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Dietary fat and coenzyme Q effects on age-associated changes of rat periodontal tissues

TESIS DOCTORAL

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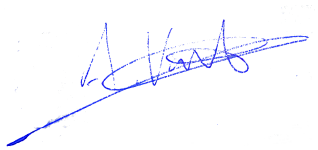

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El envejecimiento se describe como un decaimiento endógeno y progresivo en la eficacia de los procesos fisiológicos después de la fase reproductiva de la vida. Esto está relacionado con algunas enfermedades crónicas, como la periodontitis, que es un trastorno caracterizado por la descomposición de los tejidos que soportan el diente (principalmente de hueso alveolar) debido fundamentalmente al desequilibrio ecológico entre la biopelícula microbiana normal, establecida sobre los dientes, y los tejidos del hospedador. Cada vez hay más pruebas que relacionan la periodontitis con enfermedades sistémicas relacionadas con la edad como la aterosclerosis o el síndrome metabólico. El estrés oxidativo ha sido se propuesto como un posible vínculo común para explicar esta relación.

El desarrollo de intervenciones que retarden el proceso de envejecimiento, se han centrado en ampliar la vida útil máxima y/o retardar el amplio espectro de cambios biológicos asociados con la edad. Entre otras, las intervenciones dietéticas para reducir el estrés oxidativo parecen ser útiles. En este sentido, se ha demostrado que las intervenciones en relación a al tipo de grasa presente en la dieta, así como algunos antioxidantes, pueden ser utilizados como terapias dietéticas antienvejecimiento, al menos en roedores de laboratorio. Se ha demostrado que la grasa de la dieta, a través de cambios en los perfiles de lípidos de la membrana, puede ayudar a atenuar algunos aspectos perjudiciales del envejecimiento, tales como los relacionados con el estrés oxidativo o la disfunción mitocondrial. En este sentido, en tejidos y sangre de rata, se ha descrito que dietas ricas en ácidos grasos n-6 conducen a mayores niveles de peroxidación lipídica y de roturas de doble cadena en el ADN durante el envejecimiento, en comparación con las dietas basadas en ácidos grasos monoinsaturados que son menos suceptibles de oxidarse. En paralelo, se observó que las dietas ricas en ácidos grasos monoinsaturados daban lugar a mitocondrias menos dañadas que aquellas ricas en ácidos grasos poliinsaturados n-6, como reflejaba la menor frecuencia de mutaciones en el ADN mitocondrial en el hígado, el corazón y el cerebro.

Entre las terapias dietéticas con antioxidantes la suplementación con coenzima Q (CoQ), principalmente forma de CoQ_{10} , han sido ampliamente utilizados en la investigación en relación al papel de grasa de la dieta sobre el envejecimiento. El CoQ es un cofactor liposoluble esencial que participa en la cadena de transporte electónico jugando un papel central en la bionergética celular y su regulación. Por otra parte, también se ha observaado que puede ejercer actividades que difieren de su función como coenzima. En este sentido, se ha observado que es un cofactor obligatorio para la función de las proteínas desacoplantes y por lo tanto juega un papel clave en el control redox de la celula y en el funcionamiento de otros sistemas de membrana. Pero tal vez su característica más importante es que el CoQ es que actúa como un importante antioxidante dentro de las membranas biológicas gracias a su actividad como *scavenger* de especies reactivas de oxígeno protegiendo a las celulas del estrés oxidativo. Además, también se han relacionado con una mejora de los sistemas de reparación del ADN y con la modulacion de la cascada de señalización inflamatoria. Sin embargo, desde el punto de vista de la presente investigación, los estudios que comparan su efecto entre las dietas hipocalóricas con diferente perfil lipídico son mucho más interesante. En este sentido, los efectos de la suplementacion con CoQ_{10} a largo plazo en ratas alimentadas con dietas ricas en ácidos grasos monoinsaturados o ácidos grasos poliinsaturados n-6, observándose que estos últimos resultaban especialmente beneficiados por la adición de CoQ_{10} a la dieta. Entre otros efectos, se ha observado que en los animales con este tipo de dieta, el tiempo de vida se alarga, los niveles de peroxidación lipídica caen, y se consigue una capacidad antioxidante más alta que respeto a los alimentados con la misma dieta sin CoQ_{10}.

