caused a reduction in the surface expression of VCAM-1, but only CLA9,11 reduced ICAM-1 compared to control. Additionally, both CLA isomers showed a strong tendency to reduce the binding of monocytes to HUVECs (Stachowska et al, 2012), which agrees with the current findings to some degree, although the FA concentration used was much higher in the previous study.

Again, is important to consider the differences in the FA concentration and times of exposure used in the different studies, which most likely explain the different findings.

In a similar way to the findings reported for EA, the surface expression of ICAM-1 on ECs was not significantly changed by the pre-incubation with CLA9,11; however the adhesion of monocytes was reduced at both concentrations of CLA9,11 used, which suggests that this FA might be acting on other inflammatory signalling molecules to decrease the activation ECs.

### 6.5.3 Conclusions

Overall, the results presented in the current chapter show differential effects of the FAs tested on the adhesion of monocytes to cultured ECs (summary in table 6.1).

Table 6.1: Summary of effects of FAs on adhesion of THP-1 monocytes to EA.hy926 cells.

| | **Unstimulated ECs** | | | | | | |
|---|---|---|---|---|---|---|---|
| **Fatty acid** | OA | CVA | TVA | EA | LA | CLA9,11 | CLA10,12 |
| THP-1 adhesion to ECs | ↓ | ↔ | ↓ | ↑ | ↓ | ↔ | ↔ |
| | **TNF-α stimulated ECs** | | | | | | |
| | ↓ | ↓ | ↓ | ↑ | ↓ | ↓ | ↔ |

The exposure of EA.hy926 cells to EA increased the number of THP-1 cells bound, while TVA and CLA9,11 caused a reduction in adhesion.

In relation to the results on the cell surface expression of ICAM-1 in cultured ECs, mild differential effects of the FAs tested were observed (summary in table 6.2).

Table 6.2: Summary of effects of FAs on the cell surface expression of ICAM-1 in EA.hy926 cells stimulated with TNF-α.

| | **ECs expressing ICAM-1 (% gated)** | | | | | | |
|---|---|---|---|---|---|---|---|
| **Fatty acid** | OA | CVA | TVA | EA | LA | CLA9,11 | CLA10,12 |
| Cell surface expression of ICAM-1 | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↑/↓ |
| | **Level of ICAM-1 expression on ECs (MFI)** | | | | | | |
| | ↔ | ↔ | ↓ | ↔ | ↔ | ↔ | ↓ |

Based on these results, it is suggested that EA (an iTFA) and TVA (a rTFA) have different effects in the functionality of EA.hy926 cells within the model used here, both in basal conditions and when the pro-inflammatory state was induced. In a similar way, the CLA isomers compared here also seem to have differential effects, both having neutral effects under basal conditions, but in the case of CLA9,11, decreasing monocyte adhesion after TNF-α stimulation.

The exposure of EA.hy926 cells to TVA and CLA10,12 decreased the levels of ICAM-1 cell surface expression. In the case of TVA, these results are in agreement with the anti-inflammatory effects observed in THP-1 adhesion to EA.hy926 cells and to the effects reported in the previous chapters in terms of cytokine levels and relative gene expression.

Conversely, CLA10,12 usually showed evidence of acting in a pro-inflammatory way, particularly when the cells were stimulated with TNF-α, which contrasts with the results shown in this chapter in terms of monocyte adhesion to ECs and ICAM-1 cell surface expression (10 μM).

The findings of the current chapter, together with the ones described in chapters 4 and 5, support the contention that iTFAs can act in a pro-inflammatory way. These findings also highlight the possible anti-inflammatory effects of the ruminant derived TFAs TVA and CLA9,11 and suggest a complex modulatory role of the FAs of interest on EC function and their inflammation signalling pathways.

# Chapter 7 General Discussion

Among dietary fats, intake of TFAs have been related with an increased risk of CHD in epidemiological studies (Hu et al, 1997; Mozaffarian et al, 2006; Oh et al, 2005; Sun et al, 2007; Willett et al, 1993). This risk is usually associated with iTFAs rather than rTFAs (Ascherio et al, 1994; Jakobsen et al, 2006; Pietinen et al, 1997).

The proposed mechanisms that explain the risk association with iTFAs include adverse effects on blood lipids (Mensink et al, 2003; Mozaffarian & Clarke, 2009; Sun et al, 2007), pro-inflammatory effects (Baer et al, 2004; Han et al, 2002; Lichtenstein et al, 2003; Mozaffarian et al, 2004a) and endothelial dysfunction (Baer et al, 2004; de Roos et al, 2001; Lopez-Garcia et al, 2005).

In contrast, intake of rTFAs has been inversely associated to risk of CHD (Jakobsen et al, 2006; Pietinen et al, 1997). Although intake of TFAs has been reported to be decreasing as a result of legislation and food labelling efforts (Doell et al, 2012; Vesper et al, 2017), there are sub-groups of the population that show high intakes of iTFAs due to high consumption of processed and fast food (Kris-Etherton et al, 2012). The current intake recommendation for TFAs corresponds to less than 1% of daily energy intake (Lichtenstein et al, 2006), with no distinction by the type of TFA ingested. However, if iTFA and rTFA have different, perhaps opposing, biological effects and different associations with disease risk, then legislation, labelling and intake assessments will need to be more specific. If this is to be the case, then careful examination of the different properties of iTFAs and rTFAs in general and of specific members of those families is required.

With the purpose of understanding the possible differential effects and mechanisms through which TFAs could affect normal functioning of human endothelial tissues in relation to CHD development, the present study compared the effects of various 18-carbon *cis* and *trans* FA isomers on the inflammatory response in cultured EA.hy926 endothelial cells. These cells are a type of HUVEC, which are perhaps the most widely studied type of endothelial cell. The effects of the TFAs were investigated using unstimulated cells and cells stimulated with TNF-$\alpha$. The former would indicate direct pro- or anti-inflammatory effects while the latter would indicate the ability of the TFAs to modulate the response to a classic inflammatory stimulus. In all cases, effects of TFAs were compared to cells incubated without additional FAs and to cells exposed to comparator 18-carbon *cis* FAs.

All the FAs tested were incorporated into endothelial cells in culture in a dose dependent manner after 48 h exposure at 1, 10 or 50 μM, and this incorporation was accompanied by the appearance of some metabolic products, likely desaturation or elongation products. Additionally, the cells remained viable at the different FA concentrations used, with the exception of *trans*-10, *cis*-12 CLA at 50 μM.

## 7.1 Comparing the effects of 18-carbon *trans* isomers from industrial and ruminant origin

In relation to the effects of the FAs on the production of inflammatory mediators by ECs, EA increased or tended to increase the levels of most of the cytokines and adhesion molecules measured after inflammatory stimulation, while TVA caused their reduction in non-stimulated cells and showed a neutral effect in TNF-α stimulated ECs. EA also showed some anti-inflammatory effects under basal conditions when used at low concentrations, which have not been reported before.

Although EA and TVA did not induce many significant changes in the expression of genes involved in the inflammatory pathway of EA.hy926 cells, some differential effects were observed. EA induced a significant upregulation of TLR4 and COX-2 gene expression when used at 50 μM in TNF-α stimulated cells (Chapter 5, figure 5.16), which could be considered as pro-inflammatory. In contrast, TVA induced a significant reduction in the mRNA expression of NFκB1 when used at 1 μM in TNF-α stimulated cells, and tended to do the same when used at 10 μM. TVA also decreased the relative gene expression of COX-2 under basal conditions at 10 μM, showing the same trend when used at the other two concentrations. These results suggest anti-inflammatory actions of TVA.

When adhesion of monocytes (THP-1 cells) to the endothelial monolayer and the expression of ICAM-1 on the endothelial cell surface were assessed, differences between EA and TVA were also observed. EA induced a significant increase of THP-1 cell adhesion to ECs when used at 50 μM, both with and without TNF-α stimulation (Chapter 6, figure 6.1). EA also showed a trend to increase both the % of gated ICAM-1 positive cells and the level of surface expression of ICAM-1 at all the concentrations used (Chapter 6, figure 6.8). Again, these effects could be regarded as pro-inflammatory and pro-atherogenic. In contrast, TVA

reduced THP-1 monocyte adhesion when used at 1 µM, under basal conditions and after TNF-α stimulation, and tended to decrease both the % of gated ICAM-1 positive cells and the level of surface expression of ICAM-1 at all the concentrations used, reducing the latter significantly when used at 50 µM. These effects could be considered to be anti-atherogenic.

TVA is naturally occurring in dairy products while EA is industrially produced. Thus, these findings suggest that this ruminant TFA is anti-inflammatory in its own right while EA enhances the response to an inflammatory stimulus. These findings fit with the idea that iTFA and rTFA can have different and perhaps opposing biological actions.

Several studies have described that EA and other iTFAs have deleterious effects on health outcomes in humans: a high dietary intake or high blood/tissue levels were associated with CHD, systemic inflammation, endothelial dysfunction and possibly inflammation in the central nervous system (Baer et al, 2004; Han et al, 2002; Lichtenstein, 2014; Morris et al, 2006; Smit et al, 2011). Animal models have shown similar effects, with enhanced inflammatory parameters in cerebrospinal fluid and blood, insulin resistance, altered lipid profiles and hepatic damage (Longhi et al, 2017; Ruth et al, 2010). *In vitro* studies have described that EA (100 µM) induces the expression of ICAM-1 and VCAM-1 on the surface of aortic endothelial cells, increases the expression of ICAM-1 and VCAM-1 mRNA, and also, leukocyte adhesion, phosphorylation of NFκB and ROS generation in these cells (Bryk et al, 2011). Experiments with another human endothelial cell model (HMECs) showed that exposure to EA (100 µM) increased NFκB activation as measured by IL-6 levels and phosphorylation of IκBα, increased superoxide production, and impaired insulin signalling and nitric oxide production (Iwata et al, 2011).

Using a maximum concentration that is half of what other authors have used (50 µM), here it was shown that EA exposure induced increased inflammatory mediator levels (MCP-1, VEGF, RANTES and IL-8). Increased levels of these mediators is related to a pro-inflammatory state that could lead to the development of atherosclerosis and endothelial dysfunction. For example, MCP-1 is one of the key chemokines that regulate migration and infiltration of monocytes/macrophages into the subendothelial space, being considered an early indicator of endothelial dysfunction (Martynowicz et al, 2014).

EA also showed a trend to induce the surface expression of ICAM-1. This adhesion molecule is involved in the leukocyte-endothelium interaction and the regulation of vascular

permeability (Frank & Lisanti, 2008). Because adhesion is one of the earliest steps of inflammation in atherosclerosis, followed by migration of blood leukocytes into the sub-endothelial space, the increase in adhesive properties of endothelial cells may be an important mechanism by which dietary iTFAs exert their pro-inflammatory and pro-atherogenic effects (Bryk et al, 2011). In agreement with the findings of this study, EA induced a significant increase of THP-1 cell adhesion to ECs in non-stimulated and TNF-α stimulated conditions.

The evidence about the effects of rTFAs is less consistent, especially when comparing the results from animal models with outcomes in human studies. It has been shown that TVA can lower fasting triglycerides, total cholesterol, low density lipoprotein and non-esterified fatty acids in animal models of dyslipidaemia (Tyburczy et al, 2009; Wang et al, 2009; Wang et al, 2008b). Blewett et al. (2009) also reported that short-term (3 wk.) supplementation with TVA (1.5% w/w) normalized stimulated IL-2 and TNF-α production and increased IL-10 production in JCR-LA-cp rats, suggesting anti-inflammatory effects.

In humans, the literature is inconsistent. While some authors have reported beneficial effects of rTFAs, other have shown opposite results. For example, Da Silva et al. (2015) compared iTFA and rTFA in plasma phospholipids and their correlations with metabolic risk factors, including lipid profile, glycaemic profile, adiposity and blood pressure, in a cohort composed of 100 healthy non-obese and 100 obese participants. They found that plasma rTFAs (TVA and *trans* palmitoleic acid) levels were associated with lower insulin levels and blood pressure and higher adiponectin levels, unlike their industrial counterpart (EA) which was associated with higher total cholesterol, triglycerides and glycaemia (Da Silva et al, 2015). On the other hand, Gebauer et al. (2015) conducted a double-blind, randomized, crossover feeding trial in 106 healthy adults. They determined the effects of TVA, CLA9,11, and EA, in the context of highly controlled diets on lipoprotein risk factors compared with a control diet. Their findings showed that both TVA and partially hydrogenated vegetable oil adversely affect atherogenic lipoproteins, with higher concentrations of LDL cholesterol, apoB and triacylglycerol (Gebauer et al, 2015).

In contrast, studies in endothelial and other cell line models have reported beneficial effects of TVA (Da Silva et al, 2017a; Iwata et al, 2011; Jaudszus et al, 2012; Krogager et al, 2015), concordant to some extent with the results presented in this thesis. Overall, this study showed that low concentrations of TVA were enough to result in decreased levels of

MCP-1, VEGF, RANTES, IL-8 and IL-6 in non-stimulated ECs compared to the control, suggesting an anti-inflammatory preventive effect. Although after TNF-α stimulation, the levels of the inflammatory mediators measured did not change with the exposure of the ECs to TVA, the cell surface expression of ICAM-1 showed a trend to be reduced with all the concentrations used, being statistically significant at 50 μM.

Even though other studies have reported outcomes related to inflammation in relation to exposure to TVA, they are not the same outcomes as measured in the current research, which makes the comparison with other findings considerably harder in comparison to EA.

For instance, a study on HMECs reported that TVA (100 μM) did not induce any inflammatory responses in comparison to EA and linoelaidic acid, specifically in relation to NFκB activation, levels of IL-6 and superoxide production (Iwata et al, 2011). Another study on human PBMCs showed that TVA decreased the percentage of both IL-2 and TNF-α expressing Th cells induced by alloreactive stimulation (Jaudszus et al, 2012). Krogager et al. (2015) showed that TVA had no effect on cellular proliferation of HepG2-SF cells or their metabolism of cholesterol in comparison to EA, assessed by proteome analysis. The study by Da Silva et al. (2017) showed that TVA at concentrations above 25 μM significantly reduced the TNF-α induced expression of TNF-α, VCAM-1 and superoxide dismutase 2 genes in HUVECs. The same study reported that TVA induced a reduction on the gene expression of IL-8 and TNF in HepG2 cells (Da Silva et al, 2017b). To my knowledge, there are no studies describing the effect of TVA in static or flow adhesion assays or its effects on cell surface expression of ICAM-1.

