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# IMPLEMENTATION OF *IN VITRO* SCREENING ASSAYS TO TEST INFLAMMATION, THROMBOSIS AND ENDOTHELIAL FUNCTION ON CELLS

Laia PONS LLECHA

European Thesis
Doctorat en Nutrició i Metabolisme
Departament de Medicina i Cirurgia
Unitat de Recerca en Lípids i Arteriosclerosi


Reus, 2010

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| Títol de la tesi doctoral<br>**IMPLEMENTATION OF *IN VITRO* SCREENING ASSAYS TO TEST INFLAMMATION, THROMBOSIS AND ENDOTHELIAL FUNCTION ON CELLS** | |
| Doctorand/a<br>**Laia PONS LLECHA** | |
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| Departament<br>Medicina i Cirurgia | Grup de recerca<br>Unitat de Recerca en Lípids i Arteriosclerosi |
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**- Resveratrol reduces tissue factor mRNA and protein expression in human aortic endothelial cells**
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**- Improvement of endothelial dysfunction produced by alpha-tocopherol and BAY11-7082 reducing vascular cell adhesion molecule-1**
Laia Pons-Llecha MSc[1,3], Ursula Catalan MSc[1,3], Sara Fernandez-Castillejo BSc[1,3], Mercedes Heras DipTech[1], Neus Angles PhD[2], Jose Morello[2], Rosa Sola MD, PhD[1.] (sotmès a l'editor)

- **Methodological aspects to reduce variability in the study of inflammation with LPS-stimulated human THP-1 monocytes to secrete TNF-α.**
Sara Fernández-Castillejo*[&], Laia Pons*[&], Úrsula Catalán*[&], Mercedes Heras*, Cecilia González*, Roser Rosales*, Josefa Girona*, Lluís Masana*, Neus Anglés, Jose Morelló, Rosa Solà*[&] (sotmès a l'editor)

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Reus, 4 de desembre de 2009

Nom i cognoms. Rosa SOLÀ ALBERICH.
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# Resum

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## Resum

### Introducció

Les malalties cardiovasculars (MCV) són la primera causa de morbimortalitat dels països industrialitzats en el segle XXI i, l'arteriosclerosi, n'és el principal procés patològic.

Els compostos naturals o els extractes obtinguts a partir dels aliments tenen efectes beneficiosos en els mecanismes relacionats en la patogènesi de les MCV com és ara la inflamació, la funció endotelial i la trombosi. Els models experimentals cel·lulars i*n vitro* són una eina efectiva per estudiar aquests mecanismes i podrien ser útils per valorar els efectes beneficiosos dels productes bioactius naturals.

### Hipòtesi

Els models cel·lulars *in vitro* són efectius a l'hora de valorar les propietats biològiques d'ingredients, compostos o extractes obtinguts d'aliments. L'evidència científica es pot traslladar en models animals o humans.

### Objectiu

Implementar i estandaritzar metodologia experimental per monitoritzar els efectes de compostos o extractes obtinguts a partir d'aliments en un sistema anti-inflamatori basat en monòcits THP-1 com també en la funció endotelial i la resposta anti-trombòtica en cèl·lules endotelials d'aorta humanes (HAEC).

### Material i mètodes

S'han desenvolupat 4 models cel·lulars:

1. Inducció d'inflamació:

Els monòcits THP-1 s'estimulen durant 4h amb Lipopolisacàrids (LPS; 0-2000 ng/mL), amb absència o presencia de *foetal bovine serum* (FBS; 0-10%) al llarg de 6, 12 i 19 dies en cultiu. Es va determinar la secreció proteica i expressió del mRNA del factor de necrosis tumoral alfa (TNF-α). Aquestes condicions es van estudiar utilitzant 2 lots de monòcits THP-1 per valorar la influència de cadascun en l'expressió de proteïna i del mRNA del TNF-α.

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2. Inducció de la disfunció endotelial:

Les HAEC van ser estimulades amb TNF-α a diferents dosis (5-20 ng/mL o 1-10 ng/mL) durant 24h per determinar la dosi-resposta a nivell de proteïna i de mRNA de la molècula d'adhesió cel·lular vascular -1 (VCAM-1).

Les HAEC es van incubar amb BAY 11-7082 (BAY; 0.5, 1, 2 i 10 μM) i TNF-α (10 ng/mL) durant 24h per analitzar la secreció de proteïna de la VCAM-1 en el medi de cultiu cel·lular.

Les HAEC es van incubar amb BAY (0.1-0.2 μM) i TNF-α (1 ng/mL) durant 24h per determinar l'expressió del mRNA de la VCAM-1.

Les HAEC van ser incubades amb α-tocoferol (AT; 10-150 μM) durant 6h i posteriorment estimulades amb TNF-α (10 ng/mL) durant 24h, per determinar la secreció proteica de VCAM-1 i TNF-α (1 ng/mL) per analitzar l'expressió del mRNA de la VCAM-1.

3. Valoració de la funció endotelial:

Les HAEC es van preincubar amb TNF-α (5 and 10 ng/mL) durant 24h o amb TNF-α (10 ng/mL) durant 2, 4 i 24h en medi complet (suplementat amb 2% de FBS; 10 ng/mL del factor epidèrmic de creixement humà, 3 ng/mL de factors de creixement de fibroblast, 10 μg/mL heparina i 1 μg/mL hidrocortisona, 10 μg/ml gentamicina, 0.25 μg/ml solució B d'amfotericina, 100U/mL penicil·lina, 100 μg/mL estreptomicina). Es va determinar l'expressió del mRNA de la *endothelial nitric oxide synthase* (eNOS).

Les HAEC es van exposar a insulina (10, 100, 500, 1000, 5000 nM) durant 24h amb medi complet en HAEC pretractades (si és el cas) amb medi basal (suplementat només amb el 2% de FBS més els antibiòtics però sense els altres components citats anteriorment) durant 2 i 8h. Es va analitzar l'expressió del mRNA de la eNOS.

4. Estudi dels efectes trombòtics:

Les HAEC es van preincubar amb resveratrol a 5, 10, 25, 50 i 100 μM durant 16h i posteriorment es van estimular amb TNF-α (10 ng/mL) durant 6h per estudiar la proteïna i el mRNA del factor tissular (TF). Els mètodes utilitzats són la lisi cel·lular amb *tris buffered saline* (TBS) per l'evaluació de la proteïna TF, i *nucleic acid purification lysis solution* per l'anàlisi del mRNA del TF.

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La quantificació de la secreció proteica dels estudis es va fer a través del test específic ELISA i el mRNA per real-time RT-PCR.

La citotoxicitat dels compostos sobre les cèl·lules s'ha determinat pel test colorimètric de la lactat deshidrogenasa (LDH) i la viabilitat cel·lular mitjançant el test d'exclusió de *Trypan blue*.

Els coeficients de variació (CV) es presenten com el valors més alts obtinguts a partir del càlcul del CV intra- i/o inter-assaig. Es determina, la mitjana, la desviació estàndard (SD) i l'error estàndard de la mitja (SEM).

**Resultats**

1. L'òptima expressió de la secreció proteica i del mRNA del TNF-α s'han trobat en cultius amb un alt contingut de LPS i un elevat contingut de FBS. En períodes llargs de cultiu de monòcits THP-1(19 dies), hi ha un augment de 5 vegades de l'expressió de TNF-α, respecte 6 dies de cultiu ($p<0.05$). Utilitzant les mateixes condicions, la resposta observada del TNF-α varia segons el lot de cèl·lules.

Les condicions experimentals adequades per a aquest model són l'estimulació dels monòcits amb LPS (500-1000 ng/mL) depenent del lot de cèl·lules, durant 4h després de 6 dies de cultiu amb un 10% de FBS en el medi de cultiu. El CV és <20%.

2. El TNF-α, a 10 ng/mL, augmenta entre 3 i 6 vegades la concentració proteica de la VCAM-1 en el medi de cultiu, comparat amb les cèl·lules no tractades ($p<0.05$). El CV és <17%.

El TNF-α, a 1 ng/mL, incrementa 3.6 i 10 vegades l'expressió de mRNA de la VCAM-1 ($p<0.05$). El CV és <13%.

La presència de BAY (0.5 i 1μM) redueix la concentració de la secreció de la VCAM-1 en el medi de cultiu entre un 45-70% respectivament ($p<0.05$; CV <13%) i a una concentració de 0.1 μM disminueix l'expressió del mRNA al voltant del 30%, comparat amb la màxima estimulació ($p<0.05$; CV <14%).

L'AT a 75 i 150 μM redueix un 33% l'expressió proteica de la VCAM-1 i a 50, 75 i 150 μM, un 30% l'expressió del mRNA de l'esmentada molècula

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(p<0.05; CV < 9; CV < 27% respectivament) sense observar un efecte dosi-depenent.

3. Les HAEC estimulades amb TNF-α (5 i 10 ng/mL) durant 24h, disminueixen l'expressió de mRNA de la eNOS en un 40-50% respectivament, comparat amb cèl·lules no estimulades (p<0.05; CV <21%). Els nivells de mRNA de la eNOS van disminuir quan es van tractar les HAEC amb TNF-α (10 ng/mL) durant 4h però només a 24h es va aconseguir una reducció significant del 50%, comparat amb cèl·lules no tractades (p<0.05).

El pretractament perllongat (18h) amb medi basal previ a la incubació amb insulina (500 i 1000 nM), durant les següents 24h augmenta l'expressió del mRNA de la eNOS en un 40-50%, respectivament, comparat amb cèl·lules no tractades amb insulina (p<0.05). El CV és <13%.

Per altra banda, les HAEC pretractades 2h amb medi basal sense pretractament i amb medi basal seguit d'una estimulació amb Insulina (100, 500 i 1000 nM) durant 24h, no produeix l'increment del mRNA de la eNOS.

4. El resveratrol (5, 10, 25, 50 i 100 μM) redueix el TF comparat amb el TNF- α sol, de forma dosi-depenent entre 25 i 100 μM (p<0.05). El resveratrol, a 100 μM, disminueix fins a un 90% la concentració proteica de TF i un 60% l'expressió de mRNA en HAEC exposades a TNF-α amb comparació amb HAEC no estimulades (CV <22%, CV<6%; p<0.05, respectivament).

Les HAEC i els monòcits THP-1 no van mostrar citotoxicitat a cap de les condicions testades.

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**Conclusions**

1. L'activació dels monòcits THP-1 depèn de la dosi de LPS, del contingut de FBS en el medi de cultiu, dels dies que les cèl·lules estan en cultiu i del lot de monòcits utilitzades en els experiments.
2. El TNF-α és un bon candidat per induir la disfunció endotelial en HAEC i estudiar la secreció proteica i de mRNA de la VCAM-1 i el TF.
3. El BAY i l'AT es poden utilitzar com a controls positius en HAEC estimulades amb TNF-α, ja que provoquen l'efecte desitjat al reduir l'alliberació de la concentració de la proteïna VCAM-1 en el medi de cultiu i la seva expressió de mRNA.
4. El TNF-α és un control negatiu mentre que la insulina n'és un de positiu per l'estudi de l'expressió del mRNA de la eNOS en HAEC.
5. El resveratrol és un control positiu degut als seus efectes anti-trombòtics ja que redueix la concentració de proteïna i l'expressió de mRNA del TF en HAEC estimulades amb TNF-α.

Així, els models cel·lulars *in vitro* són un sistema vàlid per estudiar i seleccionar compostos o extractes amb possibles beneficis sobre mecanismes involucrats en la patogènia de les MCV.

Font de finançament: Aquest treball ha estat realitzat dins de les activitats del proyecto CENIT 2006-2009, MET-DEV-FUN, liderat per la Morella Nuts (Reus), del Centro de Desarrollo Tecnológico e Industrial (CDTI), Ministerio de Ciencia e Innovación, España.

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## Summary

**Introduction:** Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in industrialised countries in the 21st Century, and atherosclerosis is the main pathological process leading to CVD.

Natural compounds or extracts obtained from foods have beneficial effects on the mechanisms underlying the pathogenesis of CVD such as inflammation, endothelial function and thrombosis. *In vitro* cellular experimental models are effective instruments to explore. Hence, *in vitro* cellular models could be useful in assessing the putative beneficial effects of natural bioactive products.

### Hypothesis

*In vitro* cell models are effective preliminary tools for screening the biological properties of ingredients, compounds and extracts of foodstuffs. The scientific evidence could then be explored in animal models or in humans.

### Objectives

To implement and standardise experimental methodology to monitor the effect of compounds or extracts obtained from food on anti-inflammatory system of monocytes THP-1 cells, as well as the endothelial function and the anti-thrombotic responses of human aortic endothelial cells (HAECs).

### Material and Methods

Four cellular models have been developed

1. Induction of inflammation:

Monocytes THP-1 cells challenged with a dose-response of lipopolysaccharide (LPS; 0-2000 ng/mL), 4h incubation, in the absence or presence of foetal bovine serum (FBS; 0-10%) in culture medium for 6, 12 and 19 days incubation and tumour necrosis factor-alpha (TNF-$\alpha$) protein release and mRNA expression are assessed. These conditions are studied using 2 different batches of monocytes THP-1 cells to assess cell-batch influence on TNF-$\alpha$ protein release and its mRNA expression.

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2. Induction of endothelial dysfunction:

HAEC challenged with TNF-α at different doses (5-20 ng/mL or 1-10 ng/mL) for 24h, to determine the dose-response of vascular cell adhesion molecule-1 (VCAM-1) protein release or the VCAM-1 mRNA expression, respectively.

HAEC were incubated with BAY 11-7082 (BAY; 0.5, 1 and 10 μM) and TNF-α (10 ng/mL) for 24h to analyse the VCAM-1 protein release in the culture medium. HAEC were incubated with BAY (0.1-0.2 μM) and TNF-α (1 ng/mL) for 24h to determine VCAM-1 mRNA expression.

HAEC were incubated with alpha-tocopherol (AT; 10-150 μM) for 6h, then stimulated with TNF-α (10 ng/mL) for 24h, to determine VCAM-1 protein release, and TNF-α (1 ng/mL) to analyse VCAM-1 mRNA expression.

3. Assessment of endothelial function:

HAEC were incubated with TNF-$\alpha$ (5 and 10 ng/mL) for 24h, or TNF-α (10 ng/mL) for 2, 4 and 24h in complete medium (supplemented with 2% FBS, 10 ng/mL human epidermal growth factor, 3 ng/mL basic fibroblast growth factor, 10 μg/mL heparin and 1 μg/mL hydrocortisone, 10 μg/ml gentamicin, 0.25 μg/ml amphotericin B solution, 100U/mL penicillin, 100 μg/mL streptomycin) and mRNA expression of endothelial nitric oxide synthase (eNOS) was determined.

HAEC were exposed to insulin (10, 100, 500, 1000, 5000 nM) for a further 24h in complete medium in which the HAEC had (or had not) been pre-treated with basal medium (supplemented only with 2% FBS plus the antibiotics but without the other components, above) for 2 or 18h, and eNOS mRNA expression were analysed.

4. Study of thrombotic effects:

HAEC were pre-incubated with resveratrol at 5, 10, 25, 50 or 100 μM for 16h, then challenged with TNF-α (10 ng/mL) over 6h incubation, to assess the tissue factor (TF) protein or TF mRNA expression. The methods used are cell lysis with tris buffered saline (TBS) for TF protein evaluation, and nucleic acid purification lysis solution for TF mRNA expression assessment.

All protein release measurements were by specific ELISA test, and all mRNA by real-time RT-PCR.

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The cytotoxicity of compounds on cells is determined by the lactate dehydrogenase (LDH) colorimetric test, and cell viability by Trypan blue exclusion in all the above models.

The coefficients of variation (CV) are presented as the highest values obtained from intra- and/or inter-assay, mean, standard deviation (SD) and the standard error of the mean (SEM) are calculated.

**Results**

1. The optimum TNF-α protein release and mRNA expression are found in cultures containing high LPS concentrations and with the maximum FBS content. With prolonged periods of monocytes THP-1 cell cultures (19 days) there are 5-fold increases in TNF-α compared to that at 6 days of culture ($p<0.05$). Under the same conditions, the observed TNF-α response varies according to the cell batch.

Optimum experimental conditions for this model are to stimulate monocytes with LPS (500-1000 ng/mL, depending on the monocytes THP-1 cell batch) for 4h, following 6 days of culture, plus 10% FBS in the culture medium. The CVs of measurement are <20%.

2. TNF-α at 10 ng/mL produced a range from 3 to 6-fold an increase in VCAM-1 protein concentration in the cell culture medium, compared to untreated cells ($p<0.05$). CV are <17%.

At TNF-α concentration of 1 ng/mL, ranged from 3.6 to 10-fold an increases in the mRNA expression of the VCAM-1 ($p<0.05$). CV <40% are observed.

The presence of BAY (0.5-1 μM) reduces, by 45 and 70% the VCAM-1 protein release, respectively ($p<0.05$) (CV <7%) and at 0.1 μM the VCAM-1 mRNA expression is diminished by 30%, compared with maximal stimulation ($p<0.05$) (CV <14%).

AT at 75 and 150 μM reduces the VCAM-1 protein release by 33%, in both cases, and AT at 50, 75 and 150 μM reduces VCAM-1 mRNA expression by 30%, compared with maximal stimulation ($p<0.05$), with no dose-dependent effect. CV are <12% and <27%, respectively.

3. HAEC challenged with TNF-$\alpha$ (at 5 and 10 ng/mL) for 24h, causes a decrease in eNOS mRNA expression by 40 and 50%, respectively,

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compared with untreated cells ($p<0.05$). CV was <21%. eNOS mRNA levels decreased when HAEC were stimulated with TNF-$\alpha$ (10 ng/mL) for 4h but only at 24h was the reduction of 50% significant, compared to untreated cells ($p<0.05$).

HAEC prolonged pre-treatment (18h) with basal culture medium, then incubated with insulin (at 500 and 1000 nM) for 24h, caused eNOS mRNA expression increase of 40 and 50%, respectively, compared to untreated cells with insulin ($p<0.05$). CV is <13%.

However, HAEC pre-treatment for 2h with basal medium, or without pre-treatment with basal medium followed by insulin stimulation (100, 500 and 1000 nM) for 24h, produce no eNOS mRNA increase.

4. Resveratrol (5, 10, 25, 50 and 100 μM), relative to TNF-α-stimulated HAECs, reduced the TF in a dose-dependent manner from 25 to 100 μM ($p<0.05$). At 100 μM the decrease in the TF protein concentration was 90% and decrease in its mRNA expression was 60% in HAEC exposed to TNF-α ($p<0.05$). CV were <22% and <6%, respectively.

HAEC and monocytes THP-1 cells show no cytotoxicity in all experimental conditions while cell viability, assessed by trypan blue exclusion, is between 92-98%.

**Conclusions**

1. Monocytes THP-1 cells activation depends on LPS dose, on FBS content of the culture medium, on the duration of culture, and on the monocytes THP-1 cell-batch used for the experiments.

2. TNF-α is a good candidate to act as a stressor to induce endothelial dysfunction in HAEC, as well as to study VCAM-1 protein and TF protein, as well as mRNA expression.

3. BAY and AT could be used as positive controls in TNF-α-stimulated HAECs since they cause the desired effect by reducing the secretion of VCAM-1 protein in the cell culture medium, and its mRNA expression.

4. TNF-$\alpha$ is a good negative control, while insulin is a good positive control, for the study of mRNA expression of eNOS in HAEC.

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5. Resveratrol is a positive control because of its antithrombotic effect since it reduces the protein concentration and the mRNA expression of TF in TNF-α-stimulated HAECs.

Thus, these *in vitro* cellular models are valid systems for the study of novel compounds or food extracts that are considered to have potential beneficial effect on mechanisms involved in the pathogenesis of CVD.

In the implementation of the presented *in vitro* models, we have used several bioactive natural extracts given by la Morella Nuts for the screening of anti-inflammatory and anti-thrombotic activities and improvement of endothelial function. However, we cannot cite the results obtained using these extracts since they are protected by confidentiality.

Financial support: This work was supported by *Proyecto CENIT* 2006-2009, MET-DEV-FUN with the leadership of *la Morella Nuts*, and a grant from the *Centro de Desarrollo Tecnológico e Industrial* (CDTI) of the *Ministerio de Ciencia e Innovación* (Spain).

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# Abbreviations

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## Abbreviations

| | |
|---|---|
| **Akt** | Kinase protein B (PKB) |
| **AT** | Alpha tocopherol |
| **ATP** | Adenosine triphosphate |
| **BAEC** | Bovine aortic endothelial cells |
| **BAY11-7082** | (E)3-[(4-Methylphenyl)sulfonyl]-2-propenenitrile |
| **BP** | Blood pressure |
| **$Ca^{2+}$** | Ion calcium |
| **CRP** | C-reactive protein |
| **$C_t$** | Cycle Threshold |
| **CV** | Coefficients of Variation |
| **CVD** | Cardiovascular Diseases |
| **DMSO** | Dimethyl sulfoxide |
| **DSMZ** | Deutsche Sammlung von Mikroorganismen und Zellkulturen; German Collection of Microorganisms and Cell Cultures |
| **ECVAM** | European Center for Validation of Alternative Methods |
| **E-Selectin** | Cell adhesion molecule E |
| **ELISA** | Enzyme-Linked ImmunoSorbent Assay |
| **eNOS** | Endothelial nitric oxide synthase |
| **EBV** | Epstein-Barr virus |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **EtOH** | Ethanol |
| **FBS** | Fetal Bovine Serum |
| **FDA** | Federal Drug Administration |
| **GAPDH** | Glyceraldehyde 3-phosphate dehydrogenase |

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## Abbreviations

| | |
|---|---|
| **HAEC** | Human aortic endothelial cells |
| **HBV** | Hepatitis B virus |
| **HCV** | Hepatitis C virus |
| **HDL** | High density lipoprotein |
| **HHLV-II** | Human T-cell lymphotropic virus I/II |
| **HHV-8** | Human Herpesvirus 8 |
| **HIV** | Human immunodeficiency virus |
| **HRP** | Horseradish peroxidase |
| **HUVEC** | Human umbilical vein endothelial cells |
| **ICAM-1** | Intracellular Cell Adhesion Molecule 1 |
| **IL** | Interleukin |
| **IFN** | Interferon |
| **LBP** | Lipopolysaccharide Binding Protein |
| **LDH** | Lactate dehydrogenase |
| **LDL** | Low density lipoprotein |
| **LPS** | Lipopolysaccharide |
| **LSGS** | Low Serum Growth Supplement |
| **M-200** | HAEC cell culture medium |
| **MCP-1** | Monocyte chemoattractant protein 1 |
| **MD2** | Myeloid Differentiation 2 receptor |
| **Mg** | Milligram |
| **$MgCl_2$** | Magnesium chloride |
| **$Mg^{2+}$** | Ion magnesium |
| **µM** | Micromolarity |

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| | |
|---|---|
| **mRNA** | Messenger ribonucleic acid |
| **NaCl** | Sodium chloride |
| **$NAD^+$** | Nicotinamide adenine dinucleotide (oxidizing agent) |
| **NaOH** | Sodium hydroxide |
| **NF-κB** | Nuclear factor κB |
| **Ng** | Nanogram |
| **ng/mL** | Nanogram/millilitre |
| **nM** | Nanomolarity |
| **NO** | Nitric oxide |
| **OD** | Optical Density |
| **Ox-LDL** | Oxidised low density lipoprotein |
| **pM** | Picomolarity |
| **P/S** | Penicillin/Streptomycin |
| **P3** | Passage 3 |
| **P4** | Passage 4 |
| **P5** | Passage 5 |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerase chain reaction |
| **PDGF** | Platelet-derived growth factor |
| **Pg/mL** | Picogram/millilitre |
| **PROT** | Protein |
| **ROS** | Reactive oxygen species |
| **rt RT-PCR** | Real time Reverse Transcription Polymerase Chain Reaction |
| **RT-PCR** | Reverse Transcription Polymerase Chain Reaction |

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## Abbreviations

| | |
|---|---|
| **SD** | Standard Deviation |
| **SDS** | Sodium dodecyl sulfate |
| **SEM** | Standard Error of the Mean |
| **SMRV** | Squirrel monkey retrovirus |
| **STR** | Short tandem repeat |
| **sVCAM-1** | Soluble vascular cell adhesion molecule |
| **T-25** | 25-$cm^2$ culture flasks |
| **T-75** | 75-$cm^2$ culture flasks |
| **TBS** | Tris Buffered Saline |
| **TE** | Trypsin inhibitor |
| **TF** | Tissue factor |
| **TGF** | Transforming growth factor |
| **TLR4** | Toll-Like Receptor 4 |
| **TN** | Trypsin neutralizer |
| **TNF-α** | Tumor Necrosis Factor Alpha |
| **TRIS** | Tris(hydroxymethyl)aminomethane |
| **V/V** | Volume/volume |
| **VCAM-1** | Vascular cell adhesion molecule |

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# Introduction

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## 1. CARDIOVASCULAR DISEASES

Cardiovascular disease (CVD) is a term that refers to more than one disease of the circulatory system including the heart and blood vessels. These diseases are increasing in prevalence and represent a major public health burden in the 21st century (Olofsson SO, 2007). Thus, CVD, notably atherosclerosis, will become the leading global cause of the total disease burden.

The Mediterranean Region is facing a growing epidemic of CVD due to an ageing population and socioeconomic change. Because of modifications in dietary and lifestyle patterns, CVD is becoming an increasingly significant cause of disability and premature death in both developing and newly developed countries, placing additional burdens on already overtaxed national health budgets. Although many CVDs can be treated or prevented, CVDs cause 17.5 million deaths worldwide every year. By 2010, CVD is estimated to be the leading cause of death in developing countries (FAO; WHO).

Epidemiologic studies and randomised clinical trials have provided compelling evidence that CVD is largely preventable. However, there is also reason to believe that there is a heritable component to the disease (Nabel EG, 2003).

### 1.1 ATHEROSCLEROSIS

Atherosclerosis, the main pathological process leading to CVD, cerebral and peripheral artery disease, can lead to ischemia of the heart, brain or extremities, resulting in infarction. Atherosclerosis is considered to be an inflammatory disease that begins early in life and progresses gradually through adolescence and early adulthood (Hansson GK, 2005; Libby P, 2002; Packard RR, 2008; Stoll G, 2006; Tan KT, 2008; Ross R, 1999). Atherosclerosis is a pathological condition in which arteries undergo thickening of their intimal regions and lose their elasticity and their lesions

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(atheromata) are asymmetric focal thickenings of the innermost layer of the artery, the intima (Hansson GK, 2005).

However, atherosclerosis itself is rather asymptomatic (Itabe H, 2003). The aetiology of atherosclerosis is multifactorial.

### 1.1.1 Clinical manifestations of atherosclerosis

Although many generalised or systemic risk factors contribute to its development, atherosclerosis affects various regions of the circulatory system preferentially and yields distinct clinical manifestations depending on the particular circulatory bed affected. Atherosclerosis of the coronary arteries commonly causes myocardial infarction and angina pectoris.

Atherosclerosis of the arteries supplying the central nervous system frequently provokes strokes and transient cerebral ischemia. In the peripheral circulation, atherosclerosis causes intermittent claudication and gangrene and can jeopardise limb viability (Libby P, 2009).

### 1.1.2 Initiation of atherosclerosis

#### *1.1.2.1 Early phases of atherosclerosis: The "fatty streak" formation*

An integrated view of experimental results in animals and studies of human atherosclerosis suggests that the "fatty streak", a pure inflammatory lesion consisting only of monocyte-macrophages and T lymphocytes, represents the initial lesion of atherosclerosis (Hansson GK, 2005; Ross R, 1999).

These early lesions most often seem to arise from focal increases in the content of lipoproteins within regions of the intima. This accumulation of lipoprotein particles may not simply result from an increased permeability, or "leakiness," of the overlying endothelium. Rather, these lipoproteins may collect in the intima of arteries because they bind to constituents of the extracellular matrix, increasing the residence time of the lipid-rich particles

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within the arterial wall (Packard RR, 2008). Lipoproteins that accumulate in the extracellular space of the intima of arteries often associate with glycosaminoglycans of the arterial extracellular matrix, an interaction that may slow the egress of these lipid-rich particles from the intima. Lipoprotein particles in the extracellular space of the intima, particularly those retained by binding to matrix macromolecules, may undergo oxidative modifications.

Considerable evidence suggests that there is a pathogenic role for products of oxidised lipoproteins in atherogenesis. Lipoproteins sequestered from plasma antioxidants in the extracellular space of the intima become particularly susceptible to oxidative modification, giving rise to hydroperoxides, lysophospholipids, oxysterols, and aldehydic breakdown products of fatty acids and phospholipids. Modifications of the apoprotein moieties may include breaks in the peptide backbone and derivatization of certain amino acid residues. Local production of hypochlorous acid by myeloperoxidase associated with inflammatory cells within the plaque yields chlorinated species such as chlorotyrosyl of such oxidation products in atherosclerotic lesions (Libby P, 2009).

Although the fatty streak commonly precedes the development of a more advanced atherosclerotic plaque, not all fatty streaks progress to form complex atheromata.

#### *1.1.2.2 Leukocyte Recruitment*

Accumulation of leukocytes characterises the formation of early atherosclerotic lesions. Thus, from its very inception, atherogenesis involves elements of inflammation, a process that now provides a unifying theme in the pathogenesis of this disease. The inflammatory cell types typically found in the evolving atheroma include monocyte-derived macrophages and lymphocytes. A number of adhesion molecules or receptors for leukocytes expressed on the surface of the arterial endothelial cell are likely to participate in the recruitment of leukocytes to the nascent atheroma. Constituents of oxidatively modified low-density lipoprotein (LDL) can

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augment expression of leukocyte adhesion molecules (Harrison's online, 2009).

Laminar shear forces such as those encountered in most regions of normal arteries can also suppress the expression of leukocyte adhesion molecules. Sites of predilection for atherosclerotic lesions (e.g., branch points) often have disturbed laminar flow. The ordered, pulsatile laminar shear of normal blood flow augments the production of nitric oxide (NO) by endothelial cells. This molecule, in addition to its vasodilator properties, can act at the low levels constitutively produced by arterial endothelium as a local anti-inflammatory autacoid, limiting the expression of local adhesion molecules. Exposing endothelial cells to laminar shear stress increases the transcription of Kruppel-like factor 2 (KLF2) and reduces the expression of a thioredoxin-interacting protein (Txnip), which inhibits the activity of thioredoxin. KLF2 augments the activity of endothelial nitric oxide synthase (eNOS), and reduced Txnip levels boost the function of the endogenous antioxidant thioredoxin.

Laminar shear stress also stimulates endothelial cells to produce superoxide dismutase, an antioxidant enzyme. These examples indicate how haemodynamic forces may influence the cellular events that underlie atherosclerotic lesion initiation and provide a potential explanation for the favoured localization of atherosclerotic lesions at sites that experience disturbance due to laminar shear stress.

Recruitment of monocytes into the intima occurs early in the formation of the atherosclerotic plaque. Once captured on the surface of the arterial endothelial cell by adhesion receptors, monocytes and lymphocytes penetrate the endothelial layer and take up residence in the intima. In general, leukocyte extravasion initiates any inflammatory process. While leukocytes do not adhere to a normal endothelium, they do adhere to an activated endothelium. In any type of inflammation, molecules expressed on the cell surface of activated endothelial cells mediate leukocyte adhesion and transmigration in the intima (De Caterina R, 2007). In addition to

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products of modified lipoproteins, cytokines (protein mediators of inflammation) can regulate the expression of adhesion molecules involved in leukocyte recruitment. For example, interleukin 1 (IL-1) or Tumor Necrosis Factor-α (TNF-α) induces or augments the expression of leukocyte adhesion molecules on endothelial cells.

Because the products of lipoprotein oxidation can induce cytokine release from vascular wall cells, this pathway may provide an additional link between arterial accumulation of lipoproteins and leukocyte recruitment (figure 1). Chemoattractant cytokines such as monocyte chemoattractant protein 1 (MCP-1) appear to direct the migration of leukocytes into the arterial wall (Libby P, 2009). Firm adhesion of leukocytes involves adhesion molecules of the immunoglobulin superfamily, i.e., vascular-cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM) ICAM-1, -2, -3 and platelet endothelial cell adhesion molecule (PECAM). VCAM-1 principally mediates the adhesion of monocytes, lymphocytes, eosinophils, and basophils but not neutrophils, to the surface of the vascular endothelium (De Caterina R, 2007). The atherosclerosis process is shown in figure 2.

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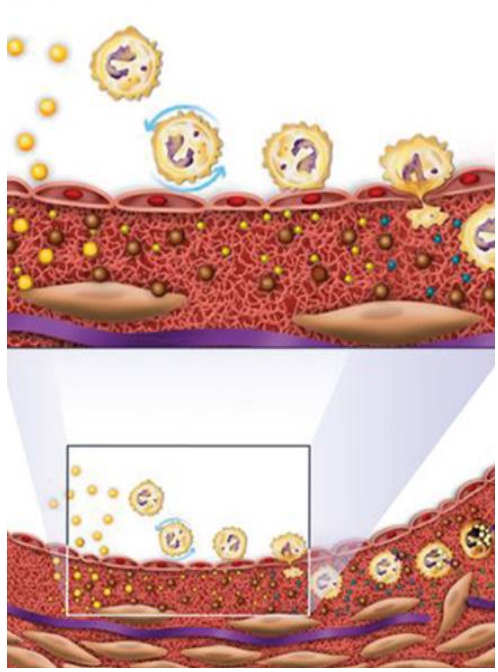
**Figure 1.** Cross-sectional view of an artery depicting the steps in the development of an atheroma, from left to right. The upper panel shows a detail of the boxed area below. The endothelial monolayer overlying the intima contacts blood.



Source: Fauci AS, Kasper DL, Braunwald E, Hauser SL, Longo DL. Harrison's Principles of Internal Medicine, 17th Edition.

### *1.1.2.3 Adhesion molecules*

The last two decades have witnessed growing interest in the mechanisms by which cells interact with their surrounding environment, which can include either other cells or components to the extracellular matrix. Most of this knowledge has been derived from an increased understanding of the structures and functions of adhesion molecules and differentiation of proteins that permit the large variety and high specificity of all possible cell-cell and cell-matrix interactions (Harrison, 2009).

Adhesion molecules are heterogeneous proteins that mediate reciprocal adhesion between different cell types, i.e., endothelial cells (EC),

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monocytes, lymphocytes, platelets and smooth muscle cells (SMC), or between cells and components of the extracellular matrix. Gianetti F and De Caterina R currently classify six types of adhesion molecules: integrins (VLA-4), selectins (L-, E- Selectins) immunoglobulins (ICAM-1, VCAM-1, PECAM-1), cadherins, proteoglycans, and mucins. Adhesion molecules may be constitutively expressed on the cell surface, or induced or amplified by various stimuli (De Caterina R, 2007).

#### *1.1.2.4 Foam cell formation*

Once resident within the intima, the mononuclear phagocytes mature into macrophages and become lipid-laden foam cells, a conversion that requires the uptake of lipoprotein particles by receptor-mediated endocytosis. One might suppose that the well-recognised "classical" receptor for LDL mediates this lipid uptake; however, patients or animals lacking effective LDL receptors due to genetic alterations (e.g., familial hypercholesterolemia) have abundant arterial lesions and extraarterial xanthomata rich in macrophage-derived foam cells. Candidates for alternative receptors that can mediate lipid loading of foam cells include a growing number of macrophage "scavenger" receptors, which preferentially endocytose modified lipoproteins and other receptors for oxidised LDL or beta very low density lipoprotein (β-VLDL). Thus, scavenger receptors were presumed to be responsible for foam cell formation (Itabe H, 2002; Libby P, 2009). Monocyte attachment to the endothelium, migration into the intima, and maturation to form lipid-laden macrophages thus represent key steps in the formation of the fatty streak, the precursor of fully formed atherosclerotic plaques.

#### *1.1.2.5 Atheroma evolution and complications*

Although the presence of a fatty streak commonly precedes the development of a more advanced atherosclerotic plaque, not all fatty streaks

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progress to form complex atheromata. By ingesting lipids from the extracellular space, the mononuclear phagocytes bearing such scavenger receptors may remove lipoproteins from the developing lesion. Some lipid-laden macrophages may leave the artery wall, exporting lipid in the process. Lipid accumulation, and hence the propensity to form an atheroma, ensues if the amount of lipid entering the artery wall exceeds that removed by mononuclear phagocytes or other pathways. Export by phagocytes may constitute one response to local lipid overload in the evolving lesion.

Some foam cells may die as a result of programmed cell death, or *apoptosis*. This death of mononuclear phagocytes results in formation of the lipid-rich centre, often called the *necrotic core*, in established atherosclerotic plaques. Macrophages loaded with modified lipoproteins may produce cytokines and growth factors, which can then further signal some of the cellular events in lesion complication.

Another mechanism, reverse cholesterol transport, which is mediated by high-density lipoproteins (HDL), probably provides an independent pathway for lipid removal from atheromata. This transfer of cholesterol from the cell to the HDL particle involves specialised cell surface molecules such as the ATP-binding cassette (ABC) transporters. ABCA1, the gene mutated in Tangier disease, a condition characterised by very low HDL levels, transfers cholesterol from cells to nascent HDL particles and ABCG1 to mature HDL particles. "Reverse cholesterol transport" mediated by these ABC transporters allows HDL loaded with cholesterol to deliver it to hepatocytes by binding to scavenger receptor B 1 or other receptors. The liver cell can metabolise the sterol to bile acids that can be excreted. This export pathway from macrophage foam cells to peripheral cells such as hepatocytes explains part of HDL's antiatherogenic action. Anti-inflammatory and antioxidant properties may also contribute to HDL's atheroprotective effects (Inder M, 2007). Thus, macrophages may play a vital role in the dynamic economy of lipid accumulation in the arterial wall during atherogenesis.