Tradicionalmente, gran parte de la investigación sobre dieta y enfermedades periodontales se ha centrado sólo en nutrientes con funciones bien establecidas en la formación y mantenimiento de los componentes estructurales de los tejidos orales, como el colágeno (vitamina C) y el hueso (calcio), la integridad del tejido epitelial (vitamina A), o en la promoción de la formación de placa que alberga patógenos periodontales (carbohidratos). Más recientemente, la investigación se ha ampliado para incluir nutrientes que atenúan los procesos inflamatorios o que tienen propiedades anti-inflamatorias, como algunos ácidos grasos poliinsaturados n-3. Sin embargo, no se ha realizado ninguna investigación sobre el papel putativo de otros ácidos grasos saludables tales como ácidos grasos monoinsaturados . Del mismo modo, el potencial de los antioxidantes ha sido probado también. En este sentido, la administración de CoQ en pacientes con periodontitis crónica, como suplemento dietético o de aplicación tópica ha mejorado algunos medidas clínicas. Varios estudios han sido llevados a cabo en modelos animales, principalmente ratas, en donde se inducían experimentalmente enfermedades periodontales. En general, estos modelos se han basado en una infección inducida por agentes patógenos para reproducir, en un corto período de tiempo, las características de la enfermedad. Sin embargo, todos estos no tienen en cuenta el deterioro de la respuesta del huésped asociado a la edad, ni las condiciones óseas alveolares relacionadas con la edad . En este contexto, el presente estudio reproduce un modelo de peridonto dependiente de la edad en para evaluar los efectos fisiológicos en los tejidos periodontales de dietas con diferente perfil de ácidos grasos, así como el posible efecto modulador que pueda ejercer la adición de CoQ_{10} a las mismas.

- Evaluar el impacto del tipo de grasa en las condiciones de los tejidos periodontales a nivel morfométrico e histológico en relación con la edad.

- Evaluar el efecto de la adición de CoQ_{10} a la dieta sobre los efectos fisiológicos que diferentes tipos de grasa tienen en las condiciones de los tejidos periodontales a nivel morfométrico e histológico en relación con la edad.

- Explorar algunos posibles mecanismos implicados en estos efectos a nivel local, en concreto aquellos que tengan que ver con cambios en metabolismo óseo y procesos immunoinflamatorios.

- Explorar la posible contribución que los cambios en el metabolismo óseo y procesos immunoinflamatorios a nivel sistémico puedan tener sobre los mismos

- Explorar como pueden contribuir los niveles de estrés oxidativo a nivel local y la posible implicación de los procesos celulares reacionados con la mitocondria y la defensa frente al estrés oxidativo en el tejido gingival.

Para abordar dichos objetivos, 72 ratas Wistar machos con un peso 80-90 g fueron alojadas en una jaula y se mantuvieron en un ciclo de 12 horas de luz y 12 de oscuridad, con libre acceso a comida y agua. Las ratas fueron asignadas al azar a seis grupos experimentales y se alimentaron desde el destete hasta los 24 meses de edad con dietas semi-sintética y isoenergética de acuerdo con los criterios AIN93 que diferían sólo en el origen de la dieta en grasas (aceites de oliva virgen, girasol o pescado) con CoQ añadido y sin él. 12 ratas por grupo fueron sacrificadas, respectivamente, a los 6 y 24 meses desde el inicio del experimento.