In terms of the mechanism of action of these FAs, it seems plausible that TLR4 and NFκB are involved. EA induced an increased mRNA expression of TLR4 and COX-2, but had no effect on NFκB1 relative expression. The TLR4 pathway eventually activates NFκB signalling (see section 1.2.3.3 and 1.2.3.4, Figure 1.5), which could be related to effects on NFκB phosphorylation or nuclear translocation; or a later (past 6 h stimulation) higher gene expression and protein level of other NFκB monomers. Other authors have also suggested that EA exerts pro-inflammatory actions in HUVECs through increased TLR4 protein expression within lipid rafts (Pan et al, 2017). Although TVA had no effect at higher concentrations, at 1 μM it was able to reduce NFκB1 relative gene expression in TNF-α

stimulated ECs. Additionally, under basal conditions, TVA reduced the levels of MCP-1, IL-6 and IL-8, together with a decreased gene expression of COX-2 and IL-6, which are all consistent with decreased NFκB signalling.

The difference between the findings shown here and those of other studies can be related to the FA concentrations and cell line used and other experimental/methodological differences. In addition, other authors may not check the concentrations of the FAs used periodically or even at all. While conducting the current research, it was seen that TFAs can be unstable when stored, so not knowing precisely the FA concentration used in *in vitro* studies is not a negligible factor when analysing the effects and possible outcomes in this type of investigation. In relation to the FA concentrations used in this study, a maximum of 50 µM was considered to avoid the toxic effects found at 100 µM. It is important to consider that most of the studies in the literature use TFA concentrations of 100 µM average, reaching maximum values of 400 µM (Qiu et al, 2012). An important discussion in this matter is if these concentrations are physiologically reachable and comparable to the amount of TFA that can be incorporated into human cells and tissues through the diet. There is limited information on this. Nevertheless, the highest TFAs levels reported in plasma in healthy adults correspond to 88 µM for EA, 74 µM for TVA, 42 µM for CLA9,11 and 18 µM for CLA10,12 (Abdelmagid et al, 2015). To my knowledge, absolute concentrations of these FAs in other tissues in humans have not being described.

## 7.2 Comparing the effect of two CLA isomers of ruminant origin and LA

In relation to the effects of the CLAs on the production of inflammatory mediators by ECs, CLA9,11 had neutral or anti-inflammatory effects in non-stimulated ECs, reducing the levels of MCP-1, RANTES, IL-8 and IL-6. CLA10,12 exposure reduced the same cytokines in basal conditions, with the exception of IL-6, which was increased. In contrast, CLA10,12 increased the levels of ICAM-1, VEGF, RANTES and IL-6 after TNF-α stimulation. CLA9,11 had no effect on any of the inflammatory mediators measured after inflammatory stimulation.

In terms of gene expression, both CLAs increased NFκB1 gene expression under basal conditions, together with a reduction of PPAR-α, COX-2 and IL-6 relative gene expression. These effects may be considered contradictory, although, given that both CLAs reduced the levels of the pro-inflammatory mediators measured under basal conditions, it could be speculated that the higher gene expression of NFκB1 did not translate into higher expression of its protein or its activity.

Instead, both CLAs decreased the mRNA appearance of NFκB1 after TNF-α stimulation when used at 1 μM, but only CLA9,11 maintained this downregulation at 10 μM. CLA10,12 induced increased levels of VEGF, RANTES and IL-6, had no effect on THP-1 adhesion while decreasing significantly the % of cells expressing ICAM-1 and also levels of ICAM-1 expression per cell when used at 10 μM. These findings suggest that CLA10,12 has a complex modulatory role in the inflammatory response of ECs, probably through different and maybe opposing mechanisms.

In the case of CLA9,11, most of the results are consistent with anti-inflammatory effects. Even though this isomer did not produce any significant changes in ICAM-1 cell surface expression, it did reduce the adhesion of THP-1 cells to the EA.hy926 cell monolayer at both concentrations used, which could play a protective role against the development of atherosclerosis. The reduction in monocyte adhesion without an effect on ICAM-1 suggests that CLA9,11 might affect other adhesion molecules not studied here.

Differential effects of CLA isomers have also been reported by others. In healthy postmenopausal women supplementation with CLA10,12 caused higher plasma levels of CRP, fibrinogen, and plasminogen activator inhibitor-1 (PAI-1) and of a urine marker of lipid

peroxidation, compared to CLA9,11 and the olive oil, although, plasma levels of IL-6, sVCAM-1, sICAM-1, MCP-1, and TNF-α were not different between the groups (Tholstrup et al, 2008). Other studies have also reported increased CRP after CLA10,12 or CLA mix supplementation, whether in obese men with metabolic syndrome (Riserus et al, 2002), obese adults (Steck et al, 2007) or healthy adults (Smedman et al, 2005).

Nevertheless, the evidence in humans is not consistent. A supplementation study in healthy young adults with CLA9,11 or CLA10,12 for 8 weeks showed that neither of the isomers affected lymphocyte subpopulations, serum concentrations of CRP or *ex vivo* cytokine production by PBMCs in response to different inflammatory stimuli. Only some differences in blood lipids and in the expression of ICAM-1 on monocytes were observed (Tricon et al, 2004a; Tricon et al, 2004b). Ramakers et al. (2005) found no effects on *ex vivo* cytokine production by isolated PBMCs or by PBMCs present in whole blood when stimulated with LPS from a small sample of moderately overweight subjects at increased risk for CHD, after daily consumption of 3 g of CLA9,11 or CLA10,12 in an enriched dairy product for 13 weeks (Ramakers et al, 2005). Another study testing an enriched butter with CLA9,11 or CLA10,12 for 5 weeks showed increases in lipid peroxidation but no effects on plasma total-, LDL-, and HDL-cholesterol and TAGs, or inflammatory and haemostatic risk markers, nor in fasting insulin and glucose concentrations, in healthy young men (Raff et al, 2008).

In an animal model of ApoE$^{-/-}$ mice, the development of atherosclerotic lesions was impaired by CLA9,11 supplemented diet, while the CLA10,12 showed pro-atherogenic effects (Arbones-Mainar et al, 2006). The authors also reported that the group fed CLA9,11 had stabilized plaques with higher content of SMCs and collagen than the CLA10,12 fed group. In the animals fed with CLA10,12 diet, the fibrous cap was replaced by an acellular mass with higher macrophage content and activation underneath the plaques. The authors suggested that the two isomers displayed a different capacity to repress or accelerate the progress of atherosclerosis in that animal model (Arbones-Mainar et al, 2006).

In C57BL/6J female mice the administration of CLA10,12 by gavage at a dose of 20 mg/day for 7 days led to the upregulation of TNF-α, MCP-1 and IL-6 gene expression in WAT without affecting their plasma levels, together with macrophage infiltration in WAT, upregulation of SOCS3 and downregulation of PPAR-γ gene expression in WAT (Poirier et al, 2006).

Another study, using diets enriched with a mix of CLA10,12 with LA (50/50), a mix of CLA10,12 with CLA9,11 (50/50) or LA alone as a control in young male mice for 6 weeks, showed that the intermediate and higher intakes of CLA10,12 reduced adiposity, increased plasma levels of MCP-1 and IL-6 and increased liver steatosis (Shen et al, 2013).

Some *in vitro* studies also agree with these results. Goua et al. (2008) showed that CLA10,12 and a CLA mix reduced ICAM-1 and VCAM-1 expression, although only the CLA mix (with CLA9, 11) used at 25 µM was able to decrease NFκB activity (by 30%) in both HUVECs and smooth muscle cells treated with TNF-α. Another study by Stachowska et al. (2012) showed that the incubation of monocytes from healthy donors with CLA9,11 and CLA10,12 at 100 µM for 7 days reduced the expression of the integrins VLA-4 and Mac-1. When HUVECs were exposed to the same concentrations of the individual CLA isomers, both caused a reduction in the surface expression of VCAM-1, but only CLA9,11 reduced ICAM-1 compared to control. The study by Poirier et al. (2006) also showed that when 3T3-L1 adipocytes were exposed to CLA10,12, this isomer induced increased nuclear localization of the p65 subunit of NFκB, upregulation of IL-6 gene expression and secretion, downregulation of the PPAR-γ gene and protein and upregulation of SOCS3 mRNA levels.

In terms of the mechanism of action of the CLAs, it seems feasible that the NFκB pathway is also involved, together with PPAR-α. Under basal conditions, both CLAs induced downregulation of PPAR-α together with upregulation of NFκB gene expression, when used at 10 µM. In contrast, in TNF-α stimulated ECs, CLA10,12 showed an opposite pattern, tending to upregulate PPAR-α gene expression and reducing the mRNA expression of NFκB1. It is known that PPAR-α can negatively regulate pro-inflammatory gene expression by antagonizing the activities of other transcription factors, including NFκB (Pontis et al, 2016). In the case of CLA9,11, changes in the adhesive properties of ECs must also be considered given that the surface expression of ICAM-1 on ECs was not significantly changed by the pre-incubation with this CLA isomer, but the adhesion of monocytes decreased significantly at both concentrations used, with no changes in the inflammatory mediators measured in the cell supernatant. This suggests that CLA9,11 might be acting on other molecules to reduce the adhesive properties of EA.hy926 cells.

## 7.3 Conclusions

Overall, this study showed differential effects of the FAs tested, in terms of ruminant vs industrialized 18-carbon TFAs, between the CLAs tested, and also some differences between the *cis* and *trans* isomers.

The exposure of EA.hy926 cells to EA increased the levels of most of the cytokines and adhesion molecules measured after inflammatory stimulation, having neutral or some anti-inflammatory effects in basal conditions. These results are consistent with the findings on gene expression. EA increased the mRNA levels of some of the inflammatory pathway genes measured after TNF-α stimulation, having neutral effects or lowering gene expression effects under basal conditions. In addition, EA exposure increased THP-1 cell adhesion to the ECs, both with and without TNF-α stimulation.

In contrast, TVA caused reduced levels of the pro-inflammatory mediators under basal conditions, which was also observed after inflammatory stimulation for some of them at the lowest TVA concentration used, having otherwise neutral effects. TVA had very few effects on the inflammatory pathway gene expression while causing a reduction in monocyte adhesion and in the level of surface expression of ICAM-1.

Most of the findings described in this research show opposite effects of these two 18-carbon TFA isomers, with the exception of the levels of pro-inflammatory mediators under basal conditions, where both caused a reduction of MCP-1, IL-8 and IL-6 levels, although always at the lowest concentrations used in the case of EA. This highlights a possible differential effect of the FAs depending on the cellular environmental context (basal vs pro-inflammatory conditions).

The *cis*-isomers OA and CVA usually showed neutral or mild anti-inflammatory effects. The anti-inflammatory effects were usually observed under basal conditions. Some differences between the effects of the *cis* and *trans* isomers were observed both with and without TNF-α stimulation.

The CLAs showed some differential effects as well. CLA9,11 showed anti-inflammatory effects in non-stimulated cells (MCP-1, RANTES, IL-8 and IL-6), with null effects after TNF-α stimulation. In contrast, CLA10,12 had neutral or some anti-inflammatory effects under basal conditions, while it increased the levels of most of the pro-inflammatory mediators

measured after TNF-α stimulation. At the gene expression level, these two CLA isomers had similar effects. Both of them caused downregulation of COX-2 and IL-6 gene expression and upregulation of NFκB1 under basal conditions, and lowered gene expression of NFκB1 after TNF-α stimulation. Additionally, both CLAs had null effect on THP-1 adhesion in non-stimulated cells, while only CLA9,11 induced a reduction in monocyte adhesion in TNF-α stimulated cells. Instead, CLA10,12 increased the % of cells expressing ICAM-1 when used at 1 μM, while causing its reduction when used at 10 μM. CLA9,11 induced null effects in terms of ICAM-1 cell surface expression.

In summary, both CLAs showed similar effects under basal conditions, acting generally in a neutral or modest anti-inflammatory way. In contrast, after TNF-α stimulation, CLA10,12 showed some pro-inflammatory effects, in terms of increasing the levels of some inflammatory mediators and increasing the % of cells expressing ICAM-1 (at 1 μM), while CLA9,11 maintained some anti-inflammatory effects (THP-1 adhesion). In a similar way as with EA, this suggests that the EC responses to some FAs may change depending on the presence of inflammation.

LA also showed some anti-inflammatory effects, particularly under basal conditions in terms of levels of inflammatory mediators, with usually null effects on gene expression. LA caused a reduction in THP-1 adhesion, both with and without TNF-α stimulation, when used at 10 μM, acting significantly different from the CLA isomers, and producing null effects in terms of ICAM-1 cell surface expression.

Overall, the results of this study indicate that the ruminant-derived TVA has the potential of reducing the inflammatory response related to atherosclerosis in ECs. In contrast, EA increased this response, which is particularly consistent after TNF-α stimulation. Similarly, CLA10,12 induced some pro-inflammatory responses, mainly related to the induction of higher levels of inflammatory mediators in the supernatant of TNF-α stimulated ECs. CLA9,11 induced some anti-inflammatory effects both under basal conditions and after TNF-α stimulation. These findings suggest differential effects induced by the TFAs tested. The mechanisms through which these FAs influence the inflammatory response in ECs need further exploration, although NFκB, TLR4 and PPAR-α are all likely to be involved.

## 7.4 Limitations, strengths and future work

One limitation that should be considered is that the TFA stocks used were in ethanol. Solvents such as DMSO or in this case ethanol are used to dissolve FAs in this type of research. With every use of the master stocks, a small amount of the solvent can get evaporated, which is the main reason why it is important to check systematically the concentration of the FAs in the master stocks.

Other limitations are inherent with the use of an *in vitro* model to mimic a patho-physiological process. Even though it will not be possible to extrapolate the observations made in this EC model to what happens in the complexity of interactions resulting in a health outcome (atherosclerosis) in human beings, *in vitro* models are useful for understanding the mechanisms involved in the biological effects of compounds such as FAs. In this case, HUVECs have been widely used as a model to study the inflammatory response of the endothelium and the effects of FAs. These cells respond to inflammatory stimuli in a very similar way to other human ECs, such as those derived from the aorta and coronary artery (Baker et al, 2018).

On the other hand, one of the strengths of this study is the consistent checking of FA concentrations before performing every set of experiments. The FA concentrations were checked periodically to ensure to be working with the planned concentrations in response to the first limitation described above.