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While accumulation of lipid-laden macrophages characterises the fatty streak, build up of fibrous tissue formed by the extracellular matrix typifies the more advanced atherosclerotic lesion. The SMC synthesise the bulk of the extracellular matrix in the complex atherosclerotic lesion. A number of growth factors or cytokines produced by mononuclear phagocytes can stimulate SMC proliferation and the production of extracellular matrix.

Cytokines found in the plaque, including IL-1 or/and TNF-$\alpha$, can induce local production of growth factors, including forms of platelet-derived growth factor (PDGF), fibroblast growth factors, and others that may contribute to plaque evolution and complication. Other cytokines, notably interferon $\gamma$ (IFN-$\gamma$) derived from activated T cells within lesions, can limit the synthesis of interstitial forms of collagen by SMCs. These examples illustrate how atherogenesis involves a complex mix of mediators that determine the characteristics of particular lesions.

The arrival of SMCs and their production of extracellular matrix probably provide a critical transition, yielding a fibrofatty lesion in place of the simple accumulation of macrophage-derived foam cells. For example, PDGF produced by activated platelets, macrophages, and endothelial cells can stimulate the migration of SMCs normally resident in the tunica media into the intima. Such growth factors and cytokines produced locally can stimulate the proliferation of resident SMCs in the intima as well as those that have migrated from the media.

Transforming growth factor $\beta$ (TGF-$\beta$), among other mediators, potently stimulates interstitial collagen production by SMCs. These alterations in SMCs, signalled by mediators acting at short distances, can hasten transformation of the fatty streak into a more fibrous SMC- and extracellular matrix-rich lesion.

In addition to locally produced mediators, products of blood coagulation and thrombosis likely contribute to atheroma evolution and complication. This

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involvement justifies the use of the term atherothrombosis to convey the inextricable links between atherosclerosis and thrombosis.

Fatty streak formation begins beneath a morphologically intact endothelium. In advanced fatty streaks, however, microscopic breaches in endothelial integrity may occur. Microthrombi rich in platelets can form at such sites of limited endothelial denudation owing to exposure of the thrombogenic extracellular matrix of the underlying basement membrane. Activated platelets release numerous factors that can promote the fibrotic response, including PDGF and TGF-β.

Thrombin itself generates fibrin, not only during coagulation, but also through protease-activated receptors that can signal SMC migration, proliferation, and extracellular matrix production. Many arterial mural microthrombi resolve without clinical manifestation by a process of local fibrinolysis, resorption, and endothelial repair. Yet, stimulation of these pro-fibrotic functions of SMCs can lead to lesion progression (Libby P, 2009).

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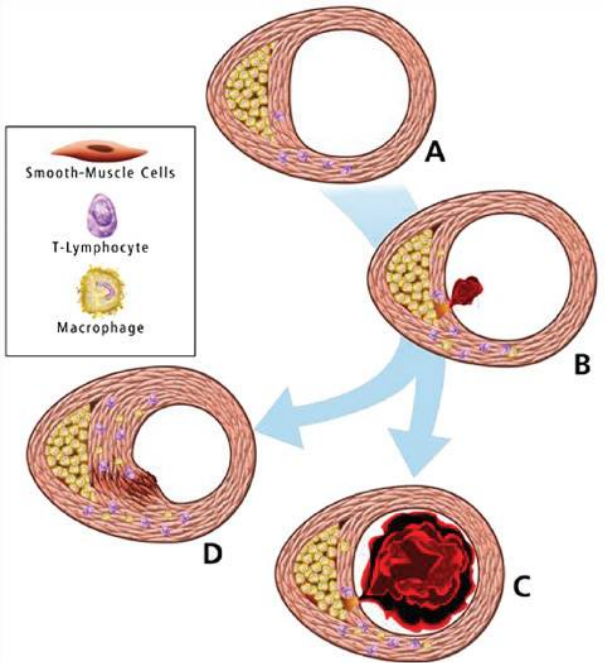
**Figure 2.** Plaque rupture, thrombosis, and healing. ***A.*** Arterial remodelling during atherogenesis. ***B.*** Rupture of the plaque's fibrous cap causes thrombosis. ***C.*** When the clot overwhelms the endogenous fibrinolytic mechanisms, it may propagate and lead to arterial occlusion. ***D.*** The subsequent thrombin-induced fibrosis and healing causes a fibroproliferative response that can lead to a more fibrous lesion, one that can produce an eccentric plaque that causes a haemodynamically significant stenosis.




Source: Fauci AS, Kasper DL, Braunwald E, Hauser SL, Longo DL. Harrison's Principles of Internal Medicine, 17th Edition.

### *1.1.2.6 Calcification*

There is often a deposition of calcium within atherosclerotic plaques. Proteins usually found in bone also localise in atherosclerotic lesions, e.g., osteocalcin, osteopontin, and bone morphogenetic proteins. These proteins

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are not normally expressed in healthy arterial tissue and are only found in atheromatous arteries. Mineralization of the atherosclerotic plaque recapitulates many aspects of bone formation. Small to moderate amounts of calcification may destabilise the plaque by making it less tolerant of shear stress (Tan KT, 2008). The extent of calcium is thought to reflect the total coronary atherosclerotic burden (Alexopoulos N, 2009; Libby P, 2009).

### *1.1.2.7 Plaque evolution*

Although atherosclerosis research has focused much attention on the proliferation of SMCs. As in the case of macrophages, SMCs can also undergo apoptosis in the atherosclerotic plaque. Indeed, complex atheromata often have a mostly fibrous character and lack the hypercellularity of less advanced lesions. This relative paucity of SMCs in advanced atheromata may result from the predominance of cytostatic mediators, such as TGF-$\beta$ or IFN-$\gamma$, which can inhibit SMC proliferation and apoptosis. Some pro-inflammatory cytokines that activate atherogenic functions of vascular wall cells can also sensitise these cells to undergo apoptosis.

Thus, during the evolution of the atherosclerotic plaque, a complex balance between entry and egress of lipoproteins and leukocytes, cell proliferation and cell death, extracellular matrix production and remodelling as well as calcification and neovascularization contribute to lesion formation. Multiple and often competing signals regulate these various cellular events. Increasingly, we appreciate links between atherogenic risk factors, inflammation, and the altered behaviour of intrinsic vascular wall cells and infiltrating leukocytes that underlie the complex pathogenesis of these lesions (Libby P, 2009).

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### 1.1.3 Plaque instability and rupture

Plaques that cause fatal thromboses tend to have thin fibrous caps, relatively large lipid cores, and a high content of macrophages. Morphometric studies of lesions show that at sites of plaque rupture, macrophages and T lymphocytes predominate and contain relatively few SMCs. Cells that concentrate at sites of plaque rupture bear markers of inflammatory activation.

A superficial erosion of the endothelium or a frank plaque rupture or fissure usually produces the thrombus that causes episodes of unstable angina pectoris or the occlusive and relatively persistent thrombus that causes acute myocardial infarction. Rupture of the plaque's fibrous cap permits contact between coagulation factors in the blood with highly thrombogenic tissue factor (TF) expressed by macrophage foam cells in the plaque's lipid-rich core. Repetitive episodes of plaque disruption and healing provide one likely mechanism of transition of the fatty streak to a more complex fibrous lesion. The healing process in arteries, as in skin wounds, involves the laying down of new extracellular matrix and fibrosis.

The presence of the transplantation, or histocompatibility, antigen HLA-DR provides one convenient gauge of the degree of inflammation in cells in atheroma. Resting cells in normal arteries seldom express this transplantation antigen. However, macrophages and SMCs at sites of human coronary artery plaque disruption do bear this inducible cell surface marker. Therefore, the presence of HLA-DR-positive macrophages and T cells indicates an ongoing inflammatory response at sites of plaque rupture. In addition, patients with active atherosclerosis and acute coronary syndromes display signs of disseminated inflammation. Inflammatory mediators regulate processes that govern the integrity of the plaque's fibrous cap and hence its propensity to rupture. For example, the T cell–derived cytokine IFN-$\gamma$, which is found in atherosclerotic plaques and is required to induce the HLA-DR present at sites of rupture, can inhibit growth and

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collagen synthesis by SMCs, as noted above. Cytokines derived from activated macrophages and lesional T cells can elicit the expression of genes encoding proteolytic enzymes that can degrade the extracellular matrix of the plaque's fibrous cap. Thus, inflammatory mediators can impair collagen synthesis required for maintenance and repair of the fibrous cap and trigger degradation of extracellular matrix macromolecules. These are processes that weaken the plaque's fibrous cap and enhance its vulnerability to rupture. In contrast to plaques with these features of vulnerability, those with a dense extracellular matrix and relatively thick fibrous cap without a substantial TF-rich lipid core seem generally resistant to rupture and unlikely to provoke thrombosis.

Features of the biology of the atheromatous plaque in addition to its degree of luminal encroachment influence the clinical manifestations of this disease. This enhanced understanding of plaque biology provides insight into the diverse ways in which atherosclerosis can present clinically and why the disease may remain silent or stable for prolonged periods, punctuated by acute complications at certain times.

Increased understanding of atherogenesis provides new insight into the mechanisms linking it to the risk factors discussed below, indicates the ways in which current therapies may improve outcomes, and also suggests new targets for future intervention (Libby P, 2009).

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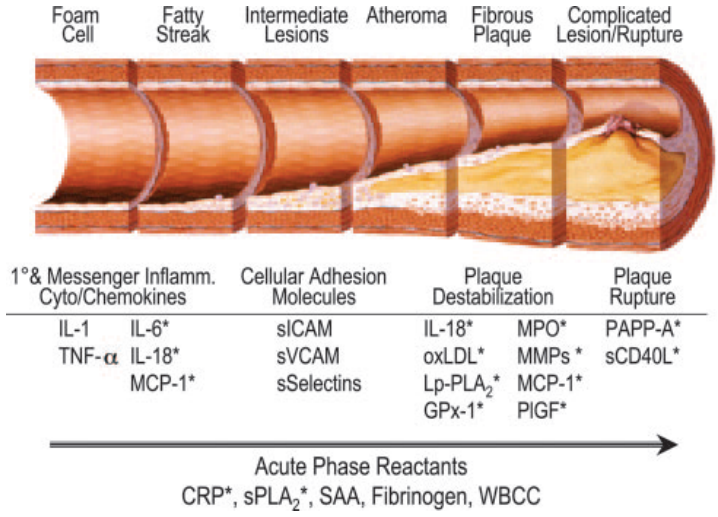
**Figure 3.** Biomarkers of inflammation and plaque instability: From cell to plaque rupture.




Source: Koenig W, Khuseyinova N. Biomarkers of atherosclerotic plaque instability and rupture. Arterioscler Thromb Vasc Biol. 2007;27(1):15-26.

### 1.1.4 Endothelial dysfunction

Alterations of endothelial physiology, also referred to an endothelial dysfunction, increase the adhesion of circulating leukocytes to the injured endothelial layer. Their subsequent extravasion into the vessel wall is a critical and early event in the development of atherosclerosis. Circulating leukocytes are also involved in plaque progression and the occurrence of atherosclerotic complications (Bonetti PO, 2003; Zhou Z, 2005; Ferroni P, 2006). By definition, endothelial dysfunction is a functional and reversible alteration of endothelial cells resulting from impairment during oxidative stress and availability of the signalling molecule NO, which has potent vasodilatory and antiatherogenic properties (Kawakami A, 2008; Federici M, 2004; Aljada A, 2000).

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The endothelium has unique functions in controlling (a) haemostasis, including the control of platelet adhesion and activation, coagulation and fibrinolysis; (b) vascular tone; (c) vascular permeability; (d) SMC growth and proliferation, and (e) leukocyte adhesion. Qualitative and quantitative alterations of endothelial functions can lead to decreased antithrombogenic properties of the endothelium and, conversely, to increased vasomotor tone, increased vascular permeability to plasma lipoproteins, increased production by cytokines and growth factors, and hyperadhesiveness toward circulating leukocytes. Overall, such alterations of the endothelium can be designated as endothelial dysfunction.

Endothelial activation indicates a group of dysfunctions characterised by antigenic changes on the surface of the vascular endothelium, particularly occurring in response to inflammatory cytokines and bacterial endotoxins. Such responses modulate the interactions of circulating leukocytes and play a pivotal role in the initiation and progression of atherosclerosis (De Caterina R, 2007).

### 1.1.5 The concept of atherosclerotic risk factors

The systematic study of risk factors for atherosclerosis emerged from the coalescence of experimental results as well as cross-sectional and ultimately longitudinal studies in humans. Some studies provided rigorous support for the concept that hypercholesterolemia, hypertension, and other factors correlated with CVD risk. Similar observational studies performed worldwide bolstered the concept of "risk factors" for CVD (Libby P, 2009).

From a practical viewpoint, the CVD risk factors that have emerged from such studies fall into two categories: those that are modifiable by lifestyle and/or pharmacotherapy and those such as age and gender that are immutable.

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The weight of evidence supporting various risk factors differs. For example, hypercholesterolemia and hypertension certainly predict coronary risk, but other so-called non-traditional risk factors, such as levels of homocysteine, lipoprotein (a) [Lp(a)], or infection, remain controversial. Moreover, the causality of some biomarkers that predict CVD risk, such as C-reactive protein (CRP), remains uncertain.

### 1.1.6 Lifestyle modification

The prevention of atherosclerosis presents a long-term challenge for all health care professionals as well as for public health policy, to help patients optimise their risk factor profile long before atherosclerotic disease becomes manifest. Moreover, patients should take personal responsibility for behaviour related to modifiable risk factors for development of premature atherosclerotic disease. All patients are given advice about healthy dietary and physical activity habits (recommend at least 30 min of moderate-intensity physical activity per day) for maintaining an ideal body weight, since it has been demonstrated that, for example, obesity, particularly the male pattern of centripetal or visceral fat accumulation, can contribute to the elements of the metabolic syndrome. Smoking cessation is also recommended.

Conscientious counselling and patient education may forestall the need for pharmacologic measures intended to reduce coronary risk.

### 1.1.7 Nutrition components in cardiovascular disease prevention

Fatty acids regulate cholesterol homeostasis and concentrations of blood lipoproteins and also affect the levels of other CVD risk factors, such as blood pressure (BP), haemostasis, and body weight, through various mechanisms. There are strong, consistent, and graded relationships among saturated fat intake, blood cholesterol levels, and the mass occurrence of CVD. The relationships are accepted as causal. n-3 fatty acids, in contrast to

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previous described factors, showed protective effects against fatal events in patients who had suffered a previous myocardial infarction (Graham I, 2007).

Sodium intake, especially in the form of sodium chloride, influences arterial BP and therefore the risk of arterial hypertension, stroke, coronary heart disease, and heart failure.

Intervention trials with vitamin supplements have failed to demonstrate any protection against CVD. Treatment with β-carotene, vitamin A, and vitamin E may even increase mortality risk. The potential roles of vitamin C and selenium on mortality warrant further investigation (Bjelakovic G, 2007).

Besides micro- and macronutrients, dietary patterns, including consumption of fruit and vegetables, monounsaturated fatty acid-rich oil (such as olive oil), and low fat dairy products, have been associated with decreased incidence of cardiovascular events (Graham I, 2007).

#### 1.1.7.1 Virgin olive oil

Virgin olive oil contains relatively high amounts of antioxidants, mainly phenolic compounds and vitamin E (Gimeno E, 2002). Diets rich in olive oil have been observed to improve flow-mediated (endothelium dependent) dilatation in hypercholesterolemic males and metabolic syndrome patients.

Such diets also reduce BP in hypertensive patients and the plasma concentrations of glucose and insulin in type 2 diabetic subjects (Covas MI; 2007). Some findings suggest that olive oil components may modulate inflammation and endothelial activation (Carluccio MA, 2003). The protective mechanism of oleic acid-rich diets against inflammation has been attributed to a decrease in the LDL content of linoleic acid.

A daily 25-mL dose of 3 types of olive oil with differents polyphenol content, similar to the daily consumption recommended by the U.S. FDA, reduced

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lipid cardiovascular risk factors and improved glutathione antioxidant status. Daily consumption of high- and medium-polyphenol olive oil decreased oxidative damage of lipids. Consumption of olive oil with high phenolic content provided the greatest benefits by increasing HDL cholesterol levels and reducing the oxidative damage of lipids (Covas MI, 2006).
In the secondary prevention of CVD, the use of olive oil can reduce systolic BP (Covas MI, 2007).

In cultured endothelial cell models, oleic acid inhibited the expression of VCAM-1 mRNA levels, the monocyte adhesion, and a key transcription factor NFκB ( Covas MI, 2007).

Rats fed with an olive oil-rich diet showed: (1) a significant delay in aortic thrombotic occlusion, a lower incidence of venous thrombosis, and a prolonged bleeding time in comparison with the control group fed the usual diet; and (2) decreased platelet hyperactivity and subendothelial trombogenicity (Covas MI, 2007).

#### 1.1.7.2 Nuts

Nuts are energy-dense foods that are rich in total fat and unsaturated fatty acids, and their skins contain several antioxidants (López-Uriarte P, 2009; Ros E, 2009). Healthy fats in nuts contribute to the beneficial effects of frequent nut intake observed in epidemiological studies (prevention of coronary heart disease, diabetes, and sudden death) and in short-term feeding trials (cholesterol lowering, LDL resistance to oxidation, and improved endothelial function). By virtue of their unique composition, nuts are likely to benefit newer cardiovascular risk biomarkers, such as LDL oxidizability, soluble inflammatory molecules, and endothelial dysfunction (Ros E, 2009; Nash SD, 2008).

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### 1.1.7.3 Cocoa

Cocoa powder and dark chocolate may favourably affect CVD risk status by modestly reducing LDL oxidation susceptibility. The total antioxidant capacity and HDL concentrations may be increased due to chocolate's high levels of plant-derived flavonoids, which have antioxidant effects (Wan Y, 2001; Erdman JW Jr, 2008).

Endothelial dysfunction and a consequent reduction in NO production play central conceptual roles in the pathogenesis of atherosclerosis and coronary artery disease, diabetes mellitus, and hypertension. Recent evidence that flavanol-rich cocoa activates vascular NO synthesis in the intact human raises an interesting possibility of a therapeutic potential (Hollenberg NK, 2004).

### 1.1.7.4 Carob bean

Carob pods also contain large amounts of polyphenols, which possess antioxidative properties *in vivo* (Kumazawa S, 2002). Insoluble fibre from carob pulp has been found to affect blood lipids in animals in a manner similar to that of soluble dietary fibre.

In rodent studies, carob pod and carob pulp preparations rich in dietary fibre demonstrated a cholesterol-lowering effect and an increased excretion of cholesterol and bile acids. In a one-arm clinical pilot study, a carob pulp preparation (carob fibre) was suggested to be effective in reducing LDL cholesterol concentrations in serum.

In humans, the intake of carob markedly reduced total cholesterol and LDL. The LDL:HDL ratio marginally decreased in the carob fibre group (Zunft HJ, 2003; Kumazawa S, 2002).

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### 1.1.8 *Other food components or extracts for a healthy heart*

#### 1.1.8.1 Antioxidant-rich diets

Diets rich in antioxidants, including plant flavonoids, can inhibit LDL oxidation and inhibit the formation of cell-cell adhesion factors, which are implicated in damage to the arterial endothelium and in the formation of blood clots. However, their importance with respect to CVD remains to be established (Graham I, 2007).

#### 1.1.8.2 Polyphenolic compounds

These compounds are widely distributed in the human diet, mainly in plant-derived foods and beverages (fruits, vegetables, nuts, seeds, herbs, spices, tea and red wine). These structurally diverse compounds exhibit a range of biological activities *in vitro* that may explain their potential cardioprotective properties, which include increased HDL, antioxidant and anti-inflammatory effects. Polyphenol intake has been associated with low cancer and coronary heart disease mortality rates and with improvements in endothelial dysfunction (Erdman J, 2007; Curin Y, 2005; Covas MI, 2006).

#### 1.1.8.3 Resveratrol

Epidemiological studies have shown an inverse relationship between moderate alcohol consumption, particularly in the form of wine, and the incidence of CVD (Klatsky AL, 1992; Fremont L, 2000; Klatsky AL, 1993).

Red wine, which is widely consumed in Mediterranean countries, is considered one of the principal factors in protection against CVD. The source of this protection is believed to derive not merely from ethanol, but also from a complex mixture of bioactive compounds, predominantly polyphenols (Cullen JP, 2007; Demrow HS, 1995; Pendurthi UR, 1999). These compounds include flavonoids (predominantly flavonols and anthocyanidins), stilbenoids and phenolic acids. The concentration of total stilbenes in red wine ranges between 0.05 and 1.8 mg/mL (Wu JM, 2001), and the main stilbene, resveratrol, has been detected in numerous types of

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wines, with concentrations dependent upon the country of production and the type of grape used (Fremont L, 2000; Wu JM, 2001). Red wine had been considered the main source of resveratrol (*trans*-3, 4', 5-tri-hydroxystilbene) in the human diet. However, resveratrol is also widely distributed in the plant world and has been identified in several fruits and vegetables that are normal constituents of the human diet (Soleas GJ,1997).

Resveratrol is synthesised in the skins of grapes in response to an injury, such as fungal infection. In the circulation, the compound acts as a phytoalexin that prevents the proliferation of pathogens and confers disease resistance (Fremont L, 2000; Soleas GJ, 1997; Brown L, 2009). Resveratrol exists in two isoforms, *cis*- and *trans*-, with the *trans*-isomer being more thermodynamically stable. A *trans*- to *cis*- isomerization can occur under the influence of ultraviolet light and high pH exposure (Camera M,1999). The concentrations of the *trans*-isomer generally range between 0.1 and 15 mg/L (Fremont L, 2000) in red wine, and it is almost non-existent in white wine.

This is because the production process for red wine involves fermentation in contact with the "must" (grape skins and seeds), and this process does not occur in the production of white wine (Brown L, 2009). Resveratrol is currently available commercially as a "nutraceutical" dietary supplement in tablets of 20 and 100 mg/dose.

*Trans*-resveratrol possesses a variety of biological activities (Brown L, 2009). With respect to CVD, the protection that is conferred by resveratrol against arteriosclerosis involves antioxidant activity, the modulation of hepatic apolipoprotein and lipid synthesis, cyclooxygenase activity, which provides anti-inflammatory effects, inhibition of platelet aggregation, and anticoagulant properties (Soleas GJ, 1997; Pendurthi UR, 2002; Zhu J, 2008).

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#### 1.1.8.4 Folate and homocysteine

Evidence suggests that beneficial modulation of risk indicators such as plasma homocysteine concentrations and BP may protect vascular integrity. Folate has the potential to reduce CVD risk by lowering the plasma level of homocysteine but it has not been confirmed in clinical trials (Graham I, 2007).

#### 1.1.8.5 Minerals

An increase in the intake of potassium and calcium and a reduction in sodium can help in reducing BP (Graham I, 2007).

#### 1.1.8.6 Peptides from milk protein

Consumption of certain peptides derived from milk proteins has also been reported to be beneficial (Graham I, 2007).

#### 1.1.8.7 Plant sterol and stanol esters

Plant sterols are natural constituents of plants, including a number of common crops such as soya and maize. Sterols play a similar role to that of cholesterol in animals, i.e., as metabolic precursors for several biologically active molecules, such as hormones, and also as cell structure molecules.

Early evidence obtained more than 50 years ago indicated that plant sterols can interact with cholesterol in the intestinal tract to induce a reduction of cholesterol absorption and a subsequent reduction in blood cholesterol. More recently, a number of studies have confirmed the ability of plant sterols and stanols (their hydrogenated derivatives) to reduce LDL cholesterol under a variety of conditions (Plana N, 2008).

They are present naturally in the diet at low levels. However, recent technological advances in the extraction and esterification of plant sterols, either as the sterols themselves or as stanols, enable them to be solublised

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in the matrix of food fat and, as such, to be easily incorporated into food products at biologically effective levels (Graham I, 2007).

#### 1.1.8.8 Soluble fibre

Dietary fibre provides many health benefits. A generous intake of dietary fibre reduces the risk of developing CVD, stroke, hypertension, diabetes, obesity, and certain gastrointestinal disorders (Anderson J, 2009; Petchetti L, 2007).

Further, increased consumption of dietary fibre improves serum lipid concentrations, reduced LDL cholesterol, or/and triglycerides (Solà R, 2007) and lowers BP. Dietary fibre also improves blood glucose control in diabetes, promotes gastrointestinal regularity, aids in weight loss, and appears to improve immune function (Anderson J, 2009; Petchetti L, 2007).

Dietary fibre was previously defined as the portion of plant foods, including polysaccharides and lignin, that are resistant to digestion by human digestive tract enzymes. The definition has been expanded to include oligosaccharides, such as inulin, and resistant starches. Fibres have been classified as soluble (such as viscous or fermentable fibres e.g., pectin), which are fermented in the colon, and insoluble (such as wheat bran), which have bulking action but may only be fermented to a limited extent in the colon (Anderson J, 2009).

#### 1.1.8.9 Soybean protein

Soybean protein reduces levels of LDL cholesterol (Graham I, 2007).

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## 2. TRANSLATIONAL RESEARCH

Translational Medicine is the process which leads from evidence based medicine to sustainable solutions for public health problems (Lean, 2008). The consensus view is that the end-target of translational research is the patient, and the goal is to improve care (Sipido KR, 2009). Biomarkers allowing for the prediction of the efficacy and safety of treatments in animals and humans are considered to account for 80-90% of the chances of success of the translation.

Fulfilment of the promise of translational research to improve the health and longevity of the world's populations depends on the development of broad-based teams of scientists and scholars to link basic scientific discoveries with the results of clinical investigation. The results of clinical trials must be translated into changes in clinical practice that are assisted by evidence from the social and political sciences (Lean ME, 2008).

Similar to other challenges in science, the keys to the success of translational medicine are based on developing methods and appropriate systems that close the gap between bench and bedside (Bermejo J, 2009).

### 2.1 Phase 1 translational research

Phase 1 in translational research refers to the research process that explores existing needs, develops potential treatments through basic laboratory research, and tests safety and efficacy, principally in randomised clinical trials. The concept arose from pharmacotherapeutic research and lead to the initial basis for evidence-based practice and clinical guidelines, which are now incorporated into Translational Medicine.

In the traditional process of translation, basic research is the first step in identification of new mechanisms of disease and potential targets for therapy. The results of these studies are elaborated in a proof of concept and eventually validated in a clinical trial. In reverse translation, this process starts with a clinical observation that is tested in basic experimental research

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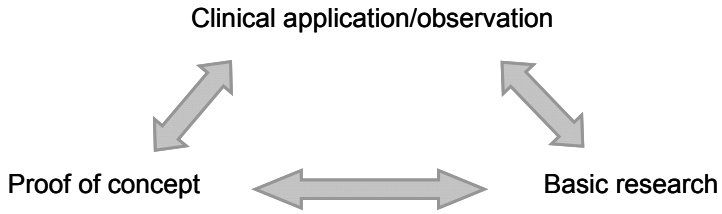
and a proof of concept with re-iterative step to validate new concepts (figure 4).

**Figure 4:** In the traditional process of translation, basic research is the first step to identification of new mechanisms of diseases and potential targets for therapy. The results of these studies are elaborated in a proof of concept and eventually validated in a clinical trial. In reverse translation, this process starts with a clinical observation that is tested in basic experimental research and a proof of concept with re-iterative steps to validate new concepts.




Source: Adapted from Sipido KR, 2009

In the cases of drug discovery and development, translational research typically refers to the translation of laboratory-based research into real therapies for real patients. This is often called the "bench to bedside" definition. Many pharmaceutical companies are building (phase 1) translational medicine groups to facilitate the interaction between basic research and clinical medicine, particularly in clinical trials.

## 2.2 Phase 2 Translational Research.

Phase 2 Translational Research examines how clinical findings that are found to be efficacious and safe treatments during Phase 1 function when they are applied in routine practice, as first described by Hiss RG in 2004. Phase 2 Translational Research informs guidelines with respect to need, acceptability, effectiveness, and cost efficiency in ecological settings, as well as policies to promote uptake for optimal management and resource use (Lean, 2008).

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### 2.3 Phase 3 Translational Research

Phase 3 Translational Research provides the necessary information to convert treatments and prevention strategies that are shown to be effective and cost-effective in Phase 2 Translational Research into sustainable solutions. Thus, governments can generate enduring evidence-based policies (Lean, 2008).

## 3. EVIDENCE-BASED NUTRITION OR PRACTICE-BASED EVIDENCE IN NUTRITION

A definition of evidence-based nutrition can be derived very simply from one well-known definition of evidence-based public health. Evidence-based nutrition is the application of the best available systematically assembled evidence in setting nutrition policy and practice (Brunner, 2001).

*In vitro* research models are essential to the early discovery of the biological effects of compounds, i.e., the kinetics of diene formation in order to determine resistance to oxidation, plasma antioxidant capacity, etc.

The use of cellular models as tools in discovery is almost universal. However, with respect to disease, it is important to determine whether they are relevant to the process and whether they add value. In terms of building target confidence, *in vitro* models often represent the only mechanistic link to human systems early in a project's life (figure 5). With respect to their predictive role and their use in decision-making processes, *in vitro* models are likely to be more valuable when used in conjunction with other tools. However, there is a rapid rate of advancement in this field and future developments hold much promise (Hallén S, 2009).

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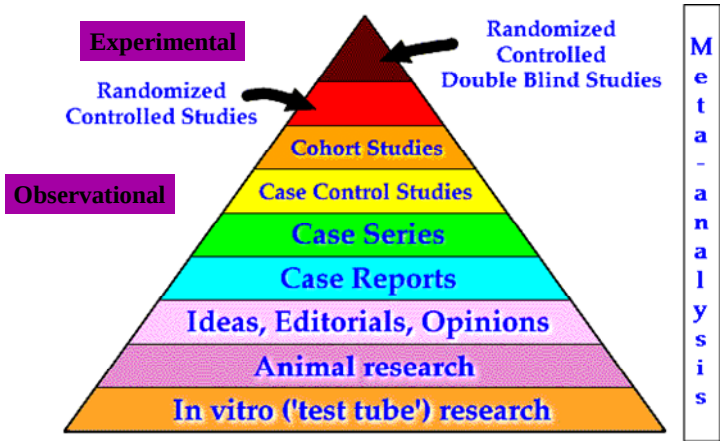
**Figure 5.**




## 4. TRANSLATIONAL MEDICINE IN ATHEROSCLEROSIS

The introduction of the term 'translational medicine' reflects the increasing demand for a successful, reproducible, and efficient method of translating results from animal- and test tube-based studies to humans. Some new aspects of translational medicine are described below (Sipido KR, 2009).

### *4.1 Atherosclerosis research*

The concept that atherosclerosis is an inflammatory disease has led to new discoveries regarding the role of specific elements of the immune system, especially innate and adaptive immunity, that orchestrate lesion development (Sipido KR, 2009).

These paradigms have been identified in experimental animal studies and validated against clinical data. Different subpopulations of T-cells have been identified, including some pro-atherogenic, mainly Th1, and some protective, regulatory T-cells.

In addition, natural antibodies against various components of oxidised phospholipids have been found to protect against atherosclerosis. These findings have opened new lines of investigation based on

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immunomodulation of atherosclerosis, by either stimulating regulatory immunity or raising protective antibody titres. The potential benefit of vaccination has been explored in several animal studies:

- active immunization using oxidised LDL (oxLDL)
- passive immunization
- induction of immune tolerance by oral oxLDL has been shown to provide protection against lesion development in atherosclerosis-prone mice.

As a specific antigen, oxLDL appears to play a major role. There is a clear opportunity here for further translation, but major questions need to be addressed. Conceptually, the approach is different from classic vaccination, where immunization is sought against foreign, hostile elements. The long-term risks of immunization against cells that participate in immune responses are currently unknown. This avenue is a novel one that must be further explored in collaborative efforts that bring together laboratories with molecular, cellular, and physiological expertise. Better disease models than transgenic mice must be tested, and large animal models such as the pig will be needed.

Further distinction between different subtypes of lymphocytes and macrophages and recognition of this cell diversity will further fine-tune current insights into the role of the immune system in atherosclerosis. Furthermore, the diversity of lymphocyte and macrophage populations has broader applicability for metabolic syndrome. The presence of these cell types may characterise the inflammatory state and provide important biomarkers. Collecting patient samples and data are a necessary aspect of this approach.

In summary, the concept of atherosclerosis as an inflammatory and immune disease offers opportunities for translation.

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Priorities are:

- exploration of the potential of vaccination and immunomodulation as treatments for atherosclerosis
- identification of new biomarkers for vascular disease using monocyte diversity
- Others (such as stem cells, early cardiovascular biomarkers as endothelial progenitor cells, etc.)

**5. CELL CULTURE**

Animal cells that have been removed from tissues will continue to grow if supplied with the appropriate nutrients and conditions. Cells can be easily purified from blood, however, only the white cells are capable of growth in culture.

When carried out in a laboratory, the process is called Cell Culture. It occurs *in vitro* as opposed to *in vivo.* The culture process allows single cells to act as independent units. The cells are capable of dividing; they increase in size and, in a batch culture, can continue to grow until limited by some culture variable such as nutrient depletion.

The cultivation of cells *in vitro* plays an important role in the biological and medical sciences and it has become an essential tool for the study of most biochemical and physiological processes. The use of large-scale animal cell culture has become increasingly important to the commercial production of specific compounds for the pharmaceutical industry and nowadays, for food industry. Animal tissue culture gives rise to many possibilities for current and future medical advancements.

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## 5.1 Historic introduction to cell culture

Animal cell culture became a common laboratory technique in the mid-1900s, but the concept of maintaining live cell lines separated from their original tissue source was discovered in the 19th century (table 1). Cell culture techniques were advanced significantly in the 1940s and 1950s.

**Table 1:** Historic evolution of cell cultures

| | |
|---|---|
| 1885 | **Roux** shows that embryonic chick cells can be maintained alive in a saline solution outside the animal body. |
| 1907 | **Harrison** cultivates amphibian spinal cord in a lymph clot, thereby demonstrating that axons are produced as extensions of single nerve cells. |
| 1910 | **Rous** induces a tumour by using a filtered extract of chicken tumour cells, later shown to contain an RNA virus (Rous sarcoma virus). |
| 1913 | **Carrel** shows that cells can grow for long periods in culture provided they are fed regularly under aseptic conditions. |
| 1948 | **Earle** and colleagues isolate single cells of the L cell line and show that they form clones of cells in tissue culture. |
| 1952 | **Gey** and colleagues establish a continuous line of cells derived from a human cervical carcinoma, which later becomes the well-known HeLa cell line. |
| 1954 | **Levi-Montalcini** and associates show that nerve growth factor (NGF) stimulates the growth of axons in tissue culture. |
| 1955 | **Eagle** makes the first systematic investigation of the essential nutritional requirements of cells in culture and finds that animal cells can propagate |

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| | |
|---|---|
| | in a defined mixture of small molecules supplemented with a small proportion of serum proteins. |
| 1956 | **Puck** and associates select mutants with altered growth requirements from cultures of HeLa cells. |
| 1958 | **Temin and Rubin** develop a quantitative assay for the infection of chick cells in culture by purified Rous sarcoma virus. In the following decade the characteristics of this and other types of viral transformation are established by **Stoker**, **Dulbecco**, **Green**, and other virologists. |
| 1961 | **Hayflick and Moorhead** show that human fibroblasts die after a finite number of divisions in culture. |
| 1964 | **Littlefield** introduces HAT medium for the selective growth of somatic cell hybrids. Together with the technique of cell fusion, this makes somatic-cell genetics accessible. |
| | **Kato and Takeuchi** obtain a complete carrot plant from a single carrot root cell in tissue culture. |
| 1965 | **Ham** introduces a defined, serum-free medium able to support the clonal growth of certain mammalian cells. |
| | **Harris and Watkins** produce the first heterocaryons of mammalian cells by the virus-induced fusion of human and mouse cells. |

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| | |
|---|---|
| 1968 | **Augusti-Tocco and Sato** adapt a mouse nerve cell tumour (neuroblastoma) to tissue culture and isolate clones that are electrically excitable and that extend nerve processes. A number of other differentiated cell lines are isolated at about this time, including skeletal muscle and liver cell lines. |
| 1975 | **Köhler and Milstein** produce the first monoclonal antibody-secreting hybridoma cell lines. |
| 1976 | **Sato** and associates publish the first of a series of papers showing that different cell lines require different mixtures of hormones and growth factors to grow in serum-free medium. |
| 1977 | **Wigler and Axel** and their associates develop an efficient method for introducing single-copy mammalian genes into cultured cells, adapting an earlier method developed by **Graham and van der Eb**. |
| 1986 | **Martin** and **Evans** and colleagues isolate and culture pluripotent embryonic stem cells from mouse. |
| 1998 | **Thomson** and **Gearhart** and their associates isolate human embryonic stem cells. |
| 2000 + | **Human Genome Project: genomics, proteomics, other –omics, genetic deficiencies, etc.** Exploitation of tissue engineering. |

Source: Alberts B, Bray D, Lewis J, Raff M, Roberts K, and Watson JD.

### 5.2 Types of Mammalian Cultures

Freshly isolated cultures derived directly from mammalian tissues are known as primary cultures. At this stage, cells represent the parent cell type with

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respect to the expression of tissue-specific properties. Most of these cultures have a limited lifespan; after sub-culturing onto fresh media, the cell line will either die out or 'transform' to become a continuous cell line. Such cell lines show many alterations from the primary culture, including changes in morphology and chromosomal variations such as artificial expression of the telomerase gene. There are numerous well-established cell lines that are representative of particular cell types.