- Medidas de las pérdida de hueso alveolar entre los 6 y 24 meses de edad

- Estudio histopatológico de las encias
- Medida de los niveles circulantes de los marcadores del metabolismo óseo, osteoprotegerina y RANKL, mediante un inmunoensayo *multiplex* basado en microesferas.
- Medida de los niveles circulantes de los marcadores de citocinas inflamatorias.
- Expresión génica de marcadores de citocinas proinflamatorias, apoptosis, autofagia, biogenesis, cadena de transporte electrónico mitocondrial,y defensas antioxidantes.
- Niveles de peroxidación lipídica en las encías medidos mediante ensayo TBARS.
- Pérfil graso de las fuentes grasas utilizadas para fabricar las dietas y del plasma

Los resultados del estudio presentado en esta tesis indican que existen diferencias en cuanto a la pérdida de hueso alveolar asociada al envejecimiento en función en la grasa de la dieta. Varios estudios han investigado los efectos potenciales de diferentes ácidos grasos, principalmente ácidos grasos poliinsaturados n-3 sobre la enfermedad periodontal, derivados de la dieta o como suplementos, tanto en los seres humanos como en modelos animales. En los animales, todos los estudios revisados se centran en ácidos grasos poliinsaturados n-3. En general, cuando la periodontitis fué inducida mediante inyecciones de lipopolisacárido bacteriano, el consumo de ácidos grasos poliinsaturados n-3 no conllevaba una reducción de la pérdida de hueso alveolar. Sin embargo, cuando se utilizaba la inoculación bacteriana, los ácidos grasos poliinsaturados n-3 reducían la pérdida de hueso alveolar de una manera significativa. En cualquier caso, estos estudios no tenian en cuenta la influencia de la edad y de la dieta en la salud periodontal. Es bien conocido que el inicio de esta enfermedad se debe a la infección bacteriana. Por ello, la mayoría de los estudios se han centrado en la inflamación, y en menor medida, en otros eventos mecanísticos como la apoptosis. En el presente estudio, en general, no se observaron diferencias entre tratamientos dietéticos en los niveles de expresión para los genes de citocinas inflamatorias en los grupos de mayor edad. En comparación con los sujetos sanos, se ha observado que los niveles de RANKL están aumentados en casos de periodontitis mientras que los de osteoprotegerina están disminuidos. Aquí, se ha encontrado que los niveles circulantes de RANKL disminuyen con el envejecimiento, así como la expresión gingival del gen que la codifica. Por otro lado, los niveles de osteoprotegerina fueron mayores para los animales viejos, pero no para aquellos alimentados con aceite de girasol. Algo similar también se observó en la expressión a nivel gingival del gen *Opg* que codifica dicha proteína. Se podría esperar que el envejecimiento condujese a un aumento de RANKL y a una disminución de osteoprotegerina, resultando en una pérdida de hueso. Sin embargo, los resultados encontrados en el presente estudio en los animales alimentados con aceite de oliva virgen y aceite de pescado son compatibles con los resultados de otros estudios que sugieren que la osteoprotegerina actúa como un mecanismo de defensa durante el envejecimiento con el fin de evitar un exceso de destrucción ósea inducida por un exceso de estimulación por parte de RANKL . Un hallazgo interesante es que este mecanismo, a su vez, depende de la dieta.Varios estudios han demostrado cambios relacionados con la edad en los niveles de proteínas y factores que regulan la apoptosis. En el presente estudio, el envejecimiento conllevaba un inrcremento en la expresión de genes pro-apoptóticos (*Bax* y/o *Bak*), pero también de *Bcl-2* que es antiapoptótico, aunque no para los animales tratados con aceite de girasol. Se