Other strengths include the use of several, including low (physiological), concentrations of FAs. Several studies use very high FA concentrations, which may be considered supra physiological when comparing with the described TFA levels in human blood described in healthy subjects (see chapter 1, section 1.1.4). In addition, the MTT test allowed the identification of the FA concentrations that were able to induce toxicity in the EC model used.

The confirmation that the FAs of interest were incorporated into the cells to be able to exert a biological effect is also part of the strengths of this research.

Additionally, different cellular processes and pathways related to inflammation were evaluated to be able to more fully understand which mechanisms are involved in the effects of the FAs observed in this cell model.

In relation to future work, the identification of the metabolic products of TVA and EA and determining whether the effects observed are due to the exposure to the TFA itself or to its metabolic products should be explored next.

Secondly, it would be interesting to measure the expression of other adhesion molecules on the cell surface through flow cytometry. Given that the findings for ICAM-1 expression on the surface of endothelial cells were not always consistent with the findings for monocyte adhesion to the monolayer of ECs, it is likely that other adhesion molecules could be involved in the effects of TFAs observed in this *in vitro* model.

Thirdly, it would also be interesting to examine the effects of the TFAs of interest on the adhesive interactions of endothelial cells and monocytes under flow conditions, to more closely mimic the blood flow in blood vessels.

Measuring the levels of the proteins in the inflammatory pathway already assessed by gene expression using Western blotting, specifically for proteins within the NFκB and TLR4 signalling pathways should also be explored. Gene expression is controlled by several transcription factors and can also be regulated post-transcriptionally, so it will be important to check if the expression of mRNA matches the levels of functional proteins in this cell model. The surface expression of TLR4 was assessed by flow cytometry under basal conditions and after inflammatory stimulation, but there were only few cells expressing TLR4 on the cell surface (data not shown). A study showed that TLR4 is located and functions intracellularly in HCAECs (Dunzendorfer et al, 2004), which may explain why it was not possible to find it at the cell surface here and also why it would be important to determine its protein expression levels.

Further experiments in other cell lines, animal models and clinical trials will be needed to completely elucidate and understand the differential role of these TFAs in the regulation of the inflammatory response, in the context of atherosclerosis.

Future research in cell lines and in experimental animals should consider that the effects of TFAs are dependent upon cell type/tissues, the species of TFA, their concentration and the duration of exposure.

Another factor to consider, in animal models and clinical trials, is the food matrix where the TFAs are found, because when a single type of TFA is tested, the effects do not always match with those when the TFA is administered in a food matrix, as consumed normally. It is also possible that other compounds generated in the industrial processes involved in the production of TFAs or in the matrix of dairy products may contribute to their detrimental or beneficial effects, respectively.

For human studies, long term randomised controlled trials, with an initial depletion period, measuring basal and post intervention levels of the TFAs tested, with long enough wash out periods, controlling TFA intake and the diet of the participants, with isocaloric interventions and an adequate nutrient replacement in the control group might be necessary to correctly identify the beneficial and detrimental effects of the different types of TFA.

Endothelial dysfunction is a pro-inflammatory state characterized by chronic activation of the endothelium, which leads to atherosclerosis and CVD. EA, the iTFA studied here, showed the capability of increasing the inflammatory response of ECs, which may contribute to the development and/or the progression of plaque formation in blood vessels. Conversely, the rTFAs TVA and CLA9,11 showed the potential to reduce inflammation in this *in vitro* model. Instead, CLA10,12 showed contradictory effects in terms of inflammation, some pro-inflammatory and some anti-inflammatory, suggesting a complex modulatory role of this CLA isomer on ECs function and their inflammation signalling pathways, which needs further exploration.

The findings described in this thesis generally support the contention that iTFAs can be pro-inflammatory while the most common rTFAs found in natural food sources may be anti-inflammatory or without effect.

# Appendix A Reagents

| Section | Reagent/Material | Supplier | Cat. No. |
|---|---|---|---|
| **Cell culture** | EA.hy926 ATCC CRL-2922 - Human, somatic cell hybrid, endothelial | LGC standards | CRL-2922 |
| | Dulbecco's Modified Eagle's Medium (DMEM) - high glucose | Sigma-Aldrich | D6546 |
| | Foetal bovine serum | Sigma-Aldrich | F7524 |
| | Penicillin/ Streptomycin/ Glutamine | Sigma-Aldrich | G6784 |
| | HAT Media Supplement | Sigma-Aldrich | H0262 |
| | Recombinant Human TNF-α, | PeproTech EC | 300-01A |
| **Fatty Acids** | Linoleic acid | Sigma-Aldrich | L1012-1G |
| | Oleic acid | Sigma-Aldrich | O1383-1G |
| | *Cis* vaccenic acid | Sigma-Aldrich | V0384-100MG |
| | *Trans* vaccenic acid | Sigma-Aldrich | V1131-100MG |
| | Elaidic acid | Sigma-Aldrich | E4637-1G |
| | *Cis*-9, *trans*-11, conjugated linoleic acid | Sigma-Aldrich | 16413-50MG |
| | *Trans*-10, *cis*-12, conjugated linoleic acid | Cayman chemical company | 90145 |
| **MTT** | Thiazolyl Blue Tetrazolium Bromide | Sigma-Aldrich | M5655-100MG |
| **Luminex Kits** | Human Magnetic Luminex Screening Assay (IL-6, IL-8, RANTES, MCP-1, VEGF and ICAM-1) | R&D Systems | LXSAHM-06 |
| **PCR** | ReliaPrep™ RNA Cell Miniprep System | Promega | Z6011 |
| | GoScript™ Reverse Transcriptase | Promega | A5003 |
| | Human geNorm- kit. 6 Double Dye Probe gene detection kits | PrimerDesign | ge-DD-6-hu |

| Section | Reagent/Material | Supplier | Cat. No. |
|---|---|---|---|
| | TaqMan® Gene Expression primers;<br>NFκB1 (Hs00765730_m1)<br>PPARα (Hs00947536_m1)<br>PPARγ (Hs00234592_m1)<br>MCP1 (Hs00234140_m1)<br>B2M (Hs00187842_m1)<br>PTGS2 (Hs00153133_m1)<br>IKBkB (Hs00233287_m1)<br>IL-6 (Hs00985639_m1)<br>TLR4 (Hs00152939_m1)<br>NFKBIA (Hs00355671_g1)<br>YWHAZ (Hs01122445_g1)<br>CYC1 (Hs00357717_m1)<br>RPL13A (Hs04194366_g1) | Thermo Fisher Scientific | 4331182 |
| | TaqMan® Gene Expression Master Mix | Thermo Fisher Scientific | 4369016 |
| **Static adhesion** | Vybrant™ Cell Adhesion Assay Kit | Fisher Scientific | VXV13181 |
| **Flow cytometry** | Foetal bovine serum | Sigma-Aldrich | F7524 |
| | Phosphate Buffer Saline (PBS) | Sigma-Aldrich | |
| | PE-Cy™5 Mouse Anti-Human CD54 | BD Biosciences | 555512 |
| | PE-Cy™5 Mouse IgG1 κ Isotype Control | BD Biosciences | 555750 |
| | BD Cell FIX | BD Biosciences | 340181 |

# Appendix B Fatty acids concentration checking (GC)

Fatty acid concentrations (GC), diluted from the master stocks in 100% ethanol to a 100 µM concentration in warm complete medium (according to concentration declared by the provider company) measured in 3 different occasions.

| **Fatty acids (µM)** | **A** | **B** | **C** |
|---|---|---|---|
| **Oleic acid** | 82.1800 | 122.3554 | 119.5034 |
| ***Cis* vaccenic acid** | 77.5372 | 105.3105 | 100.9476 |
| ***Trans* vaccenic acid** | 85.2061 | 123.4458 | 102.6642 |
| **Elaidic acid** | 96.3164 | 94.4328 | 122.6413 |
| **Linoleic acid** | 79.6407 | 85.6124 | 88.8935 |
| **Conjugated *cis*-9, *trans*-11 Linoleic acid** | 69.5242 | 82.3354 | 59.6088 |
| **Conjugated *trans*-10, *cis*-12 Linoleic acid** | 96.0992 | 109.6238 | 73.2548 |

# Appendix C Fatty acids composition of supplemented DMEM (GC)

Fatty acid composition (GC) of complete culture medium (DMEM + supplements), n=3.

| Fatty acid (µg/mL) | DMEM+supps A | DMEM+supps B | DMEM+supps C | Average |
|---|---|---|---|---|
| **16:00** | 5.95 | 5.33 | 4.09 | 5.12 |
| **16:1n-7** | 0.42 | 0.36 | 0.29 | 0.36 |
| **18:00** | 7.28 | 6.76 | 4.82 | 6.29 |
| **18:1n-9 (OA)** | 2.96 | 3.43 | 3.27 | 3.22 |
| **18:1n-9t (EA)** | 0.00 | 0.00 | 0.00 | 0.00 |
| **18:1n-7t (TVA)** | 0.00 | 0.00 | 0.00 | 0.00 |
| **18:1n-7 (CVA)** | 0.74 | 0.81 | 0.77 | 0.77 |
| **18:2n-6** | 0.92 | 0.86 | 0.58 | 0.79 |
| **18:3n-6** | 0.24 | 0.27 | 0.19 | 0.23 |
| **18:3n-3** | 0.08 | 0.06 | 0.05 | 0.06 |
| ***Cis*-9, *trans*-11 18:2n-7 (CLA9,11)** | 0.00 | 0.00 | 0.00 | 0.00 |
| ***Trans*-10, *cis*-12 18:2n-6 (CLA10,12)** | 0.00 | 0.00 | 0.00 | 0.00 |
| **20:1n-9** | 0.09 | 0.15 | 0.13 | 0.12 |
| **20:3n-6** | 0.50 | 0.89 | 0.31 | 0.57 |
| **20:4n-6** | 1.31 | 1.43 | 0.90 | 1.21 |
| **20:4n-3** | 0.04 | 0.05 | 0.03 | 0.04 |
| **20:5n-3** | 0.45 | 0.43 | 0.35 | 0.41 |
| **22:6n-3** | 0.88 | 1.03 | 0.67 | 0.86 |

# List of References

(2002) Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation*, 106(25), 3143-421.

Abdelmagid, S. A., Clarke, S. E., Nielsen, D. E., Badawi, A., El-Sohemy, A., Mutch, D. M. & Ma, D. W. (2015) Comprehensive profiling of plasma fatty acid concentrations in young healthy Canadian adults. *PLoS One*, 10(2), e0116195.

Abdelmagid, S. A., Nielsen, D. E., Badawi, A., El-Sohemy, A., Mutch, D. M. & Ma, D. W. (2017) Circulating concentrations and relative percent composition of trans fatty acids in healthy Canadian young adults between 2004 and 2010: a cross-sectional study. *CMAJ Open*, 5(1), E130-e136.

Adams, J., Goffe, L., Brown, T., Lake, A. A., Summerbell, C., White, M., Wrieden, W. & Adamson, A. J. (2015) Frequency and socio-demographic correlates of eating meals out and take-away meals at home: cross-sectional analysis of the UK national diet and nutrition survey, waves 1-4 (2008-12). *Int J Behav Nutr Phys Act*, 12(1), 51.

Adams, J. & White, M. (2015) Characterisation of UK diets according to degree of food processing and associations with socio-demographics and obesity: cross-sectional analysis of UK National Diet and Nutrition Survey (2008-12). *Int J Behav Nutr Phys Act*, 12(1), 160.

Akira, S., Uematsu, S. & Takeuchi, O. (2006) Pathogen recognition and innate immunity. *Cell*, 124(4), 783-801.

Alexopoulou, L., Holt, A. C., Medzhitov, R. & Flavell, R. A. (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature*, 413(6857), 732-8.

Allison, D. B., Egan, S. K., Barraj, L. M., Caughman, C., Infante, M. & Heimbach, J. T. (1999) Estimated intakes of trans fatty and other fatty acids in the US population. *J Am Diet Assoc*, 99(2), 166-74; quiz 175-6.

Arbones-Mainar, J. M., Navarro, M. A., Guzman, M. A., Arnal, C., Surra, J. C., Acin, S., Carnicer, R., Osada, J. & Roche, H. M. (2006) Selective effect of conjugated linoleic acid isomers on atherosclerotic lesion development in apolipoprotein E knockout mice. *Atherosclerosis*, 189(2), 318-27.

Artwohl, M., Lindenmair, A., Sexl, V., Maier, C., Rainer, G., Freudenthaler, A., Huttary, N., Wolzt, M., Nowotny, P., Luger, A. & Baumgartner-Parzer, S. M. (2008) Different mechanisms of saturated versus polyunsaturated FFA-induced apoptosis in human endothelial cells. *J Lipid Res*, 49(12), 2627-40.

Ascherio, A., Hennekens, C. H., Buring, J. E., Master, C., Stampfer, M. J. & Willett, W. C. (1994) Trans-fatty acids intake and risk of myocardial infarction. *Circulation*, 89(1), 94-101.

Ascherio, A. & Willett, W. C. (1997) Health effects of trans fatty acids. *Am J Clin Nutr*, 66(4 Suppl), 1006s-1010s.

Astrup, A. (2006) The trans fatty acid story in Denmark. *Atheroscler Suppl*, 7(2), 43-6.

Astrup, A., Dyerberg, J., Selleck, M. & Stender, S. (2008) Nutrition transition and its relationship to the development of obesity and related chronic diseases. *Obes Rev*, 9 Suppl 1, 48-52.

Bae, Y. S., Lee, J. H., Choi, S. H., Kim, S., Almazan, F., Witztum, J. L. & Miller, Y. I. (2009) Macrophages generate reactive oxygen species in response to minimally oxidized low-density lipoprotein: toll-like receptor 4- and spleen tyrosine kinase-dependent activation of NADPH oxidase 2. *Circ Res*, 104(2), 210-8, 21p following 218.

Baer, D. J., Judd, J. T., Clevidence, B. A. & Tracy, R. P. (2004) Dietary fatty acids affect plasma markers of inflammation in healthy men fed controlled diets: a randomized crossover study. *Am J Clin Nutr*, 79(6), 969-73.

Baker, E. (2018) *Identifying and Understanding Anti-inflammatory Effects of 18-Carbon Fatty Acids from Sustainable Plant Sources*UNIVERSITY OF SOUTHAMPTON.

Baker, E. J., Yusof, M. H., Yaqoob, P., Miles, E. A. & Calder, P. C. (2018) Omega-3 fatty acids and leukocyte-endothelium adhesion: Novel anti-atherosclerotic actions. *Mol Aspects Med*, 64, 169-181.