### 5.3 Monocytes THP-1 cells

There are fundamental differences between human monocytes and human monocytic THP-1 cells line. First, human monocytes undergo apoptosis when stimulated by certain cytokines, while monocytes THP-1 cells grow indefinitely. Therefore, stimulated THP-1 cells are an appropriate substitute for blood monocyte models since they easily divide and can be maintained in cell culture for long periods. (Gil A, 2007) (figure 6).

This cell line was isolated by Tsuchiya S. et al. in 1980 from the blood of a 1-year-old boy suffering from acute monocytic leukemia. Monocytes THP-1 cells resemble human monocytes with respect to numerous criteria such as morphology, secretory products, oncogene expression, expression of membrane antigens, and expression of genes involved in lipid metabolism. In contrast to native human monocytes, a cell line such as monocytes THP-1 cells offers the additional advantage of a homogeneous population, which markedly facilitates further biochemical study. Moreover, they are characterised by their phagocytic capacity. This cells line might be a useful tool for studying the role of monocytes in the human immune response.
Cell cultures of monocytes THP-1 cells have been widely used for the study of multiple molecular mechanisms involved in the development of atherosclerosis.

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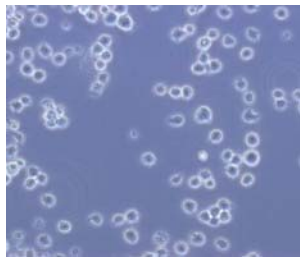

**Figure 6.** Human monocytic THP-1 cells (20x)

## 5.4 Endothelial cells

In the sphere of medicine and physiology, it was thought for a long time that the cells of the endothelium such as human aortic endothelial cells (HAEC), human umbilical endothelial cells (HUVEC), etc., were physiologically inactive and merely had the function of separating blood from tissue. It is now known that the endothelial tissue is highly active and closely involved in numerous physiological processes (figure 7).

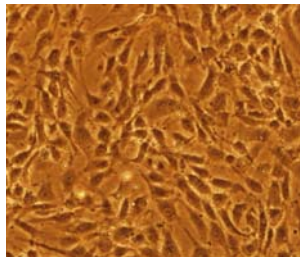

**Figure 7.** HAEC (20x)

If the functioning of the endothelial cells is impaired, there may be serious consequences with regard to the onset of numerous diseases. In particular, the two main causes of death in the western world, atherosclerosis, a disease of the arteries, and cancer, the malignant, uncontrolled proliferation of cells are associated with impairment of the endothelium. The biological techniques for the *in vivo* and *in vitro* characterisation of the endothelium have improved, making it possible to determine the extensive heterogeneity of the endothelial cells.

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### 5.5 Human cell lines and bioethics

Cell lines that originate from humans have been somewhat controversial in bioethics, as they may outlive their parent organism and later be used in the discovery of lucrative medical treatments. In a pioneering decision in this area, the Supreme Court of California ruled in Moore v. Regents of the University of California that human patients have no property rights to cell lines derived from organs removed with their consent.

One of the earliest human cell lines is descended from Henrietta Lacks. The patient died from the cancer that produced the cell line. The cultured HeLa cells shown here have been stained with Hoechst, which turns their nuclei blue.

### 5.6 Maintaining cells in culture

Mammalian cells are grown and maintained at an appropriate temperature and gas mixture (37 °C, 5% $CO_2$) in a cell incubator, either in suspension culture (such as cells that exist in the bloodstream) or attached to a solid surface (cells derived from solid tissues), and they generally grow to fill the available area or volume. Such growth can generate nutrient depletion in the growth media, accumulation of apoptotic/necrotic (dead) cells, and cell-to-cell contact, which can stimulate cellular differentiation and cell cycle arrest, causing cells to stop dividing. This is known as contact inhibition or senescence.

The most commonly varied factor in culture systems is the growth medium, which can vary in pH, glucose concentration, growth factors, and the presence of other nutrients. The supplementation of animal/human cell culture media with sera (components) derived from animal blood, such as calf serum, remains the standard and provides the necessary nutrition, shear protection, growth factors and cytokines (Falker E, 2006). One complication of these blood-derived ingredients is the potential for contamination of the culture with viruses or prions, particularly in biotechnological and medical

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applications. The current practice is to minimise or eliminate the use of these ingredients wherever possible, but this cannot always be accomplished.

During maintenance, cells undergo metabolic processes that lead to acid production and consequently, the pH of the cell culture medium decreases. For this reason, a pH predictor is added to the medium in order to measure nutrient depletion.
Culture conditions vary widely for each cell type, and variation of conditions for a particular cell type can result in the expression of different phenotypes.

### 5.7 Manipulation of cultured cells

The manipulation of culture cells is typically carried out in a biosafety hood or laminar flow cabinet to avoid contamination with bacteria, yeast, or other cell lines. Antibiotics (e.g., Penicillin and streptomycin) and antifungals (e.g., Amphotericin B) can also be added to the growth media.

### 5.8 Evolution of the cell lines

Once confluence is reached in the cell culture, many cell lines express their most characteristic aspects. In this state, the morphological and physiological similarity to the cellular model of origin is greatest. It is at this time that growth stops, and the culture must be divided (split) or re-seeded to propagate the cells.

### 5.9 Passaging cells

Subculture or splitting cells, which is known as passaging, involves transferring a small number of cells into a new vessel. Cells can be cultured for a longer time if they are split regularly, which prevents the senescence associated with prolonged high cell density. Suspension cultures are easily passaged by diluting a small amount of culture containing a few cells with a larger volume of fresh media. For adherent cultures, cells first need to be detached. This is commonly done with a mixture of trypsin-

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ethylenediaminetetraacetic acid (EDTA) , however other enzyme mixes are now available for this purpose. A small number of detached cells can then be used to seed a new culture.

### 5.10 Media

Because of the undefined composition, risk of contamination, high cost and also animal welfare considerations concerning the production of sera, the conversion to serum free alternatives is promoted by regulatory authorities, industry and the research community in general.

To support this trend and to help conserve resources and time, a data bank was compiled of commercially available formulations. This database is searchable for products, applications, cell lines and manufacturers.

### 5.11 Media changes

In the case of suspension cultures, after collecting the cells by centrifugation, the cell culture media can be refreshed with an adequate volume of fresh media depending on the desired final cellular density.

On the other hand, the media of adherent cultures can be removed directly by aspiration and replaced with fresh media.

### 5.12 Cell line cross-contamination

Cell line cross-contamination can be a problem for scientists working with cultured cells. Studies suggest that anywhere from 15–20% cells used in experiments have been misidentified or contaminated with cells from other lines. Tumour cell lines used in scientific research are affected by inter- or intraspecies cross-contamination or have been wrongly identified, thereby rendering many of the conclusions doubtful if not completely invalid (Lancet Oncology, vol. 2, July 2001, p. 393).

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Problems with cell line cross contamination have even been detected in lines from the NCI-60 panel, which are used routinely for drug-screening studies. Major cell line repositories including the American Type Culture Collection (ATCC) and the German Collection of Microorganisms and Cell Cultures (DSMZ) have received cell line submissions that were misidentified by the researcher.

Such contamination poses a problem for the quality of research produced using cell culture lines, and the major repositories are now authenticating all cell line submissions. ATCC uses short tandem repeat (STR) DNA fingerprinting to authenticate its cell lines.

To address the problem of cell line cross-contamination, researchers are encouraged to authenticate their cell lines at an early passage to establish the identity of the line. Authentication should be repeated before freezing cell line stocks, every two months during active culturing and before any publication of research data generated using the cell lines. There are many methods for identifying cell lines including isoenzyme analysis, human lymphocyte antigen (HLA) typing and STR analysis.

### 5.13 Cell integrity

The scientific community is increasingly recognizing that cell line integrity is critical for maintaining high standards in research. Initiatives have called for standardised cell culture quality, including confirmation of cell line identity (through authentication) as a condition for receipt of grant funds from major agencies (NIH, NSF, HHMI, ACS, etc.), as well as for publication of research using cultured cells.

### 5.14 Applications of cell culture

Mass culture of animal cell lines is fundamental to:

- the manufacture of viral vaccines
- the production of biotechnology

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- the understanding the pathophysiologic mechanisms of diseases such as atherosclerosis, type 2 diabetes, obesity or cancer
- the evaluation of the effects of nutritional and pharmacological products on the development of complex pathophysiological processes.

Biological products produced by recombinant DNA (rDNA) technology in animal cell cultures include enzymes, synthetic hormones, immunobiologicals (monoclonal antibodies, interleukins, lymphokines), and anticancer agents. Although many simpler proteins can be produced using rDNA in bacterial cultures, more complex proteins that are glycosylated (carbohydrate-modified) currently must be made in animal cells.

An important example of such a complex protein is the hormone erythropoietin. The cost of growing mammalian cell cultures is high, so research is underway to produce such complex proteins in insect cells or in higher plants.

### 5.15 Why Grow Cells in Culture?

There are several applications for animal cell culture, for example, to test the effects of various chemicals, natural compounds or drugs on specific cell types (normal or cancerous cells, for example).

The major advantage of using cell culture for these applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells. The disadvantage is that, after a period of continuous growth, cell characteristics can change and cultured cells may become quite different from those found in the starting population. Cells can also adapt to different culture environments (e.g., different nutrients, temperatures, salt concentrations etc.) by varying the activities of their enzymes.

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These techniques have a number of undeniable advantages, but also have some disadvantages that must be taken into consideration.

Advantages of cell culture include:

1. Precise control over the environment: In cell culture, all environmental factors can be controlled, including physical-chemical (pH, temperature, osmotic pressure, levels of $O_2$, $CO_2$, surface tension), and physiological properties (hormones, growth factors, cell density). This is only completely true for some cell lines that are grown using so-called defined media. A defined medium is one in which all the components, their concentrations and their interactions are known. Establishment of a defined medium requires knowledge of the exact nutritional requirements of the cells in question. However, defined media have not been developed for many cell lines. In these cases, the media must be supplemented with complex solutions (serum, embryo extracts, etc) containing hormones and nutritional factors that are essential for continued cultivation but whose nature is unknown.
2. Characterization and sample homogeneity: Cultured cells from a cell line are homogeneous, with uniform shape and composition. One can easily obtain a large number of identical replicas, which overcomes the serious problem inherent heterogeneity of the sample that is associated with the use of experimental animals.
3. Economy: Cell culture is economical because the use of reagents or drugs can be performed at low volume. Since cultured cells can be grown in direct contact with the drug, the concentrations required are much lower than in whole animals.
4. Ethical reasons: Biomedical research requires the sacrifice of many thousands of animals every year. Cell culture cannot always replace *in vivo* testing but is a viable alternative in many situations. Primary cell culture experiments that involve the sacrifice of one or a few animals allow for the testing of a number of experimental conditions and avoid the slaughter of tens or hundreds of animals.

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Disadvantages of cell culture include:

1. Sensitivity of the technique: The growth of animal cells is much slower than the most common contaminants (fungi, yeast, bacteria, mycoplasmas). Furthermore, cells that are derived from multicellular organisms cannot grow in the absence of a complex mixture of nutrients that simulates the plasma or interstitial fluid. This implies the need to maintain aseptic conditions at all times, which is limiting at both the instrumentation level and at the level of the qualified personnel required for handling.
2. Quantity and cost: The production cost for 1 g of tissue in culture is more than 10 times higher than in the animal. There is also a limitation of production, which is about 10 g of cells in a normal laboratory. Industrial facilities are required to produce 100 g or more of tissue.
3. Instability: Many of the continuous cell lines are unstable as a result of chromosomal aneuploidy. The cell population can change its composition if any of the cell subpopulations are able to grow with a slightly higher rate, i.e., there can be significant differences in the cell line from one generation to the next. The only solution to this problem is to replenish lines from a frozen stock every few minutes or after a certain number of generations.
4. Validity of the model *in vitro*: When we refer to a cell culture, we are referring to exactly one cell derived from a tissue of origin. The culture differs from the original tissue in that it:
    a. Has lost the three-dimensional spatial organization of the tissue itself.
    b. Has lost heterotypic interactions between different cell types, and between cells and extracellular matrix. It is noteworthy that the most exciting advances in cell function arise from the recognition of the importance of specific interactions of cells with other cells or with the substrate.

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c. Lacks the systemic components involved in the regulation of homeostasis *in vivo*, especially the nervous and endocrine systems.

When a culture is established, the cells differentiate and, among other things, become mobile and begin to proliferate. This differentiation can sometimes be reversed in cases where it was induced using hormones, confluence, or chemical inducers (phorbol esters, etc.), but it is unclear whether the state is equivalent to the differentiation state *in vivo*.

For all these reasons it is important to be cautious regarding the validity of the results obtained *in vitro* and of which observations can be made *in vivo*. There are large numbers of *in vitro* model validation studies that have been performed in the development of alternative methods to animal experiments, for example, ECVAM (European Center for Validation of Alternative Methods'), Altweb (a collection of resources for development of alternative methods to animal testing, John Hopkins University, USA), Invittox (Collection of '*in vitro*' protocols), Invitroderm (Alternatives to skin irritation testing in animals), etc.

## 6. INVESTIGATION OF NUTRITIONAL BENEFITS USING CELL CULTURE

Experimental models have been used for the clinical study of CVD and to determine the pathophysiologic mechanisms of atherosclerosis, as well as to evaluate the effects of nutritional and pharmacological products on the development of this complex inflammatory process that is present in many CVD.

Some of the cell lines most frequently used for the study of the molecular mechanisms of atherosclerosis and assessment of substances with nutritional or pharmacological interest are considered.

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To develop new strategies for dietary prevention of these conditions, it is important to examine the interactions between genetic and diet-related early biomarkers. Study of the interactions between physiological, cellular and genetic factors may allow for identification of nutrition related early biomarkers. Such biomarkers would be valuable for detection of individuals with a high risk for cardiovascular diseases at a much earlier stage of pathogenesis. This could lead to personalised dietary counselling for the prevention of disease and development of new targeted healthy foods.

Although some of the underlying technology for quantifying protein was introduced almost thirty years ago there has recently been a significant increase in the development of new tools (Greenbaum D, 2003). Concurrently, tools for analysing mRNA expression are becoming more mainstream. The quantification of both of these molecular populations is not an exercise in redundancy; measurements taken from mRNA and protein levels are complementary and both are necessary for a complete understanding of how the cell works (Hatzimanikatis V, 1999). Additionally, as mRNA is eventually translated into protein, one might assume that there should be some sort of correlation between the level of mRNA and that of protein. Alternatively, there may not be any significant correlation, which, in itself, is an informative conclusion (Greenbaum D, 2003).

Ensuring a high level of protection for human health and wildlife is the central feature of current and new regulation of substances (chemicals, cosmetics, food, functional food, pharmaceuticals, etc.) in the European Union. Europe's interest in alternative tests is evident through the many efforts made to encourage the development of non-animal methods and techniques (Bottini A.A, 2008).

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## 7. *IN VITRO* CELL MODELS

### 7.1 Induction of inflammation in monocytes THP-1 cells

Atherosclerosis is a complex inflammatory process that is characterised by the presence of monocytes/macrophages and T lymphocytes in the atheroma plaque (Lusis AJ, 2000). A large body of evidence suggests a major role for inflammation in all phases of atherosclerosis, from the initiation of the fatty streak to the culmination in acute coronary syndromes (plaque rupture) (Koening W, 2007).

The cells most typically involved in the inflammatory process are monocytes/macrophages. When they cross the endothelium to the Tunica Intima, they produce and secrete several cytokines such as Tumour Necrosis Factor alpha (TNF-α). This molecule is a multifunctional cytokine that exerts pleiotropic biological actions and has a profound influence on the progression of atherosclerosis (Koening W, 2007; Boyle JJ, 2005).

Some natural compounds or extracts of certain foods have anti-inflammatory properties toward monocytes (Dufour D, 2007; Haversen L, 2002; Kobori M, 2008; Schmelzer C, 2009; Singh U, 2005; Tripathi S, 2008). Most of *in vitro* models that currently exist to test the anti-inflammatory effects of different natural extracts or compounds are based on the study of TNF-$\alpha$ release from Lipopolysaccharide (LPS)-activated monocytes (Singh U, 2005). The human monocytic leukemia THP-1 cell line is the most commonly used model in inflammatory systems based on human cells because of its capacity to produce cytokines such as TNF-$\alpha$ in response to LPS stimulation (Song M, 2000; Yao J, 1997). However, in general, monocytes THP-1 cells human cellular models presented a high variability of about 30-40%, measured by coefficient variation (CV).

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Monocytes THP-1 cells were used rather than peripheral blood mononuclear cells isolated from humans in order to minimise the variability derived from individual variations (Schmelzer C, 2009).

The LPS molecule is a major structural constituent of the outer membrane of gram-negative bacteria and a potent activator of the immune system (Jiang Z, 2005). It consists of a polysaccharide region that is anchored in the outer bacterial membrane by a specific carbohydrate lipid moiety termed lipid A, an endotoxin responsible for the immunostimulatory activity of LPS (Leon CG, 2008). LPS and LPS-containing particles (including intact bacteria) form complexes with a plasma protein known as LPS-Binding-Protein (LBP). Human LBP is a serum glycoprotein that is synthesised by hepatocytes and intestinal epithelial cells. It is present in normal serum at concentrations of 5 to 10 μg/ml, but levels rise dramatically up to 200 μg/ml 24h after induction of an acute-phase response (Gutsmann T, 2001).

LPS requires the presence of LBP to act as a prototypical endotoxin. It binds to the CD14/TLR4/MD2 receptor complex (Jiang Z, 2005; Gutsmann T, 2001), which promotes the secretion of pro-inflammatory cytokines by an intracellular signal amplification pathway in many cell types, especially in monocytes/macrophages. This cellular pathway includes expression of cytokine mRNA and the consequent protein synthesis and release of TNF-α. These mediators in turn act on additional target cells and produce the inflammatory response (Song M, 2000; Gutsmann T, 2001; Guha M, 2001). LPS can initiate pathological reactions such as the induction of septic shock leading to an inflammatory response (Gutsmann T, 2001; Guha M, 2001). As LBP is present in the FBS of the culture medium, it is required for the activation of monocytes by LPS. Nevertheless, some monocytes THP-1 cells models are produced without FBS (Jiang Z, 2005). Moreover, because monocyte properties can change dramatically after prolonged periods in culture, inherently unstable human monocytes THP-1 cells are found (Song M, 2000).

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## 7.2 Production of endothelial dysfunction in HAECs

Atherosclerosis may be considered as the clinical endpoint of an inflammatory process and endothelial dysfunction. Alteration of endothelial physiology, also referred to as endothelial dysfunction (Bonetti PO, 2003; Ferroni P, 2006; Zhou Z, 2005).

The binding of monocytes to the vascular endothelium is mediated by cross-linkage of cell adhesion molecules such as VCAM-1. The cell surface expression of these molecules is greatly increased at sites of atherosclerosis, which is stimulated by pro-inflammatory cytokines such as TNF-α. This cytokine was selected as stressor to test the our *in vitro* model because it has been shown to induce cellular response including expression and activation of other cytokines as VCAM-1 which contribute to the inflammatory process (Amberger A, 1997; Wu D, 1999; Zhou Z, 2005; Davis P, 2006).

Furthermore, there is *in vivo* evidence of increased VCAM-1 levels in animal models of inflammation (Lee G, 2006). Induction of VCAM-1 by TNF-α is regulated at the level of gene transcription and requires binding of the transcription nuclear factor-κB (NF-κB) to the regulatory regions within the promoters of this gene (Pierce JW, 1997; Dieguez-Acuna FJ, 2004). NF-κB is located in an inactive form in the cytoplasm in association with the inhibitor IκB-α. Binding between TNF-α and NF-κB occurs via a process involving the phosphorylation and degradation of IκB-α (Pierce JW, 1997; Izban K, 2000; Spieker M, 1997).

BAY11-7082 (BAY) ((E)3-[(4-Methylphenyl)sulfonyl]-2-propenenitrile) inhibits the inducible phosphorylation of IkB-α in a selective and non-reversible way and thus prevents its degradation. This results in a high level of cytosolic NF-κB, which sequesters IkB, and in decreased transcription and surface expression of the adhesion molecules (Kamthong PJ, 2001).

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Recent studies indicate that antioxidants inhibit NF-κB by NF-kB-dependent transcription of VCAM-1 (Spieker M, 1997). It is well established that dietary factors play significant etiologic roles in the development of atherosclerosis by influencing the immune and inflammatory processes associated with the development of this disease. Vitamin E, particularly alpha-tocopherol (AT), the main fat-soluble dietary antioxidant, appears to be the most effective in reducing VCAM-1 release *in vivo* and *in vitro* (Liu L, 2004).

Several studies have demonstrated that vitamin E diminished cytokine-stimulated HAEC production of chemokines and VCAM-1 (Martin KR, 2000), sICAM-1 (Wu D, 1999) or IL-6 and IL-8 (Liu L, 2004) after stimulation with IL-1β. In parallel, it was found that VCAM-1 had a constant elevated expression level in human umbilical endothelial cells (HUVECs) that were incubated for 6 to 24h with TNF-α, which remains at high levels under these conditions (Tribolo S, 2008; Raab M, 2002). It is found that exposure to TNF-α (10 ng/mL) for 16h increased VCAM-1 expression, which was measured by means of flow cytometry. Moreover, Tribolo S et al. (Tribolo S, 2008) co-treated HUVECs with TNF-α (10 ng/mL) and LPS (10 μg/mL) for 6h and tested VCAM-1 mRNA expression. It is observed a very marked increase VCAM-1 expression. Thus, we chose a stimulation period of 24h in order to render it possible to evaluate the modulatory influences of TNF-α on the expression of VCAM-1 by HAECs.

### 7.3 Assessment of endothelial function in HAECs

Endothelial dysfunction is involved in the pathogenesis of atherosclerosis (Bonetti PO, 2003; Ferroni P, 2006; Zhou Z, 2005). Endothelial dysfunction is due to oxidative stress and reduced availability of NO (Kawakami A, 2008; Aljada A, 2000; Federici M, 2004). NO is synthesised by conversion of L-arginine as a result of eNOS mRNA expression (Li H, 2002). A reduction of eNOS expression may contribute to endothelial dysfunction (Tai SC, 2004, Dudzinski DM, 2007), and loss of NO production and its bioavailability

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(Aberle S, 1997; Gonzalez-Fernandez F, 2001). An important feature of a healthy endothelium is an adequate output of NO (Li H, 2002; Tai SC, 2004).

Several highly organised layers of regulation exist, such as the control of eNOS mRNA expression, which is controlled at the transcriptional and posttranscriptional levels, and by epigenetic mechanisms that appear to modulate tissue-specific and cell-specific eNOS expression (Dudzinski DM, 2007).

Although eNOS was initially considered to be a constitutive enzyme, subsequent studies have clearly shown that its expression is regulated by a variety of stimuli (Govers R, 2001; Grumbach IM, 2005). Extended pro-inflammatory cytokine treatment decreases eNOS activity and expression in endothelial cells (Aberle S, 1997; Tai SC, 2004; Hussein KK, 1975; Roth GA, 2006). One of the most potent inhibitory stimuli for eNOS expression is the pro-inflammatory cytokine TNF-$\alpha$ (Yan G, 2008; Seidel M, 2006; Suwannaprapha P, 2005). Several reports have demonstrated that TNF-$\alpha$ reduces endothelium-dependent vasorelaxation in vivo and ex vivo (Tai SC, 2004; Aljada A, 2002; Roth GA, 2006; Yoshizumi M, 1993; Kosmidou I, 2007; Anderson HD, 2004). Indeed, TNF-$\alpha$ treatment reduced the eNOS mRNA half-life from 48h under basal conditions to 3h in Bovine Aortic Endothelial Cells (BAECs) and in HUVECs (Li H, 2002; Gonzalez-Fernandez F, 2001; Hussein KK, 1975; Seidel M, 2006; Arriero MM, 2000). In BAECs, a maximal decrease in eNOS protein expression was observed by western blot analysis between 12 and 24h after TNF-$\alpha$ incubation (10 ng/mL). Expression reached a plateau phase between 8 and 48h (Gonzalez-Fernandez F, 2001; Anderson HD, 2004). Long-term incubations of HAECs (from 2 to 6 days) with different TNF-$\alpha$ concentrations (5 and 10 ng/mL) resulted in a dose-dependent inhibition of eNOS protein levels, as shown by western blot. This result was corroborated in BAECs incubated with increasing concentrations of TNF-$\alpha$ (from 0-10000 pg/mL) (Aljada A, 2002).

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Vascular endothelium is a target tissue of insulin, which promotes bioavailability of NO by activating the signalling pathway involving the insulin receptor (IR), the IR substrate-1 (IRS-1), phosphatidylinositol 3-kinase (PI3K), and Akt, which leads to the activation of eNOS (Kawakami A, 2008; Zeng G, 2000), which finally increases NO levels (Aberle S, 1997). Thus, insulin has an important vascular role in activating the eNOS protein (Muniyappa R, 2007). Insulin increases eNOS expression in both HAECs and HUVECs (Aljada A, 2002). In BAECs, insulin (100 nM) significantly augmented eNOS mRNA expression and eNOS protein levels at 1h by 33 ± 9%. eNOS expression attained a maximum stimulation of 2-fold at 12h, which was maintained for 24h (Kuboki K, 2000).

Some of the current *in vitro* models have been used to analyse eNOS mRNA expression in HUVECs (Federici M, 2004; Aberle S, 1997) and BAECs, since eNOS was first identified and isolated from this cell type (Li H, 2002). Interestingly, higher eNOS protein levels have been observed in HAECs than in HUVECs, indicating that there may be differences in eNOS expression between endothelial cells of different origin (Aberle S, 1997). We selected HAECs instead of HUVECs or BAECs due to our interest in studying several aspects of human CVD.

Previous reports had taken into account the importance of the cell culture medium composition for endothelial cells, i.e., growth factors and serum deprivation. Confluent endothelial cells have been grown in medium lacking insulin in order to determine insulin's effects (Kawakami A, 2008; Wang XL, 2006; Iantorno M, 2007). Incubation of BAECs overnight in medium lacking serum prior to insulin treatment provided the ideal situation to evaluate NO production by immunoblotting (Iantorno M, 2007). Thus, to propose an *in vitro* model, insulin treatments were performed either in complete medium or in medium deprived of hydrocortisone, human epidermal growth factor, basic fibroblast growth factor and heparin.

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Studies show that the number of cell passages plays an important role in the expression of eNOS by endothelial cells, as does age. Older cells achieve less eNOS expression. Indeed, almost all *in vitro* models have been produced using cells at the same passage to avoid instability found in cell culture (Matsushita H, 2001).

### 7.4 Study of thrombosis in HAECs

Thrombus formation is associated with rupture of the atherosclerotic plaque (Pendurthi UR, 2002) leading to an acute coronary event or to a stroke (Meerarani P, 2006). Accumulation of TF in atherosclerotic plaques is thought to play a critical role in determining plaque thrombogenicity (Taubman MB, 1997). Inflammation, the key component in the pathogenesis of atherosclerosis, promotes thrombosis by the induction of TNF-α.

TF is a 47-kDa transmembrane glycoprotein that activates the coagulation cascade and is considered a major regulator of coagulation, haemostasis, and thrombosis (Camera M, 1999). TF expression is regulated in a cell-specific manner. Under physiological conditions, TF is constitutively expressed in many extravascular cell types including fibroblasts and pericytes, but it is not expressed by cells that are in direct contact with blood such as circulating monocytes or the endothelial cells that line blood vessels (Fleck RA, 1990; Rao LV, 2005). However, certain pathological conditions, such as sepsis and cancer, can induce TF expression in monocytes and endothelial cells (Camera M, 1999).

TF is the cellular receptor for coagulation factor VII (FVII) and its activated form, FVIIa (Jude B, 2005), is the primary trigger of the blood coagulation cascade. This complex leads to production of thrombin, which subsequently stimulates platelet activation and fibrinogen cleavage (Fleck RA, 1990; Rao LV, 2005). In this context, the expression of TF may be relevant to pathophysiological conditions if the outcome of the condition is an increased surface expression of TF. Since expression of TF is thought to play a critical

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role in pathogenesis of CVD, the consumption of red wine could be associated with a decreased risk of CVD because of its potential ability to suppress the expression of TF in the arterial wall (Pendurthi UR, 1999).

Many authors have focused their investigations on the effects of resveratrol on TF activity (Pendurthi UR, 1999; Pendurthi UR, 2002; Di Santo A, 2003; Kaur G, 2007). The regulation of TF by resveratrol occurs predominantly at the level of transcription, mRNA stabilisation and the subsequent expression of the TF protein (Camera M, 1999).

Resveratrol can exert an antithrombotic effect by reducing TF gene expression in endothelial cells that have been previously exposed to pathophysiological stimuli produced by TNF-α.

## 8. VALIDATION OF A PROCESS

Method validation is a continuous process used to confirm that the analytical procedure employed, through scientific studies, for a specific test is suitable for its intended use. The results of the method validation can be used to judge the quality, reliability and consistency of the experimental process. Method validation has received considerable attention in the literature and from industrial committees and regulatory agencies.

The steps involved in method development and its validation depend upon the type of method being developed. Although, there are a lack of information related to cell culture *in vitro* validation processes applied to atherosclerosis (i.e., http://ecvam.jrc.it).
However, the following steps are common to most types of projects (Breaux J, 2003; Breier S, 2008):

- Method development plan definition
- Background information gathering
- Laboratory method development
- Generation of test procedure

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- Method validation protocol definition
- Laboratory method validation
- Validated test method generation
- Validation report

Optimal validation of a continuous analytical method involves a gold standard or reference method (Huysmans MC, 2004) and its validity are unknown in reference to *in vitro* cell models.

The proper use of cells, whether in research or for commercial exploitation, requires that they are validated. In an industrial environment, this is a legal obligation if the ultimate product is to be accepted by the FDA in the United States or the National Institute for Clinical Excellence (NICA) in the United Kingdom. However, in an academic research laboratory the requirement may be well-defined and the obligation left to individual conscience (Culture of animal cells; a manual of basic technique. 2005. chapter 7, 103).

There are three major elements to validation:

- *Authentication of the cells*: DNA fingerprinting and profiling are probably the best techniques, but require that DNA is available from the donor or, at least from an earlier generation of the cells to be authenticated at the time that the sample was preserved.
- *Provenance:* It is important to document what has happened to the cells since their original isolation. The validation process that requires that there be a record of how the cells were isolated and what has happened to the sample since isolation.
- *Contamination*: The cells should be free from all known forms of microbial contamination. The results would be compromised if the cells were shown to be contaminated with one or more microorganisms (Freshney R, 2005).
-

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## 8.1 Parameters for Method Validation

The parameters for method validation have been defined in different working groups of national and international committees and are described in the literature.

Validation requirements depend upon the type of test method, including the following:

- Specificity: ability to measure the desired analyte in a complex mixture.
- Accuracy: agreement between the measured and real value.
- Linearity: proportionality of the measured value to the concentration.
- Precision: agreement between a series of measurements
- Range: concentration interval where the method is precise, accurate, and linear.
- Detection limit: lowest amount of analyte that can be detected.
- Quantitation limit: lowest amount of analyte that can be measured.
- Robustness: reproducibility under normal but variable laboratory conditions.

The transfer of analytical methods from one group to another then becomes an important step for ensuring that the proper validation is in place to justify its intended use (Breaux J, 2003).

Moreover, statistical methods can aid in decisions of what and how much to measure, and it can provide a useful assessment of the process capabilities and conformity of the product to requirements.

## 8.2 Reference results

The results of control experiments are an integral part of the test itself and are obtained in individual experiments to safeguard proper test performance. Usually positive control is a molecule that produces the expected effect while

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negative control produces the contrary effect are studied, Also, vehicle of molecules dissolution are analysed. Sometimes additional benchmark controls are included, i.e., substances that induce a well-defined response in a test.

The controls serve as a basis for interpreting responses to other substances. In addition to controls for test performance, control results are often used to normalise the results and/or to support the interpretation of the results obtained with test substances.

In any validation exercise that does not include a reference test to be carried out in parallel, it is mandatory to define the reference results of the substances to be tested prior to the actual testing. This is one driving factor in the selection of such chemicals/substances (Bottini A.A, 2008).

In addition to the above-mentioned issues, results of testing using reference substances should be selected and established by considering all the available data and information (figure 8). This would require systematic, transparent guidance based on independent processes of data collection and integration similar to those described by Balls et al. (2006).

In a case where no or insufficient reference substances can be identified that meet the pre-defined criteria, it might be necessary to generate more toxicological information on candidate chemical/substances in order to come to a decision regarding reference results (Hoffmann S, 2008).

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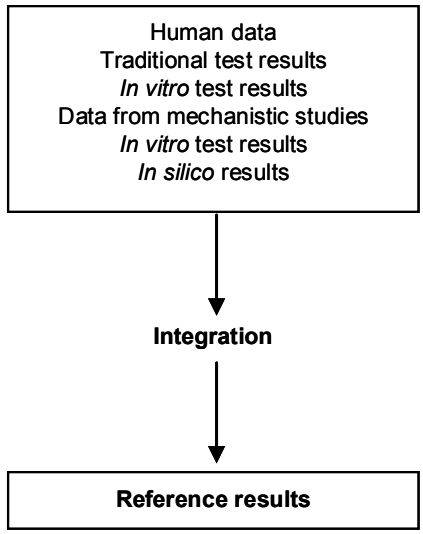


**Figure 8:** Integration of data and information to generate reference results.
Adapted from*:* Hoffman S et al., Altern Lab Anim. 2008;36(3):343-52.

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## 9. ASSESSMENT OF ANALYTICAL QUALITY FOR BIOMEDICAL RESEARCH

The analytical quality measurements are large described for biomedical research, and these criteria can be used for cell culture methods in order to improve quality of data (Altman DG, 1983).

**9.1**. Quality of the laboratory measurement
**9.2**. Setting up and testing a method

### 9.1 Quality of a laboratory measurement

**9.1.1** Precision (vs Error)
**9.1.2** Variability
**9.1.3** Analytical range, limit of detection and limit of quantification
9.1.3.1 Limit of detection
9.1.3.2 Limit of quantitation
9.1.3.2 Sensitivity
**9.1.4** Robustness/Specificity
**9.1.5** Accuracy (*vs* Bias)
**9.1.6** Diagnostic power

Control of analytical performance of measurements is a prerequisite for a good and true study result.

**9.1.1 Precision (vs Error):**

Refers to the consistency of measurement. It is the closeness of a series of measurements and is calculated by means of:

- Standard deviation (SD)
- Coefficient of variation (CV)
- SEM: describes the uncertainty of how the sample mean represent the population mean.

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Assessment of Precision:

Repeating measurement on the same sample can be subdivided into two values:

repeatability and reproducibility, which are calculated using simplified methods or ANOVA.

On one hand, repeatability (within-day; intra-assay) is obtained by repeating measurements using the same sample:

- within-batch/run/assay (intra-assay)
- between-batch

On the other hand, reproducibility (between-day; inter-day), which represents the precision obtained between different days, or laboratories, could be affected by various experimental parameters for which we must account:

- Operator with different experience and thoroughness
- Instruments and pipettes
- Recalibration of instrument solutions
- Differences in room temperature and humidity
- Laboratory conditions
- Variations in material and instrument conditions
- Variation in experimental details not specified by the method
- Equipment and consumables of different ages
- Solvents, reagents and other materials with varying quality

The objective is to verify that the method will provide the same results in different laboratories and operators.

### 9.1.2 Variability

In order to calculate precision, some questions have to be answered:

- Normal distribution?
- Same variance? Determine at various concentrations
- Batch-difference? Decide what is acceptable
- Total within-assay CV at various concentrations

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- Between-day CV at various concentrations
- Total analytical variability

Variability one must take into account that there are many sources of errors:

- The Gaussian theory concluded that errors are additive, so:
  - Total SD2= SD21+SD22+SD23+SD24...

- Total variability = Analytical + Biological +…, which, in turn :
  - Analytical: within-day + between-day + dilution

The final calculated value would be acceptable if it is < 50% of total.

High repeatability and reproducibility can reduce the number of measurements that need to be done.

### 9.1.3 Analytical Range, Limit of detection and Limit of quantification

The range of concentrations for which the test is applicable; the interval between the upper and lower levels (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity. The range is normally expressed in the same units as the test results (i.e., percentage, parts per million) obtained by the analytical method.

Ideal: - Linear, Accurate, Precise
- Through Zero
- Includes 99% of expected undiluted samples

#### 9.1.3.1 Limit of detection

The smallest concentration that will produce a response higher than the noise level.

#### 9.1.3.2 Limit of quantitation

The smallest concentration that is detected with reasonable certainty, accuracy and precision. It depends on:

- Blanks (background); noise; drift

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- Precision
- Probability level

#### 9.1.3.3 Sensitivity

In evaluating and selecting markers, the sensitivity of the marker is important.

Change in signal per change in concentration.

To check for linearity, range, limits, sensitivity

- Repeat calibration > 3 times.
- Take > 5 concentrations, evenly spaced, at:

  50-150% of target analyte

  80-120%
- Determine $R^2$, slope, intercept, SEs. Intercept < 10%of target
- Scatter plot: check linearity at extremes; Z residuals -2 to + 2
- Check for outliers
- Determine CV at all concentrations
- Limit of Detection = mean blank + 2*SD lowest measured concentration.

  + 2*SD blank (95% prob.)

  + 3*SD blank (99% prob.)

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# Hypothesis and Objectives

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## HYPOTHESIS

*In vitro* cell models are effective preliminary tools for screening the biological properties of ingredients, compounds and extracts of foodstuffs. The scientific evidence could then be explored in animal models or in humans.

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# OBJECTIVES

## 1. GENERAL OBJECTIVE

To implement and standardise experimental methodology to monitor the effect of compounds or extracts obtained from food on anti-inflammatory system of monocytes THP-1 cells, as well as the endothelial function and the anti-thrombotic responses of HAEC.