encontraron mayores de expresión para los marcadores de autofagia (*Maplc3a* y *Atg5*) sólo en los grupos alimenados con aceite de oliva virgen y aceite de pescado. Como para los genes relacionados con la apoptosis y la autofagia, *Tfam*, un marcador del proceso de biogénesis implicado en la renovación natural de la mitocondria, no mostraba diferencias en relación a la edad en los animales alimentados con aceite de girasol, aunque los animales alimentados con aceite de oliva virgen y aceite de pescado presenaron valores más altos a edad avanzada. A su vez, los otros dos marcadores de biogénesis estudiados, *Ppargc1a* y *Sirt1,* refuerzaba esta observación en *Tfam*. Además, se investigaron elementos de la cadena de transporte electrónico mitocondrial junto con del sistema antioxidante y un marcador de oxidación. En todas las situaciones, las moléculas estudiadas reportaron resultados similares a los encontrados para los marcadores previamente discutidos. Si se consideran los niveles de TBARS a los 24 meses, los valores más altos fueron encontrados en los animales con el porcentaje más alto de acidos grasos poliinsaturados n-6 , es decir, los animales alimentados con aceite de girasol. Es bien sabido, que a pesar de aceite de pescado que tiene un contenido de ácidos grasos poliinsaturados totales más alto que el aceite de girasol, el hecho de que la mayoría de ellos sean acidos grasos poliinsaturados n-3, que están dotados de propiedades anti-inflamatorias, da lugar a valores más bajos de la peroxidación lipídica que los aceites de semillas *in vivo*, más ricos en acidos grasos poliinsaturados n-6, con alta capacidad pro-inflamatoria. Eso, junto con una inducción potencialmente diferente de enzimas desintoxicantes podría explicar el único hallazgo aparentemente contradictorio con respecto a los niveles de TBARS y la composición de ácidos grasos. Algo similar se puede aplicar para los 6 meses de edad los animales, aunque a esta edad las diferencias no son significativas en relación con los niveles de TBARS con respecto a los animales alimentados con aceite de girasol.

En el caso de los grupos que tambien recibieron CoQ_{10} en sus dietas, no se encontraron diferencias en cuanto a la la pérdida ósea alveolar asociada a la edad. Por lo tanto, a largo plazo, la alimentación con dosis bajas de CoQ_{10}, parece ser eficaz para reducir la pérdida de hueso alveolar asociada la edad que se deriva del uso de girasol aceite como fuente de grasa en la dieta, ya que los animales alimentados con dieta basada en esta mostraban pérdidas óseas similares a los mantenidos con dietas suplementadas basadas en aceite de oliva virgen. En este sentido, también se observó que la adicion de CoQ_{10} al aceite de girasol consiguió reducir la fibrosis a nivel gingival frente a lo que ocurría cuando esta grasa de la dieta se administró sin CoQ_{10}, y algo similar se encontró para la activación endotelial, tanto en los animales alimentados con aceite de girasol como con aceite de pescado. Dichas variaciones histológicas podrían ayudar también a explicar cómo CoQ_{10} mejora la salud periodonto durante el envejecimiento. En una serie de organismos, se ha demostrado que el CoQ_{10} administrado en la dieta causa múltiples efectos fenotípicos y podría regular la expresión de genes implicados en procesos múltiples. La inflamación puede afectar la formación del hueso y a los procesos de resorción a través de la vía RANK-RANKL-OPG. En relación a los marcadores utilizados para evaluarla, sólo se observaron cambios asociados a la edad en la expresión gingival del gen *Il6*, que fué mayor de los grupos alimentados viejos con aceite pescado y aceite de girasol con respecto a los jóvnes. Los altos niveles de IL-6 estimulan la resorción ósea, pero a niveles fisiológicos no, a menos que se asocien con otras citoquinas. Por lo tanto, la diferencia encontrada aquí no debería tener consecuencias importantes sobre el hueso alveolar. Por otra parte, el hecho de que la inflamación periodontal no estuviese claramente promovida como en nuestro estudio anterior podría estar relacionado con la ausencia de variaciones en la respecto a la expresion del gen *Il1b* en las encías. En relación a la mitocondria, uno de los datos más relevantes fué que el CoQ_{10} inducía un incremento en expresión de *Tfam*