Bannenberg, G. & Serhan, C. N. (2010) Specialized pro-resolving lipid mediators in the inflammatory response: An update. *Biochim Biophys Acta*, 1801(12), 1260-73.

Banni, S., Carta, G., Angioni, E., Murru, E., Scanu, P., Melis, M. P., Bauman, D. E., Fischer, S. M. & Ip, C. (2001) Distribution of conjugated linoleic acid and metabolites in different lipid fractions in the rat liver. *J Lipid Res*, 42(7), 1056-61.

Bassaganya-Riera, J., Reynolds, K., Martino-Catt, S., Cui, Y., Hennighausen, L., Gonzalez, F., Rohrer, J., Benninghoff, A. U. & Hontecillas, R. (2004) Activation of PPAR γ and δ by conjugated linoleic acid mediates protection from experimental inflammatory bowel disease. *Gastroenterology*, 127(3), 777-791.

Bendsen, N. T., Stender, S., Szecsi, P. B., Pedersen, S. B., Basu, S., Hellgren, L. I., Newman, J. W., Larsen, T. M., Haugaard, S. B. & Astrup, A. (2011) Effect of industrially produced trans fat on markers of systemic inflammation: evidence from a randomized trial in women. *J Lipid Res*, 52(10), 1821-8.

Blewett, H. J., Gerdung, C. A., Ruth, M. R., Proctor, S. D. & Field, C. J. (2009) Vaccenic acid favourably alters immune function in obese JCR:LA-cp rats. *Br J Nutr*, 102(4), 526-36.

Bonanome, A., Pagnan, A., Biffanti, S., Opportuno, A., Sorgato, F., Dorella, M., Maiorino, M. & Ursini, F. (1992) Effect of dietary monounsaturated and polyunsaturated fatty acids on the susceptibility of plasma low density lipoproteins to oxidative modification. *Arterioscler Thromb*, 12(4), 529-33.

Brown, J. M., Boysen, M. S., Jensen, S. S., Morrison, R. F., Storkson, J., Lea-Currie, R., Pariza, M., Mandrup, S. & McIntosh, M. K. (2003) Isomer-specific regulation of metabolism and PPARγ signaling by CLA in human preadipocytes. *Journal of Lipid Research*, 44(7), 1287-1300.

Bryk, D., Zapolska-Downar, D., Malecki, M., Hajdukiewicz, K. & Sitkiewicz, D. (2011) Trans fatty acids induce a proinflammatory response in endothelial cells through ROS-dependent nuclear factor-kappaB activation. *J Physiol Pharmacol*, 62(2), 229-38.

Burdge, G. C. & Calder, P. C. (2015) Introduction to fatty acids and lipids. *World Rev Nutr Diet*, 112, 1-16.

Burdge, G. C., Tricon, S., Morgan, R., Kliem, K. E., Childs, C., Jones, E., Russell, J. J., Grimble, R. F., Williams, C. M., Yaqoob, P. & Calder, P. C. (2005) Incorporation of cis-9, trans-11 conjugated linoleic acid and vaccenic acid (trans-11 18 : 1) into plasma and leucocyte lipids in healthy men consuming dairy products naturally enriched in these fatty acids. *Br J Nutr*, 94(2), 237-43.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J. & Wittwer, C. T. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*, 55(4), 611-22.

Butz, D. E., Li, G., Huebner, S. M. & Cook, M. E. (2007) A mechanistic approach to understanding conjugated linoleic acid's role in inflammation using murine models of rheumatoid arthritis. *Am J Physiol Regul Integr Comp Physiol*, 293(2), R669-76.

Calder, P. C. (2011) Fatty acids and inflammation: the cutting edge between food and pharma. *Eur J Pharmacol*, 668 Suppl 1, S50-8.

Calder, P. C. (2013a) Long chain fatty acids and gene expression in inflammation and immunity. *Curr Opin Clin Nutr Metab Care*, 16(4), 425-33.

Calder, P. C. (2013b) Omega-3 polyunsaturated fatty acids and inflammatory processes: nutrition or pharmacology? *Br J Clin Pharmacol*, 75(3), 645-62.

Calder, P. C. (2015a) Functional Roles of Fatty Acids and Their Effects on Human Health. *JPEN J Parenter Enteral Nutr*, 39(1 Suppl), 18S-32S.

Calder, P. C. (2015b) Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms and clinical relevance. *Biochim Biophys Acta*, 1851(4), 469-84.

Calder, P. C. (2017) Omega-3 fatty acids and inflammatory processes: from molecules to man. *Biochem Soc Trans*, 45(5), 1105-1115.

Calder, P. C., Ahluwalia, N., Albers, R., Bosco, N., Bourdet-Sicard, R., Haller, D., Holgate, S. T., Jonsson, L. S., Latulippe, M. E., Marcos, A., Moreines, J., M'Rini, C., Muller, M., Pawelec, G., van Neerven, R. J., Watzl, B. & Zhao, J. (2013) A consideration of biomarkers to be used for evaluation of inflammation in human nutritional studies. *Br J Nutr*, 109 Suppl 1, S1-34.

Calder, P. C., Albers, R., Antoine, J. M., Blum, S., Bourdet-Sicard, R., Ferns, G. A., Folkerts, G., Friedmann, P. S., Frost, G. S., Guarner, F., Lovik, M., Macfarlane, S., Meyer, P. D., M'Rabet, L., Serafini, M., van Eden, W., van Loo, J., Vas Dias, W., Vidry, S., Winklhofer-Roob, B. M. & Zhao, J. (2009) Inflammatory disease processes and interactions with nutrition. *Br J Nutr*, 101 Suppl 1, S1-45.

Calder, P. C. & Yaqoob, P. (2007) Lipid rafts--composition, characterization, and controversies. *J Nutr*, 137(3), 545-7.

Calder, P. C., Yaqoob, P., Harvey, D. J., Watts, A. & Newsholme, E. A. (1994) Incorporation of fatty acids by concanavalin A-stimulated lymphocytes and the effect on fatty acid composition and membrane fluidity. *Biochem J*, 300 ( Pt 2), 509-18.

Calder, P. C. B. G. C. (2004) Fatty Acids, *Bioactive Lipids*The Oily Press, 25-31.

Chan, J., Prado-Lourenco, L., Khachigian, L. M., Bennett, M. R., Di Bartolo, B. A. & Kavurma, M. M. (2010) TRAIL promotes VSMC proliferation and neointima formation in a FGF-2-, Sp1 phosphorylation-, and NFkappaB-dependent manner. *Circ Res*, 106(6), 1061-71.

Chardigny, J.-M., Clouet, P., Combe, N., Quignard-Boulangé, A., Schmitt, B., Lagarde, M. & Léger, C.-L. (2007) Metabolism of trans and conjugated fatty acids. *European Journal of Lipid Science and Technology*, 109(9), 930-934.

Chowdhury, R., Warnakula, S., Kunutsor, S., Crowe, F., Ward, H. A., Johnson, L., Franco, O. H., Butterworth, A. S., Forouhi, N. G., Thompson, S. G., Khaw, K. T., Mozaffarian, D., Danesh, J. & Di

Angelantonio, E. (2014) Association of dietary, circulating, and supplement fatty acids with coronary risk: a systematic review and meta-analysis. *Ann Intern Med*, 160(6), 398-406.

Crisafulli, C. & Cuzzocrea, S. (2009) The role of endogenous and exogenous ligands for the peroxisome proliferator-activated receptor alpha (PPAR-alpha) in the regulation of inflammation in macrophages. *Shock*, 32(1), 62-73.

Cullberg, K. B., Larsen, J. O., Pedersen, S. B. & Richelsen, B. (2014) Effects of LPS and dietary free fatty acids on MCP-1 in 3T3-L1 adipocytes and macrophages in vitro. *Nutr Diabetes*, 4, e113.

Da Silva, M. S., Bilodeau, J. F., Larose, J., Greffard, K., Julien, P., Barbier, O. & Rudkowska, I. (2017a) Modulation of the biomarkers of inflammation and oxidative stress by ruminant trans fatty acids and dairy proteins in vascular endothelial cells (HUVEC). *Prostaglandins Leukot Essent Fatty Acids*, 126, 64-71.

Da Silva, M. S., Julien, P., Bilodeau, J. F., Barbier, O. & Rudkowska, I. (2017b) Trans Fatty Acids Suppress TNF-alpha-Induced Inflammatory Gene Expression in Endothelial (HUVEC) and Hepatocellular Carcinoma (HepG2) Cells. *Lipids*, 52(4), 315-325.

Da Silva, M. S., Julien, P., Perusse, L., Vohl, M. C. & Rudkowska, I. (2015) Natural Rumen-Derived trans Fatty Acids Are Associated with Metabolic Markers of Cardiac Health. *Lipids*, 50(9), 873-82.

Damas, P., Ledoux, D., Nys, M., Vrindts, Y., De Groote, D., Franchimont, P. & Lamy, M. (1992) Cytokine serum level during severe sepsis in human IL-6 as a marker of severity. *Annals of surgery*, 215(4), 356-362.

Daynes, R. A. & Jones, D. C. (2002) Emerging roles of PPARS in inflammation and immunity. *Nat Rev Immunol*, 2(10), 748-759.

de Roos, N. M., Bots, M. L. & Katan, M. B. (2001) Replacement of dietary saturated fatty acids by trans fatty acids lowers serum HDL cholesterol and impairs endothelial function in healthy men and women. *Arterioscler Thromb Vasc Biol*, 21(7), 1233-7.

de Winther, M. P., Kanters, E., Kraal, G. & Hofker, M. H. (2005) Nuclear factor kappaB signaling in atherogenesis. *Arterioscler Thromb Vasc Biol*, 25(5), 904-14.

Deckelbaum, R. J., Worgall, T. S. & Seo, T. (2006) n-3 fatty acids and gene expression. *Am J Clin Nutr*, 83(6 Suppl), 1520S-1525S.

Dehghan, M., Mente, A., Zhang, X., Swaminathan, S., Li, W., Mohan, V., Iqbal, R., Kumar, R., Wentzel-Viljoen, E., Rosengren, A., Amma, L. I., Avezum, A., Chifamba, J., Diaz, R., Khatib, R., Lear, S., Lopez-Jaramillo, P., Liu, X., Gupta, R., Mohammadifard, N., Gao, N., Oguz, A., Ramli, A. S., Seron, P., Sun, Y., Szuba, A., Tsolekile, L., Wielgosz, A., Yusuf, R., Hussein Yusufali, A., Teo, K. K., Rangarajan, S., Dagenais, G., Bangdiwala, S. I., Islam, S., Anand, S. S. & Yusuf, S. (2017) Associations of fats and carbohydrate intake with cardiovascular disease and mortality in 18 countries from five continents (PURE): a prospective cohort study. *Lancet*, 390(10107), 2050-2062.

Delerive, P., Gervois, P., Fruchart, J. C. & Staels, B. (2000) Induction of IkappaBalpha expression as a mechanism contributing to the anti-inflammatory activities of peroxisome proliferator-activated receptor-alpha activators. *J Biol Chem*, 275(47), 36703-7.

Dentener, M. A., Louis, R., Cloots, R. H., Henket, M. & Wouters, E. F. (2006) Differences in local versus systemic TNFalpha production in COPD: inhibitory effect of hyaluronan on LPS induced blood cell TNFalpha release. *Thorax*, 61(6), 478-84.

DiNicolantonio, J. J. & O'Keefe, J. H. (2018) Effects of dietary fats on blood lipids: a review of direct comparison trials. *Open Heart*, 5(2), e000871.

Doell, D., Folmer, D., Lee, H., Honigfort, M. & Carberry, S. (2012) Updated estimate of trans fat intake by the US population. *Food Additives & Contaminants: Part A*, 29(6), 861-874.

Duffy AM, B.-H. D., Harmey JH *Vascular Endothelial Growth Factor (VEGF) and Its Role in Non-Endothelial Cells: Autocrine Signalling by VEGF*. Madame Curie Bioscience Database: Landes Bioscience.

Dumas, J. A., Bunn, J. Y., Nickerson, J., Crain, K. I., Ebenstein, D. B., Tarleton, E. K., Makarewicz, J., Poynter, M. E. & Kien, C. L. (2016) Dietary saturated fat and monounsaturated fat have reversible effects on brain function and the secretion of pro-inflammatory cytokines in young women. *Metabolism*, 65(10), 1582-8.

Dunzendorfer, S., Lee, H. K., Soldau, K. & Tobias, P. S. (2004) Toll-like receptor 4 functions intracellularly in human coronary artery endothelial cells: roles of LBP and sCD14 in mediating LPS responses. *Faseb j*, 18(10), 1117-9.

Eckel, R. H., Borra, S., Lichtenstein, A. H. & Yin-Piazza, S. Y. (2007) Understanding the complexity of trans fatty acid reduction in the American diet: American Heart Association Trans Fat Conference 2006: report of the Trans Fat Conference Planning Group. *Circulation*, 115(16), 2231-46.

Ecker, J., Liebisch, G., Scherer, M. & Schmitz, G. (2010) Differential effects of conjugated linoleic acid isomers on macrophage glycerophospholipid metabolism. *J Lipid Res*, 51(9), 2686-94.

Eder, K., Schleser, S., Becker, K. & Korting, R. (2003) Conjugated linoleic acids lower the release of eicosanoids and nitric oxide from human aortic endothelial cells. *J Nutr*, 133(12), 4083-9.

EFSA (2018) Scientific and technical assistance on trans fatty acids. *EFSA Supporting Publications*, 15(6), 1433E.

Eicher-Miller, H. A., Fulgoni, V. L. & Keast, D. R. (2015) Processed Food Contributions to Energy and Nutrient Intake Differ among US Children by Race/Ethnicity. *Nutrients*, 7(12), 10076-88.

Elias, S. L. & Innis, S. M. (2002) Bakery foods are the major dietary source of trans-fatty acids among pregnant women with diets providing 30 percent energy from fat. *J Am Diet Assoc*, 102(1), 46-51.

Emken, E. A. (1984) Nutrition and biochemistry of trans and positional fatty acid isomers in hydrogenated oils. *Annu Rev Nutr*, 4, 339-76.

Enig, M. G., Atal, S., Keeney, M. & Sampugna, J. (1990) Isomeric trans fatty acids in the U.S. diet. *J Am Coll Nutr*, 9(5), 471-86.

Esmon, C. T. (2004) The impact of the inflammatory response on coagulation. *Thrombosis Research*, 114(5–6), 321-327.