In the implementation of the presented *in vitro* models, we have used several bioactive natural extracts given by la Morella Nuts for the screening of anti-inflammatory and anti-thrombotic activities and improvement of endothelial function. However, we cannot cite the results obtained using these extracts since they are protected by confidentiality.

## 2. SPECIFIC OBJECTIVES

1. To implement an *in vitro* cell model system to assess inflammation based on TNF-$\alpha$ protein or mRNA expression in LPS-stimulated monocytes THP-1 cells.
2. To study endothelial dysfunction measured by VCAM-1 protein and mRNA production in TNF-α-stimulated HAECs.
3. To determine the effect of BAY and AT on VCAM-1 protein and mRNA production in TNF-α-stimulated HAECs.
4. To assess endothelial function based on eNOS mRNA expression induced by TNF-$\alpha$ or by insulin in HAECs.
5. To evaluate thrombogenic consequences measured by TF in TNF-α-stimulated HAECs.
6. To assess the effects of resveratrol on TF protein or mRNA expression in TNF-α-stimulated HAECs.

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# Material and Methods

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# 1. DEVELOPMENT OF AN *IN VITRO* MODEL TO SCREEN INFLAMMATION IN MONOCYTES THP-1 CELLS

## 1.1 MODEL RATIONALE

This model is based on the induction of inflammation of monocytes THP-1 cells by LPS stimulation, which provokes an increase of the inflammatory cytokine, TNF-α.

It is predicted an inhibition of TNF-α at the protein or at mRNA levels caused by compounds that reduce the inflammation state caused by the LPS stimulation.

Thus, we developed an *in vitro* cell-based system to test the anti-inflammatory effects on monocytes monocytes THP-1 cells of different compounds at several concentrations .

a) The blank conditions were as follows:

- Untreated cells: non-stimulated cells.
- Vehicle alone control: not necessary in these experiments since the LPS vehicle was pyrogen free sterile water.

b) The controls of the model were:

- *Positive control*: a compound or molecule that produces the expected effect. In the present case, we used a compound that produced an anti-inflammatory effect and significantly reduced the TNF-$\alpha$ protein and mRNA expression.
- *Negative control*: a compound or molecule that produces an effect not expected to be observed in the experiment; in this case, an inflammatory effect.

Thus, we developed an *in vitro* cell-based system to test the inflammation in LPS-stimulated monocytes THP-1 cells.

***Expression of results***

The effect of the tested compound was calculated by comparing TNF-$\alpha$ protein or mRNA expression in monocytes THP-1 cells pre-incubated with each compound to the results obtained when cells were stimulated only with LPS.

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TNF-α protein release was as a percentage of that released by monocytes THP-1 cells that were stimulated with LPS alone.

TNF-α mRNA expression was reported as an expression by monocytes THP-1 cells that were stimulated with LPS alone.

In both cases, it refered to increases or decreases of expression or release TNF-α as a percentage, comparing each condition tested to the maximum stimulation achieved by LPS, which was considered to be 100%.

All data were obtained from duplicate or triplicate experiments.

## 1.2 CELL ORIGIN

The monocytic leukemia cell line THP-1 was isolated by Tsuchiya S. et al, in 1980 from the blood of a 1-year-old boy suffering from acute monocytic leukemia.

Each cell batch used tested negative for mycoplasma, Epstein-Barr virus (EBV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Human Herpes virus 8 (HHV-8), Human immunodeficiency virus (HIV), Human T-cell lymphotropic virus I/II (HTLV-I/II), and squirrel monkey retrovirus (SMRV) according to the manufacturer.

## 1.3 EXPERIMENTAL PROCESS

**Materials and Reagents:**

1. The THP-1 cell line was provided as cryo-preserved cultures from DSMZ.
2. Complete RPMI 1640 + GlutaMAX-I culture medium (Gibco, Invitrogen; Paisley, UK).
3. Heat-inactivated FBS Gold (PAA, Labclinics; Barcelona, Spain).
4. Penicillin/Streptomycin (P/S) (PAA, Labclinics; Barcelona, Spain).
5. Sterile plastic tubes of 15 mL (Nunc, Labclinics; Madrid, Spain).
6. Sterile 75-$mm^2$ flasks (Nunc, Labclinics; Madrid, Spain).
7. Pasteur pipette (Sarstedt; Barcelona, Spain).
8. Dimethyl sulfoxide (DMSO; Sigma-Aldrich, Madrid, Spain).
9. Nunclon Δ surface Multidishes of 12 wells (Nunc, Labclinics; Madrid, Spain).

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10. LPS from *Escherichia coli* (O55:B5) (Sigma-Aldrich; Madrid, Spain) dissolved in sterile water and stored at -70 ºC.
11. Pyrogen-free sterile water (Braun Medical; Barcelona,Spain).
12. TNF-α ELISA kit (R&D Systems; Minneapolis, MN, USA), which contains *Escherichia coli*-derived recombinant human TNF-α and antibodies against this protein. The analytical range of this ELISA was 0 – 1000 pg/mL.
13. For mRNA expression studies, $MgCl_2$, nucleic acid purification lysis solution, RNA purification wash solutions, nucleic acid purification elution solution, Taq Polymerase, and pre-designed primers for TNF-α and for GAPDH were obtained from Applied Biosystems (Foster City, CA, USA). The Quant-it™ RNA-Assay kit, SuperScript II reverse transcriptase, RNAse Out, DTT, and dNTPs were purchased from Invitrogen (Paisley, UK).
14. Cytotoxicity Detection Kit for LDH (Roche Applied Science; Mannheim, Germany).
15. Trypan blue (Merck & Co., Inc.; Darmstadt, Germany).

**Equipment:**

1. Olympus IMT2 microscope (Olympus; Barcelona, Spain).
2. Centrifuge Multifuge 3L-R (Heraeus; Madrid, Spain).
3. ABI PRISM 7900 Detection System (Applied Biosystems; Foster City, CA, USA).
4. ABI PRISM® 6100 Nucleic Acid PrepStation extraction system (Applied Biosystems; Foster City, CA, USA).
5. QubitTM flourometer (Invitrogen; Paisley, UK).
6. Laminar flow cabinet (CV-100,Telstar Industrials S.L; Barcelona, Spain)
7. ELISA reader Bio Whittaker (Inverness Medical; Barcelona, Spain).
8. Multiwash plate washers (Tecan; Barcelona, Spain).
9. Vortex HEIDOLPH (Merck; Barcelona, Spain).
10. 2720 Thermal Cycler (Applied Biosystems; Foster City, CA, USA).
11. Liquid Nitrogen tank (Air Liquide; Madrid, Spain).
12. Heated Bath (Selecta, Afora; Barcelona, Spain).
13. Incubator HERAcell 150 (Heraeus, Control técnica; Madrid, Spain)

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**Preparation of cell stocks:**

1) Monocytes THP-1 cells were thawed and resuspended in complete RPMI 1640 + GlutaMAX-I (1x) culture medium supplemented with 20% (v/v) heat-inactivated FBS Gold, 100 U/mL of Penicillin and 100 mg/mL of Streptomycin in 15 mL sterile plastic tubes, according to the manufacturer's instructions supplied by DSMZ.
2) The cellular suspension was mixed gently several times, using a Pasteur pipette, to homogenise the preparation.
3) Monocytes THP-1 cells were centrifuged (200 g, 5 min at room temperature) to separate them from the cryopreservation solution (DMSO), which is toxic to cells after long periods in culture.
4) The pellet was resuspended with RPMI 1640 medium (20% FBS) to assess cell viability and to count the cells.
5) The obtained cellular density (cells/mL) was adjusted with RPMI 1640 medium (20% FBS) (see section **A**; *cell counting*).
6) Cells were maintained and grown in suspension in 75-$mm^2$ flasks for the first 48h at 37 ºC in a humidified atmosphere with 5% $CO_2$.
7) Cells were split 1:2 every two days with RPMI 1640 medium supplemented with 10% FBS rather than 20%, and maintained at 37 ºC (5% $CO_2$) until the desired cellular density (cell/mL).

*The normal cellular division rate was every two days but it could be accelerated after one week of maintenance. Once the cell density exceeded 3x$10^5$ cells/mL, the cells divided every day. The cellular density must be controlled, and it must not exceed 1x$10^6$ cells/Ll. If this occurs, it is recommended to eliminate the cellular stock for the experiment. Finally, the cell culture medium must always be used at 37 ºC.*

Once 4-5x$10^6$ cells/mL was achieved:

a) Monocytes THP-1 were frozen for 24h at -80 ºC in a freezing container.

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b) The recommended guidelines for cellular cryopreservation were followed. Monocytes THP-1 cells were stored within 24-48h at -196 ºC in the gas phase of the liquid Nitrogen ($N_2$) tank.

c) Then, cells were replaced and maintained in the liquid phase of the $N_2$ tank until their use.

This protocol was repeated until we reached the desired cellular density to perform all the planned experiments.

**a) Monocytes THP-1 cells stimulation and response**

1) Following the detailed protocol described above, cells from previously frozen stocks were thawed, maintained and plated with in RPMI 1640 + GlutaMax medium (10% FBS) in Nunclon Δ surface Multidishes of 12 wells (1 mL/well) at a density of ~ $5x10^5$ cells/mL (see section **A**: *example of cellular density calculation*) for the experimental process.

The cells must be seeded by moving the culture plate in a circular motion to guarantee a good cellular dispersion in each individual well.

**a)** To study the effect of the FBS content of culture medium to distinguish whether it affects monocytes activation.

Monocytes THP-1 cells ($5x10^5$ cells/mL) were challenged for 4h with LPS (0 - 2000 ng/mL, mixed previously for 1 min) in the absence or presence of FBS in the culture medium (0, 2%, 5% or 10%) at 37 ºC.

**b)** To study the behaviour of the monocytes THP-1 cells kept in culture for prolonged periods, cells were thawed and cultured for 6, 12 and 19 days.

Then the cells were challenged for 4h with different concentrations of LPS (0 - 2000 ng/mL) in culture medium supplemented with 5% FBS at 37 ºC.

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**c)** To study the response of monocytes THP-1 cells derived from different batches were also tested. Cells were thawed and grown for 6 days to study activation with different doses of LPS (0, 100, 500 and 1000 ng/mL) over 4h in culture medium supplemented with 10% FBS at 37 ºC.

2) Cells were maintained at 37 ºC in a humidified incubator and atmosphere 95% to 5% (v/v) mixture of air and $CO_2$.
3) After each experiment, cell suspensions were collected and centrifuged (200 g, 5 min at room temperature), and the supernatants were discarded.
4) Cell pellets were resuspended in fresh RPMI 1640 medium, and cells were counted using the Trypan blue exclusion assay.

Cell-free, supernatants were separated from the pellets and each specimen was processed differently:

*Supernatants*:

1) Supernatants were collected with sterile micropipettes with filter tips, transferred to sterile plastic tubes (1 mL) and centrifuged (300 g, 5 min at room temperature) to ensure that there was no cell debris in the solution. It was necessary to remove the majority of the cell culture medium from each well since it contained $Ca^{2+}$ and $Mg^{2+}$, which can interfere with posterior cellular lysis and consequently, it can affect the final results.
2) Afterwards, monocytes THP-1 free supernatants were harvested as described previously until the cytotoxicity and ELISA analyses.

The performance characteristics for TNF-α ELISA assay were:

- ***Sensitivity***

  The minimum detectable dose (MDD) of TNF-α ranged from 0.5 - 5.5 pg/mL. The mean MDD was 1.6 pg/mL.

- ***Precision***

  CV intra-assay (5.3%) and CV inter-assay (8.7%).

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*Pellet:*

We extracted the mRNA and proceeded as indicated previously (see *quantification of the mRNA expression*).

**b) Study of the anti-inflammatory effects of natural bioactive compounds in monocytes THP-1 cells**

1) THP-1 cells ($5x10^5$ cells/mL with 10% FBS) were seeded in Nunclon Δ surface Multidishes of 12 wells for the experimental procedure (1 mL/well).
2) Each well was treated individually with either natural bioactive compounds at different non-cytotoxic concentrations or with the vehicle of the compound (vehicle alone control) for 1h. A blank control and vehicle alone control were run in all experiments.
3) Cells were maintained at 37 ºC in a humidified incubator and atmosphere 95% to 5% (v/v) mixture of air and $CO_2$.
4) Monocytes THP-1 cells were incubated with LPS (100 ng/mL) in all wells except the untreated cells and vehicle controls, for 4h at 37 ºC.
5) Then, the cell suspension was collected with sterile micropipettes with filter tips, transferred to RNAase-free sterile tubes, and centrifuged (200 g, 5 min at room temperature).

*The wells were washed with the cellular suspension in order to collect the cells that remained on the plate.*

6) THP-1 cell-free supernatants were stored at -20 ºC and used either for testing the cytotoxicity or to quantify the TNF-α released into the medium by ELISA.
7) Cells were lysed with 400 μL of nucleic acid purification lysis solution and processed as stated previously (see section **C** *quantification of mRNA expression*).

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# 2. DEVELOPMENT OF AN *IN VITRO* MODEL TO SCREEN ENDOTHELIAL FUNCTION IN HAECs.

## VCAM-1 protein and mRNA expression in TNF-α-stimulated HAECs

### 2.1 MODEL RATIONALE

This model was based on endothelial dysfunction in HAECs caused by TNF-α, which provokes an increase of VCAM-1.

These studies were focused either on protein or mRNA expression.

a) The blank for these experiments was the vehicle alone control (DMSO or Ethanol; EtOH)

b) Controls of the model were:

- *Positive control:* a compound or molecule that produces the expected effect; a reduction of VCAM-1 protein and mRNA expression.
- *Negative control:* a compound or molecule that produces the undesired effect; an increase of VCAM-1 protein and mRNA expression.

Thus, we developed an *in vitro* cell-based system to test the endothelial function in TNF-α-stimulated HAECs.

***Expression of results***

To validate the effect of the tested compound, we compared VCAM-1 protein or mRNA expression in HAECs pre-treated with the compounds to cells stimulated with TNF-α alone.

The soluble VCAM-1 protein (VCAM-1) results were assessed using a Bradford assay and normalised per protein. Protein levels were reported as ng VCAM-1/mg of total protein.

VCAM-1 mRNA expression was reported as fold-increase or decrease. In both cases, it refers to increased or decreased expression as a percentage, comparing all conditions to the maximum stimulation by TNF-α, which was considered as 100%.

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A significant diminution of the VCAM-1 expression was considered to represent improvement of endothelial function.

All data were obtained from duplicate or triplicate experiments.

## 2.2 CELL ORIGIN

HAECs are primary endothelial cells isolated from normal human aorta.

HAECs have been cryopreserved at the end of the tertiary culture stage and can be cultured and propagated for at least 16 population doublings. They respond to cytokine stimulation by expressing cell adhesion molecules, by modifying their growth characteristics and by producing cytokines. Aortic endothelial cells have been used as an *in vitro* model for the study of morphological and ultra-structural changes (Antonor A.S, 1986) in the development of atherosclerosis.

Each lot of HAECs tested negative for HIV, HBV and HCV and negative for mycoplasma, bacteria, yeast and fungi.

## 2.3 EXPERIMENTAL PROCESS

### Materials and Reagents

1. HAECs (P5) (Cascade Biologics$^{TM}$, Invitrogen; Portland, USA).
2. Low serum growth supplement (LSGS): LSGS was an ionically balanced supplement containing FBS, hydrocortisone, human epidermal growth factor, basic fibroblast growth factor and heparin, required for a correct cell growth pattern.
3. Gentamicin/Amphotericin B solution ($G/A_B$) and P/S (Labclinics; Barcelona, Spain).
4. Trypsin/EDTA (TE) and Trypsin Neutralizer (TN) (Cascade Biologics$^{TM}$, Invitrogen; Portland, USA).
5. Synth-a-freeze (Cascade Biologics$^{TM}$, Invitrogen; Portland, USA).
6. Sterile plastic tubes of 15 mL (Nunc, Labclinics; Madrid, Spain).
7. Pasteur pipette (Sarstedt; Barcelona, Spain).
8. Phosphate buffered saline without $Mg^{2+}$ and $Ca^{2+}$ (PBS) (Gibco, Invitrogen; Paisley, UK).

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9. Nunclon Δ surface Multidishes of 12 or 24 wells (Nunc, Labclinics; Madrid, Spain).
10. Sterile 25/75-$mm^2$ flasks (Nunc, Labclinics; Madrid, Spain).
11. Human CD106 VCAM-1 ELISA (Diaclone; Besançon, France).
12. AT (Sigma; Madrid, Spain)
13. BAY11-7082 (Merk; Darmstadt, Germany).
14. Dimethyl sulfoxide (DMSO; Sigma-Aldrich, Madrid, Spain).
15. Ethanol (Panreac; Madrid, Spain).
16. For mRNA expression studies, $MgCl_2$, nucleic acid purification lysis solution, RNA purification wash solutions, nucleic acid purification elution solution, Taq Polymerase and pre-designed primers for GAPDH were obtained from Applied Biosystems (Foster City, CA, USA). The Quant-it™ RNA-Assay kit, SuperScript II reverse transcriptase, RNAse Out, DTT, and dNTPs were purchased from Invitrogen (Paisley, UK).
17. Trypan blue (Merck & Co., Inc.; Darmstadt, Germany).

**Equipment:**

1. Olympus IMT2 Microscope (Olympus; Barcelona, Spain).
2. Centrifuge Multifuge 3L-R (Heraeus; Madrid, Spain).
3. ABI PRISM 7900 Detection System (Applied Biosystems, Foster City, CA, USA).
4. ABI PRISM® 6100 Nucleic Acid PrepStation extraction system (Applied Biosystems; Foster City, CA, USA).
5. QubitTM flourometer (Invitrogen; Paisley, UK).
6. Laminar flow cabinet (CV-100; Telstar Industrials S.L, Barcelona, Spain).
7. Vortex HEIDOLPH (Merck, Barcelona, Spain).
8. 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA).
9. Liquid Nitrogen tank (Air Liquide; Madrid, Spain).
10. Heated Bath (Selecta; Afora, Barcelona, Spain).
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**Preparation of cells stocks at the 4th passage**

1) HAECs were provided as cryo-preserved tertiary cultures (P3).
2) Cells were thawed with medium 200 (M-200) supplemented with 2% of LSGS, G/$A_B$ solution (10 μg/ml Gentamicin; 0.25 μg/ml Amphotericin B) and with 100 U/mL penicillin and 100 mg/mL streptomycin according to the manufacturer's protocol supplied by Cascade Biologics™. This medium is referred to through the thesis as Complete Medium (CM).
3) The cellular suspension was mixed gently several times using a Pasteur pipette, to ensure a good homogenization of the cellular suspension prior to plating.
4) The cell suspension was seeded either on several 75-$cm^2$ flasks (T-75) or 25-$cm^2$ flasks (T-25). HAECs were maintained at 37 ºC in a humidified atmosphere with 5% $CO_2$.
5) After 24h in culture, the cell culture medium was removed from the flasks and cells were refreshed with CM to wash out the cryopreservation solution (Synth-a-freeze), which was toxic at 37 ºC after long periods in culture.
6) Then, the cell culture medium was changed every two days until HAECs achieved 80-90% of confluence (5th day, approximately).
7) By microscopic examination, HAECs confluent monolayers display a cobblestone phenotype typical of quiescent endothelial cells.
8) HAECs at ~ 80-90% of confluence were trypsinised with TE and TN following the manufacture's instructions (Cascade Biologics™).
9) As a result, cells were detached from the flasks, collected in sterile plastic tubes of 15 or 30 mL, and centrifuged (180 g, 7 min at room temperature).
10) The pellet was resuspended in CM. The cellular suspension was homogenised and counted using the Trypan blue exclusion assay (see *cell counting*).
11) Cells were centrifuged again (180 g, 7 min at room temperature) and resuspended with Synth-a Freeze solution, which ensures a gradual decrease in temperature of the cellular preparation. The volume of the

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solution was calculated depending either on the number of cells counted or on the desired final cellular density.

12) Afterwards and following the recommended guidelines for the cellular cryopreservation:
- HAECs were frozen for 24h at -80 ºC in a freezing container
- Cells were stored within 24-48h at -196 ºC in the gas phase of a liquid Nitrogen ($N_2$) tank.
- Then, cells were replaced and maintained in the liquid phase of the $N_2$ tank until their use.

**Preparation of cells stocks at 5$^{th}$ passage**

We followed the same protocol as the indicated below to prepare the HAEC stocks (P5) for the experimental process.

**Preparation of cells for the experiment**

1) HAECs at P5 were thawed in CM in a sterile plastic tube of 15 mL. The cellular suspension was mixed gently using a Pasteur pipette several times.
2) HAECs were seeded (~12x10$^3$ cells/cm$^2$) using circular movements on Nunclon Δ surface Multidishes of 24 wells (0.5 mL/well for protein studies) and 12 wells (1 mL/well for mRNA studies).

3) After 24h of maintenance at 37 ºC (5% $CO_2$), the culture medium was replaced with fresh CM to wash out the cryo-preservation solution (Synth-a-freeze) containing 10% of DMSO, which is toxic to cells at 37 ºC after long periods in culture.
4) The media was refreshed every two days until HAECs reached confluence (5$^{th}$ day in culture) (figure 9).

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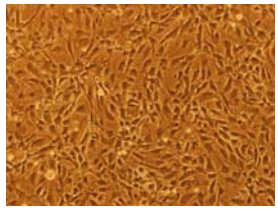

**Figure 9.** HAEC (100% confluence)

*The experimental procedure for the protein assays differed from that for the mRNA studies with respect to the number of wells per plate and the selected compound concentrations Nunclon Δ surface Multidishes of 24 wells required 0.5 mL/well (for the protein assays) and of 12 wells required 1mL/well (for the mRNA experiments).*

**TNF-α-stimulated HAECs on VCAM-1**

TNF-α was dissolved in pyrogen-free sterile water (stored at -80 ºC). All the TNF-α conditions were prepared individually and in excess (+ 0.5 mL) in sterile plastic tubes of 15 mL.

**a) Dose-response effect of TNF-α on VCAM-1 protein and mRNA expression in HAECs**

The blank was the TNF-α vehicle alone control (pyrogen free sterile water).

For **protein expression** assessment:

1) Cells were incubated with different dosages of TNF-α (5, 10 and 20 ng/mL) or vehicle for 24h at 37 ºC
2) After the treatment, cell culture medium was aspirated and centrifuged (3000 rpm, 10 min at room temperature) to separate cells debris from culture medium.

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*Supernatants*:

Cell-free culture medium was used to test cytotoxicity and VCAM-1 by ELISA.

The performance characteristics for this assay were:

- ***Sensitivity***

The minimum detectable dose of VCAM-1 (< 0.6 ng/mL)

- ***Precision***

CV intra-assay (2.27 %) and CV inter-assay (5.94 %)

*Pellets:*

1. The adhered cells were washed twice with PBS without $Mg^{2+}$ or $Ca^{2+}$, to clean the wells of culture medium.
2. Then, 0.4 mL/well of NaOH (500 mM) was added to cells in order to assess the total protein content by Bradford analysis.
3. Results were normalised per milligram of total protein.

For **mRNA expression** assessment:

1) A dose response time-course experiment was performed by stimulating HAECs with TNF-α (1, 2, 5 and 10 ng/mL) or vehicle control for 2, 4 and 24h. Once the appropriate experimental conditions were fine-tuned allowing for measurement of minimal increases in VCAM-1 mRNA expression, we opted for 1 ng/mL of TNF-α over a stimulation period of 24h.
2) After the treatment, cells were disrupted and lysed with 650 µL nucleic acid purification lysis solution.
3) We proceed as indicated previously (see section **C** *quantification of the mRNA expression*).

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**b) Dose-response of BAY on VCAM-1 protein and mRNA expression in TNF-α-stimulated HAECs**

The blank was the vehicle alone control (DMSO).

BAY was dissolved in absolute DMSO in amounts below the limit of cytotoxicity (<1 µL DMSO/mL of cell culture medium) and stored at -20 ºC.

For **protein expression** assessment:

1) HAECs were pre-treated with:
    a. BAY (2 µM) for 30 min followed by incubation with TNF-α (10 ng/mL) for an additional 24h.
    b. BAY (0.5, 1 and 10 µM) in combination with TNF-α (10 ng/mL) for 24h.
2) When the experiment was complete, cell supernatants were siphoned and centrifuged (3000 rpm, 10 min at room temperature).
3) The cell-free supernatants were harvested at -20 ºC until the determination of the VCAM-1 (ELISA) and cytotoxicity (LDH release).
4) The cells were washed twice with PBS without $Mg^{2+}$ or $Ca^{2+}$, to clean the wells of culture medium.
5) Afterwards, HAECs were lysed with 0.4 mL/well of NaOH (500 mM) to extract the protein.
6) The total protein content was determined by Bradford.

For **mRNA expression** assessment:

Since the TNF-α/BAY ratio used in protein assessments was 10:1 (10 ng/mL vs 1µM) and TNF-α implemented concentration for VCAM-1 mRNA studies, was at 1 ng/mL, BAY concentrations were 0.1 and 0.2 µM.

1) HAEC were treated with BAY (0.1 and 0.2 µM) with the presence of TNF-α (1 ng/mL) or with the vehicle alone control (DMSO) for 24h at 37 ºC.
2) Cell culture medium was aspired and centrifuged (3000 rpm, 10 min at room temperature).
3) Cell-free supernatants were stored at -20 ºC to test cytotoxicity.

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4) We extracted the mRNA from cells and proceed as indicated previously (see section **C**: *quantification of the mRNA expression*).

**c) Dose-response of α-Tocopherol on VCAM-1 protein and mRNA expression in TNF-α-stimulated HAECs**

The blank was the vehicle alone control (EtOH).

AT was prepared in absolute EtOH and stored at -80 ºC, protected from light. In order to avoid cytotoxicity, the EtOH concentration was not allowed to exceed 0.1% (v/v) in the culture medium.

1) HAECs were treated with AT (10, 25, 50, 75 and 150 μM) or the vehicle alone control, for 6h.
2) The cell culture medium was aspirated, centrifuged (3000 rpm for 10 min at room temperature), and stored at -20 ºC until the cytotoxicity test.

For **protein expression** assessment:

1) HAECs were treated for 24 h with:
    a. 0.4 mL of fresh CM with TNF-α (10 ng/mL) for 24h in the case where cells were pre-treated with AT. (The volume was reduced from 0.5 to 0.4 mL in order to increase the concentration of released VCAM-1 in the cell culture medium. Thus, experimental values were obtained within the analytical range of the selected ELISA Kit.)
    b. 0.4 mL of TNF-α (10 ng/mL) alone, to achieve the maximal stimulation.
    c. CM alone was added with the vehicle alone control.
    d. TNF-α (10 ng/mL) with BAY (1 μM) for 24h.

After the treatment, the cell culture medium was processed as described above.

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For **mRNA expression** assessment:

It was extracted the mRNA from cells as stated previously (see section **C** *quantification of mRNA expression*).

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# 3. DEVELOPMENT OF AN *IN VITRO* MODEL TO SCREEN ENDOTHELIAL FUNCTION IN HAECs.

## *eNOS mRNA* expression in HAECs

### 3.1 MODEL RATIONALE

HAECs were incubated with the selected molecules (insulin or TNF-α) at different concentrations in order to test their capacity to induce or reduce eNOS expression.

a) The blanks for these experiments were:
- Vehicle alone control (sterile water) for TNF-α experiments
- Untreated cells for insulin experiments

b) The controls of the model were:
- *Positive control* a compound or molecule that produces the expected effect, the increase of eNOS mRNA expression.
- *Negative control* a compound or molecule that produces the opposite effect, the reduction of eNOS mRNA expression.

Thus, we developed an *in vitro* cell-based system to test the endothelial function in HAECs.

***Expression of results***

It was compared eNOS mRNA expression in HAECs treated with each compound to eNOS mRNA expression in untreated cells and cells incubated with the vehicle alone control.

eNOS mRNA expression was reported as fold-increases or decreases.

It referes to increases or decreases expression of eNOS mRNA as a percentage of the expression level observed in untreated cells or cells treated with the vehicle alone.

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Thus, the effects of insulin or TNF-α on endothelial function were defined as the increase or decrease in eNOS mRNA levels in insulin- or TNF-α-treated HAECs relative to untreated cells.

An improvement of endothelial function was defined as a significant increase of the percentage of eNOS mRNA expression compared with control HAECs.
All data were obtained from duplicate or triplicate experiments.

## 3.2 CELL ORIGIN

It is described in 2.2.

## 3.3 EXPERIMENTAL PROCESS

**Materials and reagents**

1) HAECs (P5); (Cascade Biologics™; Invitrogen; Portland, USA).
2) LSGS
3) Basal medium (BM): M-200 with FBS.
4) $G/A_B$ solution, P/S (Labclinics; Barcelona, Spain).
5) TNF-α (Calbiochem; Darsmsdat, Germany).
6) Pyrogen-free sterile water (Braun Medical; Barcelona,Spain).
7) Insulin (Sigma; Madrid, Spain).
8) Sterile plastic tubes of 15 mL (Nunc, Labclinics; Madrid, Spain).
9) Sterile 75-$mm^2$ flasks (Nunc, Labclinics; Madrid, Spain).
10) Pasteur pipettes (Sarstedt; Barcelona, Spain).
11) Nunclon Δ surface Multidishes of 12 wells (Nunc, Labclinics; Madrid, Spain).
12) For mRNA expression studies, $MgCl_2$, nucleic acid purification lysis solution, RNA purification wash solutions, nucleic acid purification elution solution, Taq Polymerase and pre-designed primers for eNOS and for GAPDH were obtained from Applied Biosystems (Foster City, CA, USA). The Quant-it™ RNA-Assay kit, SuperScript II reverse transcriptase, RNAse Out, DTT and dNTPs were purchased from Invitrogen (Paisley, UK).

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13) Cytotoxicity Detection Kit of LDH (Roche Applied Science, Mannheim, Germany).
14) Trypan blue (Merck & Co., Inc., Darmstadt, Germany).

**Equipment**

12. Olympus IMT2 Microscope (Olympus; Barcelona, Spain).
13. Centrifuge Multifuge 3L-R (Heraeus; Madrid, Spain).
14. ABI PRISM 7900 Detection System (Applied Biosystems; Foster City, CA, USA).
15. ABI PRISM® 6100 Nucleic Acid PrepStation extraction system (Applied Biosystems; Foster City, CA, USA).
16. QubitTM flourometer (Invitrogen; Paisley, UK).
17. Laminar flow cabinet (CV-100; Telstar Industrials S.L, Barcelona, Spain).
18. Vortex HEIDOLPH (Merck, Barcelona, Spain).
19. 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA).
20. Liquid Nitrogen tank (Air Liquide; Madrid, Spain).
21. Heated Bath (Selecta; Afora, Barcelona, Spain).
22. Incubator HERAcell 150 (Heraeus, Control técnica; Madrid, Spain)

**a) Dose-response of TNF-α on eNOS mRNA expression in HAECs**

Each experimental condition was be prepared in excess (+ 0.5 mL) in order to have enough volume for all wells.

TNF-α stock solution (stored at -70 ºC at 10 μg/mL) was diluted with pyrogen free sterile water.

1) Confluent HAECs were treated with TNF-α (5 and 10 ng/mL) for 24h. We replaced the medium with 1mL/well of TNF-α (5 or 10 ng/mL).
2) HAECs incubated with cell culture medium alone (untreated cells) were designated as blank control and were included in all experiments.
3) After 24h, HAECs were observed by microscopy to evaluate cell morphology and to estimate viability.

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4) Cell culture medium was aspirated and centrifuged (3000 rpm, 10 min at room temperature).
5) Cell-free supernatants were stored at -20 ºC until cytotoxicity testing.
6) HAECs were disrupted and lysed with 650 µL nucleic acid purification lysis solution.
7) We proceeded as indicated previously (see section **C***quantification of the mRNA expression*).

**b) Time- and dose-response of insulin on eNOS mRNA expression in HAECs**

*Procedure A*

1) Confluent HAECs were treated with insulin at 10 - 5000 nM in CM or with CM alone (untreated cells) for 24 or 72h.
2) After treatment, the cell culture medium was removed carefully and centrifuged (3000 rpm, 10 min, at room temperature).
3) Cell-free supernatants were stored at -20 ºC until the cytotoxicity of insulin was tested at the selected dosages.
4) Cells were disrupted and lysed with 650 µL of nucleic acid purification lysis solution.
5) We proceeded as indicated previously (see section **C** *quantification of the mRNA expression*).

*Procedure B*

To avoid the effects of the different components of CM ((supplemented with 2% FBS, 10 ng/mL human epidermal growth factor, 3 ng/mL basic fibroblast growth factor, 10 µg/mL heparin and 1 µg/mL hydrocortisone, 10 µg/ml gentamicin, 0.25 µg/ml amphotericin B solution, 100U/mL penicillin, 100 µg/mL streptomycin), confluent HAEC starved of these components by incubation in BM (2% FBS) for either 2h or 18h prior to the experiment.

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This allowed for the removal of these factors from cells.

1) Then, HAECs were exposed with insulin (100, 500 and 1000 nM) for 24h in CM.
2) Untreated cells were included as blanks in all the experiments.
3) After the incubation, cell culture medium was removed carefully and centrifuged (3000 rpm, 10 min at room temperature).
4) eNOS mRNA expression was assessed by real time RT-PCR as described below.
5) The cell free supernatants were stored at -20 ºC until testing of the cytotoxicity of insulin at the selected dosages was performed.
6) Cells were disrupted and lysed with 650 µL nucleic acid purification lysis solution and eNOS mRNA expression was assessed by real time RT-PCR as described below.

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# 4. DEVELOPMENT OF AN *IN VITRO* MODEL TO SCREEN THROMBOSIS IN HAECs.

## 4.1 MODEL RATIONALE

Thrombosis was induced in HAECs by using TNF-α stimulation, which causes an increase of TF protein and mRNA expression as and produces a thrombogenic state.

a) The blank for these experiments was a vehicle alone control (EtOH).

b) The controls were:

- *Positive control* was a compound or molecule, resveratrol, that produced the desired effect, the reduction of TF protein and mRNA expression.
- *Negative control* was not found.

Thus, it is developed an *in vitro* cell-based system to test anti-thrombotic effects in TNF-α-stimulated HAECs.

***Expression of results***

The effect of the tested compound, was obtained comparing TF protein or mRNA expression in HAECs that had been pre-treated with each compound to TF protein or mRNA expression in HAECs that were stimulated with TNF-α alone.

The TF protein levels were reported as pg TF/mL and TF mRNA expression is presented as the fold-increase or decrease.

In both cases, it referes to increases or decreases expression comparing all the conditions with the maximum stimulation by TNF-α which was considered as 100%. An improvement of the thrombotic state was considered to be a significant reduction in the percentage of TF protein and mRNA expression compared with HAECs stimulated by TNF-α.

All data were obtained from duplicate or triplicate experiments.

## 4.2 CELL ORIGIN

Is is described at 2.2

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## 4.3 EXPERIMENTAL PROCESS

### Materials and reagents

1. HAECs (P5); (Cascade Biologics™; Invitrogen; Portland, USA).
2. LSGS supplement.
3. G/$A_B$ solution, P/S (Labclinics; Barcelona, Spain).
4. TNF-$\alpha$ (Calbiochem; Darsmsdat, Germany).
5. Pyrogen-free sterile water (Braun Medical; Barcelona,Spain).
6. Resveratrol was obtained from Sigma-Aldrich (Madrid, Spain).
7. The IMUBIND® Tissue Factor ELISA Kit (American Diagnostica inc, Stamford, USA).
8. Dimethyl sulfoxide (DMSO; Sigma-Aldrich, Madrid, Spain).
9. Sterile plastic tubes of 15 mL (Nunc, Labclinics; Madrid, Spain).
10. Sterile 75-$mm^2$ flasks (Nunc, Labclinics; Madrid, Spain).
11. Pasteur pipettes (Sarstedt; Barcelona, Spain).
12. Nunclon Δ surface Multidishes of 12 wells (Nunc, Labclinics; Madrid, Spain).
13. Cell scrapers (Nunc, Labclinics; Madrid, Spain).
14. PBS (Gibco, Invitrogen; Paisley, UK).
15. For mRNA expression studies, $MgCl_2$, nucleic acid purification lysis solution, RNA purification wash solutions, nucleic acid purification elution solution, Taq Polymerase and pre-designed primers for TF and for GAPDH were obtained from Applied Biosystems (Foster City, CA, USA). The Quant-it™ RNA-Assay kit, SuperScript II reverse transcriptase, RNAse Out, DTT and dNTPs were purchased from Invitrogen (Paisley, UK).
16. Cytotoxicity Detection Kit of LDH (Roche Applied Science, Mannheim, Germany).
17. Trypan blue (Merck & Co., Inc., Darmstadt, Germany).
18. Tris Buffered Saline (TBS) was generated at our laboratory with Tris (Sigma-Aldrich; Madrid, Spain), NaCl (Panreac; Barcelona, Spain) and Triton X-100 (Bio-Rad; Barcelona, Spain).

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**Equipment**

1. Olympus IMT2 Microscope (Olympus; Barcelona, Spain).
2. Centrifuge Multifuge 3L-R (Heraeus; Madrid, Spain).
3. ABI PRISM 7900 Detection System (Applied Biosystems; Foster City, CA, USA).
4. ABI PRISM® 6100 Nucleic Acid PrepStation extraction system (Applied Biosystems; Foster City, CA, USA).
5. QubitTM flourometer (Invitrogen; Paisley, UK).
6. Laminar flow cabinet (CV-100; Telstar Industrials S.L, Barcelona, Spain).
7. Vortex HEIDOLPH (Merck, Barcelona, Spain).
8. ELISA reader Bio Whittaker (Inverness Medical; Barcelona, Spain).
9. Multiwash plate washers (Tecan; Barcelona, Spain).
10. 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA).
11. Liquid Nitrogen tank (Air Liquide; Madrid, Spain).
12. Heated Bath (Selecta; Afora, Barcelona, Spain).
13. Incubator HERAcell 150 (Heraeus, Control técnica; Madrid, Spain).