*Commission Regulation (EU) 2019/649 of 24 April 2019 amending Annex III to Regulation (EC) No 1925/2006 of the European Parliament and of the Council as regards trans fat, other than trans fat naturally occurring in fat of animal origin* (2019) Chapter Official Journal of the European Union:

Evans, N. P., Misyak, S. A., Schmelz, E. M., Guri, A. J., Hontecillas, R. & Bassaganya-Riera, J. (2010) Conjugated linoleic acid ameliorates inflammation-induced colorectal cancer in mice through activation of PPARgamma. *J Nutr*, 140(3), 515-21.

FAO (2010) Fats and fatty acids in human nutrition. Report of an expert consultation. *FAO Food Nutr Pap*, 91, 1-166.

Fatkhullina, A. R., Peshkova, I. O. & Koltsova, E. K. (2016) The Role of Cytokines in the Development of Atherosclerosis. *Biochemistry (Mosc)*, 81(11), 1358-1370.

Fernandez-Real, J. M., Broch, M., Vendrell, J. & Ricart, W. (2003) Insulin resistance, inflammation, and serum fatty acid composition. *Diabetes Care*, 26(5), 1362-8.

Field, C. J., Blewett, H. H., Proctor, S. & Vine, D. (2009) Human health benefits of vaccenic acid. *Appl Physiol Nutr Metab*, 34(5), 979-91.

Fonsatti, E., Altomonte, M., Coral, S., Cattarossi, I., Nicotra, M. R., Gasparollo, A., Natali, P. G. & Maio, M. (1997) Tumour-derived interleukin 1alpha (IL-1alpha) up-regulates the release of soluble intercellular adhesion molecule-1 (sICAM-1) by endothelial cells. *Br J Cancer*, 76(10), 1255-61.

Fox, T. E., Houck, K. L., O'Neill, S. M., Nagarajan, M., Stover, T. C., Pomianowski, P. T., Unal, O., Yun, J. K., Naides, S. J. & Kester, M. (2007) Ceramide recruits and activates protein kinase C zeta (PKC zeta) within structured membrane microdomains. *J Biol Chem*, 282(17), 12450-7.

Frank, P. G. & Lisanti, M. P. (2008) ICAM-1: role in inflammation and in the regulation of vascular permeability. *Am J Physiol Heart Circ Physiol*, 295(3), H926-H927.

Frantz, S., Ertl, G. & Bauersachs, J. (2007) Mechanisms of disease: Toll-like receptors in cardiovascular disease. *Nat Clin Pract Cardiovasc Med*, 4(8), 444-54.

Galli, C. & Calder, P. C. (2009) Effects of fat and fatty acid intake on inflammatory and immune responses: a critical review. *Ann Nutr Metab*, 55(1-3), 123-39.

Ganguly, R. & Pierce, G. N. (2015) The toxicity of dietary trans fats. *Food Chem Toxicol*, 78, 170-6.

Gebauer, S. K., Chardigny, J. M., Jakobsen, M. U., Lamarche, B., Lock, A. L., Proctor, S. D. & Baer, D. J. (2011) Effects of ruminant trans fatty acids on cardiovascular disease and cancer: a comprehensive review of epidemiological, clinical, and mechanistic studies. *Adv Nutr*, 2(4), 332-54.

Gebauer, S. K., Destaillats, F., Dionisi, F., Krauss, R. M. & Baer, D. J. (2015) Vaccenic acid and trans fatty acid isomers from partially hydrogenated oil both adversely affect LDL cholesterol: a double-blind, randomized controlled trial. *Am J Clin Nutr*, 102(6), 1339-46.

Genolet, R., Wahli, W. & Michalik, L. (2004) PPARs as drug targets to modulate inflammatory responses? *Curr Drug Targets Inflamm Allergy*, 3(4), 361-75.

Ghebreyesus, T. A. & Frieden, T. R. (2018) REPLACE: a roadmap to make the world trans fat free by 2023. *Lancet*, 391(10134), 1978-1980.

Glass, C. K. & Witztum, J. L. (2001) Atherosclerosis. the road ahead. *Cell*, 104(4), 503-16.

Goua, M., Mulgrew, S., Frank, J., Rees, D., Sneddon, A. A. & Wahle, K. W. (2008) Regulation of adhesion molecule expression in human endothelial and smooth muscle cells by omega-3 fatty acids and conjugated linoleic acids: involvement of the transcription factor NF-kappaB? *Prostaglandins Leukot Essent Fatty Acids*, 78(1), 33-43.

Gregor, M. F. & Hotamisligil, G. S. (2011) Inflammatory mechanisms in obesity. *Annu Rev Immunol*, 29, 415-45.

Griinari, J. M., Corl, B. A., Lacy, S. H., Chouinard, P. Y., Nurmela, K. V. & Bauman, D. E. (2000) Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by Delta(9)-desaturase. *J Nutr*, 130(9), 2285-91.

Grosch, S., Schiffmann, S. & Geisslinger, G. (2012) Chain length-specific properties of ceramides. *Prog Lipid Res*, 51(1), 50-62.

Hallenbeck, J. M., Hansson, G. K. & Becker, K. J. (2005) Immunology of ischemic vascular disease: plaque to attack. *Trends Immunol*, 26(10), 550-6.

Han, S. N., Leka, L. S., Lichtenstein, A. H., Ausman, L. M., Schaefer, E. J. & Meydani, S. N. (2002) Effect of hydrogenated and saturated, relative to polyunsaturated, fat on immune and inflammatory responses of adults with moderate hypercholesterolemia. *J Lipid Res*, 43(3), 445-52.

Hansson, G. K. (2005) Inflammation, Atherosclerosis, and Coronary Artery Disease. *New England Journal of Medicine*, 352(16), 1685-1695.

Hansson, G. K., Libby, P. & Tabas, I. (2015) Inflammation and plaque vulnerability. *J Intern Med*, 278(5), 483-93.

Harris, W. S., Pottala, J. V., Vasan, R. S., Larson, M. G. & Robins, S. J. (2012) Changes in erythrocyte membrane trans and marine fatty acids between 1999 and 2006 in older Americans. *J Nutr*, 142(7), 1297-303.

Hartman, J. & Frishman, W. H. (2014) Inflammation and atherosclerosis: a review of the role of interleukin-6 in the development of atherosclerosis and the potential for targeted drug therapy. *Cardiol Rev*, 22(3), 147-51.

Harvey, K. A., Walker, C. L., Pavlina, T. M., Xu, Z., Zaloga, G. P. & Siddiqui, R. A. (2010) Long-chain saturated fatty acids induce pro-inflammatory responses and impact endothelial cell growth. *Clin Nutr*, 29(4), 492-500.

Harvey, K. A., Walker, C. L., Xu, Z., Whitley, P. & Siddiqui, R. A. (2012) Trans fatty acids: induction of a pro-inflammatory phenotype in endothelial cells. *Lipids*, 47(7), 647-57.

Healy, D. A., Wallace, F. A., Miles, E. A., Calder, P. C. & Newsholm, P. (2000) Effect of low-to-moderate amounts of dietary fish oil on neutrophil lipid composition and function. *Lipids*, 35(7), 763-8.

Hotamisligil, G. S. (2006) Inflammation and metabolic disorders. *Nature*, 444(7121), 860-7.

Hu, F. B., Stampfer, M. J., Manson, J. E., Rimm, E., Colditz, G. A., Rosner, B. A., Hennekens, C. H. & Willett, W. C. (1997) Dietary fat intake and the risk of coronary heart disease in women. *N Engl J Med*, 337(21), 1491-9.

Huebner, S. M., Campbell, J. P., Butz, D. E., Fulmer, T. G., Gendron-Fitzpatrick, A. & Cook, M. E. (2010) Individual isomers of conjugated linoleic acid reduce inflammation associated with established collagen-induced arthritis in DBA/1 mice. *J Nutr*, 140(8), 1454-61.

Hulshof, K. F., van Erp-Baart, M. A., Anttolainen, M., Becker, W., Church, S. M., Couet, C., Hermann-Kunz, E., Kesteloot, H., Leth, T., Martins, I., Moreiras, O., Moschandreas, J., Pizzoferrato, L., Rimestad, A. H., Thorgeirsdottir, H., van Amelsvoort, J. M., Aro, A., Kafatos, A. G., Lanzmann-Petithory, D. & van Poppel, G. (1999) Intake of fatty acids in western Europe with emphasis on trans fatty acids: the TRANSFAIR Study. *Eur J Clin Nutr*, 53(2), 143-57.

Huxford, T., Hoffmann, A. & Ghosh, G. (2011) Understanding the logic of IkappaB:NF-kappaB regulation in structural terms. *Curr Top Microbiol Immunol*, 349, 1-24.

Iwata, N. G., Pham, M., Rizzo, N. O., Cheng, A. M., Maloney, E. & Kim, F. (2011) Trans fatty acids induce vascular inflammation and reduce vascular nitric oxide production in endothelial cells. *PLoS One*, 6(12), e29600.

Jacome-Sosa, M. M., Borthwick, F., Mangat, R., Uwiera, R., Reaney, M. J., Shen, J., Quiroga, A. D., Jacobs, R. L., Lehner, R., Proctor, S. D. & Nelson, R. C. (2014) Diets enriched in trans-11 vaccenic acid alleviate ectopic lipid accumulation in a rat model of NAFLD and metabolic syndrome. *J Nutr Biochem*, 25(7), 692-701.

Jakobsen, M. U., Bysted, A., Andersen, N. L., Heitmann, B. L., Hartkopp, H. B., Leth, T., Overvad, K. & Dyerberg, J. (2006) Intake of ruminant trans fatty acids in the Danish population aged 1-80 years. *Eur J Clin Nutr*, 60(3), 312-8.

Jakobsen, M. U., Overvad, K., Dyerberg, J. & Heitmann, B. L. (2008) Intake of ruminant trans fatty acids and risk of coronary heart disease. *Int J Epidemiol*, 37(1), 173-82.

Jaudszus, A., Jahreis, G., Schlormann, W., Fischer, J., Kramer, R., Degen, C., Rohrer, C., Roth, A., Gabriel, H., Barz, D. & Gruen, M. (2012) Vaccenic acid-mediated reduction in cytokine production is independent of c9,t11-CLA in human peripheral blood mononuclear cells. *Biochim Biophys Acta*, 1821(10), 1316-22.

Jaudszus, A., Krokowski, M., Mockel, P., Darcan, Y., Avagyan, A., Matricardi, P., Jahreis, G. & Hamelmann, E. (2008) Cis-9,trans-11-conjugated linoleic acid inhibits allergic sensitization and airway inflammation via a PPARgamma-related mechanism in mice. *J Nutr*, 138(7), 1336-42.

Juaneda, P. & Sebedio, J. L. (1999) Combined silver-ion and reversed-phase high-performance liquid chromatography for the separation and identification of C20 metabolites of conjugated linoleic acid isomers in rat liver lipids. *J Chromatogr B Biomed Sci Appl*, 724(2), 213-9.

Jump, D. B. (2008) N-3 polyunsaturated fatty acid regulation of hepatic gene transcription. *Curr Opin Lipidol*, 19(3), 242-7.

Kagan, J. C., Su, T., Horng, T., Chow, A., Akira, S. & Medzhitov, R. (2008) TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat Immunol*, 9(4), 361-8.

Kalinski, P. (2012) Regulation of immune responses by prostaglandin E2. *J Immunol*, 188(1), 21-8.

Kant, A. K. & Graubard, B. I. (2004) Eating out in America, 1987-2000: trends and nutritional correlates. *Prev Med*, 38(2), 243-9.

Kawai, T. & Akira, S. (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol*, 11(5), 373-84.

Kitagawa, K., Matsumoto, M., Sasaki, T., Hashimoto, H., Kuwabara, K., Ohtsuki, T. & Hori, M. (2002) Involvement of ICAM-1 in the progression of atherosclerosis in APOE-knockout mice. *Atherosclerosis*, 160(2), 305-10.

Klein-Platat, C., Drai, J., Oujaa, M., Schlienger, J. L. & Simon, C. (2005) Plasma fatty acid composition is associated with the metabolic syndrome and low-grade inflammation in overweight adolescents. *Am J Clin Nutr*, 82(6), 1178-84.

Kleinbongard, P., Heusch, G. & Schulz, R. (2010) TNFα in atherosclerosis, myocardial ischemia/reperfusion and heart failure. *Pharmacology & Therapeutics*, 127(3), 295-314.

Koenen, R. R., von Hundelshausen, P., Nesmelova, I. V., Zernecke, A., Liehn, E. A., Sarabi, A., Kramp, B. K., Piccinini, A. M., Paludan, S. R., Kowalska, M. A., Kungl, A. J., Hackeng, T. M., Mayo, K.

H. & Weber, C. (2009) Disrupting functional interactions between platelet chemokines inhibits atherosclerosis in hyperlipidemic mice. *Nat Med*, 15(1), 97-103.

Kondo, K., Ishida, T., Yasuda, T., Nakajima, H., Mori, K., Tanaka, N., Mori, T., Monguchi, T., Shinohara, M., Irino, Y., Toh, R., Rikitake, Y., Kiyomizu, K., Tomiyama, Y., Yamamoto, J. & Hirata, K. (2015) Trans-fatty acid promotes thrombus formation in mice by aggravating antithrombogenic endothelial functions via Toll-like receptors. *Mol Nutr Food Res*, 59(4), 729-40.

Krey, G., Braissant, O., L'Horset, F., Kalkhoven, E., Perroud, M., Parker, M. G. & Wahli, W. (1997) Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay. *Mol Endocrinol*, 11(6), 779-91.

Kris-Etherton, P. M., Innis, S., Ammerican Dietetic, A. & Dietitians of, C. (2007) Position of the American Dietetic Association and Dietitians of Canada: dietary fatty acids. *J Am Diet Assoc*, 107(9), 1599-611.

Kris-Etherton, P. M., Lefevre, M., Mensink, R. P., Petersen, B., Fleming, J. & Flickinger, B. D. (2012) Trans Fatty Acid Intakes and Food Sources in the U.S. Population: NHANES 1999–2002. *Lipids*, 47(10), 931-940.

Krogager, T. P., Nielsen, L. V., Kahveci, D., Dyrlund, T. F., Scavenius, C., Sanggaard, K. W. & Enghild, J. J. (2015) Hepatocytes respond differently to major dietary trans fatty acid isomers, elaidic acid and trans-vaccenic acid. *Proteome Sci*, 13, 31.

Kruithof, E. K. (2008) Regulation of plasminogen activator inhibitor type 1 gene expression by inflammatory mediators and statins. *Thromb Haemost*, 100(6), 969-75.