**a) Dose-response of TF protein expression to resveratrol**

Each experimental condition was prepared in excess (+ 0.5 mL) in order to have enough volume for all wells.

Resveratrol (stored at -70 ºC) was dissolved with EtOH (its concentration never exceeded 0.1 % (v/v) in culture medium in order to avoid cytotoxicity).

1) HAECs were treated with resveratrol (5, 10, 25, 50 and 100 µM) or with vehicle (EtOH) for 16h, at 37 ºC (5% $CO_2$).
2) Then, cell culture media were removed and the cells were challenged with TNF-$\alpha$ (10 ng/mL), for 6h.
3) The cell culture medium again collected and then centrifuged (3000 rpm, 10 min at 4 ºC).
4) Cell-free supernatants were stored at -20 ºC until cytotoxicity testing.

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5) After the complete treatment and depending on the analysis that we were interested in (protein or gene expression of TF), we proceeded as described below.

6) For **protein expression** assessment:
   a. It was important to maintain protein stability and structure in order to avoid protein degradation. For this purpose, the following precautions were taken:
      - All material was maintained at 4 ºC
      - Cellular extracts were collected on ice
      - Samples were frozen at -80 ºC as soon as possible after collection
   b. After the treatment, the cells that were adhered to the well surface of the culture plates were washed twice with cold PBS (4 ºC). This was performed in order to clean the wells and to avoid interference with the ELISA spectrophotometer reader since the medium is coloured.
   c. PBS was added directly to the wall of each well, instead of to the cells in order to avoid disrupting adherence of the cells to the culture plate. Moreover, it was recommended to work in a maximum of two or three wells simultaneously in order to preserve the adherence of the cells. We observed that cells could be released from the plate easily as a consequence of prolonged exposure to PBS.
   d. Then, cells were lysed with 110 µL of Tris Buffered Saline (TBS; 50 mM Tris and 100 mM NaCl) at pH 7.4 containing 0.1% Triton X-100.
   e. It was necessary to gently scrape each well in a circular motion to detach the adherent cells and to displace the entire volume at the bottom of the well to facilitate the recollection.
   f. The suspension was mixed gently several times with a micropipette.

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g. The samples from each well were collected and placed into a cryotube of 1.5 mL.

h. HAECs were disrupted by three repeated freeze-thaw cycles of 10 min each one (10 min at 37 ºC followed by 10 min at -196 ºC in $N_2$).

i. To facilitate the extraction, it was recommended to store the lysed cells at 2-8 ºC overnight (o/n for 18h).

j. Afterwards, cell lysates were centrifuged at 3000 rpm for 10 min at 4 ºC in order to remove cell debris and stored at -80 ºC until the TF ELISA assay was performed.

7) For assessment of **mRNA expression**:

A total of 650 µL of nucleic acid purification lysis were added directly to the cells. The solution was mixed gently by a micropipette using filter tips.

a. We proceed as indicated (see section **C** *quantification of the mRNA expression*).

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## GENERAL METHODS

### A. Cells

#### Cell counting

To determine the number of cells per unit of volume of the suspension we used the Neubauer chamber and the Trypan blue exclusion assay:

a. The suspension was diluted (1:1) in a sterile plastic tube so that the cells were uniformly distributed, i.e., 50 µL of the cellular suspension with 50 µL of Trypan blue reagent.
b. The suspension was gently mixed with the micropipette to avoid foaming.
c. The cells were counted within 3 to 5 min, since longer incubations can lead to cell death and reduced viability counts.
d. A drop of the suspension was applied to the Neubauer chamber.
e. Cells were counted (viable or not).
f. The media of the viable cells (M) was calculated.

$$M \times 2^{a} \times 16^{b} \times 10000^{c} = \text{Total cells/mL}$$

*a = Dilution of 1: 1 (50 µL cell sample: 50 µL dye)*

*b = M corresponds to one square of the Neubauer chamber. The surface of 16 squares corresponds to 0.1 µL. So, 16 is the factor that will give the M for 0.1 µL.*

*c = the final result in mL*

g. The obtained cellular density (cells/mL) from the formula must be multiplied by the resuspension volume (mL) to determine the total number of cells contained in the sterile plastic tube.

h. According to the desired cellular density (i.e., ~$2 \times 10^5$ viable cells/mL in the case of monocytes THP-1 cells or the adequated for the HAECs), the cellular suspension was adjusted to this density with cell culture medium.

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*Example of cellular density calculation:*

$26x10^6$ cells in total ⟶ 10 mL cell culture medium
If we need: $13x10^6$ cells in total ⟶ X mL
**X = 5 mL** of cellular suspension

We decided to plate $5x10^5$ cells/mL per well, so:
$5x10^5$ cells ⟶ 1 mL
$13x10^6$ cells ⟶ Y mL
**Y =** 26 mL of cell culture medium

*In summary, to reach 26 mL, we must add 21 mL of cell culture medium to the 5 mL of the cellular suspension to reach a final density of $5x10^5$ cells/mL.*

**Cell viability**

Cell viability was assessed based on morphology by the Trypan blue exclusion assay.

The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that cannot be penetrated by certain dyes, such as Trypan blue, whereas dead cells do not. Therefore, a viable cell has clear cytoplasm in this assay, whereas a non-viable cell has blue cytoplasm.

The percentage of viability is calculated by the following formula:

$$\% \text{ viability} = (\text{viable cells}/\text{total cells}) \times 100$$

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**Cell cytotoxicity**

The cytotoxicity was determined by measuring the activity of the enzyme LDH according to the manufacturer's protocol. This enzyme, present in a wide variety of organisms, is released into the culture medium when its expression is induced as a result of disruption or permeabilization of the plasmatic membrane. The activity of this enzyme is detected in culture medium and is directly proportional to the cellular cytotoxicity since it increases as the number of dead cells or cells with damaged plasma membranes increases.

To assay cytotoxicity, culture supernatant is collected and cells are removed. The cell-free supernatant is incubated with the substrate mixture from the kit.

In the first step, released LDH reduces $NAD^+$ to $NADH^+ H^+$ by oxidation of lactate to pyruvate. In the second step, the catalyst (diaphorase) transfers H/H+ from NADH/H+ to the tetrazolium salt INT [2-[4-iodophenyl]-3-[4-nitropheyl]-5-phenyltetrazolium chloride] by a catalyst, which in turn, is reduced to formazan (figure 10). The amount of colour formed in the assay is proportional to the number of lysed cells, so, the increase in supernatant LDH activity directly correlates to the amount of formazan formed over time.

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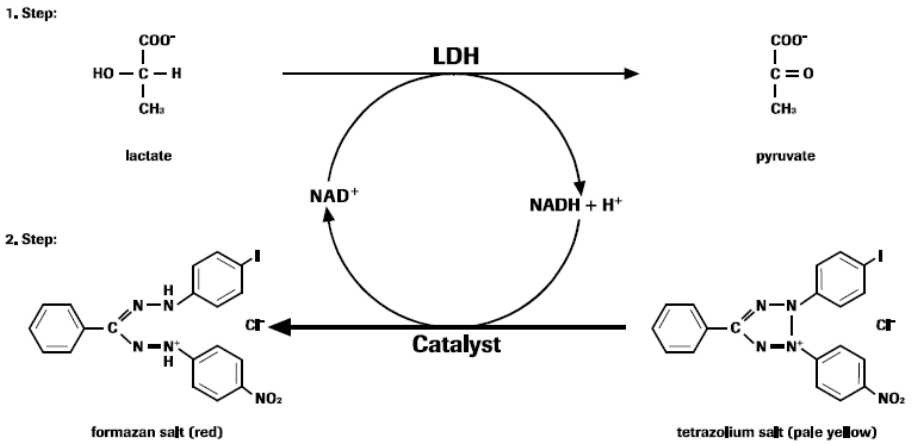
**Figure 10.** Reaction steps




The catalytic activity of LDH was determined by measuring the increase of the absorbance at an optical density (OD) of 492 nm (reference wavelength; 600 nm). The results are expressed as arbitrary Units of Absorbance.

The cytotoxicity of the compounds (at different concentrations) used for the implementation of the *in vitro* models was tested using supernatants collected at the key points of each experimental process on all models.

The experimental conditions that are statistically different from the blank are discarded.

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## B. Determination of protein concentration of biomarkers TNF-α, VCAM-1, eNOS and TF concentration by ELISA

### History:

This brief explanation spotlights the importance of immunoassays from the invention of this method in the 1960s through its development and early use during the 1970s and 1980s. The ELISA technique was conceptualised and developed by Peter Perlmann and Eva Engvall at Stockholm University, Sweden (Lequin RM, 2005).

### Principle:

This assay was based on a quantitative immunoenzymatic technique sandwich ELISA. The ELISA system was used to determine the secretion of TNF-α, VCAM-1 and TF protein following the protocol provided by commercial kits (R&D Systems, Minneapolis, MN, USA; Diaclone, Besançon, France; American Diagnostica, Stamford, CT, USA; consecutively).

*Procedure:*

1. The kit provides a plate of 96 wells. In each well, a monoclonal antibody against a selected biomarker (TNF-α, VCAM-1 and TF) was affixed.
2. In each well, we dispensed standards (with known concentrations) or samples (cell culture medium or cell lysed extracts) to test for the presence of a complementary antigen.
3. After washing the wells to exclude proteins from the sample that were not linked, we added a polyclonal antibody specific for each biomarker and an enzyme conjugate (horseradish peroxidase), which binds the biomarkers.
4. After another wash to remove excess reagent, we added a substrate solution, which resulted in development of colour in proportion to the amount of biomarker that had been bound in the first step.
5. Finally, the colourimetric reaction was stopped with $H_2SO_4$ and the intensity of the colour was measured in an ELISA plate reader at 450 nm (each ELISA kit has its own reference wavelength).

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By comparing the OD of each sample to the standard curve, we determined the concentration of each biomarker in pg/mL in the case of TNF-α and TF and in ng/mL for the VCAM-1 study.

## C. Quantification of biomarkers mRNA expression

### RT-PCR of TNF-α, VCAM-1, eNOS and TF

The amplification of single molecules of mRNA was performed by the real time polymerase chain reaction (RT-PCR) method, which combines the reverse transcription (RT) of RNA to DNA and the polymerase chain reaction (PCR).

Real-time RT-PCR was becoming widely used to quantify molecules from cells, body fluids, tissues or tissues biopsies. At present, a variety of methods are used to quantify mRNA expression, such as northern blotting, *in situ* hybridization, Rnase protection assays, sDNA arrays and RT-PCR. However, quantitative RT-PCR is the most sensitive and accurate of the quantification methods.

TaqMan real-time PCR is the selected technique to study the mRNA expression. This method uses a fluorogenic probe, which is a single stranded oligonucleotide of 20-26 nucleotides and is designed to bind only the DNA sequence between the two PCR primers. Only a specific PCR product can generate the fluorescent signal in TaqMan PCR.

TaqMan PCR requires:
- Real-time PCR equipment (ABI PRISM 7900 Detection System; Applied Biosystems).
- Two Taqman PCR primers with a preferred product size of 50-150 bp, a probe with a fluorescent reporter or fluorophore such as 6-carboxyfluorescein (FAM) covalently attached to its 5' and 3' ends, respectively.

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## Protocol for analysis of mRNA expression

1) **Cell lysis**: The first step differs according to the cell type used.
   - **a.** In the case of monocytes THP-1 cells, we added 400 µL of the nucleic acid purification lysis solution directly to the RNAase-free sterile plastic tubes that contained the previously collected the cells.
   - **b.** HAECs were lysed with 650 µL of the nucleic acid purification lysis solution in the well containing the adhered cells.

   In both cases, following the manufacturers recommendations, cells were disrupted using a micropipette (with filter tips) to ensure a good lysis and maintained at 4 ºC for 1h to facilitate the mRNA extraction. Then, the samples were stored at – 80 ºC.

2) **Total mRNA extraction:** Once the samples were thawed, total RNA was isolated from cells using the ABI PRISM® 6100 Nucleic Acid PrepStation extraction system (Applied Biosystems; Foster City, CA, USA) following the manufacture's instructions. The ABI PRISM™ 6100 isolates and purifies nucleic acids, including total RNA, from a variety of biological samples. The integrated vacuum system and embedded firmware perform all the operations.

3) **Quantification of the RNA concentration**: Total RNA was quantified by the Quant-it™ RNA Assay Kit and Qubit™ flourometer (Invitrogen).

4) **Reverse transcription polymerase chain reaction (RT- PCR)**: Using a 2720 Thermal Cycler (Applied Biosystems), a quantity of 0.5 µg of total RNA was reverse transcribed to total cDNA using Random Hexamers, SuperScript II reverse transcriptase and RNAse Out according to the manufacturer's protocol. Reverse transcription lasted 65 min and included three phases:
   -10 min (25 °C) — RT enzyme activation,
   - 50 min (42 °C) — PCR transcription,

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- 5 min (95 °C) — denaturation.

5) **Real-time RT-PCR:** To determine the amount of TNF-α, VCAM-1, eNOS and TF mRNA, quantitative real-time PCR was performed using the ABI PRISM 7900 Detection System (Applied Biosystems) with the following profile:

   Step 1: 95 ºC denaturing (20 s)

   Step 2: 40 cycles of extension at 95 ºC (1 s) and 60 ºC (20 s).

## Normalisation

The normalisation to a housekeeping gene is currently the most acceptable method to correct for minor variations due to differences in input RNA amount or in efficiencies of reverse transcription. An ideal “housekeeping” gene should be expressed at a constant level among different tissues of an organism. It should be expressed at all stages of development and should not be affected by the experimental treatment itself. Choosing the ideal housekeeping gene for each experiment is very important to ensure the credibility of the results.

It should be noted that the optimal choice of housekeeping gene is dependent on the specific experimental treatment and tissue. Prior to each set of experiments a suitable housekeeping should be chosen that gives the most reproducible results. Sometimes, however, finding a relevant housekeeping gene can be difficult. For our studies, we selected the GAPDH, an abundant glycolytic enzyme. This gene is widely used as a “housekeeping” gene and is present in most cell types.

TaqMan primers for TNF-α, VCAM-1, eNOS, TF and GAPDH were obtained from validated and pre-designed gene expression assay products (Applied Biosystems) and were used in the RT-PCR amplifications.

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## Relative quantification

One of the methods used were the comparative Ct (cycle threshold) method. In this method, mathematical formulas were used to calculate relative expression levels compared to a calibrator, which can be, for instance, a control (non-treated cells) sample. The amount of target, normalised to an endogenous housekeeping gene and relative to the calibrator, is then given by $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct$ (sample) $-\Delta Ct$ (calibrator), and $\Delta Ct$ is the Ct of the target gene subtracted from the Ct of the housekeeping gene. The equation thus represents the normalised expression of the target gene in the unknown sample, relative to the normalised expression of the calibrator sample.

Advantages of using this system were that no standards have to be constructed and that 96 wells can be fully utilised for unknown samples, saving time and money. Moreover, in comparison to the ELISA technique, many different target genes can be quantified from a single RNA sample. A disadvantage however, was that the efficiencies of amplification of the housekeeping and the target gene have to be similar to obtain reliable results.

## Expression of the results

Each sample was analysed in triplicate and the Ct was averaged from the values obtained in each reaction.

The mRNA expression results were reported as fold-increases or decreases using the recommended $2^{-\Delta\Delta Ct}$ mathematical method with GAPDH as a housekeeping gene.

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## STATISTICAL ANALYSIS

Data were expressed as the mean (SD) for protein release and for mRNA expression.

Unless otherwise stated, the experiments were performed at least twice and each condition was run in duplicate or triplicate.

Variables between groups were compared using one way analysis of variance (ANOVA) on normally distributed data. To determine the statistical significance of our results, we performed an ANOVA with Bonferroni's correction for multiple comparisons.

A value of $P < 0.05$ was considered statistically significant.

All the results were statistically analysed by SPSS (Statistical Package for the Social Sciences) v17.0 program.

## EVALUATION OF THE ANALYTICAL QUALITY OF THE MODEL

The precision of the cell models are evaluated by the SD, the intra- and inter-assay CV and the SEM calculation.

Repeatability is determined by intra-assay CV and reproducibility by inter-assay CV (McClure FD, A statistical model to evaluate analyte homogeneity for a material, 2002).

The coefficients of variation (CV) are presented as the highest values obtained from intra- and/or inter-assay, mean, standard deviation (SD) and the standard error of the mean (SEM) are calculated.

## FINANCIAL SUPORT

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# Results

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UNIVERSITAT ROVIRA I VIRGILI
IMPLEMENTATION OF IN VITRO SCREENING ASSAYS TO TEST INFLAMMATION.
THROMBOSIS AND ENDOTHELIAL FUNCTION ON CELLS.
Laia Pons Llecha
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## 1. INDUCTION OF INFLAMMATION

### Effect on TNF-α of FBS content in culture medium, in LPS-stimulated monocytes THP-1 cells

We observed different responses to TNF-α protein release depending on FBS content in the culture medium after 4h of stimulation with LPS at different concentrations (0, 50, 100 and 500 ng/mL).

Unstimulated monocytes THP-1 cells secrete no TNF-α protein, even with the highest FBS content in culture medium tested. When LPS stimulation was performed in the absence of FBS, monocytes showed no TNF-α protein release, except at 1000 and 2000 ng/mL of LPS: mean (SD) 9.57 (2.18) and 9.25 (1.50) pg/mL, respectively (table 2). This activation was not dose-dependent, as the different values obtained from 1000 and 2000 ng/mL of LPS were not statistically significant.

The same level of stimulation was achieved regardless of LPS dose when it was performed in culture medium containing different amounts of FBS (2% or 5%). A slight but statistically significant level of TNF-α protein release was observed when the stimulation was performed in medium supplemented with 2% FBS (figure 11 A). Moreover, a maximum release of TNF-α protein was achieved when monocytes THP-1 cells were stimulated with LPS doses of 1000 and 2000 ng/mL, in the presence of 5% of FBS: 480.8 (25.31) and 557.45 (61.45) pg/mL respectively (table 2). These differences were not statistically significant between them.

When comparing monocyte stimulation at the same LPS doses, the highest FBS concentration resulted in the highest TNF-α protein release. For example, in the presence of 5% FBS, LPS 500 ng/mL induced 321.70 (0.0) pg/mL compared with 2% FBS, which induced 31.59 pg/mL (2.50) ($p<0.05$). Some of these differences were statistically significant (figure 11 A, table 2) between them.

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For TNF-α mRNA expression, in addition to testing LPS stimulation (0, 50, 100, 500, 1000 and 2000 ng/mL) in culture medium containing 0% and 5% FBS, higher FBS content effect was assessed (10%) for 100, 500 and 1000 ng/mL of LPS.

When the activation was carried out in the absence of FBS, we observed a 2-fold increase in TNF-α mRNA expression in monocytes THP-1 cells activated with 2000 ng/mL of LPS, and this increase was statistically significant (figure 11 B, table 2). In the presence of FBS at the tested concentrations (5 or 10%), any dose of LPS (from 50 to 2000 ng/mL) resulted a significant increase of TNF-α mRNA ($p<0.05$). This activation was LPS-dose–dependent and it was higher in the experiments performed in medium supplemented with 10% FBS (15 fold-increase compared with non stimulated monocytes THP-1 cells or FBS 0%) (table 2).
None of the conditions tested in these experiments showed cytotoxicity.

**Effect on TNF-α of different periods of time in culture, in LPS-stimulated monocytes THP-1 cells**

Under the same stimulus, LPS activation, monocytes THP-1 cells present different behaviour depending on their post-thaw day (6, 12 and 19).
As shown in figure 12 A and table 3, unstimulated monocytes THP-1 cells secrete no TNF-α protein at any post-thaw day. However, all LPS concentrations tested stimulated TNF-α protein release compared with untreated cells ($p<0.05$). At day 6 and day 19, increasing LPS concentrations (50, 100, 500, 1000 and 2000 ng/mL) showed a statistically significant dose-dependent trend. Nevertheless, at day 12, some of the LPS doses showed the same response: 50 and 100 ng/mL (138.65 (4.74) and 108.90 (5.37) pg/mL, respectively) and 1000 and 2000 ng/mL (480.80 (25.31) and 557.45 (61.45) pg/mL, respectively).

Moreover, TNF-α protein release by LPS-activated monocytes was greater when cells were kept in culture for prolonged periods (figure 12 A). The

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differences between stimulation by the same LPS dose at different post-thaw days were higher between day 6 and days 12 and 19 (table 3).

We found that maximal monocytes THP-1 cells stimulation was observed with increasing post-thaw day (D+19) and with the maximal LPS dose-tested (500 ng/mL): 432.23 (2.89) pg/mL.

All LPS concentrations tested (50, 100, 500, 1000 and 2000 ng/mL) stimulated TNF-α mRNA expression ($p<0.05$) at any post-thaw day, as shown in table 3. Although this stimulation was greater with increasing LPS doses, it was not dose-dependent in a statistically significant way. It was also observed that with the same LPS concentration applied to monocytes THP-1 cells resulted in increased TNF-α mRNA expression, depending on the post-thaw day ($p<0.05$) (table 3). Maximum TNF-α mRNA expression was observed in monocytes stimulated with 500 ng/mL of LPS at post-thaw day 19, 10.97(0.67) - fold increase compared with non stimulated cells ($p<0.05$). The conditions tested in these experiments showed no cytotoxicity.

**Effect on TNF-α of different batches of monocytes THP-1 cells stimualted by LPS.**

The response of monocytes to LPS was different with respect to TNF-α protein levels, depending on the batch used (figure 13 A, table 4). Batch A seemed to be more sensitive to LPS than batch B. Batch A produced the same TNF-α protein release at an LPS concentration of 500 ng/mL; similar results were observed for batch B at an LPS concentration of 1000 ng/mL, with values of 119,40 (1,41) and 114,50 (7,97) pg/mL, respectively.

The opposite was shown with respect to TNF-α mRNA expression, since batch B showed higher mRNA expression when compared to batch A in monocytes activated with the same LPS dose (figure 13 B, table 4).

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### Evaluation of precision

Under the conditions tested, the intra-assay CV (within-day variation) of the protein and mRNA experiments was lower than 20% with the exception of some isolated values that were lower than 25%.

Regarding between-day variation, CVs were similar independently of the day on which experiments were performed or the person who carried the experiments out. Results presented in tables 2, 3 and 4 correspond to experiments performed on different days and some of them were performed by different technicians. Moreover, the CV inter-assay was higher in batch B than batch A.

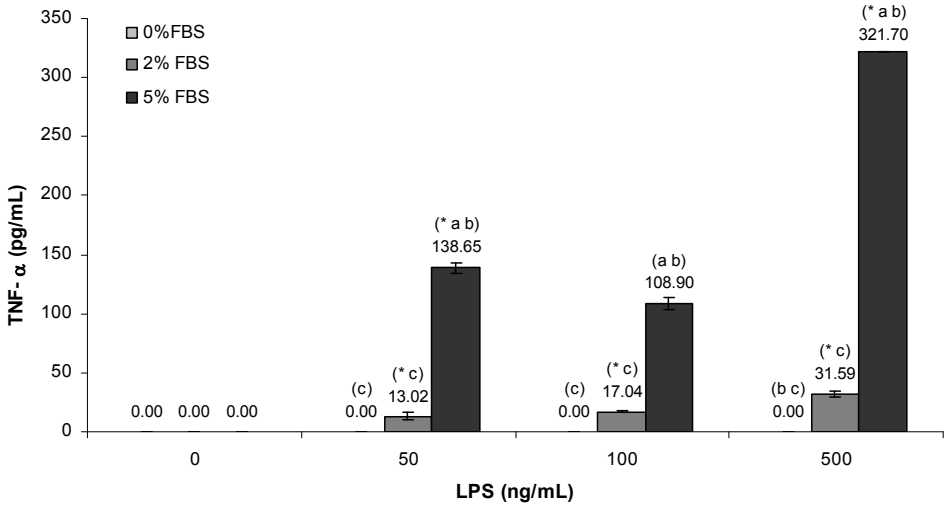


**Figure 11 A. Effect on TNF-α protein release of FBS content in culture medium, in LPS-stimulated monocytes THP-1cells.**

Data are the mean (SD: error bars) of two experiments performed in triplicate. *= $p<0.05$ compared with unstimulated monocytes. Letters = $p<0.05$ compared with the same LPS dose at 0% (a), 2% (b) and 5% (c) of FBS.

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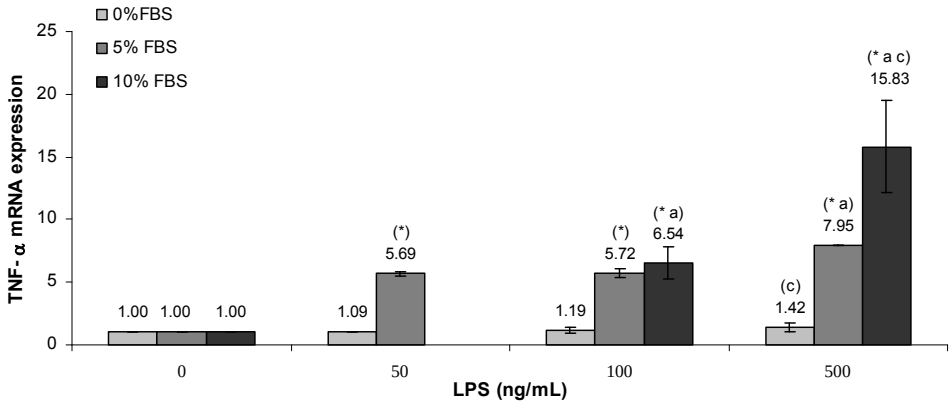


**Figure 11 B. Effect on TNF-α mRNA expression of FBS content, in LPS-stimulated monocytes THP-1cells.**

Data are the mean (SD: error bars) of two experiments performed in triplicate. *= $p<0.05$ compared with unstimulated monocytes. Letters = $p<0.05$ compared with the same LPS dose at 0% (a), 5% (b) and 10% (c) of FBS.

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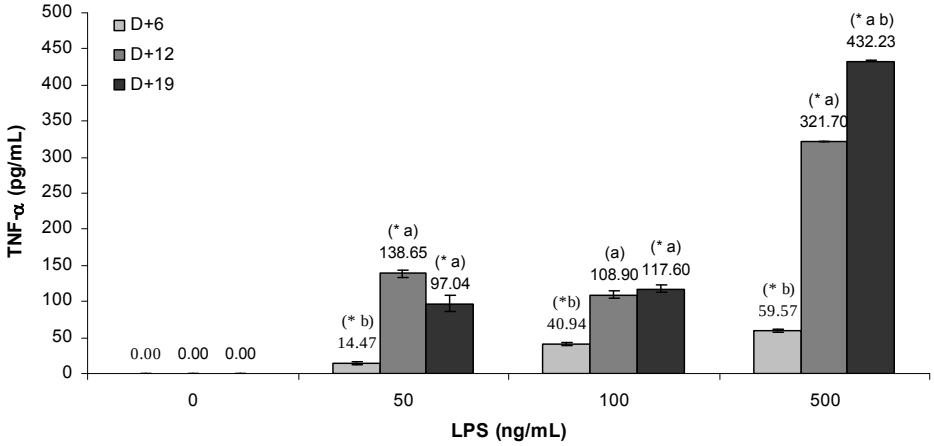


**Figure 12 A. Effect on TNF-α protein release of different periods in culture, in LPS-stimulated monocytes THP-1 cells.**

Data are the mean (SD: error bars) of two ex,periments performed in triplicate. *= p<0.05 compared with unstimulated monocytes. Letters= p<0.05 compared with the same LPS dose at D+6 (a), D+12 (b) and D+19.

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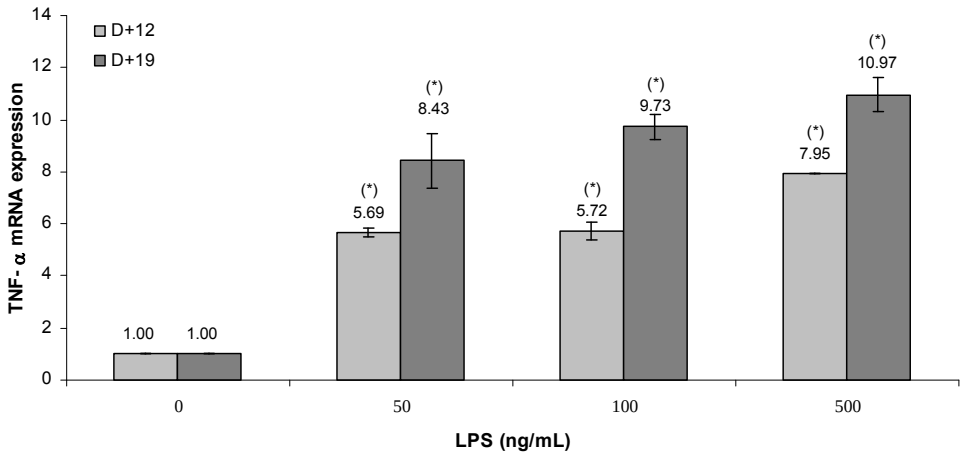


**Figure 12 B. Effect on TNF-α mRNA expression of different periods in culture, in LPS-stimulated monocytes THP-1 cells.**

Data are the mean (SD: error bars) of two experiments performed in triplicate. *= p<0.05 compared with unstimulated monocytes. Letters = p<0.05 compared with the same LPS dose at D+12 (a) and D+19.

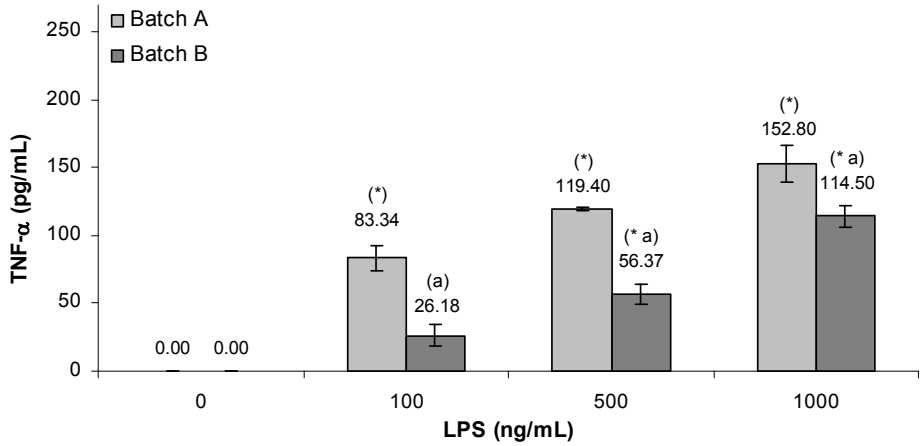


**Figure 13 A. Effect on TNF-α protein release of different batches, in LPS-stimulated monocytes THP-1.**

Data are the mean (SD: error bars) of two experiments performed in triplicate. *= p<0.05 compared with unstimulated monocytes. Letters = p<0.05 compared with the same LPS dose batch A and batch B.

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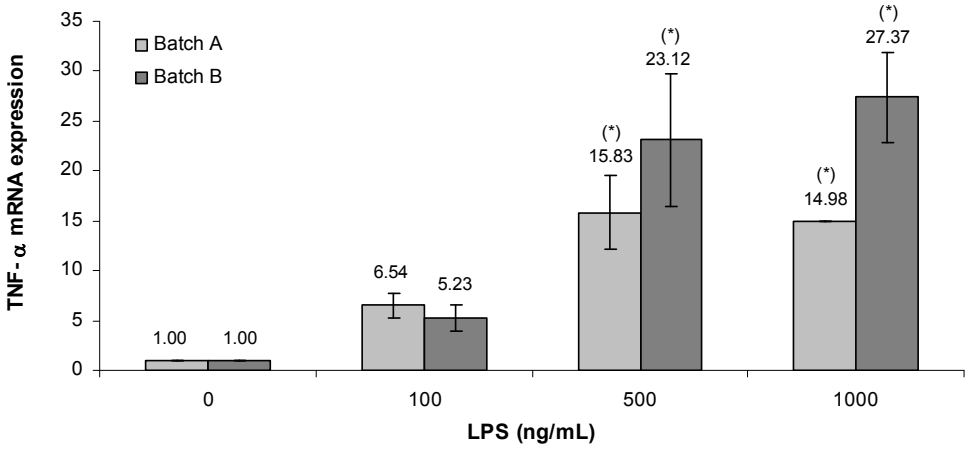


**Figure 13 B. Effect on TNF-α mRNA expression of different batches, in LPS-stimulated monocytes THP-1.**

Data are the mean (SD: error bars) of two experiments performed in triplicate. *= $p<0.05$ compared with unstimulated monocytes. Letters= $p<0.05$ compared with the same LPS dose batch A and batch B.

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**Table 2.** Effect on THP-1 monocytes activation of FBS content in culture medium and LPS dose, on TNF-α protein release and TNF-α mRNA expression

| % of FBS | LPS dose (ng/mL) | Reproducibility (between-day) INTER-ASSAY | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | TNF- α protein expression (pg/mL) | | | | TNF- α mRNA expression (relative values) | | | |
| | | Mean (SD) | P* | CV % | SEM | Mean (SD) | P* | CV % | SEM |
| 0% | 0 | 0.00 (0.00) | | 0.00 | 0.00 | 1.00 (0.00) | | 0.00 | 0.00 |
| | 50 | 0.00 (0.00) | c | 0.00 | 0.00 | 1.09 (0.00) | | 0.00 | 0.00 |
| | 100 | 0.01 (0.00) | c | 0.00 | 0.00 | 1.19 (0.23) | d | 19.43 | 0.16 |
| | 500 | 0.02 (0.00) | c | 0.00 | 0.00 | 1.42 (0.33) | c d | 23.58 | 0.24 |
| | 1000 | 9.57 (2.18) | c | 22.77 | 1.54 | 1.59 (0.04) | c d | 2.78 | 0.03 |
| | 2000 | 9.25 (1.50) | c | 16.16 | 1.06 | 2.06 (0.11) | † c | 5.50 | 0.08 |
| 2% | 0 | 0.00 (0.00) | | 0.00 | 0.00 | | | | |
| | 50 | 13.02 (3.15) | † c | 24.21 | 1.82 | | | | |
| | 100 | 17.04 (0.96) | † c | 5.64 | 0.68 | | | | |
| | 500 | 31.59 (2.50) | † c | 7.90 | 1.77 | | | | |
| 5% | 0 | 0.00 (0.00) | | 0.00 | 0.00 | 1.00 (0.00) | | 0.00 | 0.00 |
| | 50 | 138.65 (4.74) | † a b | 3.42 | 3.35 | 5.69 (0.16) | † | 2.89 | 0.12 |
| | 100 | 108.90 (5.37) | a b | 4.93 | 3.80 | 5.72 (0.36) | † | 6.22 | 0.25 |
| | 500 | 321.70 (0.00) | † a b | 0.00 | 0.00 | 7.95 (0.00) | † ad | 0.00 | 0.00 |
| | 1000 | 480.80 (25.31) | † a | 5.27 | 17.90 | 8.17 (0.00) | † ad | 0.00 | 0.00 |
| | 2000 | 557.45 (61.45) | † a | 11.02 | 43.45 | 7.40 (1.51) | † a | 20.42 | 1.07 |
| 10% | 0 | | | | | 0.00 (0.00) | | 0.00 | 0.00 |
| | 100 | | | | | 6.45 (1.25) | † a | 19.15 | 0.89 |
| | 500 | | | | | 15.83 (3.69) | † ac | 23.31 | 2.61 |
| | 1000 | | | | | 14.98 (0.03) | † ac | 0.18 | 0.02 |

†P<0.05 compared to blank (unstimulated monocytes)

Letters (a, b, c, d) P<0.05 compared with the same LPS dose at 0% (a), 2% (b), 5% (c), 10% (d) of FBS

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**Table 3.** Effect on THP-1 monocytes activation of different periods in culture and LPS dose, on TNF-α protein release and TNF-α mRNA expression

| Post-thaw day | LPS dose (ng/mL) | Reproducibility (between-day) INTER-ASSAY | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | TNF- α protein expression (pg/mL) | | | | TNF- α mRNA expression (relative values) | | | |
| | | Mean (SD) | P* | CV % | SEM | Mean (SD) | P* | CV % | SEM |
| D+6 | 0 | 0.00 (0.00) | | 0.00 | 0.00 | | | | |
| | 50 | 14.47 (1.69) | † bc | 11.04 | 1.13 | | | | |
| | 100 | 40.94 (2.77) | † bc | 6.77 | 1.96 | | | | |
| | 500 | 59.57 (2.70) | † bc | 4.53 | 1.91 | | | | |
| | 1000 | 87.84 (2.96) | † b | 3.36 | 2.09 | | | | |
| | 2000 | 132.60 (4.10) | † b | 3.09 | 2.90 | | | | |
| D+12 | 0 | 0.00 (0.00) | | 0.00 | 0.00 | 1.00 (0.00) | | 0.00 | 0.00 |
| | 50 | 138.65 (4.74) | † a | 3.42 | 3.35 | 5.69 (0.16) | † | 2.89 | 0.12 |
| | 100 | 108.90 (5.37) | a | 4.93 | 3.80 | 5.72 (0.36) | † | 6.22 | 0.25 |
| | 500 | 321.70 (0.00) | † ac | 0.00 | 0.00 | 7.95 (0.00) | † | 0.00 | 0.00 |
| | 1000 | 480.80 (25.31) | † c | 5.27 | 17.90 | 8.17 (0.00) | † | 0.00 | 0.00 |
| | 2000 | 557.45 (61.45) | † c | 11.02 | 43.45 | 7.40 (1.51) | † | 20.42 | 1.07 |
| D+19 | 0 | 0.00 (0.00) | | 0.00 | 0.00 | 1.00 (0.00) | | 0.00 | 0.00 |
| | 50 | 97.04 (10.65) | † a | 10.98 | 6.15 | 8.43 (1.05) | † | 12.47 | 0.61 |
| | 100 | 117.60 (5.48) | † a | 4.66 | 3.17 | 9.73 (0.48) | † | 4.90 | 0.28 |
| | 500 | 432.23 (2.89) | † ab | 0.67 | 1.67 | 10.97 (0.67) | † | 6.09 | 0.47 |

†P<0.05 compared to blank (unstimulated monocytes).