Kuhnt, K., Degen, C. & Jahreis, G. (2016) Evaluation of the Impact of Ruminant Trans Fatty Acids on Human Health: Important Aspects to Consider. *Crit Rev Food Sci Nutr*, 56(12), 1964-80.

Kuhnt, K., Kraft, J., Vogelsang, H., Eder, K., Kratzsch, J. & Jahreis, G. (2007) Dietary supplementation with trans-11- and trans-12-18 : 1 increases cis-9, trans-11-conjugated linoleic acid in human immune cells, but without effects on biomarkers of immune function and inflammation. *Br J Nutr*, 97(6), 1196-205.

Kułdo, J. M., Westra, J., Ásgeirsdóttir, S. A., Kok, R. J., Oosterhuis, K., Rots, M. G., Schouten, J. P., Limburg, P. C. & Molema, G. (2005) Differential effects of NF-κB and p38 MAPK inhibitors and combinations thereof on TNF-α- and IL-1β-induced proinflammatory status of endothelial cells in vitro. *American Journal of Physiology-Cell Physiology*, 289(5), C1229-C1239.

Lawson, C. & Wolf, S. (2009) ICAM-1 signaling in endothelial cells. *Pharmacol Rep*, 61(1), 22-32.

Leeuwenberg, J. F., Smeets, E. F., Neefjes, J. J., Shaffer, M. A., Cinek, T., Jeunhomme, T. M., Ahern, T. J. & Buurman, W. A. (1992) E-selectin and intercellular adhesion molecule-1 are released by activated human endothelial cells in vitro. *Immunology*, 77(4), 543-9.

Lehmann, J. C., Jablonski-Westrich, D., Haubold, U., Gutierrez-Ramos, J. C., Springer, T. & Hamann, A. (2003) Overlapping and selective roles of endothelial intercellular adhesion molecule-1 (ICAM-1) and ICAM-2 in lymphocyte trafficking. *J Immunol*, 171(5), 2588-93.

Lemaire-Ewing, S., Lagrost, L. & Neel, D. (2012) Lipid rafts: a signalling platform linking lipoprotein metabolism to atherogenesis. *Atherosclerosis*, 221(2), 303-10.

Lewis, R. A., Austen, K. F. & Soberman, R. J. (1990) Leukotrienes and other products of the 5-lipoxygenase pathway. Biochemistry and relation to pathobiology in human diseases. *N Engl J Med*, 323(10), 645-55.

Ley, K., Laudanna, C., Cybulsky, M. I. & Nourshargh, S. (2007) Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol*, 7(9), 678-689.

Li, J., Rao, H., Bin, Q., Fan, Y.-W., Li, H.-Y. & Deng, Z.-Y. (2017) Linolelaidic acid induces apoptosis, cell cycle arrest and inflammation stronger than elaidic acid in human umbilical vein endothelial cells through lipid rafts. *European Journal of Lipid Science and Technology*, 119(7), 1600374.

Lichtenstein, A. H. (2014) Dietary Trans Fatty Acids and Cardiovascular Disease Risk: Past and Present. *Current Atherosclerosis Reports*, 16(8), 1-7.

Lichtenstein, A. H., Appel, L. J., Brands, M., Carnethon, M., Daniels, S., Franch, H. A., Franklin, B., Kris-Etherton, P., Harris, W. S., Howard, B., Karanja, N., Lefevre, M., Rudel, L., Sacks, F., Van Horn, L., Winston, M. & Wylie-Rosett, J. (2006) Diet and lifestyle recommendations revision 2006: a scientific statement from the American Heart Association Nutrition Committee. *Circulation*, 114(1), 82-96.

Lichtenstein, A. H., Erkkila, A. T., Lamarche, B., Schwab, U. S., Jalbert, S. M. & Ausman, L. M. (2003) Influence of hydrogenated fat and butter on CVD risk factors: remnant-like particles, glucose and insulin, blood pressure and C-reactive protein. *Atherosclerosis*, 171(1), 97-107.

Livak, K. J. & Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, 25(4), 402-8.

Livingstone, K. M., Givens, D. I., Jackson, K. G. & Lovegrove, J. A. (2014) Comparative effect of dairy fatty acids on cell adhesion molecules, nitric oxide and relative gene expression in healthy and diabetic human aortic endothelial cells. *Atherosclerosis*, 234(1), 65-72.

Longhi, R., Almeida, R. F., Machado, L., Duarte, M. M., Souza, D. G., Machado, P., de Assis, A. M., Quincozes-Santos, A. & Souza, D. O. (2017) Effect of a trans fatty acid-enriched diet on biochemical and inflammatory parameters in Wistar rats. *Eur J Nutr*, 56(3), 1003-1016.

Lopez-Garcia, E., Schulze, M. B., Meigs, J. B., Manson, J. E., Rifai, N., Stampfer, M. J., Willett, W. C. & Hu, F. B. (2005) Consumption of trans fatty acids is related to plasma biomarkers of inflammation and endothelial dysfunction. *J Nutr*, 135(3), 562-6.

Lopez-Moreno, J., Garcia-Carpintero, S., Jimenez-Lucena, R., Haro, C., Rangel-Zuniga, O. A., Blanco-Rojo, R., Yubero-Serrano, E. M., Tinahones, F. J., Delgado-Lista, J., Perez-Martinez, P., Roche, H. M., Lopez-Miranda, J. & Camargo, A. (2017) Effect of Dietary Lipids on Endotoxemia Influences Postprandial Inflammatory Response. *J Agric Food Chem*, 65(35), 7756-7763.

Loscher, C. E., Draper, E., Leavy, O., Kelleher, D., Mills, K. H. G. & Roche, H. M. (2005) Conjugated Linoleic Acid Suppresses NF-κB Activation and IL-12 Production in Dendritic Cells through ERK-Mediated IL-10 Induction. *The Journal of Immunology*, 175(8), 4990-4998.

Lu, Y., Zhu, X., Liang, G. X., Cui, R. R., Liu, Y., Wu, S. S., Liang, Q. H., Liu, G. Y., Jiang, Y., Liao, X. B., Xie, H., Zhou, H. D., Wu, X. P., Yuan, L. Q. & Liao, E. Y. (2012) Apelin-APJ induces ICAM-1, VCAM-1 and MCP-1 expression via NF-kappaB/JNK signal pathway in human umbilical vein endothelial cells. *Amino Acids*, 43(5), 2125-36.

Ludwig, D. S. (2011) Technology, diet, and the burden of chronic disease. *Jama*, 305(13), 1352-3.

Martynowicz, H., Janus, A., Nowacki, D. & Mazur, G. (2014) The role of chemokines in hypertension. *Adv Clin Exp Med*, 23(3), 319-25.

Matejcic, M., Lesueur, F., Biessy, C., Renault, A. L., Mebirouk, N., Yammine, S., Keski-Rahkonen, P., Li, K., Hemon, B., Weiderpass, E., Rebours, V., Boutron-Ruault, M. C., Carbonnel, F., Kaaks, R., Katzke, V., Kuhn, T., Boeing, H., Trichopoulou, A., Palli, D., Agnoli, C., Panico, S., Tumino, R.,

Sacerdote, C., Quiros, J. R., Duell, E. J., Porta, M., Sanchez, M. J., Chirlaque, M. D., Barricarte, A., Amiano, P., Ye, W., Peeters, P. H., Khaw, K. T., Perez-Cornago, A., Key, T. J., Bueno-de-Mesquita, H. B., Riboli, E., Vineis, P., Romieu, I., Gunter, M. J. & Chajes, V. (2018) Circulating plasma phospholipid fatty acids and risk of pancreatic cancer in a large European cohort. *Int J Cancer*, 143(10), 2437-2448.

Mazidi, M., Gao, H. K. & Kengne, A. P. (2017) Inflammatory Markers Are Positively Associated with Serum trans-Fatty Acids in an Adult American Population. *J Nutr Metab*, 2017, 3848201.

Mendis, S., Cruz-Hernandez, C. & Ratnayake, W. M. (2008) Fatty acid profile of Canadian dairy products with special attention to the trans-octadecenoic acid and conjugated linoleic acid isomers. *J AOAC Int*, 91(4), 811-9.

Mensink, R. P., Zock, P. L., Kester, A. D. & Katan, M. B. (2003) Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. *Am J Clin Nutr*, 77(5), 1146-55.

Mestas, J. & Ley, K. (2008) Monocyte-endothelial cell interactions in the development of atherosclerosis. *Trends Cardiovasc Med*, 18(6), 228-32.

Michelsen, K. S., Wong, M. H., Shah, P. K., Zhang, W., Yano, J., Doherty, T. M., Akira, S., Rajavashisth, T. B. & Arditi, M. (2004) Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E. *Proc Natl Acad Sci U S A*, 101(29), 10679-84.

Miller, Y. I., Choi, S. H., Wiesner, P., Fang, L., Harkewicz, R., Hartvigsen, K., Boullier, A., Gonen, A., Diehl, C. J., Que, X., Montano, E., Shaw, P. X., Tsimikas, S., Binder, C. J. & Witztum, J. L. (2011) Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity. *Circ Res*, 108(2), 235-48.

Minihane, A. M., Vinoy, S., Russell, W. R., Baka, A., Roche, H. M., Tuohy, K. M., Teeling, J. L., Blaak, E. E., Fenech, M., Vauzour, D., McArdle, H. J., Kremer, B. H., Sterkman, L., Vafeiadou, K., Benedetti, M. M., Williams, C. M. & Calder, P. C. (2015) Low-grade inflammation, diet composition and health: current research evidence and its translation. *Br J Nutr*, 114(7), 999-1012.

Mitchell, S., Vargas, J. & Hoffmann, A. (2016) Signaling via the NFkappaB system. *Wiley Interdiscip Rev Syst Biol Med*, 8(3), 227-41.

Monteiro, C. A., Moubarac, J. C., Cannon, G., Ng, S. W. & Popkin, B. (2013) Ultra-processed products are becoming dominant in the global food system. *Obes Rev*, 14 Suppl 2, 21-8.

Moore, K. J., Sheedy, F. J. & Fisher, E. A. (2013) Macrophages in atherosclerosis: a dynamic balance. *Nat Rev Immunol*, 13(10), 709-721.

Morris, M. C., Evans, D. A., Tangney, C. C., Bienias, J. L., Schneider, J. A., Wilson, R. S. & Scherr, P. A. (2006) Dietary copper and high saturated and trans fat intakes associated with cognitive decline. *Arch Neurol*, 63(8), 1085-8.

Mosley, E. E., McGuire, M. K., Williams, J. E. & McGuire, M. A. (2006a) Cis-9, trans-11 conjugated linoleic acid is synthesized from vaccenic acid in lactating women. *J Nutr*, 136(9), 2297-301.

Mosley, E. E., Shafii Dagger, B., Moate, P. J. & McGuire, M. A. (2006b) cis-9, trans-11 conjugated linoleic acid is synthesized directly from vaccenic acid in lactating dairy cattle. *J Nutr*, 136(3), 570-5.

Moubarac, J. C., Martins, A. P., Claro, R. M., Levy, R. B., Cannon, G. & Monteiro, C. A. (2013) Consumption of ultra-processed foods and likely impact on human health. Evidence from Canada. *Public Health Nutr*, 16(12), 2240-8.

Moya-Camarena, S. Y., Heuvel, J. P. V., Blanchard, S. G., Leesnitzer, L. A. & Belury, M. A. (1999) Conjugated linoleic acid is a potent naturally occurring ligand and activator of PPARα. *Journal of Lipid Research*, 40(8), 1426-1433.

Mozaffarian, D. & Clarke, R. (2009) Quantitative effects on cardiovascular risk factors and coronary heart disease risk of replacing partially hydrogenated vegetable oils with other fats and oils. *Eur J Clin Nutr*, 63 Suppl 2, S22-33.

Mozaffarian, D., Katan, M. B., Ascherio, A., Stampfer, M. J. & Willett, W. C. (2006) Trans fatty acids and cardiovascular disease. *N Engl J Med*, 354(15), 1601-13.

Mozaffarian, D., Pischon, T., Hankinson, S. E., Rifai, N., Joshipura, K., Willett, W. C. & Rimm, E. B. (2004a) Dietary intake of trans fatty acids and systemic inflammation in women. *Am J Clin Nutr*, 79(4), 606-12.

Mozaffarian, D., Rimm, E. B., King, I. B., Lawler, R. L., McDonald, G. B. & Levy, W. C. (2004b) trans fatty acids and systemic inflammation in heart failure. *Am J Clin Nutr*, 80(6), 1521-5.

Mozes, T., Barath, I., Gornicsar, K., Grosz, A., Mozes, T., Gondocs, C., Szephalmi, P., Gaal, K. & Madarasz, E. (2011) Deviations in circulating TNFalpha levels and TNFalpha production by mononuclear cells in healthy human populations. *Mediators Inflamm*, 2011, 972609.

Muhlenbeck, J. A., Butz, D. E., Olson, J. M., Uribe-Cano, D. & Cook, M. E. (2017) Dietary Conjugated Linoleic Acid-c9t11 Prevents Collagen-Induced Arthritis, Whereas Conjugated Linoleic Acid-t10c12 Increases Arthritic Severity. *Lipids*, 52(4), 303-314.

Myers, C. L., Wertheimer, S. J., Schembri-King, J., Parks, T. & Wallace, R. W. (1992) Induction of ICAM-1 by TNF-alpha, IL-1 beta, and LPS in human endothelial cells after downregulation of PKC. *Am J Physiol*, 263(4 Pt 1), C767-72.

Naska, A., Fouskakis, D., Oikonomou, E., Almeida, M. D., Berg, M. A., Gedrich, K., Moreiras, O., Nelson, M., Trygg, K., Turrini, A., Remaut, A. M., Volatier, J. L. & Trichopoulou, A. (2006) Dietary patterns and their socio-demographic determinants in 10 European countries: data from the DAFNE databank. *Eur J Clin Nutr*, 60(2), 181-90.

Naughton, S. S., Mathai, M. L., Hryciw, D. H. & McAinch, A. J. (2015) Australia's nutrition transition 1961-2009: a focus on fats. *Br J Nutr*, 114(3), 337-46.

Newby, A. C. (2005) Dual Role of Matrix Metalloproteinases (Matrixins) in Intimal Thickening and Atherosclerotic Plaque Rupture. *Physiological Reviews*, 85(1), 1-31.