Letters (a, b, c) P<0.05 compared with the same LPS dose at D+6 (a), D+12 (b), D+19 (c)

FBS content 5%

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**Table 4.** Effect on THP-1 monocytes activation of different batches and LPS dose, on TNF-α protein release and TNF-α mRNA expression

| Batch | LPS dose (ng/mL) | Reproducibility (between-day) INTER-ASSAY | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | TNF-α protein expression (pg/mL) | | | | TNF-α mRNA expression (relative values) | | | |
| | | Mean (SD) | P* | CV % | SEM | Mean (SD) | P* | CV % | SEM |
| A | 0 | 0.00 (0.00) | | 0.00 | 0.00 | 1.00 (0.00) | | 0.00 | 0.00 |
| | 100 | 83.34 (9.63) | † b | 11.56 | 6.81 | 6.54 (1.25) | | 19.15 | 0.89 |
| | 500 | 119.4 (1.41) | † b | 1.18 | 1.00 | 15.83 (3.69) | † | 23.31 | 2.61 |
| | 1000 | 152.8 (13.86) | † b | 9.07 | 9.80 | 14.98 (0.03) | † | 0.18 | 0.02 |
| B | 0 | 0.00 (0.00) | | 0.00 | 0.00 | 1.00 (0.00) | | 0.00 | 0.00 |
| | 100 | 26.18 (8.04) | a | 30.72 | 5.69 | 5.23 (1.34) | | 25.54 | 0.95 |
| | 500 | 56.37 (7.65) | † a | 13.58 | 5.41 | 23.12 (6.62) | † | 28.62 | 4.68 |
| | 1000 | 114.50 (7.97) | † a | 6.96 | 5.64 | 27.37 (4.48) | † | 16.38 | 3.17 |

†P<0.05 compared to blank (unstimulated monocytes).

Letters (a, b) P<0.05 compared with the same LPS dose in batch A (a), B (b)

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## 2. INDUCTION OF ENDOTHELIAL DYSFUNCTION

### Cytotoxicity

HAECs did not show any sign of cytotoxicity after exposure to AT (10, 25, 50, 75 and 150 μM) or TNF-α (from 1 to 20 ng/mL) even at higher doses. However, there was evidence of morphological changes in HAECs that were incubated with the highest BAY concentrations (2 and 10 μM), as observed under a phase contrast microscope and were discarded. BAY (0.1, 0.2, 0.5, 1 μM) did not show cytotoxicity as assessed by LDH release.

### Dose-response effects on VCAM-1 protein release of TNF-α in HAECs

HAECs stimulated for 24h with TNF-α (5, 10 and 20 ng/mL) demonstrated a ~3-4-fold enhancement of VCAM-1 expression compared with vehicle control-treated cells ($p<0.05$), but the response was not dose-dependent (figure 14, table 6).

We opted for TNF-α at 10 ng/mL because it was used on previous reports (Tribolo S, 2008; Shaw DI, 2007) in HUVECs or in HAECs (Amberger A, 1997; Szekanecz Z, 1994; Chen YH, 2001; Schleser S, 2006; Chen JW, 2003).

### Dose-response effects of BAY on VCAM-1 protein release in TNF-α-stimulated HAECs

Stimulation of HAECs for 24h with TNF-α (10 ng/mL) demonstrated increases in VCAM-1 protein release of 341.69 (11.88), nearly 6-fold, compared with vehicle control.

This concentration was reduced by about 45-70 % by BAY (0.5 and 1 μM) when it was added simultaneously with the TNF-α followed by incubation for 24h ($p<0.05$) (figure 15). In addition, we decided to evaluate the importance of a pre-incubation time (30 min) with BAY (2 μM) compared with cells

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treated simultaneously with BAY (2 μM) and TNF-α (10 ng/mL) which were discarded due to the cytotoxicity observed.

Following the analyses of these results, we proposed establishing the BAY concentration at 1 μM since this achieved the highest reduction of VCAM-1 expression ~ 70% in HAECs ($p<0.05$). Treatment of HAECs with TNF-α (10 ng/mL) in combination with BAY (1 μM) for 24h resulted in a significantly lower VCAM-1 protein concentration by ~57% ($p<0.05$) compared with the TNF-α alone, as shown in figure 15. and table 5.

**Dose-response effects of AT on VCAM-1 protein release in TNF-α-stimulated HAECs**

A 6h pre-treatment with AT significantly reduced VCAM-1 protein expression at 75 and 150 μM by 33% compared to the maximal TNF-α stimulation ($p<0.05$), as shown in figure 16 and table 6. No dose-response effect was observed.

**Dose- and time-response effects of TNF-α on VCAM-1 mRNA expression in HAECs**

A dosages of TNF-α (1, 2, 5 and 10 ng/mL) at 2, 4 and 24h showed that all the concentrations increased VCAM-1 mRNA expression relative to vehicle control in an excessive manner which was unable to detect small differences between the experimental conditions. (figure 17).

TNF-α (1 ng/mL) for 24h was selected since it achieved a 3.59-fold increase compared to vehicle control.

**Dose-response effect of BAY on VCAM-1 mRNA expression in TNF-α-stimulated HAECs**

BAY treatment (0.1 and 0.2 μM) significantly reduced TNF-α-induced VCAM-1 mRNA expression by ~25-30% compared to vehicle control in HAEC, but not in a dose-dependent manner ($p<0.05$; figure 18).

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**Dose-response effect of AT on VCAM-1 mRNA expression in TNF-α-stimulated HAECs**

AT at 50, 75 and 150 μM significantly reduced VCAM-1 mRNA expression by ~30% (figure 19) in a non-dose-dependent manner ($p<0.05$) in TNF-α-stimulated HAECs. Theses results showed the same tendency as that observed in the study of VCAM-1 protein release.

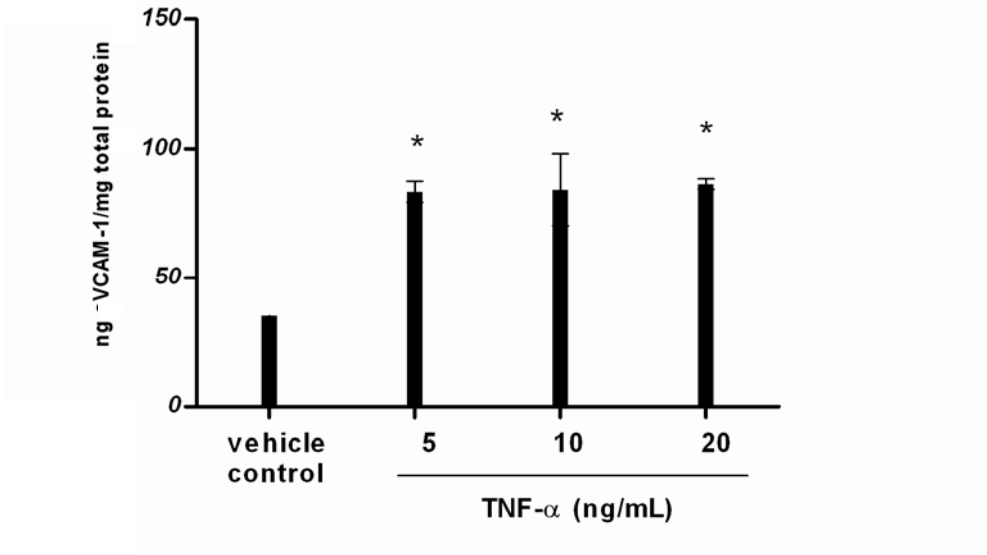


**Figure. 14. Dose-response effects on VCAM-1 protein release of TNF in HAECs.**
Each condition was run in duplicate or triplicate. Data represent the means (SD). *P<0.05 compared to TNF-α vehicle control (sterile water) group. The CV intra-assay was < 17% and the SEM < 8.1.

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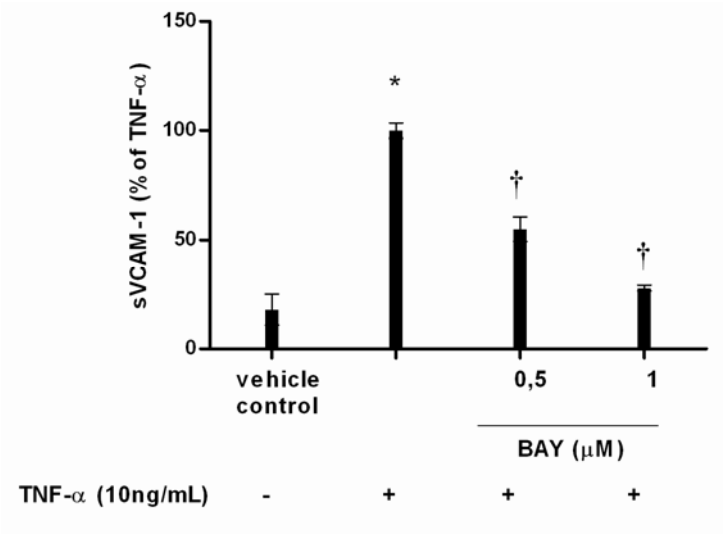


**Figure 15. Dose-response effects of BAY on VCAM-1 protein release in TNF-α-stimulated HAECs.**

Results are representative of three independent experiments and each condition was run in triplicate. Data represent the means (SD). *P<0.05 compared to BAY vehicle control (DMSO) group. †P<0.05 compared to groups treated with TNF-α alone. CV intra-assay < 20% and CV inter-assay was < 7% and the SEM < 12

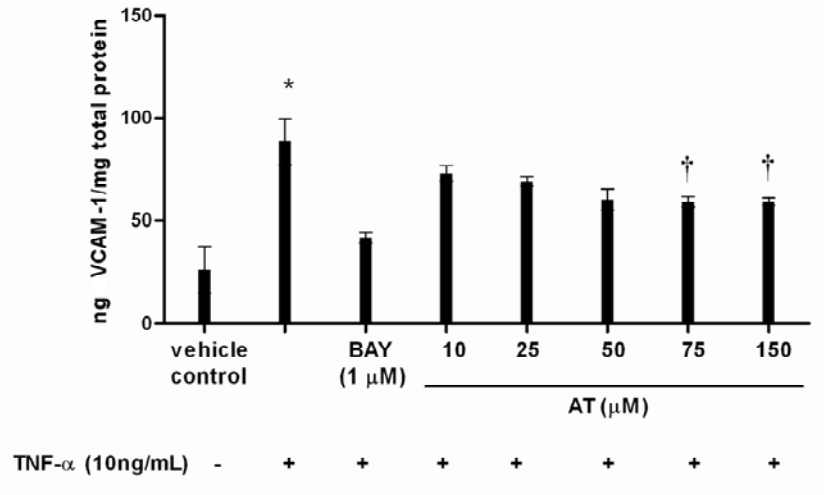


**Figure 16. Dose-response effects of AT on VCAM-1 protein release in TNF-α-stimulated HAECs.**

Each condition was run in duplicate. Data represent the means (SD). *P<0.05 compared to AT vehicle control (EtOH) group. †P<0.05 compared to groups treated with TNF-α alone. The CVs were <12% (exceptionally <44%), and the SEM was <8.1.

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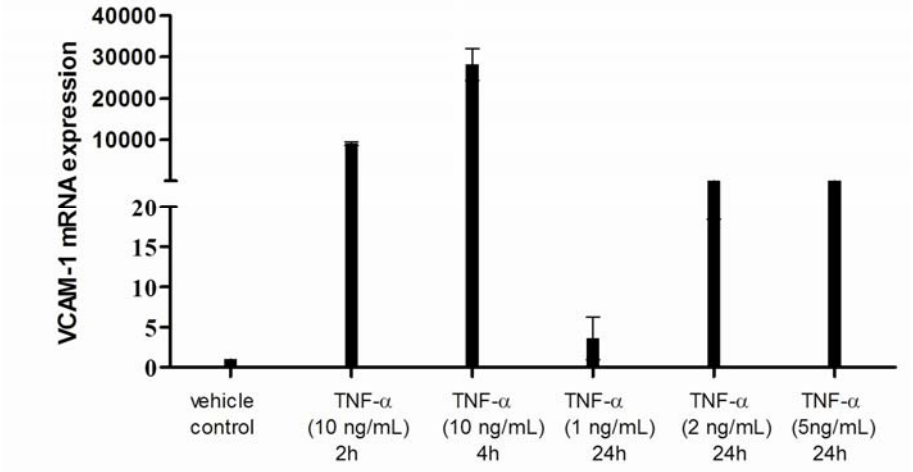


**Figure 17. TNF-α dose- and time-response effects on VCAM-1 mRNA expression in HAECs.**

Each experimental set of condition was run in duplicate. Data represent the means (SD). The intra-assay CV was <40%.

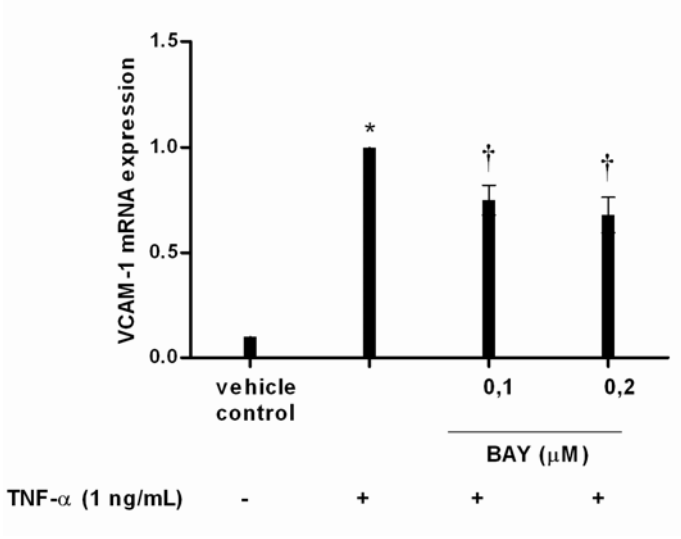


**Figure 18. Dose-response effect of BAY on VCAM-1 mRNA expression in TNF-α-stimulated HAECs**

Results are representative of two independent experiments and each condition was run in duplicate. Data represent the means (SD). *P<0.05 compared to BAY vehicle control (DMSO) group. †P<0.05 compared to groups treated with TNF-α alone. The CV intra-assay was <14%, the inter-assay <13%, and the SEM was <0.06.

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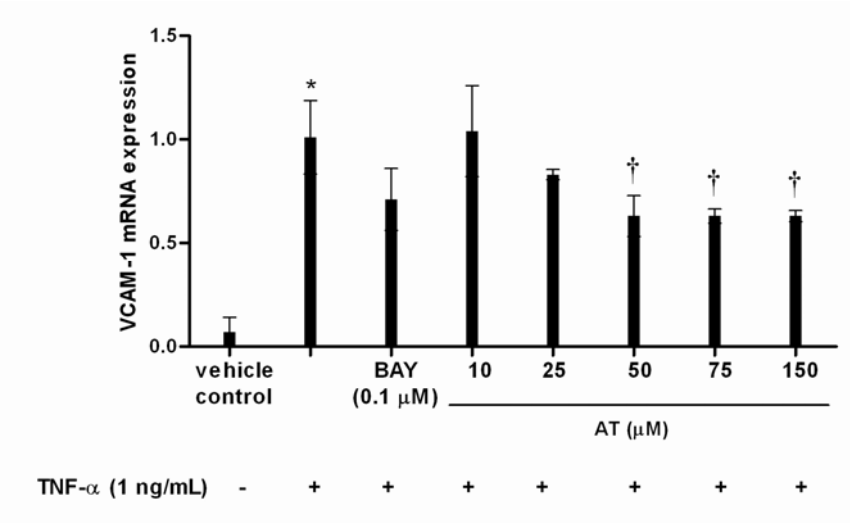


**Figure 19. Dose-response effect of AT on VCAM-1 mRNA expression in TNF-α-stimulated HAECs.**

Results are representative of two independent experiments and each condition was run in triplicate. Data represent the means (SD). *P<0.05 compared to AT vehicle control (EtOH) group. †P<0.05 compared to groups treated with TNF-α alone. The CV intra- and inter-assay were <27% and the SEM was <0.15.

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**Table 5.** Effects of BAY on VCAM-1 protein and mRNA expression and percentages of VCAM-1 reduction on TNF-α-stimulated HAEC

| Experimental conditions | Concentration | Reproducibility (between-day) INTER-ASSAY | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | VCAM-1 protein expression (ng/mL) | | | | | VCAM-1 mRNA expression (relative values) | | | | |
| | | Mean (SD) | P* | CV % | SEM | % Reduction | Mean (SD) | P* | CV % | SEM | % Reduction |
| **Protein** | | | | | | | | | | | |
| Vehicle control (DMSO) | | 61.00 (4.29) | | 7.04 | 3.03 | | | | | | |
| TNF-α | 10ng/mL | 341.69 (11.88) | † | 3.48 | 5.94 | | | | | | |
| TNF-α + BAY | 0.5 μM | 188.20 (10.51) | ‡ | 5.59 | 7.43 | 44.92 | | | | | |
| TNF-α + BAY | 1 μM | 94.65 (1.24) | | 1.31 | 0.88 | 72.30 | | | | | |
| **mRNA** | | | | | | | | | | | |
| Vehicle control (DMSO) | | | | | | | 0.10 | (0.00) | 3.92 | 0.00 | |
| TNF-α | 1ng/mL | | | | | | 1.00 | (0.00) † | 0.23 | 0.00 | |
| TNF-α + BAY | 0,1 μM | | | | | | 0.75 | (0.07) ‡ | 9.17 | 0.05 | 25.01 |
| TNF-α + BAY | 0,2 μM | | | | | | 0.68 | (0.09) ‡ | 12.66 | 0.06 | 31.99 |

†P<0.05 compared to control group

‡P<0.05 compared to groups treated with TNF-α alone

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**Table 6.** Effects of AT on VCAM-1 protein and mRNA expression and percentages of VCAM-1 reduction on TNF-α-stimulated HAEC

| Experimental conditions | Concentration | Reproducibility (between-day) INTER-ASSAY | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | VCAM-1 protein expression (ng/mL) | | | | | VCAM-1 mRNA expression (relative values) | | | | |
| | | Mean (SD) | P* | CV % | SEM | % Reduction | Mean (SD) | P* | CV % | SEM | % Reduction |
| Vehicle control (EtOH) | 0 | 26.11 (11.50) | | 44.07 | 8.14 | | 0.13 (0.02) | | 15.12 | 0.01 | |
| TNF-α | 10ng/mL | 88.42 (11.11) | † | 12.57 | 7.86 | | 1.01 (0.14) | † | 13.69 | 0.07 | -0.64 |
| TNF-α + BAY | 1 μM | 41.90 (2.57) | ‡ | 6.14 | 1.82 | 52.61 | | | | | |
| TNF-α + AT | 10 μM | 73.19 (3.79) | | 5.18 | 2.68 | 17.23 | 1.18 (0.22) | | 18.41 | 0.11 | -18.22 |
| TNF-α + AT | 25 μM | 69.17 (2.28) | | 3.30 | 1.61 | 21.77 | 1.11 (0.31) | | 27.69 | 0.15 | -10.89 |
| TNF-α + AT | 50 μM | 60.17 (5.26) | | 8.75 | 3.72 | 31.95 | 0.76 (0.16) | ‡ | 20.82 | 0.08 | 23.75 |
| TNF-α + AT | 75 μM | 59.11 (2.52) | ‡ | 4.27 | 1.78 | 33.14 | 0.74 (0.11) | ‡ | 14.85 | 0.05 | 26.49 |
| TNF-α + AT | 150 μM | 59.21 (1.86) | ‡ | 3.15 | 1.32 | 33.04 | 0.79 (0.15) | ‡ | 18.78 | 0.07 | 21.24 |

†P<0.05 compared to control group

‡P<0.05 compared to groups treated with TNF-α alone

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## 3. ASSESSMENT OF ENDOTHELIAL FUNCTION.

### Effects of insulin on eNOS mRNA expression in HAECs

HAECs challenged with insulin in CM that had not been previously washed with BM, had no statistically significant increase of eNOS mRNA expression was observed in figure 20 and table 8

This effect was observed at all time points (24 and 72h). Surprisingly, a decrease in mRNA levels was observed rather than the expected increase at 1000 nM at 24h by 0.72-fold (0.01) compared untreated cells ($p<0.05$).

Further, short-term pre-treatment of HAECs (2h) with BM followed by insulin stimulation (100, 500 and 1000 nM) for 24h in CM resulted in a dose-dependent decrease of eNOS mRNA levels, but once again these changes were statistically significant at only the maximum dose tested (1000 nM): 0.64 (0.11), compared to untreated cells ($p<0.05$; figure 21).

Longer periods of pre-treatment of HAECs with BM (18h) followed by insulin incubation at 100, 500 and 1000 nM for 24h in CM considerably augmented the eNOS mRNA levels compared with untreated cells. This increase in eNOS mRNA levels was significant only at 500 and 1000 nM: 1.48 (0.08) and 1.42 fold (0.11), respectively compared with untreated cells ($p<0.05$) (figure 21, table 9).
None of the conditions tested were cytotoxic.

### Effects of TNF-α on eNOS mRNA expression in HAECs

eNOS mRNA levels decreased in a dose-dependent manner when HAECs were stimulated with 10 ng/mL of TNF-$\alpha$ for 4 or 24h: 0.83- (0.38) and 0.53-fold (0.02) respectively but only at 24h was the reduction significant, nearly 50% ($p < 0.05$; figure 22 and table 7).
In dose-response experiments, HAECs responded to TNF-$\alpha$ stimulation (5 and 10 ng/mL) after 24h. eNOS mRNA levels decreased by 0.61-fold (0.12)

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and 0.53-fold (0.02), i.e., about 40% and 50%, respectively, compared with untreated cells ($p<0.05$) (figure 23 and table 7) but it was not dose-dependent.

None of the conditions tested were cytotoxic.

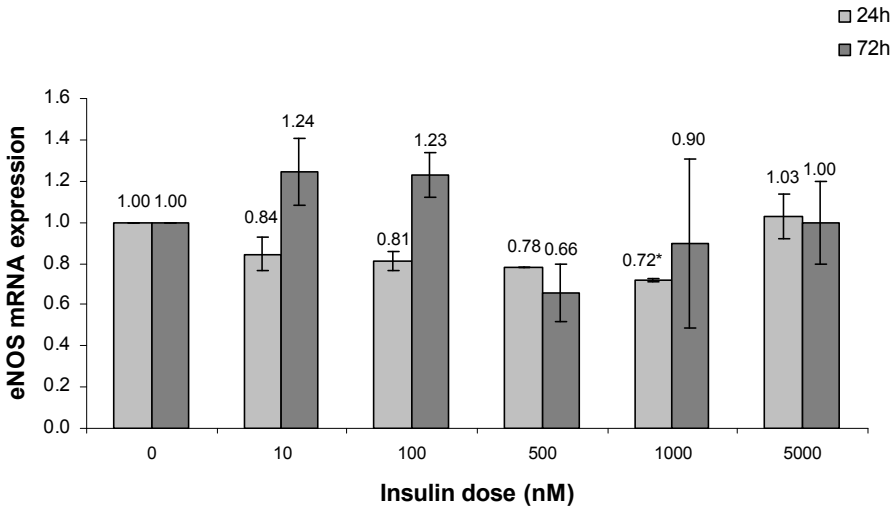


**Figure 20. Time and dose-dependent effects of insulin on eNOS mRNA expression in HAECs.**

Cells were treated with insulin (10 to 5000 nM) for 24h or 72h. Untreated cells are also represented as the 0 dose point. At the end of the experiments, eNOS mRNA expression was monitored by real time RT-PCR. Results, expressed in relative units, are representative of two independent experiments and each incubation condition was run in triplicate. Data represent the means (SD) of three separate experiments. * $p < 0.05$ compared to untreated cells. The CVs <21% (exceptionally <46%) and the SEM was 0.14.

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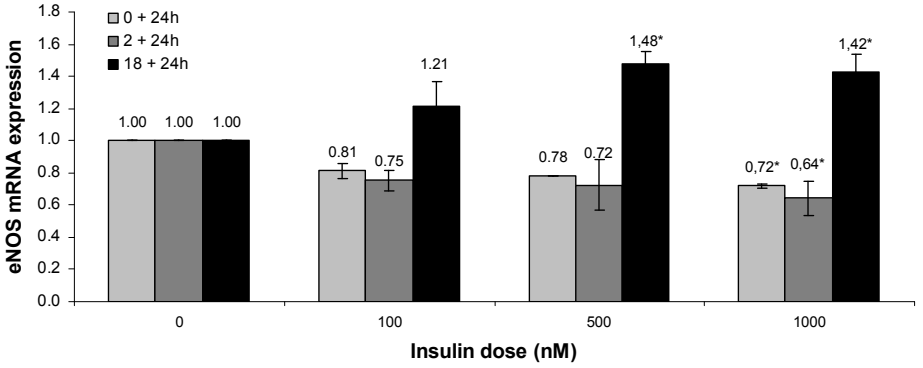


**Figure 21. Effect of a previous wash with BM on eNOS mRNA expression in HAECs.**
Results are representative of two independent experiments and each incubation condition was run in duplicate or triplicate. Data represent the means (SD). * $p < 0.05$ compared to untreated cells. The CVs were <22% and the SEM was <0.09.

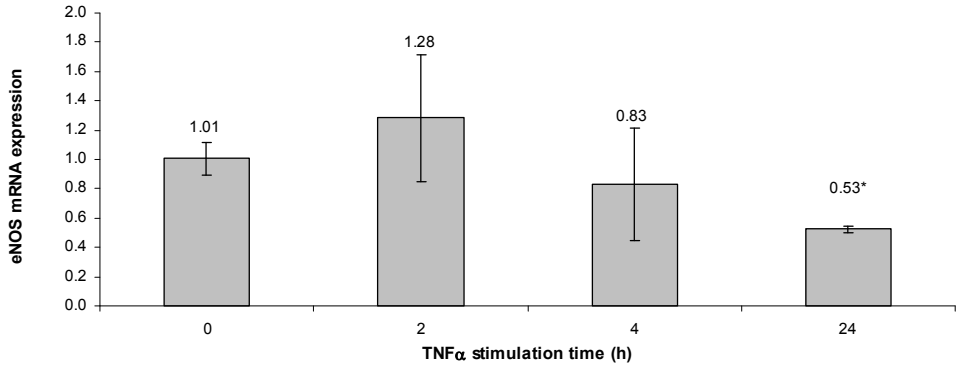


**Figure 22. Time-response of TNF-α on eNOS mRNA expression in HAECs.**
Results are representative of two independent experiments and each incubation condition was run in duplicate or triplicate. Data represent the means (SD). * $p < 0.05$ compared to untreated cells. The CVs were <34% and the SEM was 0.3.

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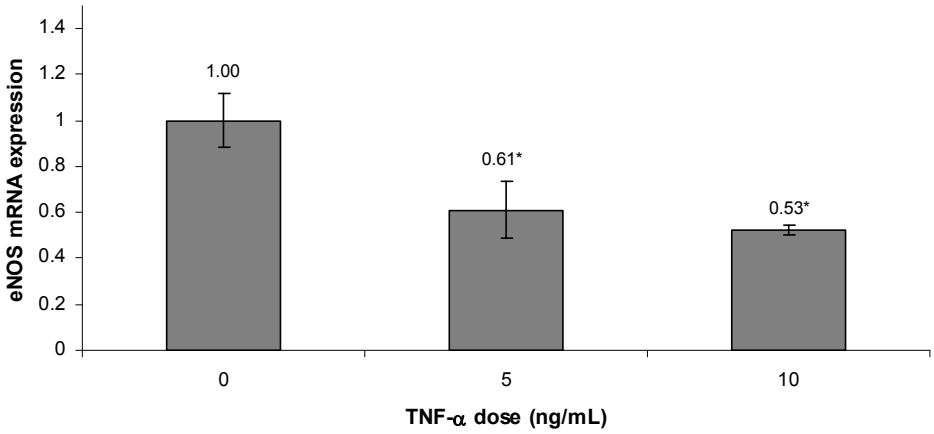


**Figure 23. Dose-dependent effects of TNF-α on eNOS mRNA expression in HAECs.**
Results correspond to two independent experiments, and each condition was run in duplicate or triplicate. Data represent the means (SD). * $p < 0.05$ compared to untreated cells. The CVs were < 21% and the SEM was 0.06.

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**Table 7.** Time and dose-response to TNF-α stimulation on eNOS mRNA expression in HAEC

| Experimental conditions | Concentration | Reproducibility (between-day) INTER-ASSAY<br>eNOS mRNA expression (relative values)<br>Mean (SD) | P* | CV % | SEM | % Reduction |
|---|---|---|---|---|---|---|
| Vehicle control (water) | | 1.01 (0.12) | | 11.45 | 0.05 | |
| TNF-α | 5ng/mL | 0.61 (0.12) | † | 20.29 | 0.06 | 39.09 |
| TNF-α | 10ng/mL | 0.53 (0.02) | † | 4.17 | 0.01 | 47.40 |
| Vehicle control (water) | | 1.01 (0.11) | | 11.07 | 0.04 | |
| TNF-α (2h) | 10ng/mL | 1.28 (0.43) | | 33.64 | 0.31 | -28.32 |
| TNF-α (4h) | 10ng/mL | 0.83 (0.38) | | 46.41 | 0.27 | 17.20 |
| TNF-α (24h) | 10ng/mL | 0.53 (0.02) | † | 4.17 | 0.01 | 47.00 |

†P<0.05 compared to control group

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**Table 8.** Time and dose-response to Insulin stimulation on eNOS mRNA expression in HAEC

| Experimental conditions | Concentration | Reproducibility (between-day) INTER-ASSAY eNOS mRNA expression (relative values) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Mean (SD) | | P* | CV % | SEM | % Increment |
| **24h** | | | | | | | |
| Unteated cells | | 1.00 | (0.00) | | 0.00 | 0.00 | |
| Insulin | 10 nM | 0.84 | (0.08) | | 9.72 | 0.06 | -15.57 |
| Insulin | 100 nM | 0.81 | (0.05) | | 6.05 | 0.03 | -18.80 |
| Insulin | 500 nM | 0.78 | (0.00) | | 0.28 | 0.00 | -21.83 |
| Insulin | 1000 nM | 0.72 | (0.01) | † | 1.42 | 0.01 | -28.24 |
| Insulin | 5000 nM | 1.03 | (0.11) | | 10.86 | 0.08 | 2.81 |
| **72h** | | | | | | | |
| Unteated cells | | 1.00 | (0.00) | | 0.00 | 0.00 | |
| Insulin | 10 nM | 1.24 | (0.16) | | 13.19 | 0.12 | 24.34 |
| Insulin | 100 nM | 1.23 | (0.11) | | 8.73 | 0.08 | 22.70 |
| Insulin | 500 nM | 0.66 | (0.14) | | 20.86 | 0.10 | -34.17 |
| Insulin | 1000 nM | 0.90 | (0.41) | | 45.69 | 0.29 | -10.45 |
| Insulin | 5000 nM | 1.00 | (0.20) | | 20.05 | 0.14 | -0.24 |

†P<0.05 compared to untreated cells

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**Table 9.** Effect of a previous wash with BM on eNOS mRNA expression in HAEC

| Experimental conditions | Concentration | Reproducibility (between-day) INTER-ASSAY eNOS mRNA expression (relative values) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Mean (SD) | | P* | CV % | SEM | % Increment |
| **0 + 24h** | | | | | | | |
| Unteated cells | | 1.00 | (0.00) | | 0.00 | 0.00 | |
| Insulin | 100 nM | 0.81 | (0.05) | | 6.05 | 0.03 | -18.80 |
| Insulin | 500 nM | 0.78 | (0.00) | | 0.28 | 0.00 | -21.83 |
| Insulin | 1000 nM | 0.72 | (0.01) | † | 1.42 | 0.01 | -28.24 |
| **2 + 24h** | | | | | | | |
| Unteated cells | | 1.00 | (0.00) | | 0.00 | 0.00 | |
| Insulin | 100 nM | 0.75 | (0.06) | | 8.13 | 0.04 | -24.81 |
| Insulin | 500 nM | 0.72 | (0.16) | | 21.96 | 0.09 | -27.62 |
| Insulin | 1000 nM | 0.64 | (0.11) | † | 16.83 | 0.06 | -35.78 |
| **18 + 24h** | | | | | | | |
| Unteated cells | | 1.00 | (0.00) | | 0.00 | 0.00 | |
| Insulin | 100 nM | 1.21 | (0.15) | | 12.59 | 0.09 | 21.49 |
| Insulin | 500 nM | 1.48 | (0.08) | † | 5.13 | 0.04 | 47.91 |
| Insulin | 1000 nM | 1.42 | (0.11) | † | 8.07 | 0.07 | 42.49 |

†P<0.05 compared to untreated cells

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## 4. STUDY OF THE THROMBOTIC EFFECTS OF RESVERATROL EFFECTS ON TF EXPRESSION IN HAECs

### Cytotoxicity

An increase of LDH was observed under all the tested conditions compared to cells incubated with culture medium (figure 24). These results were irrespective of resveratrol dose or duration of incubation. Microscopic morphological alterations were noted when cells were incubated with 100 μM of resveratrol for 16h compared with blank control (figures 25a and 25b). These altered cells appeared to recover morphologically after removal of resveratrol and incubation with TNF-α for 6h (figures 25c and 25d).

### Effect of resveratrol on TF protein release in TNF-α-stimulated HAECs

TNF-α-stimulated HAECs (10 ng/mL) for 6h expressed higher TF protein in their membranes than those non-stimulated (figure 26).

Pre-incubation of HAECs with EtOH (vehicle-alone control) showed no statistically significant differences compared with untreated cells, indicating that resveratrol vehicle had no effect per se on HAEC stimulation.

Cells pre-incubated for 16h with resveratrol (5, 10, 25, 50 and 100 μM) resulted in an inhibition of this stimulation.

The minimal resveratrol dose tested (5 μM), TF protein was inhibited by 20.84%, and at 100 μM achieved 91.76% compared to TNF-α alone ($p<0.05$) as shown in figure 26. A statistically significant dose-dependent trend was observed from 25 to 100 μM ($p<0.05$; figure 26 and table 10).

Regarding the TF protein results, vehicle control and resveratrol (100 μM) had achieved similar low values indicating a total inhibition of TNF-α stimulation (table 10).

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**Effect of resveratrol on TF mRNA expression in TNF-α-stimulated HAECs**

Stimulation of HAECs with TNF-α resulted in an approximately 8-fold increase relative to untreated cells- and vehicle control (EtOH) (figure 27).

EtOH showed no effect on mRNA expression in unstimulated cells compared with the blank control.

HAECs pre-incubated for 16h with resveratrol (25, 50 and 100 μM) and an additional 6h with TNF-α demonstrated a statistically significant dose-dependent inhibition of TF (22.24, 37.35 and 60.42% respectively) compared to TNF-α alone ($p<0.05$) (table 10).

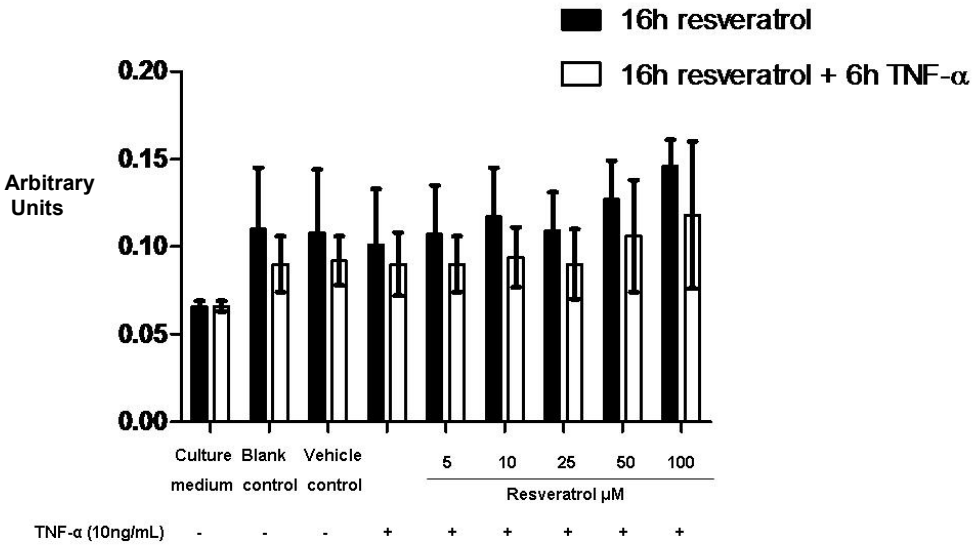


**Figure 24. Cytotoxicity of resveratrol on HAEC cells as assessed by LDH secretion**.
There were no differences between resveratrol concentrations relative to the vehicle control incubation or with respect to the TNF-α stimulation.

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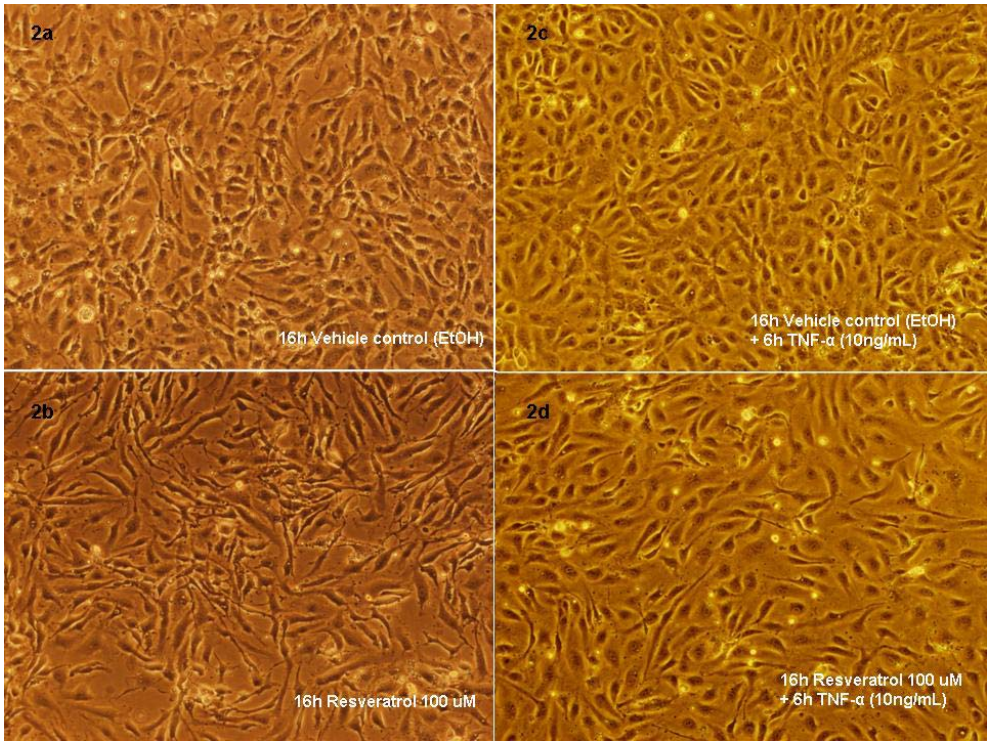


**Figure 25. HAEC images under experimental conditions**. (2a): HAEC cells incubated with vehicle control-alone (EtOH) after 16h; (2b): HAEC cells incubated with resveratrol (100 μM) after 16h; (2c): HAEC cells incubated with vehicle control-alone (EtOH) after 16h + 6h with TNF-α (10 ng/mL); (2d): HAEC cells incubated with resveratrol 100 μM + 6h with TNF-α (10 ng/mL). Scale bar = 10 μm.

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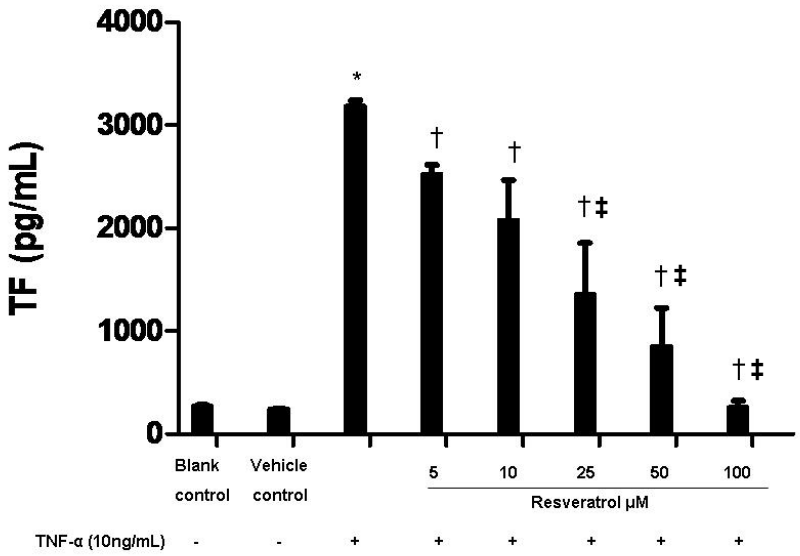


**Figure 26. The effect of resveratrol on TF protein in TNF-α-activated HAEC cells**.
Data represent the means (SD) of two separate experiments. *P<0.05 compared to blank control (untreated cells) and vehicle control; †P<0.05 compared to HAECs treated with TNF-α alone. ‡ P<0.05 compared to each other. The CVs were <43% and the SEM was <251.5.

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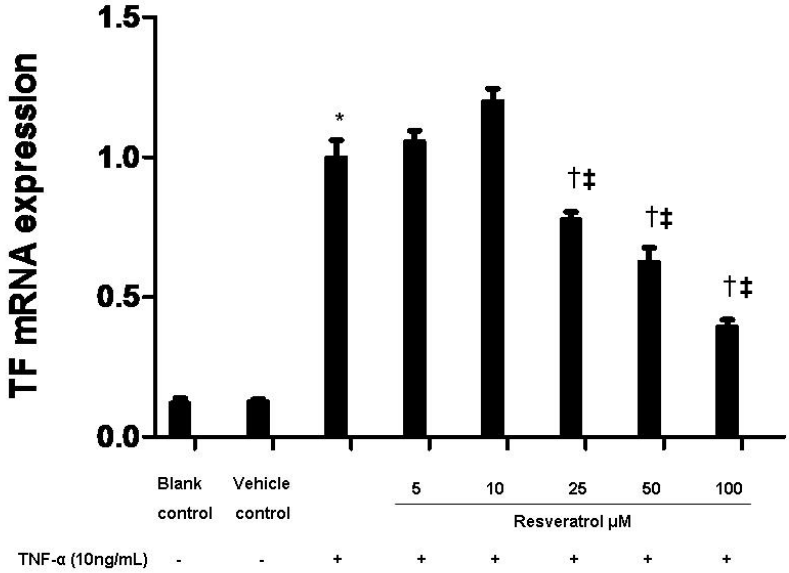


**Figure 27. The effect of resveratrol on TF mRNA expression in TNF-α-activated HAEC cells.**

Data represent the means (SD) of two separate experiments. *P<0.05 compared to blank control (untreated cells) and vehicle control; †P<0.05 compared to HAECs treated with TNF-α alone. ‡ P<0.05 compared to each other.The CVs were <13% and the SEM was <0.04.

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**Table 10.** Effects of Resveratrol on TF protein and mRNA expression and percentages of TF reduction on TNF-α-stimulated HAEC

| Experimental conditions | Concentration | Reproducibility (between-day) INTER-ASSAY | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | TF protein expression (pg/mL) | | | | | TF mRNA expression (relative values) | | | | |
| | | Mean (SD) | P* | CV % | SEM | % Reduction | Mean (SD) | P* | CV % | SEM | % Reduction |
| Blank control | 0 | 271.61 (9.88) | | 3.63 | 6.99 | 91.48 | 0.12 (0.02) | | 12.97 | 0.01 | 88.00 |
| Vehicle control (EtOH) | 0 | 244.46 (2.65) | | 1.08 | 1.87 | 92.33 | 0.13 (0.01) | | 5.84 | 0.00 | 87.00 |
| TNF-α | 10ng/mL | 3188.17 (51.85) | † | 1.62 | 36.67 | 0.00 | 1 (0.06) | † | 6.13 | 0.04 | 0.00 |
| TNF-α +Resveratrol | 5 μM | 2523.67 (92.39) | ‡ | 3.66 | 65.33 | 20.84 | 1.06 (0.04) | | 3.62 | 0.02 | -6.00 |
| TNF-α +Resveratrol | 10 μM | 2083.33 (384.43) | ‡ | 18.45 | 271.83 | 34.65 | 1.20 (0.04) | | 3.59 | 0.02 | -20.23 |
| TNF-α +Resveratrol | 25 μM | 1357.21 (497.04) | ‡& | 36.62 | 351.46 | 57.43 | 0.78 (0.03) | ‡& | 3.35 | 0.02 | 22.24 |
| TNF-α +Resveratrol | 50 μM | 854.83 (370.29) | ‡& | 43.32 | 261.83 | 73.19 | 0.63 (0.05) | ‡& | 8.02 | 0.04 | 37.35 |
| TNF-α +Resveratrol | 100 μM | 262.68 (57.78) | ‡& | 21.99 | 40.85 | 91.76 | 0.40 (0.02) | ‡& | 5.99 | 0.02 | 60.42 |

†P<0.05 compared to blank control (untreated cells)

‡P<0.05 compared to HAECs treated with TNF-α alone

&P<0.05 compared to each other

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# Discussion

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## General discussion

In the present work, we have implemented four *in vitro* cellular models in order to rapidly test the benefits of natural bioactive compounds or extracts on the expression of biomarkers and possible pathological mechanisms involved in atherosclerosis. The biomarkers selected are related to several aspects of the process of atherosclerosis; inflammation, for which we chose TNF-$\alpha$, endothelial function (VCAM-1 and eNOS) and thrombosis (TF) (De Caterina R, 2007).

Although the most effective therapies of atherosclerosis, such as statins, lower LDL cholesterol significantly (20-60%), there is still a high incidence of coronary events in these patients. Nowadays, the research is focused on the need for additional therapeutic strategies, either dietary or pharmacological, to increase HDL, decrease triglycerides, reduce vascular inflammation, improve endothelial function and attenuate the prothrombotic state (Hausenloy DJ, 2008).

The cellular models described constitute a preliminary step to screening the biological activities of natural extracts. The scientific evidence could then be explored in animals or humans for determining their possible used in human nutrition (Sipido KR, 2009; Lean ME, 2008; http://tmrc.co.uk/translational-medicine).

We tested several bioactive natural extracts provided by la Morella Nuts S.A, to study the anti-inflammatory and anti-thrombotic properties and the improvement of endothelial function by the proposed *in vitro* models. However, we cannot cite the results obtained using these extracts since they are protected by confidentiality.

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**Challenges of Cell-Based Assays**

Cell-based assays are finding numerous applications in target identification and validation, monitoring cellular events, as well as the screening of compounds for efficacy and biosafety. Although, most research activities currently concentrate on drug discovery, cell-based assays are expected to gain popularity in diagnostics, molecular biology, biochemistry, genetics, toxicology, bioengineering, and proteomics, among other fields as they become more cost-effective and biologically significant (http://scq.ubc.ca). Cell-based assays have developed into a rapid and cost-effective way of determining the challenges and issues surrounding all molecules in drug or food ingredients design and development (Freshney, RI, 2005;http://ub.edu/biomed/cas/progs).

However, the lack of an ideal cellular reference method has been circumvented by comparing an alternative test method with a traditional test method (Hoffmann S, 2008).

Cell culture, referred to as an *ex vivo* study, of the cellular milieu is problematic because the cell is not in its original, normal physiological environment. Therefore, introduction of cells to screening strategies inevitably introduces a potential source of variability in assays. Moreover, we do not have sufficient knowledge of cell biology to guarantee that all cells in a population are identical, nor can we absolutely control all the variables of cell culture. Even stably transfected cell lines can change over time in culture, such that growth rates may slow or increase and functional expression of proteins can also diminish. It is important to recognise that while not all sources of variability can be controlled, the impact can be minimised by rigorous adherence to standard operating procedures.

These data are useful only if they provide information about what we want to know. Knowledge gained from data is usually more informative and more

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accurate if the data are quantitative and analysed by statistics adequated test (Altman DG, 1983).

Quality control measures can be introduced in the design of the experimental process (including the tracking of growth parameters such as the correlation between doubling time and time in culture, documenting cell morphology, and carefully monitoring cell viability), in order to help the scientist to control the maximum number of parameters possible to guarantee viable and reliable results. An attractive approach to minimizing variability is the application of automated cell culture to the work-flow for screening. This is now an established component in many pharmaceutical environments (Altman DG, 1983).

For all of these reasons, statistical analysis is an important tool to assess the validity of the results and can guide decisions regarding future experimental decisions.

We can affirm that the results of our *in vitro* studies are acceptable because the predominant CV were <20%. It is known that cell culture experiments are subject to high variability (Freshney RI, 2005). However, this variability can be reduced after having standardised the experimental parameters involved in the development of four cell models (monocytes THP-1 cells and HAECs).

**Cell culture as a tool for screening bioactive compounds or extracts**

Cell culture allows the study of cells under controlled conditions, allowing researchers to examine the effects of specific conditions on cell physiology, toxicity of the effect of various natural or chemical compounds on a given cell type.

**Limitations of Manual Cell Culture**

Manual cell culture is labour intensive and time consuming, and the most significant limitation is that it is inherently variable.

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Tasks are repetitive and it is challenging to maintain the high standards of sterility, consistency and observation that are essential for successful cell culture (Freshney RI, 2005). At even the simplest level, the standard five day working week does not allow for consistent cell culture methods.

Additional sources of variability, as manipulation, often emerge when cell culture protocols are transferred from one group to another (or even between cell culture scientists within a group). Cell culture protocols are complex, difficult to describe precisely, and frequently contain subjective parameters such as cell confluence and morphological descriptions. As an example, one scientist's judgment of 45% cell confluence may be assessed as 65% by another, for this reason, to reduce this variability, at least, two scientist's judgment are required.

Manual cell counts derived using traditional Trypan blue exclusion methods and haemocytometer grids could vary between scientists. Automated cell counters give a cellular density more accurated than manual counting.

A useful tool for counting cells or studying cells confluence, is the routine capture of cell images using microscope-mounted cameras. The images can also be used to document single cell suspensions prior to plating when clumping of cells is an issue.

In general, cell manual process will always have variables that cannot be documented (american drug discovery on line).

**1. INDUCTION OF INFLAMMATION**

A standardised and precise *in vitro* monocytes THP-1 cells assay has been developed based on the detection of TNF-$\alpha$ as a proinflammatory biomarker, whose production is induced by LPS (Leon CG, 2008, Gutsmann T, 2001, Guha M, 2001; Song M 2000; Kobori M, 2008).

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However, these cell models in general presented high variability of 30-40% due to the inherent instability found in monocytes and the other factors such as FBS content in culture medium. It is necessary to study and control many variables presented in these models, particularly those related to monocytes THP-1 cells activation by LPS, FBS content in the culture medium, days in culture, and the cell batch used.

As reported control subjects were found to have 1.7±0.5 pg/mL of TNF-α in their plasma (Tomiyama H, 2008). However, patients with obstructive sleep apnoea reached nearly 2 pg/mL, subjects with systemic lupus erythematosus, 4.9±12.8 pg/mL and patients suffering from rheumatoid arthritis, an inflammatory disease, presented 20±36.2 pg/mL (Vadacca M, 2008).

In our *in vitro* case, LPS-stimulated monocytes THP-1 cells secreted 26-150 pg/mL of TNF-α into the cell culture. Therefore, we can affirm that we created a severe inflammatory state in cells.

A different aspect of the model proposed is the addition of FBS to the culture medium because, as it contains LBP, it participates in LPS stimulation activity (Schmelzer C, 2009). Our results showed that monocytes THP-1 cells stimulation was achieved at any LPS dose when it was performed in culture medium containing different amounts of FBS (2, 5, 10 %). This stimulation was dependent not only on LPS dose but also on FBS content. We have considered fixing FBS content at 10% since this is the same content used to keep cells in growth avoiding another source of variability.

These results agree with data presented by other authors that have demonstrated the role of LBP in monocyte activation (Schmelzer C, 2009). For instance, the immunosuppression of LBP from whole blood lowers the sensitivity of monocytes to activation by LPS by at least two orders of magnitude. In LBP-deficient mice, it is not required for the clearance of LPS

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from the circulation, but it essential for the rapid induction of an inflammatory response by small amounts of LPS or Gram-negative bacteria (Yao J, 1997).

Nevertheless, our data show that monocyte stimulation performed in the absence of FBS was effective only with the highest LPS doses tested (1000 and 2000 ng/mL). Although monocytes THP-1 cells activation by LPS is well described (Haversen L, 2002), its is known that monocyte activation can occur without the presence of LBP, and that it requires several orders of magnitude more endotoxin (Lipid A of LPS molecule; Visintin A, 2005).
These data agree with our results, since activated monocytes in the absence of FBS demonstrated increased TNF-$\alpha$ protein release and an increase of mRNA expression.

Our results revealed that TNF-$\alpha$ protein release and mRNA expression were greater when the monocytes were kept in culture for prolonged periods of time. These findings agree with studies that show that the number of cell passages plays an important role in the expression of different molecules (Tripathi S, 2008; Klein CL, 1995). Indeed, almost all *in vitro* models utilise cells at the same passage and at the same post-thaw day, to avoid the inherent instability found in cells, specifically in monocyte cell lines (Jiang Z, 2005, Leon CG, 2008, Gutsmann T, 2001, Guha M, 2001; Song M 2000; Kobori M, 2008). As LBP can be anchored transmembranously into the cell membrane, the different responses observed in monocytes THP-1 cells that were cultured for different numbers of days before being stimulated with LPS could be due to the fact that monocytes could have acquired different amounts of LBP in their cellular bilayers while they were in contact with FBS.

Nevertheless, additional studies are necessary to elucidate the complete mechanism by which the monocyte response depends on the period of time that the cells are kept in culture.

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Besides, when a monocyte THP-1 cell batch needs to be changed, it becomes necessary to optimise the experiment conditions, including the LPS dose used to activate monocytes at the same day in culture and FBS content. This tactic was necessary when studying TNF-α response in LPS-stimulated monocytes THP-1 to avoid potential problems derived from the inherent differences in their behaviour.

We decide to keep our monocytes batch in growth for six days and activate them with 500 or 1000 ng/mL of LPS, since higher LPS doses and more days in culture could result in higher TNF-α protein release that was beyond the analytical range.

The natural extracts obtained by La Morella, M107 and M114, produced a significant reduction of TNF-α protein and mRNA expression suggesting an anti-inflammatory effect. Consequently, they can be used as positive controls for the experimental process.

**2. TNF-α-stimulated HAECS**

In our cellular models, endothelial dysfunction and thrombosis were induced in HAECs by TNF-α at 10 ng/mL, which corresponds to 10000 pg/mL. This concentration is 10000-fold higher in comparison to TNF-α plasma concentrations 1.7±0.5 pg/mL of control subjects (Tomiyama H, 2008). In the present work, we chose a TNF-α concentration of 10 ng/mL, an adequate dose according to several *in vitro* reports (Tribolo S, 2008; Raab M, 2002).

**2.1 Induction of endothelial dysfunction**

We applied TNF-α at 10 ng/mL for 24h to determine the protein VCAM-1 release, based on previous reports in HUVECs (Tribolo S, 2008; Shaw DI, 2007) and in HAECs (Szekanecz Z, 1994; Amberger A, 1997). In other recent experiments, a range of concentrations of TNF-α from 2 to 10 ng/mL has been used (Chen YH, 2001; Schleser S, 2006; Chen JW, 2003).

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Once a significant increase of the VCAM-1 mRNA levels was achieved by the lower doses of TNF-α, we decided to treat the cells with 1 ng/mL for 24h, since 10 ng/ml treatment for to 2 or 4h produced an over stimulation of VCAM-1 mRNA levels. Consequently, it was more difficult to determine small changes in VCAM-1 mRNA expression caused by the selected compounds, BAY or AT.

We found that treatment with AT (75 and 150 μM) was moderately inhibitory, resulting in a nearly the 33% inhibition of VCAM-1 mRNA and protein expression by TNF-α-stimulated HAECs. Thus, AT could reduce vascular dysfunction. AT serum concentrations in humans are higher than 23.2 μM (Gámez C, 1996). The results obtained from the selected working cellular concentrations of 10, 25 and 50 could be similar to human serum values. However, the concentration of AT in human cells is unknown and is possibly higher than serum. We studied the effects of 75 and 150 μM AT in HAECs. If the AT concentrations in human cells are lower than in serum, <10μM, they had no effect as expected.

We also reported that BAY decreased the expression of VCAM-1 protein, at 1 μM, by 60-70% and mRNA expression, 0.1 μM, by 30% in stimulated HAECs. We used low BAY concentrations due to the apparent cytotoxicity at the highest doses (2 and 10 μM). Moreover, BAY can be added to the cell culture medium at the same time as TNF-α.

The production of adhesion molecules by endothelial cells may be subjected to modulation by oxidants and antioxidants. AT is a potent antioxidant which provoke a reduction of VCAM-1 protein and mRNA expression in activated HAECs, may be mainly due to its antioxidative properties. Thus, AT inhibits expression of adhesion molecules *in vitro* suggesting an improvement of endothelial dysfunction in HAECs and in various endothelial cell types as described previously (Liu L, 2004).

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We suggest that BAY, can be used as a positive control of these experiment at the proposed working concentration. It is effective in inhibiting TNF-α–induced phosphorylation of IαB-α, resulting in a decreased expression of VCAM-1. The precise molecular target for this agent is not yet clear (Pierce JW, 1997; Izban KF, 2000).

In conclusion, AT and BAY significantly reduced VCAM-1 protein and mRNA induced by TNF-α in HAECs. BAY and AT can be used as a positive control for the HAEC *in vitro* model in studies for screening the effects of compounds on endothelial function.

**3. ASSESSMENT OF ENDOTHELIAL FUNCTION**

We proposed to evaluate the eNOS mRNA expression as an endothelial dysfunction biomarker because loss of eNOS expression may contribute to endothelial dysfunction. In the present study we demonstrated that the important pro-inflammatory cytokine TNF-α, at concentrations of 5 or 10 ng/mL for 24h, is able to downregulate eNOS mRNA expression by 40% to 50% in HAECs. Previous reports have shown a reduction of eNOS protein levels by ~40 % in BAECs by TNF-α (1000 pg/mL) (Anderson HD, 2004).

TNF-α (at 5 or 10 ng/mL; safe and not cytotoxic doses) causes dysfunction of HAECs and, as such, it can be used as a negative control for the proposed cellular model.

When HAECs were incubated with increasing concentrations of insulin (500 and 1000 nM) for 24h after incubation with BM for 18h, we found that HAECs increased eNOS mRNA levels by approximately 40-50%. These data suggest that 18h of deprivation of different compounds such as growth factors is enough to obtain non-stimulated cells that are ready to be used for testing molecules or compounds such as components of food items for their effects on endothelial function.

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Insulin human plasma concentration is about 50 pM (pmol/L) (Solà R, submitted), which is nearly 2000-22000-fold higher in comparison to the working *in vitro* concentrations in HAECs, which ranged from 100 to 5000 nM.

However, with respect to the effects of insulin on HAECs, we found no significant increase in eNOS mRNA levels when the experiment was performed in CM. These data suggest that cells could be stimulated *a priori*, during the growth phase, by some components present in the CM. As mentioned previously, the cell culture medium composition could affect an increase in the eNOS mRNA expression in endothelial cells (Iantorno M, 2007; Kawakami A, 2008; Wang XL, 2006).

Detailed analysis of the CM composition indicated that human epidermal growth factor, basic fibroblast growth factor, heparin and hydrocortisone were components of the medium. Li et al. observed that both these growth factors were directly related to enhancement of eNOS mRNA expression in BAECs or HUVECs (Li H, 2002).

At the same time, M-200 medium, which includes L-arginine, plays an important role in NO production. NO is synthesised by an enzymatic conversion of L-arginine by eNOS. This amino acid cannot be excluded from the culture medium since it is essential for cell growth. As such, if we expect to achieve an increase in eNOS mRNA levels, the composition of the cell culture medium is important. Our findings suggest that insulin may not be able to stimulate cells further when the cells have previously been stimulated.

In evaluating the implications of eNOS mRNA expression, we selected HAECs as an appropriate human model since BAECs and HUVECs are bovine and umbilical cells, respectively. As shown in previous studies, there are differences in eNOS expression between endothelial cells of different

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origin that, in turn, lead to differences in eNOS regulation (Aljada A, 2000; Aberle S, 1997).

We chose eNOS mRNA expression because detection of NO as a gas in the cell culture medium requires quantifying nitrites ($NO^{2-}$) by the Griess chemical reaction, which produces highly variable results. Further, many studies of eNOS have employed activity measurements that can limit the conclusions that can be drawn regarding eNOS protein expression. This protein expression is generally measured by Western blot analysis, a semi-quantitative and relatively insensitive method requiring concentrated cell lysates for the detection of the protein. Therefore, we decided to evaluate eNOS mRNA expression as an endothelial function biomarker by means real time RT-PCR. It has been found that a decrease in eNOS mRNA expression in endothelial cells contributes to a further decrease in NO production (Aberle S, 1997).

These methodological aspects must be standardised with the purpose of developing a precise *in vitro* cellular model. Indeed, almost all in vitro models are performed with cells at the same passage in order to avoid instability found in cell culture (Matsushita H, 2001).

In summary,our HAECs model could be used to screen for the effects on endothelial dysfunction of some natural extracts or compounds by measuring eNOS mRNA expression. TNF-α is a good negative control while insulin is a good positive control to determine mRNA expression.

**4. STUDY OF THE THROMBOTIC EFFECT**

In the present work, we proposed a HAEC model to study thrombosis by TF protein and mRNA expression induced by TNF-α. However, bioactive compounds with anti-thrombotic effects are predicted to result in a reduction of TF.

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Thus, the present findings showed, for the first time, that resveratrol significantly inhibited TF production induced by TNF-α in HAECs. This is a desired anti-thrombotic effect.

The concentrations of resveratrol used in TF mRNA and protein expression studies ranged from 5 to 100 μM, and this attenuation was observed to be dose-dependent. There were significant effects of resveratrol on TF at low concentrations (5 μM). This level is likely to be higher than serum concentrations achievable with moderate red wine consumption, which in humans, is estimated to be in the nano-molar range (Ishisaka R, 1999; Markus MA, 2008). It is estimated that a concentration of resveratrol of 5 mg/l of red wine, or two glasses (375 ml), would equal a dose of 27 μg/Kg in a 70 Kg person. This would result in a peak serum concentration of 2.4 nM of free resveratrol and 180 nM of modified resveratrol. A recent Spanish study estimated that total resveratrol intake for men and women was 1629 μg/d and 235 μg/d, respectively. A pharmacologically relevant dose would be approximately 100 mg/Kg body weight or about 9 μM of free resveratrol and 680 μM of total resveratrol (Markus MA, 2008). Thus, our study suggests that resveratrol inhibits TF production and, as such, shows anticoagulant properties, but the physiological relevance of these *in vitro* observations and the effect in humans needs to be validated.

These properties need to be explored using the metabolised forms such as glucoronides and sulfates, since the human oral bioavailability of resveratrol is almost zero (Markus MA, 2008). This is due to rapid and extensive metabolism and the consequent formation of various metabolites such as resveratrol glucuronides and resveratrol sulfates (Markus MA, 2008). The potential biologic activity of resveratrol conjugates should be considered in future investigations (Jackson R, 1991). Such doses have so far shown no adverse side effects in animal models (Eilertsen KE, 2004; Frankel EN, 1993).

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It is well documented that an increased and aberrant expression of TF contributes to the pathogenesis of atherosclerosis and CVD (Taubman MB, 1997; Pace-Asciak CR, 1995) and thus, resveratrol could be useful as a new protective factor in addition to its antioxidant (Fiebich BL, 2002) and antiplatelet activities (Bi XL, 2005).

The concentration of resveratrol required to inhibit TF mRNA and TF protein expression in HAECs was comparable to the concentration of resveratrol required to affect other cellular responses (Bi XL, 2005; Feng YH, 2004). However, to determine in vivo benefits for humans, the studies of resveratrol bioavailability and clinical benefits need to be conducted to demonstrate its long-term antithrombotic efficiency.

The mode of action of resveratrol is not completely understood. It seems that resveratrol-induced inhibition of NF-Kb activation is not cell type specific (Gao X, 2001; Kawada N, 1998; Wadsworth TL, 1999). However, some research groups have argued that resveratrol can exert a cell type specific mechanism (Zhu J, 2008). Moreover, the activity of TF needs to be elucidated in order to improve our cellular model.

In conclusion, resveratrol can attenuate the TNF-α induced TF mRNA and protein expression in HAEC and can be used as a positive control of the implemented model to study anti-thombotic effect of compounds.

Finally, using table 11 provides the summary of cellular models mplemented in the present work.

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**Table 11.** Cellular models simulating mechanisms involved in the atherosclerosis process for screening the properties of bioactive compounds

| Cellular model | | Biomarker | Exposed stimuli to produce maximal effect | Positive control *(desired effect)* | Negative control *(no-desired effect)* | Results expressed as |
|---|---|---|---|---|---|---|
| **Inflammation** | THP-1 | TNF-α extracellular protein concentration<br><br>TNF-α mRNA expression | LPS | M-107<br>M-14<br><br>*Reduction of TNF-α* | | % reduction or increase compared to maximal induction by stimuli |
| **Endothelial dysfunction** | HAEC | VCAM-1 extracellular protein concentration<br><br>VCAM-1 mRNA expression | TNF-α | BAY<br>*α-tocopherol*<br><br>*Reduction of VCAM-1* | | % reduction or increase compared to maximal induction by stimuli |
| | HAEC | eNOS mRNA expression | - | Insulin<br><br>*Increase of eNOS* | TNF-α<br><br>*Reduction of eNOS* | % reduction or increase compared to control (vehicle) |
| **Thrombosis** | HAEC | TF protein concentration<br><br>TF mRNA expression | TNF-α | Resveratrol<br><br>*Reduction of TF* | | % reduction or increase compared to maximal induction by stimuli |

HAEC= Human Aortic Endothelial Cells

TNF-α= Tumor Necrosis Factor alpha

VCAM-1=Vascular Cell Adhesion Molecule-1

eNOS= endothelial Nitric Oxide Synthase

TF= Tissue Factor

LPS= Lipopolysaccharide molecule

M-107=extract obtained from natural food by La Morella Nuts

M-114= extract obtained from natural food by La Morella Nuts

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**Applicability of the proposed *in vitro* cellular models**

The quantification of protein and mRNA are complementary and necessary for a complete understanding of how the cell works (Hatzimanikatis V, 1999). Additionally, as mRNA is eventually translated into protein, one might assume that there should be some sort of correlation between the level of mRNA and that of protein. Alternatively, there may not be any significant correlation, which, in itself, is an informative conclusion (Greenbaum D, 2003).

We have proposed an algorithm of decisions to study the effects of natural bioactive extracts or compounds.

First the experimental conditions must be optimised. We have included the selection of the controls for the experiment (positive or negative), dilutions of the compounds, the study of the cytotoxicity of the vehicle or compound and the dose-dependent effects of the compound on cells in order to determine the appropriate range of concentrations for each compound.

After having decided the experimental conditions, the cellular model can be used to determine levels of the secreted protein in the cell culture medium.

Have we observed any effect? If not, the study is concluded here since it is not necessary to go ahead with the study of mRNA expression. On the contrary, if there is any expected effect, the mRNA expression of the selected biomarker may be tested.

If the results show the same tendency as the protein quantification, the next step recommended is to test the transcription factors or other signalling pathways that could be affected by the molecule. However, if the mRNA analysis does not yield the expected results, new perspectives have to be taken into account to determine the appropriated methodology to find out the mechanisms involved in CVD.

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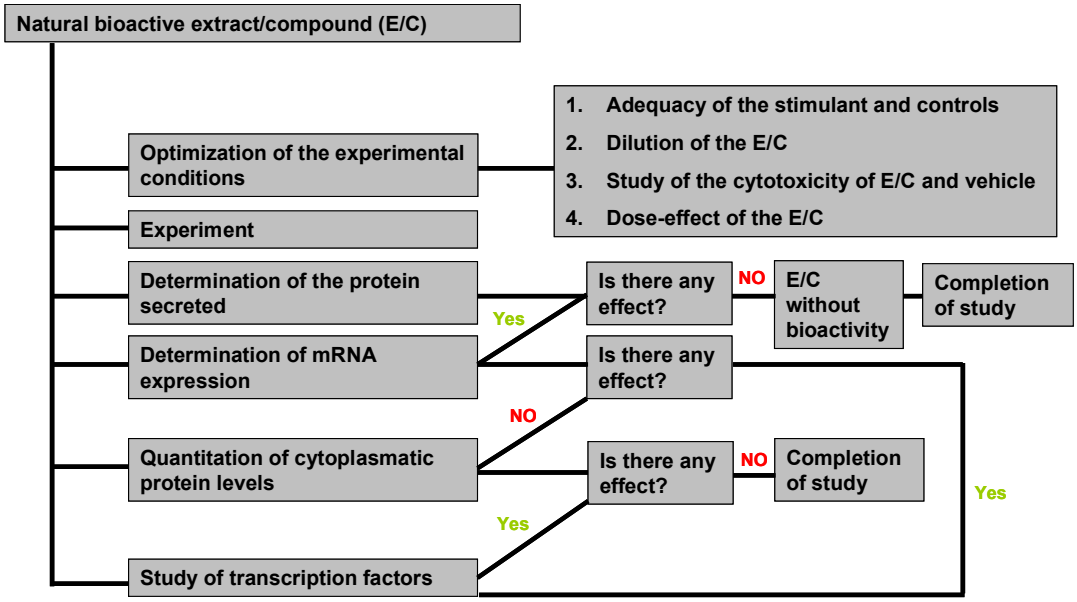


**Figure 28.** Proposal of an algorithm to study the effect of bioactive natural extracts or compounds (original source).

### Perspectives

Assay technologies have advanced to enable high density screening of cells in 1536 and 3456 well formats, increasing the accessibility of cell-based assays for screening large compound collections. Beyond traditional screening platforms using bulk cell assays, the addition of high-throughput electrophysiology and high content imaging platforms to screening strategies allows access to an increased array of assay formats and complexity of information down to the level of individual cell responses.

Lead and target identification strategies incorporating high throughput screening increasingly leverage cell-based assays as a tool to probe targets *in vitro*. Intact cells, either recombinantly or endogenously expressing proteins of interest, allow access to down-stream signalling pathways in a physiological context that cannot be addressed in biochemical (non-cell based) assays.

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# Conclusions

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1. Monocytes THP-1 cells activation depends on LPS dose, on FBS content of the culture medium, on the duration of culture, and on the THP-1 cell-batch used for the experiments.

2. TNF-α is a good candidate to act as a stressor to induce endothelial dysfunction in HAEC, as well as to study VCAM-1 protein and TF protein, as well as mRNA expression.

3. BAY and AT could be used as positive controls in TNF-α-stimulated HAEC since they cause the desired effect by reducing the secretion of VCAM-1 protein in the cell culture medium, and its mRNA expression.

4. TNF-$\alpha$ is a good negative control, while insulin is a good positive control, for the study of mRNA expression of eNOS in HAEC.

5. Resveratrol is a positive control because of its antithrombotic effect since it reduces the protein concentration and the mRNA expression of TF in TNF-α-stimulated HAECs.

Thus, these *in vitro* cellular models are valid systems for the study of novel compounds or food extracts that are considered to have potential beneficial effect on mechanisms involved in the pathogenesis of CVD.

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# References

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Aberle S, Young TA, Medberry P, Parkinson J, Rubanyi GM, Kauser K. Quantitative measurement for endothelial constitutive nitric oxide synthase in cultured human endothelial cells. Nitric Oxide. 1997;1(3):226-33.

Alexopoulos N, Raggi P. Calcification in atherosclerosis. Nat Rev Cardiol. 2009;6(11):681-8.

Aljada A, Ghanim H, Assian E, Dandona P. Tumor necrosis factor-alpha inhibits Insulin-induced increase in endothelial nitric oxide synthase and reduces Insulin receptor content and phosphorylation in human aortic endothelial cells. Metabolism. 2002;51(4):487-91.

Aljada A, Saadeh R, Assian E, Ghanim H, Dandona P. Insulin inhibits the expression of intercellular adhesion molecule-1 by human aortic endothelial cells through stimulation of nitric oxide. J Clin Endocrinol Metab. 2000;85(7):2572-5.

Altman DG, Gore SM, Gardner MJ, Pocock SJ. Statistical guidelines for contributors to medical journals. Br Med J. 1983;286: 1489-1493.

Amberger A, Maczek C, Jurgens G, Michaelis D, Schett G, Trieb K, Eberl T, Jindal S, Xu Q, and Wick G. Cell Stress Chaperones. 1997;2(2):94-103.

Anderson HD, Rahmutula D, Gardner DG. Tumor necrosis factor-alpha inhibits endothelial nitric-oxide synthase gene promoter activity in bovine aortic endothelial cells. J Biol Chem. 2004;279(2):963-9.

Anderson J, Baird P, Davis R, Ferreri S, Knudtson M, Koraym A, et al. Health benefits of dietary fiber. Nutr Rev. 2009; 67:188-205.

Arriero MM, Rodriguez-Feo JA, Celdran A, Sanchez de Miguel L, Gonzalez-Fernandez F, Fortes J, Reyero A, Frieyro O, de la Pinta JC, Franco A, Pastor C, Casado S, López-Farré A. Expression of endothelial nitric oxide synthase in human peritoneal tissue: regulation by Escherichia coli lipopolysaccharide. J Am Soc Nephrol. 2000;11:1848-56.

Balls M, Amcoff P, Bremer S, Casati S, Coecke S, Clothier R, Combes R, Corvi R,
Curren R, Eskes C, Fentem J, Gribaldo L, Halder M, Hartung T, Hoffmann S, Schectman L, Scott L, Spielmann H, Stokes W, Tice R, Wagner D, Zuang V; ECVAM. The principles of weight of evidence validation of test methods and testing strategies. The report and recommendations of ECVAM workshop 58. Altern Lab Anim. 2006;34(6):603-20.

Bermejo J, Heras M , Segovia J, Alfonso F. Medicina cardiovascular traslacional (I). Medicina cardiovascular traslacional. Ahora o nunca. Rev Esp Cardiol. 2009;62(1):66-8.