Nielsen, B. M., Nielsen, M. M., Jakobsen, M. U., Nielsen, C. J., Holst, C., Larsen, T. M., Bendsen, N. T., Bysted, A., Leth, T., Hougaard, D. M., Skogstrand, K., Astrup, A., Sorensen, T. I. & Jess, T. (2011) A cross-sectional study on trans-fatty acids and risk markers of CHD among middle-aged men representing a broad range of BMI. *Br J Nutr*, 106(8), 1245-52.

Novak, T. E., Babcock, T. A., Jho, D. H., Helton, W. S. & Espat, N. J. (2003) NF-kappa B inhibition by omega -3 fatty acids modulates LPS-stimulated macrophage TNF-alpha transcription. *Am J Physiol Lung Cell Mol Physiol*, 284(1), L84-9.

O'Dea, E. & Hoffmann, A. (2009) NF-kappaB signaling. *Wiley Interdiscip Rev Syst Biol Med*, 1(1), 107-15.

O'Dea, E. & Hoffmann, A. (2010) The regulatory logic of the NF-kappaB signaling system. *Cold Spring Harb Perspect Biol*, 2(1), a000216.

Oh, K., Hu, F. B., Manson, J. E., Stampfer, M. J. & Willett, W. C. (2005) Dietary fat intake and risk of coronary heart disease in women: 20 years of follow-up of the nurses' health study. *Am J Epidemiol*, 161(7), 672-9.

Ohta, H., Wada, H., Niwa, T., Kirii, H., Iwamoto, N., Fujii, H., Saito, K., Sekikawa, K. & Seishima, M. (2005) Disruption of tumor necrosis factor-alpha gene diminishes the development of atherosclerosis in ApoE-deficient mice. *Atherosclerosis*, 180(1), 11-7.

Oomen, C. M., Ocké, M. C., Feskens, E. J. M., Erp-Baart, M.-A. J. v., Kok, F. J. & Kromhout, D. (2001) Association between trans fatty acid intake and 10-year risk of coronary heart disease in the Zutphen Elderly Study: a prospective population-based study. *The Lancet*, 357(9258), 746-751.

Otsui, K., Inoue, N., Kobayashi, S., Shiraki, R., Honjo, T., Takahashi, M., Hirata, K., Kawashima, S. & Yokoyama, M. (2007) Enhanced expression of TLR4 in smooth muscle cells in human atherosclerotic coronary arteries. *Heart Vessels*, 22(6), 416-22.

Pacheco, Y. M., Lopez, S., Bermudez, B., Abia, R., Villar, J. & Muriana, F. J. (2008) A meal rich in oleic acid beneficially modulates postprandial sICAM-1 and sVCAM-1 in normotensive and hypertensive hypertriglyceridemic subjects. *J Nutr Biochem*, 19(3), 200-5.

Pan, Y., Liu, B., Deng, Z., Fan, Y., Li, J. & Li, H. (2017) Lipid Rafts Promote trans Fatty Acid-Induced Inflammation in Human Umbilical Vein Endothelial Cells. *Lipids*, 52(1), 27-35.

Pant, S., Deshmukh, A., Gurumurthy, G. S., Pothineni, N. V., Watts, T. E., Romeo, F. & Mehta, J. L. (2014) Inflammation and atherosclerosis--revisited. *J Cardiovasc Pharmacol Ther*, 19(2), 170-8.

Penedo, L. A., Nunes, J. C., Gama, M. A., Leite, P. E., Quirico-Santos, T. F. & Torres, A. G. (2013) Intake of butter naturally enriched with cis9,trans11 conjugated linoleic acid reduces systemic inflammatory mediators in healthy young adults. *J Nutr Biochem*, 24(12), 2144-51.

Penkov, D. N., Egorov, A. D., Mozgovaya, M. N. & Tkachuk, V. A. (2013) Insulin resistance and adipogenesis: role of transcription and secreted factors. *Biochemistry (Mosc)*, 78(1), 8-18.

Pietinen, P., Ascherio, A., Korhonen, P., Hartman, A. M., Willett, W. C., Albanes, D. & Virtamo, J. (1997) Intake of fatty acids and risk of coronary heart disease in a cohort of Finnish men. The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. *Am J Epidemiol*, 145(10), 876-87.

Poirier, H., Shapiro, J. S., Kim, R. J. & Lazar, M. A. (2006) Nutritional supplementation with trans-10, cis-12-conjugated linoleic acid induces inflammation of white adipose tissue. *Diabetes*, 55(6), 1634-41.

Pontis, S., Ribeiro, A., Sasso, O. & Piomelli, D. (2016) Macrophage-derived lipid agonists of PPAR-α as intrinsic controllers of inflammation. *Critical Reviews in Biochemistry and Molecular Biology*, 51(1), 7-14.

Poston, R. N., Haskard, D. O., Coucher, J. R., Gall, N. P. & Johnson-Tidey, R. R. (1992) Expression of intercellular adhesion molecule-1 in atherosclerotic plaques. *Am J Pathol*, 140(3), 665-73.

Pot, G. K., Prynne, C. J., Roberts, C., Olson, A., Nicholson, S. K., Whitton, C., Teucher, B., Bates, B., Henderson, H. & Pigott, S. (2012) National Diet and Nutrition Survey: fat and fatty acid intake from the first year of the rolling programme and comparison with previous surveys. *British Journal of Nutrition*, 107(03), 405-415.

Poti, J. M., Mendez, M. A. & Ng, S. W. (2015) Is the degree of food processing and convenience linked with the nutritional quality of foods purchased by US households?, 101(6), 1251-62.

Qiu, B., Hu, J. N., Liu, R., Fan, Y. W., Li, J., Li, Y. & Deng, Z. Y. (2012) Caspase pathway of elaidic acid (9t-C18:1)-induced apoptosis in human umbilical vein endothelial cells. *Cell Biol Int*, 36(3), 255-60.

Radtke, T., Schmid, A., Trepp, A., Dahler, F., Coslovsky, M., Eser, P., Wilhelm, M. & Saner, H. (2017) Short-term effects of trans fatty acids from ruminant and industrial sources on surrogate markers of cardiovascular risk in healthy men and women: A randomized, controlled, double-blind trial. *Eur J Prev Cardiol*, 24(5), 534-543.

Raff, M., Tholstrup, T., Basu, S., Nonboe, P., Sorensen, M. T. & Straarup, E. M. (2008) A diet rich in conjugated linoleic acid and butter increases lipid peroxidation but does not affect atherosclerotic, inflammatory, or diabetic risk markers in healthy young men. *J Nutr*, 138(3), 509-14.

Ramakers, J. D., Plat, J., Sebedio, J. L. & Mensink, R. P. (2005) Effects of the individual isomers cis-9,trans-11 vs. trans-10,cis-12 of conjugated linoleic acid (CLA) on inflammation parameters in moderately overweight subjects with LDL-phenotype B. *Lipids*, 40(9), 909-18.

Rao, Y. P. & Lokesh, B. R. (2017) Down-regulation of NF-kappaB expression by n-3 fatty acid-rich linseed oil is modulated by PPARgamma activation, eicosanoid cascade and secretion of cytokines by macrophages in rats fed partially hydrogenated vegetable fat. *Eur J Nutr*, 56(3), 1135-1147.

Ratnayake, W. M., L'Abbe, M. R., Farnworth, S., Dumais, L., Gagnon, C., Lampi, B., Casey, V., Mohottalage, D., Rondeau, I., Underhill, L., Vigneault, M., Lillycrop, W., Meleta, M., Wong, L. Y., Ng, T., Gao, Y., Kwong, K., Chalouh, S., Pantazopoulos, P., Gunaratna, H., Rahardja, A., Blagden, R., Roscoe, V., Krakalovich, T., Neumann, G. & Lombaert, G. A. (2009) Trans fatty acids: current contents in Canadian foods and estimated intake levels for the Canadian population. *J AOAC Int*, 92(5), 1258-76.

Rees, D., Miles, E. A., Banerjee, T., Wells, S. J., Roynette, C. E., Wahle, K. W. & Calder, P. C. (2006) Dose-related effects of eicosapentaenoic acid on innate immune function in healthy humans: a comparison of young and older men. *Am J Clin Nutr*, 83(2), 331-42.

Reiss, Y. & Engelhardt, B. (1999) T cell interaction with ICAM-1-deficient endothelium in vitro: transendothelial migration of different T cell populations is mediated by endothelial ICAM-1 and ICAM-2. *Int Immunol*, 11(9), 1527-39.

Remig, V., Franklin, B., Margolis, S., Kostas, G., Nece, T. & Street, J. C. (2010) Trans fats in America: a review of their use, consumption, health implications, and regulation. *J Am Diet Assoc*, 110(4), 585-92.

Reynolds, C. M., Draper, E., Keogh, B., Rahman, A., Moloney, A. P., Mills, K. H., Loscher, C. E. & Roche, H. M. (2009) A conjugated linoleic acid-enriched beef diet attenuates lipopolysaccharide-induced inflammation in mice in part through PPARgamma-mediated suppression of toll-like receptor 4. *J Nutr*, 139(12), 2351-7.

Ringseis, R., Muller, A., Dusterloh, K., Schleser, S., Eder, K. & Steinhart, H. (2006) Formation of conjugated linoleic acid metabolites in human vascular endothelial cells. *Biochim Biophys Acta*, 1761(3), 377-83.

Riserus, U., Basu, S., Jovinge, S., Fredrikson, G. N., Arnlov, J. & Vessby, B. (2002) Supplementation with conjugated linoleic acid causes isomer-dependent oxidative stress and elevated C-reactive protein: a potential link to fatty acid-induced insulin resistance. *Circulation*, 106(15), 1925-9.

Ross, R. (1999) Atherosclerosis — An Inflammatory Disease. *New England Journal of Medicine*, 340(2), 115-126.

Ruiz-Nunez, B., Dijck-Brouwer, D. A. & Muskiet, F. A. (2016) The relation of saturated fatty acids with low-grade inflammation and cardiovascular disease. *J Nutr Biochem*, 36, 1-20.

Ruth, M. R., Wang, Y., Yu, H. M., Goruk, S., Reaney, M. J., Proctor, S. D., Vine, D. F. & Field, C. J. (2010) Vaccenic and elaidic acid modify plasma and splenocyte membrane phospholipids and mitogen-stimulated cytokine production in obese insulin resistant JCR: LA-cp rats. *Nutrients*, 2(2), 181-97.

Santora, J. E., Palmquist, D. L. & Roehrig, K. L. (2000) Trans-vaccenic acid is desaturated to conjugated linoleic acid in mice. *J Nutr*, 130(2), 208-15.

Schaefer, M. B., Schaefer, C. A., Schifferings, S., Kuhlmann, C. R., Urban, A., Benscheid, U., Fischer, T., Hecker, M., Morty, R. E., Vadasz, I., Herold, S., Witzenrath, M., Seeger, W., Erdogan, A. & Mayer, K. (2016) N-3 vs. n-6 fatty acids differentially influence calcium signalling and adhesion of inflammatory activated monocytes: impact of lipid rafts. *Inflamm Res*, 65(11), 881-894.

Sebedio, J. L., Juaneda, P., Dobson, G., Ramilison, I., Martin, J. C., Chardigny, J. M. & Christie, W. W. (1997) Metabolites of conjugated isomers of linoleic acid (CLA) in the rat. *Biochim Biophys Acta*, 1345(1), 5-10.

Serhan, C. N. & Chiang, N. (2013) Resolution phase lipid mediators of inflammation: agonists of resolution. *Curr Opin Pharmacol*, 13(4), 632-40.

Serhan, C. N., Chiang, N. & Van Dyke, T. E. (2008) Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol*, 8(5), 349-61.

Shashkin, P., Dragulev, B. & Ley, K. (2005) Macrophage Differentiation to Foam Cells. *Current Pharmaceutical Design*, 11(23), 3061-3072.

Shen, W., Chuang, C. C., Martinez, K., Reid, T., Brown, J. M., Xi, L., Hixson, L., Hopkins, R., Starnes, J. & McIntosh, M. (2013) Conjugated linoleic acid reduces adiposity and increases markers of browning and inflammation in white adipose tissue of mice. *J Lipid Res*, 54(4), 909-22.

Siddiqui, R. A., Harvey, K. A., Ruzmetov, N., Miller, S. J. & Zaloga, G. P. (2009) n-3 fatty acids prevent whereas trans-fatty acids induce vascular inflammation and sudden cardiac death. *Br J Nutr*, 102(12), 1811-9.

Simons, K. & Toomre, D. (2000) Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol*, 1(1), 31-9.

Singh, A., Gupta, V., Ghosh, A., Lock, K. & Ghosh-Jerath, S. (2015) Quantitative estimates of dietary intake with special emphasis on snacking pattern and nutritional status of free living adults in urban slums of Delhi: impact of nutrition transition. *BMC Nutr*, 1, 22.

Siri-Tarino, P. W., Sun, Q., Hu, F. B. & Krauss, R. M. (2010) Meta-analysis of prospective cohort studies evaluating the association of saturated fat with cardiovascular disease. *Am J Clin Nutr*, 91(3), 535-46.

Skeoch, S. & Bruce, I. N. (2015) Atherosclerosis in rheumatoid arthritis: is it all about inflammation? *Nat Rev Rheumatol*, 11(7), 390-400.

Smedman, A., Basu, S., Jovinge, S., Fredrikson, G. N. & Vessby, B. (2005) Conjugated linoleic acid increased C-reactive protein in human subjects. *Br J Nutr*, 94(5), 791-5.

Smit, L. A., Katan, M. B., Wanders, A. J., Basu, S. & Brouwer, I. A. (2011) A high intake of trans fatty acids has little effect on markers of inflammation and oxidative stress in humans. *J Nutr*, 141(9), 1673-8.

Smith, K. J., McNaughton, S. A., Gall, S. L., Blizzard, L., Dwyer, T. & Venn, A. J. (2009) Takeaway food consumption and its associations with diet quality and abdominal obesity: a cross-sectional study of young adults. *Int J Behav Nutr Phys Act*, 6, 29.

Sneddon, A. A., McLeod, E., Wahle, K. W. & Arthur, J. R. (2006) Cytokine-induced monocyte adhesion to endothelial cells involves platelet-activating factor: suppression by conjugated linoleic acid. *Biochim Biophys Acta*, 1761(7), 793-801.

Soehnlein, O., Drechsler, M., Döring, Y., Lievens, D., Hartwig, H., Kemmerich, K., Ortega-Gómez, A., Mandl, M., Vijayan, S., Projahn, D., Garlichs, C. D., Koenen, R. R., Hristov, M., Lutgens, E., Zernecke, A. & Weber, C. (2013) Distinct functions of chemokine receptor axes in the atherogenic mobilization and recruitment of classical monocytes. *EMBO Molecular Medicine*, 5(3), 471-481.

Sofi, F., Buccioni, A., Cesari, F., Gori, A. M., Minieri, S., Mannini, L., Casini, A., Gensini, G. F., Abbate, R. & Antongiovanni, M. (2010) Effects of a dairy product (pecorino cheese) naturally rich in cis-9, trans-11 conjugated linoleic acid on lipid, inflammatory and haemorheological variables: a dietary intervention study. *Nutr Metab Cardiovasc Dis*, 20(2), 117-24.

Solberg, S. L., Terragni, L. & Granheim, S. I. (2015) Ultra-processed food purchases in Norway: a quantitative study on a representative sample of food retailers. *Public Health Nutr*, 1-12.

Soto-Vaca, A., Losso, J. N., McDonough, K. & Finley, J. W. (2013) Differential effect of 14 free fatty acids in the expression of inflammation markers on human arterial coronary cells. *J Agric Food Chem*, 61(42), 10074-9.

Stachowska, E., Siennicka, A., Baśkiewcz-Hałasa, M., Bober, J., Machalinski, B. & Chlubek, D. (2012) Conjugated linoleic acid isomers may diminish human macrophages adhesion to endothelial surface. *International Journal of Food Sciences and Nutrition*, 63(1), 30-35.

Steck, S. E., Chalecki, A. M., Miller, P., Conway, J., Austin, G. L., Hardin, J. W., Albright, C. D. & Thuillier, P. (2007) Conjugated linoleic acid supplementation for twelve weeks increases lean body mass in obese humans. *J Nutr*, 137(5), 1188-93.

Stender, S., Astrup, A. & Dyerberg, J. (2008) Ruminant and industrially produced trans fatty acids: health aspects. *Food Nutr Res*, 52.

Stewart, C. R., Stuart, L. M., Wilkinson, K., van Gils, J. M., Deng, J., Halle, A., Rayner, K. J., Boyer, L., Zhong, R., Frazier, W. A., Lacy-Hulbert, A., El Khoury, J., Golenbock, D. T. & Moore, K. J. (2010) CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. *Nat Immunol*, 11(2), 155-61.

Stoneman, V. E. A. & Bennett, M. R. (2009) Role of Fas/Fas-L in Vascular Cell Apoptosis. *Journal of Cardiovascular Pharmacology*, 53(2), 100-108.

Sun, Q., Ma, J., Campos, H., Hankinson, S. E., Manson, J. E., Stampfer, M. J., Rexrode, K. M., Willett, W. C. & Hu, F. B. (2007) A prospective study of trans fatty acids in erythrocytes and risk of coronary heart disease. *Circulation*, 115(14), 1858-65.

Szmitko, P. E., Wang, C. H., Weisel, R. D., de Almeida, J. R., Anderson, T. J. & Verma, S. (2003) New markers of inflammation and endothelial cell activation: Part I. *Circulation*, 108(16), 1917-23.

Tabas, I. (2010) Macrophage death and defective inflammation resolution in atherosclerosis. *Nat Rev Immunol*, 10(1), 36-46.

Tabas, I., Garcia-Cardena, G. & Owens, G. K. (2015) Recent insights into the cellular biology of atherosclerosis. *J Cell Biol*, 209(1), 13-22.

Tanimura, N., Saitoh, S., Matsumoto, F., Akashi-Takamura, S. & Miyake, K. (2008) Roles for LPS-dependent interaction and relocation of TLR4 and TRAM in TRIF-signaling. *Biochem Biophys Res Commun*, 368(1), 94-9.

Tasan, M. & Demirci, M. (2003) Trans FA in sunflower oil at different steps of refining. *Journal of the American Oil Chemists' Society*, 80(8), 825-828.

Tedgui, A. & Mallat, Z. (2006) Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev*, 86(2), 515-81.

Teruel, T., Hernandez, R. & Lorenzo, M. (2001) Ceramide mediates insulin resistance by tumor necrosis factor-alpha in brown adipocytes by maintaining Akt in an inactive dephosphorylated state. *Diabetes*, 50(11), 2563-71.

Tholstrup, T., Raff, M., Straarup, E. M., Lund, P., Basu, S. & Bruun, J. M. (2008) An oil mixture with trans-10, cis-12 conjugated linoleic acid increases markers of inflammation and in vivo lipid peroxidation compared with cis-9, trans-11 conjugated linoleic acid in postmenopausal women. *J Nutr*, 138(8), 1445-51.

Tilley, S. L., Coffman, T. M. & Koller, B. H. (2001) Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. *J Clin Invest*, 108(1), 15-23.

Tricon, S., Burdge, G. C., Jones, E. L., Russell, J. J., El-Khazen, S., Moretti, E., Hall, W. L., Gerry, A. B., Leake, D. S., Grimble, R. F., Williams, C. M., Calder, P. C. & Yaqoob, P. (2006) Effects of dairy products naturally enriched with cis-9,trans-11 conjugated linoleic acid on the blood lipid profile in healthy middle-aged men. *Am J Clin Nutr*, 83(4), 744-53.

Tricon, S., Burdge, G. C., Kew, S., Banerjee, T., Russell, J. J., Grimble, R. F., Williams, C. M., Calder, P. C. & Yaqoob, P. (2004a) Effects of cis-9,trans-11 and trans-10,cis-12 conjugated linoleic acid on immune cell function in healthy humans. *Am J Clin Nutr*, 80(6), 1626-33.

Tricon, S., Burdge, G. C., Kew, S., Banerjee, T., Russell, J. J., Jones, E. L., Grimble, R. F., Williams, C. M., Yaqoob, P. & Calder, P. C. (2004b) Opposing effects of cis-9,trans-11 and trans-10,cis-12 conjugated linoleic acid on blood lipids in healthy humans. *Am J Clin Nutr*, 80(3), 614-20.

Tsatsanis, C., Androulidaki, A., Venihaki, M. & Margioris, A. N. (2006) Signalling networks regulating cyclooxygenase-2. *Int J Biochem Cell Biol*, 38(10), 1654-61.

Tsimikas, S., Philis-Tsimikas, A., Alexopoulos, S., Sigari, F., Lee, C. & Reaven, P. D. (1999) LDL isolated from Greek subjects on a typical diet or from American subjects on an oleate-supplemented diet induces less monocyte chemotaxis and adhesion when exposed to oxidative stress. *Arterioscler Thromb Vasc Biol*, 19(1), 122-30.

Tsuzuki, W., Matsuoka, A. & Ushida, K. (2010) Formation of trans fatty acids in edible oils during the frying and heating process. *Food Chemistry*, 123(4), 976-982.

Turpeinen, A. M., Mutanen, M., Aro, A., Salminen, I., Basu, S., Palmquist, D. L. & Griinari, J. M. (2002) Bioconversion of vaccenic acid to conjugated linoleic acid in humans. *Am J Clin Nutr*, 76(3), 504-10.

Tyburczy, C., Major, C., Lock, A. L., Destaillats, F., Lawrence, P., Brenna, J. T., Salter, A. M. & Bauman, D. E. (2009) Individual trans octadecenoic acids and partially hydrogenated vegetable oil differentially affect hepatic lipid and lipoprotein metabolism in golden Syrian hamsters. *J Nutr*, 139(2), 257-63.

Usman, A., Ribatti, D., Sadat, U. & Gillard, J. H. (2015) From Lipid Retention to Immune-Mediate Inflammation and Associated Angiogenesis in the Pathogenesis of Atherosclerosis. *J Atheroscler Thromb*, 22(8), 739-49.

Vadiveloo, M., Scott, M., Quatromoni, P., Jacques, P. & Parekh, N. (2014) Trends in dietary fat and high-fat food intakes from 1991 to 2008 in the Framingham Heart Study participants. *Br J Nutr*, 111(4), 724-34.

Valenzuela, A. & Morgado, N. (1999) Trans fatty acid isomers in human health and in the food industry. *Biol Res*, 32(4), 273-87.

van der Wal, A. C., Das, P. K., Tigges, A. J. & Becker, A. E. (1992) Adhesion molecules on the endothelium and mononuclear cells in human atherosclerotic lesions. *Am J Pathol*, 141(6), 1427-33.

Vesper, H. W., Caudill, S. P., Kuiper, H. C., Yang, Q., Ahluwalia, N., Lacher, D. A. & Pirkle, J. L. (2017) Plasma trans-fatty acid concentrations in fasting adults declined from NHANES 1999-2000 to 2009-2010. *Am J Clin Nutr*, 105(5), 1063-1069.

Vestweber, D. (2015) How leukocytes cross the vascular endothelium. *Nat Rev Immunol*, 15(11), 692-704.

Viladomiu, M., Hontecillas, R. & Bassaganya-Riera, J. (2015) Modulation of inflammation and immunity by dietary conjugated linoleic acid. *Eur J Pharmacol*.

Vink, A., Schoneveld, A. H., van der Meer, J. J., van Middelaar, B. J., Sluijter, J. P., Smeets, M. B., Quax, P. H., Lim, S. K., Borst, C., Pasterkamp, G. & de Kleijn, D. P. (2002) In vivo evidence for a role of toll-like receptor 4 in the development of intimal lesions. *Circulation*, 106(15), 1985-90.

Viola, J. & Soehnlein, O. (2015) Atherosclerosis - A matter of unresolved inflammation. *Semin Immunol*, 27(3), 184-93.

Walker, C. G., West, A. L., Browning, L. M., Madden, J., Gambell, J. M., Jebb, S. A. & Calder, P. C. (2015) The Pattern of Fatty Acids Displaced by EPA and DHA Following 12 Months Supplementation Varies between Blood Cell and Plasma Fractions. *Nutrients*, 7(8), 6281-93.

Wall, R., Ross, R. P., Fitzgerald, G. F. & Stanton, C. (2010) Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. *Nutr Rev*, 68(5), 280-9.

Wang, L., Lim, E. J., Toborek, M. & Hennig, B. (2008a) The role of fatty acids and caveolin-1 in tumor necrosis factor alpha-induced endothelial cell activation. *Metabolism*, 57(10), 1328-39.

Wang, T., Lim, J. N., Lee, J. S., Lee, S. B., Hwang, J. H., Jung, U. S., Kim, M. J., Hwang, D. Y., Lee, S. R., Roh, S. G. & Lee, H. G. (2015) Effects of dietary trans-9 octadecenoic acid, trans-11 vaccenic acid and cis-9, trans-11 conjugated linoleic acid in mice. *Mol Med Rep*, 12(2), 3200-6.

Wang, Y., Jacome-Sosa, M. M., Ruth, M. R., Goruk, S. D., Reaney, M. J., Glimm, D. R., Wright, D. C., Vine, D. F., Field, C. J. & Proctor, S. D. (2009) Trans-11 vaccenic acid reduces hepatic lipogenesis and chylomicron secretion in JCR:LA-cp rats. *J Nutr*, 139(11), 2049-54.

Wang, Y., Jacome-Sosa, M. M., Ruth, M. R., Lu, Y., Shen, J., Reaney, M. J., Scott, S. L., Dugan, M. E. R., Anderson, H. D., Field, C. J., Proctor, S. D. & Vine, D. F. (2012) The intestinal bioavailability of vaccenic acid and activation of peroxisome proliferator-activated receptor-α and -γ in a rodent model of dyslipidemia and the metabolic syndrome. *Molecular Nutrition & Food Research*, 56(8), 1234-1246.

Wang, Y., Lu, J., Ruth, M. R., Goruk, S. D., Reaney, M. J., Glimm, D. R., Vine, D. F., Field, C. J. & Proctor, S. D. (2008b) Trans-11 vaccenic acid dietary supplementation induces hypolipidemic effects in JCR:LA-cp rats. *J Nutr*, 138(11), 2117-22.

Wassall, S. R., Leng, X., Canner, S. W., Pennington, E. R., Kinnun, J. J., Cavazos, A. T., Dadoo, S., Johnson, D., Heberle, F. A., Katsaras, J. & Shaikh, S. R. (2018) Docosahexaenoic acid regulates the formation of lipid rafts: A unified view from experiment and simulation. *Biochim Biophys Acta Biomembr*, 1860(10), 1985-1993.

Weber, C., Zernecke, A. & Libby, P. (2008) The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. *Nat Rev Immunol*, 8(10), 802-815.

WHO (2015) *Eliminating trans fats in Europe. A policy brief. Copenhagen: World Health Organization Regional Office for Europe*.

WHO (2018) *Global Health Estimates 2016: Deaths by Cause, Age, Sex, by Country and by Region, 2000-2016. Geneva, World Health Organization.*

Willett, W. C., Stampfer, M. J., Manson, J. E., Colditz, G. A., Speizer, F. E., Rosner, B. A., Sampson, L. A. & Hennekens, C. H. (1993) Intake of trans fatty acids and risk of coronary heart disease among women. *Lancet*, 341(8845), 581-5.

Wong, S. W., Kwon, M. J., Choi, A. M., Kim, H. P., Nakahira, K. & Hwang, D. H. (2009) Fatty acids modulate Toll-like receptor 4 activation through regulation of receptor dimerization and recruitment into lipid rafts in a reactive oxygen species-dependent manner. *J Biol Chem*, 284(40), 27384-92.

Xu, X. H., Shah, P. K., Faure, E., Equils, O., Thomas, L., Fishbein, M. C., Luthringer, D., Xu, X. P., Rajavashisth, T. B., Yano, J., Kaul, S. & Arditi, M. (2001) Toll-like receptor-4 is expressed by macrophages in murine and human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL. *Circulation*, 104(25), 3103-8.

Yadav, R., Larbi, K. Y., Young, R. E. & Nourshargh, S. (2003) Migration of leukocytes through the vessel wall and beyond. *Thromb Haemost*, 90(4), 598-606.

Yu, Y., Correll, P. H. & Vanden Heuvel, J. P. (2002) Conjugated linoleic acid decreases production of pro-inflammatory products in macrophages: evidence for a PPARγ-dependent mechanism. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1581(3), 89-99.

Zapolska-Downar, D., Kosmider, A. & Naruszewicz, M. (2005) Trans fatty acids induce apoptosis in human endothelial cells. *J Physiol Pharmacol*, 56(4), 611-25.

Zernecke, A. & Weber, C. (2014) Chemokines in atherosclerosis: proceedings resumed. *Arterioscler Thromb Vasc Biol*, 34(4), 742-50.