ISBN:978-84-693-1544-6/DL:T-637-2010

Bi XL, Yang JY, Dong YX, Wang JM, Cui YH, Ikeshima T, Zhao YQ, Wu CF. Resveratrol inhibits nitric oxide and TNF-alpha production by lipopolysaccharide-activated microglia. Int Immunopharmacol. 2005;5(1):185-193.

Bjelakovic G, Nikolova D, Gluud L, Simonetti R, Gluud C. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. JAMA. 2007;297:842-57.

Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction: a marker of atherosclerotic risk. Ateriosclerosis Thromb Vasc Biol. 2003;23(2):168-75.

Bottini A, Alepee N, Phillips B, Gribaldo L, De Silva O, Hartung T, Hendriksen C, Kuil J, Pazos P, Rhein C, Schiffelers MJ, Stokes W, Theobald A, Vidal JM, de Sandt HV, Boyle JJ. Macrophage activation in atherosclerosis: pathogenesis and pharmacology of plaque rupture. Curr Vasc Pharmacol. 2005;3:63-8.

Bottini AA, Alepee N, Phillips B, Gribaldo L, De Silva O, Hartung T, Hendriksen C, Kuil J, Pazos P, Rhein C, Schiffelers MJ, Stokes W, Theobald A, Vidal JM, Van de Sandt H, Breier S, Sintes JR, Blaauboer B. Optimisation of the post-validation process: the report and recommendations of ECVAM Workshop 67. Altern Lab Anim. 2008;36(3):353-66.

Breier S, Riego Sintes J, Blaauboer B. Optimisation of the Post-validation Process. ATLA. 2008;36:353–366.

Breaux J, Jones K, Boulas P. Analytical Methods Development and Validation. Pharmaceutical Technology Analytical Chemistry & Testing. 2003.

Brown L, Kroon PA, Das DK, Das S, Tosaki A, Chan V, Singer MV, Feick P. The Biological Responses to Resveratrol and Other Polyphenols From Alcoholic Beverages. Alcohol Clin Exp Res. 2009;33(9):1513-1523.

Brunner E, Rayner M, Thorogood M, Margetts B, Hooper L, Summerbell C, Dowler E, Hewitt G, Robertson A, Wiseman M. Making Public Health Nutrition relevant to evidence-based action. Public Health Nutr. 2001;4(6):1297-9.

Camera M, Giesen PL, Fallon J, Aufiero BM, Taubman M, Tremoli E, Nemerson Y. Cooperation between VEGF and TNF-alpha is necessary for exposure of active tissue factor on the surface of human endothelial cells. Arterioscler Thromb Vasc Biol. 1999;19(3):531-537.

Carluccio MA, Siculella L, Ancora MA, Massaro M, Scoditti E, Storelli C, Visioli F, Distante A, De Caterina R. Olive Oil and Red Wine Antioxidant Polyphenols Inhibit Endothelial Activation. Antiatherogenic Properties of

ISBN:978-84-693-1544-6/DL:T-637-2010

Mediterranean Diet Phytochemicals. Arterioscler Thromb Vasc Biol. 2003;23:622-629.

Chen JW, Chen YH, Lin FY, Chen YL, Lin SJ. Ginkgo biloba extract inhibits tumor necrosis factor-alpha-induced reactive oxygen species generation, transcription factor activation, and cell adhesion molecule expression in human aortic endothelial cells. Arterioscler Thromb Vasc Biol 23. 2003;(9):1559-1566.

Chen YH, Lin SJ, Ku HH, Shiao MS, Lin FY, Chen J, Chen YL. Salvianolic acid B attenuates VCAM-1 and ICAM-1 expression in TNF-alpha-treated human aortic endothelial cells. J Cell Biochem. 2001;82(3):512-521.

Covas MI. Olive oil and the cardiovascular system. Pharmacol Res. 2007;55(3):175-86.

Covas MI, Nyyssönen K, Poulsen HE, Kaikkonen J, Zunft HJ, Kiesewetter H, Gaddi A, de la Torre R, Mursu J, Bäumler H, Nascetti S, Salonen JT, Fitó M, Virtanen J, Marrugat J, EUROLIVE Study Group. The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial. Ann Intern Med. 2006;145(5):333-41.

Cullen JP, Morrow D, Jin Y, von Offenberg Sweeney N, Sitzmann JV, Cahill PA, Redmond EM. Resveratrol inhibits expression and binding activity of the monocyte chemotactic protein-1 receptor, CCR2, on THP-1 monocytes. Atherosclerosis. 2007;195(1):e125-133.

Curin Y, Andriantsitohaina R. Polyphenols as potential therapeutical agents against cardiovascular diseases. Pharmacological reports. 2005; 57 Suppl:97-107.

Davis PA, Polagruto JA, Valacchi G, Phung A, Soucek K, Keen CL, Gershwin ME. Exp Biol Med (Maywood). 2006;231(5):594-598.

De Caterina R, Libby P. Endothelial dysfunctions and vascular disease. 1st ed. Oxford, UK. Ed. Blacwell Futura 2007.

Demrow HS, Slane PR, Folts JD. Administration of wine and grape juice inhibits in vivo platelet activity and thrombosis in stenosed canine coronary arteries. Circulation. 1995;91(4):1182-1188.

Di Santo A, Mezzetti A, Napoleone E, Di Tommaso R, Donati MB, De Gaetano G, Lorenzet R. Resveratrol and quercetin down-regulate tissue factor expression by human stimulated vascular cells. J Thromb Haemost. 2003;1(5):1089-1095.

Dieguez-Acuña FJ, Polk WW, Ellis ME, Simmonds PL, Kushleika JV, Woods JS. Nuclear factor kappaB activity determines the sensitivity of kidney

ISBN:978-84-693-1544-6/DL:T-637-2010

epithelial cells to apoptosis: implications for mercury-induced renal failure. Toxicol Sci. 2004;82(2):361-2.
Dudzinski DM, Michel T. Life history of eNOS: partners and pathways. Cardiovasc Res. 2007;75:247-60.

Dufour D, Pichette A, Mshvildadze V, Bradette-Hebert ME, Lavoie S, Longtin A, Laprise C, Legault J. Antioxidant, anti-inflammatory and anticancer activities of methanolic extracts from Ledum groenlandicum Retzius. J Ethnopharmacol. 2007;111:22-8.

Eilertsen KE, Osterud B. Tissue factor: (patho)physiology and cellular biology. Blood Coagul Fibrinolysis. 2004;15(7):521-538.

Erdman J, Balentine D, Arab L, Beecher G, Dwyer J, Folts J, et al. Flavonoids and heart health: Proceedings of the ILSI North America Flavonoids Workshop, May31-June-1, 2005, Washington, DC. J Nutr. 2007;137:718S-737S.

Erdman JW Jr, Carson L, Kwik-Uribe C, Evans EM, Allen RR. Effects of cocoa flavanols on risk factors for cardiovascular disease. Asia Pac J Clin Nutr. 2008;17 Suppl 1:284-7.

Falkner E, Appl H, Eder C, Losert UM, Schöffl H, Pfaller W. Serum free cell culture: the free access online database. Toxicol In Vitro. 2006;395-400.

Federici M, Pandolfi A, De Filippis EA, Pellegrini G, Menghini R, Lauro D, Cardellini M, Romano M, Sesti G, Lauro R, Consoli A. G972R IRS-1 variant impairs Insulin regulation of endothelial nitric oxide synthase in cultured human endothelial cells. Circulation. 2004;109(3):399-405.

Feng YH, Zhu YN, Liu J, Ren YX, Xu JY, Yang YF, Li XY, Zou JP. Differential regulation of resveratrol on lipopolysacchride-stimulated human macrophages with or without IFN-gamma pre-priming. Int Immunopharmacol. 2004;4(6):713-720.

Ferroni P, Basili S, Paoletti V, Davì G. Endothelial dysfunction and oxidative stress in arterial hypertension. Nutr Metab Cardiovasc Dis. 2006;16(3):222-33.

Fiebich BL, Lieb K, Engels S, Heinrich M. Inhibition of LPS-induced p42/44 MAP kinase activation and iNOS/NO synthesis by parthenolide in rat primary microglial cells. J Neuroimmunol. 2002;132(1-2):18-24.

Fleck RA, Rao LV, Rapaport SI, Varki N. Localization of human tissue factor antigen by immunostaining with monospecific, polyclonal anti-human tissue factor antibody. Thromb Res. 1990;59(2):421-437.

ISBN:978-84-693-1544-6/DL:T-637-2010

Frankel EN, Waterhouse AL, Kinsella JE. Inhibition of human LDL oxidation by resveratrol. Lancet. 1993;341(8852):1103-1104.

Fremont L. Biological effects of resveratrol. Life Sci. 2000;66(8):663-673.

Freshney RI. Culture of animal cells; a manual of basic technique. 5th ed. New Jersey, USA. Ed Wiley-Liss. 2005.

Gámez C, Artacho R, Ruiz-López MD, Puerta A, López MC. Nutritional status of vitamin A and E in institutionalized elderly people in Granada, J Nutr Sci Vitaminol. 1996;42(5):397-405.

Gao X, Xu YX, Janakiraman N, Chapman RA, Gautam SC. Immunomodulatory activity of resveratrol: suppression of lymphocyte proliferation, development of cell-mediated cytotoxicity, and cytokine production. Biochem Pharmacol. 2001;62(9):1299-1308.

Gil A, Ramírez MC, Aguilera MC, Mesa MD. Modelos experimentales de enfermedad cardiovascular. Nutr Hosp. 2007;22(2):169-77.

Gimeno E, Fitó M, Lamuela-Raventós RM, Castellote AI, Covas M, Farré M, de La Torre-Boronat MC, López-Sabater MC. Effect of ingestion of virgin olive oil on human low-density lipoprotein composition. Eur J Clin Nutr. 2002;56(2):114-20.

Girona J, La Ville AE, Solà R, Motta C, Masana L. HDL derived from the different phases of conjugated diene formation reduces membrane fluidity and contributes to a decrease in free cholesterol efflux from human THP-1 macrophages. Biochim Biophys Acta. Molecular and Cell Biology of Lipids 2003;1633:143-148.

González-Fernández F, Jiménez A, López-Blaya A, Velasco S, Arriero MM, Celdrán A, Rico L, Farré J, Casado S, López-Farré A. Cerivastatin prevents tumor necrosis factor-alpha-induced downregulation of endothelial nitric oxide synthase: role of endothelial cytosolic proteins. Atherosclerosis. 2001;155(1):61-70.

Govers R, Rabelink TJ. Cellular regulation of endothelial nitric oxide synthase. Am J Physiol Renal Physiol. 2001;280(2):193-206.

Graham I, Atar D, Borch-Johnsen K, Boysen G, Burell G, Cifkova R, et al. European guidelines on cardiovascular disease prevention in clinical practice: executive summary. Fourth Joint Task Force of the European Society of Cardiology and other societies on cardiovascular disease prevention in clinical practice. Eur Heart J. 2007;28:2375-2414.

Graham I, Atar D, Borch-Johnsen K, Boysen G, Burell G, Cifkova R, et al. European guidelines on cardiovascular disease prevention in clinical

ISBN:978-84-693-1544-6/DL:T-637-2010

practice: full text. Fourth Joint Task Force of the European Society of0 Cardiology and other societies on cardiovascular disease prevention in clinical practice. Eur J Cardiovasc Prev Rehabil. 2007;14:S1-S113.

Greenbaum D, Colangelo C, Williams K, Gerstein M. Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol. 2003;4(9):117.

Grumbach IM, Chen W, Mertens SA, Harrison DG. A negative feedback mechanism involving nitric oxide and nuclear factor kappa-B modulates endothelial nitric oxide synthase transcription. J Mol Cell Cardiol. 2005;39(4):595-603.

Grundy SM. Implications of Recent Clinical Trials for the National Cholesterol Education Program Adult Treatment Panel III Guidelines. JACC. 2004;44:720-32.

Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cell Signal. 2001;13:85-94.

Gutsmann T, Muller M, Carroll SF, MacKenzie RC, Wiese A, Seydel U. Dual role of lipopolysaccharide (LPS)-binding protein in neutralization of LPS and enhancement of LPS-induced activation of mononuclear cells. Infect Immun 2001;69:6942-50.

Hallén S, Clapham JC. Cell based *in vitro* and *ex vivo* models in metabolic disease drug discovery: nice to have or critical path? Expert Opin Drug Discov.2009;4:417-428.

Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med. 2005;352(16):1685-95.

Hatzimanikatis V, Choe LH, Lee KH: Proteomics: theoretical and experimental considerations. Biotechnol Prog 1999, 15:312-318

Hausenloy DJ, Yellon DM. Targeting residual cardiovascular risk: raising high-density lipoprotein cholesterol levels. Heart. 2008; 94(6):706-14.

Haversen L, Ohlsson BG, Hahn-Zoric M, Hanson LA, Mattsby-Baltzer I. Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF-kappa B. Cell Immunol. 2002;220:83-95.

Hiss RG. Fundamental issues in translational research. Translational research—two phases of a continuum. In: From clinical trials to community: the science of translating diabetes and obesity research. Natcher Conference Center, National Institutes of Health, Bethesda, Maryland, USA. 2004:11-4.
(www.niddk.nih.gov/fund/other/Diabetes-Translation/conf-publication.pdf).

ISBN:978-84-693-1544-6/DL:T-637-2010

Hoffmann S, Edler L, Gardner I, Gribaldo L, Hartung T, Klein C, Liebsch M, Sauerland S, Schechtman L, Stammati A, Nikolaidis E. Points of reference in the validation process: the report and recommendations of ECVAM Workshop 66. Altern Lab Anim. 2008;36(3):343-52.

http://www.ncbi.nlm.nih.gov/books/bv.fcgi?db=Books&rid=mboc4.table.1516

http://www.scq.ubc.ca/cell-culture

http://www.fao.org (Food and Agriculture Organization of the United Nations)

http://www.who.int (World Health Organization)

http://ecvam.jrc.it

http://www.tmrc.co.uk/translational-medicine/discovery-process

Hollenberg NK, Schmitz H, Macdonald I, Poulter N. Cocoa, Flavanols and Cardiovascular Risk. Br J Cardiol. 2004;11(5).

Hussein KK, Hafez TA, el-Banna MH, el-Nady A. Serum glycoproteins in hepatic bilharziasis. J Egypt Med Assoc. 1975;58(1-2):45-51.

Huysmans MC, Longbottom C. The challenges of validating diagnostic methods and selecting appropriate gold standards. J Dent Res. 2004;83 Spec No C:C48-52.

Iantorno M, Chen H, Kim JA, Tesauro M, Lauro D, Cardillo C, Quon MJ. Ghrelin has novel vascular actions that mimic PI 3-kinase-dependent actions of insulin to stimulate production of NO from endothelial cells. Am J Physiol Endocrinol Metab. 2007;292:E756-64.

Inder M. Singh IM, Shishehbor MH, Ansell BJ. High-Density Lipoprotein as a Therapeutic Target. A Systematic Review. JAMA. 2007;298(7):786-798.

Ishisaka R, Sato N, Tanaka K, Takeshige T, Iwata H, Klostergaard J, Utsumi T. A part of the transmembrane domain of pro-TNF can function as a cleavable signal sequence that generates a biologically active secretory form of TNF. J Biochem. 1999;126(2):413-420.

Itabe H. Oxidized low-density lipoproteins: what is understood and what remains to be clarified. Biol Pharm Bull. 2003;26(1):1-9.

Izban KF, Ergin M, Qin JZ, Martinez RL, Pooley RJ JR, Saeed S, Alkan S. Constitutive expression of NF-kappa B is a characteristic feature of mycosis fungoides: implications for apoptosis resistance and pathogenesis. Hum Pathol. 2000;31(12):1482-90.

ISBN:978-84-693-1544-6/DL:T-637-2010

Jackson R, Scragg R, Beaglehole R. Alcohol consumption and risk of coronary heart disease. Br Med J. 1991;303(6796):211-216.

Jiang Z, Georgel P, Du X, Shamel L, Sovath S, Mudd S, Huber M, Kalis C, Keck S, Galanos C, Freudenberg M, Beutler B. CD14 is required for MyD88-independent LPS signaling. Nat Immunol. 2005;6:565-70.

Jude B, Zawadzki C, Susen S, Corseaux D. Relevance of tissue factor in cardiovascular disease. Arch Mal Coeur Vaiss. 2005;98(6):667-671.

Kamthong PJ, Wu M. Inhibitor of nuclear factor-kappaB induction by cAMP antagonizes interleukin-1-induced human macrophage-colony-stimulating-factor expression. Biochem J. 2001;356(Pt 2):525-30.

Kaur G, Roberti M, Raul F, Pendurthi UR. Suppression of human monocyte tissue factor induction by red wine phenolics and synthetic derivatives of resveratrol. Thromb Res. 2007;119(2):247-256.

Kawada N, Seki S, Inoue M, Kuroki T. Effect of antioxidants, resveratrol, quercetin, and N-acetylcysteine, on the functions of cultured rat hepatic stellate cells and Kupffer cells. Hepatology. 1998;27(5):1265-1274.

Kawakami A, Osaka M, Tani M, Azuma H, Sacks FM, Shimokado K, Yoshida M. Apolipoprotein CIII links hyperlipidemia with vascular endothelial cell dysfunction. Circulation. 2008;118(7):731-42.

Klatsky AL, Armstrong MA, Friedman GD. Alcohol and mortality. Ann Intern Med. 1992;117(8):646-654.

Klatsky AL, Armstrong MA. Alcoholic beverage choice and risk of coronary artery disease mortality: do red wine drinkers fare best? Am J Cardiol. 1993;71(5):467-469.

Klein CL, Bittinger F, Kohler H, Wagner M, Otto M, Hermanns I, Kirkpatrick CJ. Comparative studies on vascular endothelium in vitro. 3. Effects of cytokines on the expression of E-selectin, ICAM-1 and VCAM-1 by cultured human endothelial cells obtained from different passages. Pathobiology. 1995;63:83-92.

Kobori M, Nakayama H, Fukushima K, Ohnishi-Kameyama M, Ono H, Fukushima T, Akimoto Y, Masumoto S, Yukizaki C, Hoshi Y, Deguchi T, Yoshida M. Bitter gourd suppresses lipopolysaccharide-induced inflammatory responses. J Agric Food Chem. 2008;56:4004-11.

Koenig W, Khuseyinova N. Biomarkers of atherosclerotic plaque instability and rupture. Arterioscler Thromb Vasc Biol. 2007;27(1):15-26.

ISBN:978-84-693-1544-6/DL:T-637-2010

Kosmidou I, Moore JP, Weber M, Searles CD. Statin treatment and 3' polyadenylation of eNOS mRNA. Arterioscler Thromb Vasc Biol. 2007;27(12):2642-9.

Kuboki K, Jiang ZY, Takahara N, Ha SW, Igarashi M, Yamauchi T, Feener EP, Herbert TP, Rhodes CJ, King GL. Regulation of endothelial constitutive nitric oxide synthase gene expression in endothelial cells and in vivo: a specific vascular action of insulin. Circulation. 2000;101:676-81.

Kumazawa S, Taniguchi M, Suzuki Y, Shimura M, Kwon MS, Nakayama T. Antioxidant activity of polyphenols in carob pods. J Agric Food Chem. 2002;50(2):373-7.

Lean ME, Mann JI, Hoek JA, Elliot RM, Schofield G.Translational Research: from evidence-based medicine to sustainable solutions for public health problems. BMJ. 2008;337.

Lee G, Na HJ, Namkoong S, Jeong Kwon H, Han S, Ha KS, Kwon YG, Lee H, Kim YM. Eur J Pharmacol. 2006;551(1-3):143-151.

Leon CG, Tory R, Jia J, Sivak O, Wasan KM. Discovery and development of toll-like receptor 4 (TLR4) antagonists: a new paradigm for treating sepsis and other diseases. Pharm Res. 2008;25:1751-61.

Li H, Wallerath T, Förstermann U. Physiological mechanisms regulating the expression of endothelial-type NO synthase. Nitric oxide. 2002;7(2):132-47.

Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation. 2002;105(9):1135-43.

Libby P. Molecular and cellular mechanisms of the thrombotic complications of atherosclerosis. J Lipid Res. 2009;50:S352-7.

Libby P. Chapter 235: The pathogenesis, prevention and treatment of atherosclerosis. Harrison's Online. McGraw-Hill's. Access Medicine. http://www.accessmedicine.com

Liu L, Zubik L, Collins FW, Marko M, Meydani M. The antiatherogenic potential of oat phenolic compounds. Atherosclerosis. 2004;175(1):39-49.

López-Uriarte P, Bulló M, Casas-Agustench P, Babio N, Salas-Salvadó J. Nuts and oxidation: a systematic review. Nutr Rev. 2009;67(9):497-508.

Lusis AJ. Atherosclerosis. Nature 2000;407:233-41.

Markus MA, Morris BJ. Resveratrol in prevention and treatment of common clinical conditions of aging. Clin Interv Aging. 2008;3:331-339.

ISBN:978-84-693-1544-6/DL:T-637-2010

Martin KR, Wu D, Meydani M. Atherosclerosis. 2000;150(2), 265-274.

Matsushita H, Chang E, Glassford AJ, Cooke JP, Chiu CP, Tsao PS. eNOS activity is reduced in senescent human endothelial cells: Preservation by hTERT immortalization. Circ Res. 2001;89(9):793-8.

Meerarani P, Badimon JJ, Zias E, Fuster V, Moreno PR. Metabolic syndrome and diabetic atherothrombosis: implications in vascular complications. Curr Mol Med. 2006;6(5):501-514.

Miller DT, Ridker PM, Libby P, Kwiatkowski DJ. Atherosclerosis: the path from genomics to therapeutics. J Am Coll Cardiol 2007;49:1589-99.

Muniyappa R, Montagnani M, Koh KK, Quon MJ. Cardiovascular actions of insulin. Endocr Rev. 2007;28:463-91.

Nabel EG. Cardiovascular disease. N Engl J Med. 2003;349 (1):60-72.
Nash SD, Nash DT. Nuts as part of a healthy cardiovascular diet. Curr Atheroscler Rep. 2008;10(6):529-35.

No authors listed. Contamination of cell lines--a conspiracy of silence. Lancet Oncology. 2001;2:393.

Olofsson SO, Wiklund O, Borén J. Apolipoproteins A-I and B: biosynthesis, role in the development of atherosclerosis and targets for intervention against cardiovascular disease. Vasc Health Risk Manag. 2007;3(4):491-502.

Pace-Asciak CR, Hahn S, Diamandis EP, Soleas G, Goldberg DM. The red wine phenolics trans-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: implications for protection against coronary heart disease. Clin Chim Acta. 1995;235(2):207-219.

Packard RR, Libby P. Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. Clin Chem. 2008;54(1):24-38.

Panagiotakos DB, Pitsavos C, Stefanadis C. Alpha-priori and alpha-posterior dietary pattern analyses have similar estimating and discriminating ability in predicting 5-Y incidence of cardiovascular disease: methodological issues in nutrition assessment. J Food Sci. 2009;74(7):H218-24.

Pendurthi UR, Williams JT, Rao LV. Resveratrol, a polyphenolic compound found in wine, inhibits tissue factor expression in vascular cells : A possible mechanism for the cardiovascular benefits associated with moderate consumption of wine. Arterioscler Thromb Vasc Biol. 1999;19(2):419-426.

Pendurthi UR, Meng F, Mackman N, Rao LV. Mechanism of resveratrol-mediated suppression of tissue factor gene expression. Thromb Haemost. 2002;87(1):155-162.

Petchetti L, Frishman W, Petrillo R, Raju K. Nutriceuticals in Cardiovascular Disease: psyllium. Cardiol Rev. 2007;15:116–122.

Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T, Gerritsen ME. Novel inhibitors of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. J Biol Chem. 1997;272(34):21096-103.

Plana N, Nicolle C, Ferre R, Camps J, Cos R, Villoria J, Masana L; DANACOL group. Plant sterol-enriched fermented milk enhances the attainment of LDL-cholesterol goal in hypercholesterolemic subjects. Eur J Nutr. 2008;47:32-39.

Raab M, Daxecker H, Markovic S, Karimi A, Griesmacher A, Mueller MM. Variation of adhesion molecule expression on human umbilical vein endothelial cells upon multiple cytokine application. Clin Chim Acta. 2002;321(1-2):11-6.

Rao LV, Pendurthi UR. Tissue factor-factor VIIa signaling. Arterioscler Thromb Vasc Biol. 2005;25(1):47-56.

Ros E. Nuts and novel biomarkers of cardiovascular disease. Am J Clin Nutr. 2009;89(5):1649S-56S.

Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med. 1999;340(2):115-26.

Roth GA, Moser B, Huang SJ, Brandt JS, Huang Y, Papapanou PN, Schmidt AM, Lalla E. Infection with a periodontal pathogen induces procoagulant effects in human aortic endothelial cells. J. Thromb Haemost. 2006;4(10):2256-61.

Schleser S, Ringseis R, Eder K. Atherosclerosis. 2006;186(2), 337-344.

Schmelzer C, Lorenz G, Rimbach G, Doring F. In Vitro Effects of the Reduced Form of Coenzyme Q(10) on Secretion Levels of TNF-alpha and Chemokines in Response to LPS in the Human Monocytic Cell Line THP-1. J Clin Biochem Nutr. 2009;44:62-6.

Scott RC, Batten PL, Clowes HM, Jones BK, Ramsey JD. Further validation of an in vitro method to reduce the need for in vivo studies for measuring the absorption of chemicals through rat skin. Fundam Appl Toxicol. 1992;19(4):484-92.

Seidel M, Billert H, Kurpisz M. Regulation of eNOS expression in HCAEC cell line treated with opioids and proinflammatory cytokines. Kardiol Pol. 2006;64(2):153-8.

Shaw DI, Hall WL, Jeffs NR, Williams CM. Eur J Nutr. 2007;46(6):321-328.

Singh U, Tabibian J, Venugopal SK, Devaraj S, Jialal I. Development of an in vitro screening assay to test the antiinflammatory properties of dietary supplements and pharmacologic agents. Clin Chem. 2005;51:2252-6.

Sipido KR, Tedgui A, Kristensen SD, Pasterkamp G, Schunkert H, Wehling M, Steg PG, Eisert W, Rademakers F, Casadei B, Fuster V, Cerbai E, Hasenfuss G, Fernandez-Aviles F, Garcia-Dorado D, Vidal M, Hallen M, Dambrauskaite V. Identifying needs and opportunities for advancing translational research in cardiovascular disease. Cardiovasc Res. 2009;83(3):425-35.

Solà R, Godàs G, Ribalta J, Vallvé JC, Girona J, Anguera A, et al. Effects of soluble fiber (Plantago ovata husk) on plasma lipids, lipoproteins, and apolipoproteins in men with ischemic heart disease. Am J Clin Nutr. 2007; 85:1157-1163.

Solà R, Bruckert E, Valls RM, Narejos S, Luque X, Castro-Cabezas M, Doménech G, Torres F, Heras M, Farrés X, Vaquer JV, Martínez JM[i], Almaraz MC, Anguera A. Soluble fibre (*Plantago ovata* husk) reduces plasma low density lipoprotein (LDL) cholesterol, triglycerides, insulin, oxidised LDL and systolic blood pressure in hypercholesterolaemic patients: A randomised trial. Submitted.

Soleas GJ, Diamandis EP, Goldberg DM. Resveratrol: a molecule whose time has come? And gone? Clin Biochem. 1997;30(2):91-113.

Song M, Phelps DS. Comparison of SP-A and LPS effects on the THP-1 monocytic cell line. Am J Physiol Lung Cell Mol Physiol. 2000;279:L110-7.

Spiecker M, Peng HB, Liao JK. Inhibition of endothelial vascular cell adhesion molecule-1 expression by nitric oxide involves the induction and nuclear translocation of IkappaBalpha. J Biol Chem. 1997;272(49):30969-74.

Stoll G, Bendszus M. Inflammation and atherosclerosis: novel insights into plaque formation and destabilization. Stroke. 2006;37(7):1923-32.

Suwannaprapha P, Chaisri U, Riyong D, Maneerat Y. Improvement of function and morphology of tumor necrosis factor-alpha treated endothelial cells with 17-beta estradiol: a preliminary study for a feasible simple model for atherosclerosis. Circ J. 2005;69:730-8.

ISBN:978-84-693-1544-6/DL:T-637-2010

Szekanecz Z, Shah MR, Pearce WH, Koch AE. Clin Exp Immunol. 1994; 98(2):337-343.

Tai SC, Robb GB, Marsden PA. Endothelial nitric oxide synthase: a new paradigm for gene regulation in the injured blood vessel. Arterioscler Thromb Vasc Biol. 2004;24(3):405-12.

Tan KT, Lip GY. Imaging of the unstable plaque. Int J Cardiol. 2008;127(2):157-65.
Taubman MB, Fallon JT, Schecter AD, Giesen P, Mnedlowitz M, Fyfe BS, Marmur JD, Nemerson Y. Tissue factor in the pathogenesis of atherosclerosis. Thromb Haemost. 1997;78(1):200-204.

Tomiyama H, Okazaki R, Inoue D, Ochiai H, Shiina K, Takata Y, Hashimoto H, Yamashina A. Link between obstructive sleep apnea and increased bone resorption in men. Osteoporos Int. 2008;19(8):1185-92.

Tribolo S, Lodi F, Connor C, Suri S, Wilson VG, Taylor MA, Needs PW, Kroon PA, Hughes DA. Comparative effects of quercetin and its predominant human metabolites on adhesion molecule expression in activated human vascular endothelial cells. Atherosclerosis. 2008;197(1):50-6.

Tripathi S, Bruch D, Kittur DS. Ginger extract inhibits LPS induced macrophage activation and function. BMC Complement Altern Med. 2008;8:1.

Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer. 1980;26(2):171-6.

Vadacca M, Bruni R, Cacciapaglia F, Serino F, Arcarese L, Buzzulini F, Coppolino G, Rigon A, Terminio N, Afeltra A. Alexithymia and immunoendocrine parameters in patients affected by systemic lupus erythematosus and rheumatoid arthritis. Reumatismo. 2008;60(1):50-6.

Vallve JC, Uliaque K, Girona J, Cabre A, Ribalta J, Heras M, Masana L. Unsaturated fatty acids and their oxidation products stimulate CD36 gene expression in human macrophages. Atherosclerosis 2002;164:45-56.

Visintin A, Halmen KA, Latz E, Monks BG, Golenbock DT. Pharmacological inhibition of endotoxin responses is achieved by targeting the TLR4 coreceptor, MD-2. J Immunol. 2005;175:6465-72.

Wadsworth TL, Koop DR. Effects of the wine polyphenolics quercetin and resveratrol on pro-inflammatory cytokine expression in RAW 264.7 macrophages. Biochem Pharmacol. 1999;57(8):941-949.

ISBN:978-84-693-1544-6/DL:T-637-2010

Wan Y, Vinson JA, Etherton TD, Proch J, Lazarus SA, Kris-Etherton PM. Effects of cocoa powder and dark chocolate on LDL oxidative susceptibility and prostaglandin concentrations in humans. Am J Clin Nutr. 2001;74(5):596-602.

Wang XL, Zhang L, Youker K, Zhang MX, Wang J, LeMaire SA, Coselli JS, Shen YH. Free Fatty Acids Inhibit Insulin Signaling–Stimulated Endothelial Nitric Oxide Synthase Activation Through Upregulating PTEN or Inhibiting Akt Kinase. Diabetes. 2006;55(8):2301-10.

Wu JM, Wang ZR, Hsieh TC, Bruder JL, Zou JG, Huang YZ. Mechanism of cardioprotection by resveratrol, a phenolic antioxidant present in red wine (Review). Int J Mol Med. 2001;8(1):3-17.

Wu D, Koga T, Martin KR, Meydani M. Effect of vitamin E on human aortic endothelial cell production of chemokines and adhesion to monocytes. Atherosclerosis. 1999;147(2):297-307.

Yan G, You B, Chen SP, Liao JK, Sun J. Tumor necrosis factor-alpha downregulates endothelial nitric oxide synthase mRNA stability via translation elongation factor 1-alpha 1. Circ Res. 2008;103(6):591-7.

Yao J, Mackman N, Edgington TS, Fan ST. Lipopolysaccharide induction of the tumor necrosis factor-alpha promoter in human monocytic cells. Regulation by Egr-1, c-Jun, and NF-kappaB transcription factors. J Biol Chem. 1997;272:17795-801.

Yoshizumi M, Perrella MA, Burnett JC Jr, Lee ME. Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. Circ Res. 1993;73(1):205-9.

Zeng G, Nystrom FH, Ravichandran LV, Cong LN, Kirby M, Mostowski H, Quon MJ. Roles for insulin receptor, PI3-kinase, and Akt in insulin-signaling pathways related to production of nitric oxide in human vascular endothelial cells. Circulation. 2000;101:1539-45.

Zhou Z, Liu Y, Wang SQ. Protocatechuic aldehyde suppresses TNF-alpha-induced ICAM-1 and VCAM-1 expression in human umbilical vein endothelial cells. Eur J Pharmacol. 2005;513(1-2):1-8.

Zhu J, Yong W, Wu X, Yu Y, Lv J, Liu C, Mao X, Zhu Y, Xu K, Han X, Liu C. Anti-inflammatory effect of resveratrol on TNF-alpha-induced MCP-1 expression in adipocytes. Biochem Biophys Res Commun. 2008;369(2):471-477.

Zunft HJ, Lüder W, Harde A, Haber B, Graubaum HJ, Koebnick C, Grünwald J. Carob pulp preparation rich in insoluble fibre lowers total and LDL cholesterol in hypercholesterolemic patients. Eur J Nutr. 2003;42(5):235-42.

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# APPENDIX A:Contributions at Congresses and Conferences

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## POSTERS:

Pons L, Girona J, Ribalta J, Vallvé JC, Masana L, Solà R. **Metodología para la evaluación y validación de los alimentos funcionales en la prevención de enfermedades cardiovasculares**. Valencia, May 2007.

Valls RM, Godàs G, Ribalta J, Girona J, Pons L, Vallvé JC, Ameijide A, Masana L, Solà R, Anglès N, Reguant J, Ramírez B, Barriach JM. **Effects of cocoa cream and cocoa with hazelnuts cream on cardiovascular risk factors.** International Symposium on Functional Foods in Europe – International Developments in Science and Health Claims. Malta, May 2007.

Valls RM, Godàs G, Ribalta J, Girona J, Pons L, Vallvé JC, Ameijide A, Masana L, Solà R, Anglès N, Reguant J, Ramírez B, Barriach JM. **Effects of cocoa cream and cocoa with hazelnuts cream on cardiovascular risk factors.** 76$^{th}$ Annual European Atherosclerosis Society Congress. Helsinki (Finland), June 2007.

Pons L, Girona J, Ribalta J, Vallvé JC, Masana L, Solà R. **Methodologies for the evaluation and validation of functional food in cardiovascular disease prevention. Study aims and design.** Graz, March 2008.

Pons L, Fernández-Castillejo S, Catalán U, Heras M, Girona J, Masana L, Solà R. **Desarrollo metodológico de un modelo celular in vitro para el estudio de los efectos en la expresión génica de la Endothelial Nitric Oxide Synthase de compuestos bioactivos naturales y de la Insulina**. Madrid, June 2008.

Fernández-Castillejo S, Catalán U, Pons L, Heras M, Masana L, Sola R. **Desarrollo metodológico de un modelo celular in vitro para el estudio de los efectos anticoagulantes de extractos o compuestos bioactivos naturales**. Madrid, June 2008.

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## Contributions at congresses and conferences

### ORAL COMMUNICATIONS:

Pons L, Girona J, Ribalta J, Vallvé JC, Masana L, Solà R. **Methodologies for the evaluation and validation of functional food in cardiovascular disease prevention. Study aims and design.** Erasmus Intensive Programme Combating Obesity: Strategies for Prevention and Intervention “COSPI”. Graz (Austria), March 2008.

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# APPENDIX B: Scientific papers

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## Scientific papers

Pons-Llecha L, Fernández-Castillejo S, Catalán U, Girona J, Rosales R, Anglés N, Morelló J, Solà R. **Development of an *in vitro* Screening Assay to test the improvement of endothelial function on human aortic endothelial cells by the evaluation of nitric oxide synthase mRNA levels.** (editor submitted).

Ursula Catalan MSc*, Sara Fernandez-Castillejo BSc*, Laia Pons-Llecha MSc, Rosa Sola MD, PhD. **Resveratrol reduces tissue factor mRNA and protein expression in human aortic endothelial cells.** . (editor submitted).

Laia Pons-Llecha MSc[1,3], Ursula Catalan MSc[1,3], Sara Fernandez-Castillejo BSc[1,3], Mercedes Heras DipTech[1], Neus Angles PhD[2], Jose Morello[2], Rosa Sola MD, PhD[1]. **Improvement of endothelial dysfunction produced by alpha-tocopherol and BAY11-7082 reducing vascular cell adhesion molecule-1.** (editor submitted).

Sara Fernández-Castillejo*[&], Laia Pons*[&], Úrsula Catalán*[&], Mercedes Heras*, Cecilia González*, Roser Rosales*, Josefa Girona*, Lluís Masana*, Neus Anglés, Jose Morelló, Rosa Solà*[&]. **Methodological aspects to reduce variability in the study of inflammation with LPS-stimulated human THP-1 monocytes to secrete TNF-α.** (editor submitted).

Solà R, Valls RM, Godàs G, Pérez G, Ribalta J, Girona J, Pons L, Heras M, Cabré A, Castro A, Masana L, Anglès N, Reguant J, Ramírez B, Barriach JM. **Effects of dietary supplements of cocoa cream on cardiovascular risk factors. A Randomized Trial** (editor submitted).

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