en los animales alimentados con aceite de girasol conforme estos envejecen. Estos resultados podrían reforzar la importancia de la biogénesis para el correcto funcionamiento del metabolismo mitocondrial durante el envejecimiento y su potencial correlación con el mantenimiento del hueso alveolar. En relación con el otro elemento asociado al recambio mitocondrial, es decir, la autofagia, los datos del presente estudio parecen indicar que este proceso, por lo menos bajo nuestras condiciones experimentales, no es tan relevante como la biogénesis para los efectos del CoQ_{10} en animales alimentados con aceite de girasol. A este respecto, el CoQ_{10} ha equilibrado las diferencias entre fuentes grasas contrarrestando las perturbaciones producidas por algunas de ellas. En otras palabras, cuando las grasas son suministradas sin CoQ_{10}, se observaba una respuesta diferente de componentes de la cadena de transporte electónico mitocondria estudiados (*mt-Nd1, mt-Nd4*, y *Ndfus1*), presentando los animales alimentados con aceite de oliva virgen, tanto a los 6 ya los 24 meses de edad un mayor nivel de expresión génica. Después de añadir CoQ_{10}, se suprimieron estas diferencias entre grasas dietéticas. Por lo tanto el CoQ_{10} , ya sea por su papel en el trasporte de electrones, o por su papel como antioxidante, es capaz de equilibrar el efecto de la grasa de la dieta en la cadena respiratoria. Estos hallazgos sobre el estrés oxidativo podrían ayudarnos a entender que, al menos en nuestras condiciones experimentales, el papel de la CoQ sobre el estrés oxidativo no debe ser un mecanismo directo a través del cual el CoQ_{10} añadido a la dieta conducía una pérdida de hueso alveolar inferior en los animales alimentados con aceite de girasol. Por tanto, se ha encontrado que la terapia dietética con CoQ_{10} ha sido eficaz para reducir la pérdida de hueso alveolar relacionado con el envejecimiento exacerbado por las dietas ricas en ácidos grasos poliinsaturados n-6. Entre los posible mecanismos previstos que expliquen este hallazgo, el incremento asociado a la edad en los niveles circulantes de OPG parece ser un factor clave para efectos de la CoQ_{10} en la pérdida de hueso alveolar en estos animales. Además, el CoQ_{10} parece ser capaz de restaurar parcialmente los procesos en las células que fueron suprimidos de la encía por la dieta rica en ácidos grasos poliinsaturados n-6, a saber, la biogénesis y el aumento asociado a la edad en algunos componentes de la maquinaria respiratoria mitocondrial (por lo menos las codificadas por los genes mitocondriales), que se produjo probablemente como consecuencia del efecto sobre la biogénesis (puesto que *Tfam* también actúa como factor de transcripción del ADN mitocondrial). Sin embargo, el vínculo entre estos procesos y la producción de OPG necesita ser aclararado. Los resultados de la presente investigación refuerzan la idea de que otros tejidos y la influencia sistémica son muy importantes para entender laCoQ_{10} efectos sobre la resorción ósea alveolar. Desde el punto de vista nutricional y clínico, hay que destacar que la alimentación a largo plazo en los niveles bajos de CoQ_{10} podría contrarrestar los efectos negativos de dietas ricas en ácidos grasos poliinsaturados n-6 sobre la pérdida de hueso alveolar (una característica importante de la periodontitis) en relación con años. Este efecto positivo de la CoQ_{10} en periodontitis también se ha descrito para algunos aspectos de las enfermedades cardiovasculares, un grupo de enfermedades que están siendo cada vez más asociada con periodontitis relacionada con la edad.

Contents

Acronyms and abbreviations

8-OHdG: 8-hydroxy-2'-deoxyguanosine
8-OHG: 8-hydroxy-2'-deoxyguanidine
ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ADP: adenosine diphosphate
AGE: advanced glycation end-product
ALA: $\alpha-$linolenic acid
AP-1: activator protein-1
APAF1: apoptotic protease-activating factor 1
ATP: adenosine triphosphate
BrdU: 5-bromo-2'-deoxyuridine
CoA: coenzyme A
cDNA: complementary DNA
CoQ: coenzyme Q
CoQH: partially reduced coenzyme Q
$CoQH_2$: fully reduced Coenzyme Q
COX-2: ciclooxigenase-2
CPITN: Community Periodontal Index of Treatment Needs
CRP: C-reactive protein
Ct: threshold cycle
DHA: docosahexaenoic acid
EDTA: ethylenediaminetetraacetic acid
EPA: eicosapentaenoic acid
ERK: extracellular signal-regulated kinase
FAD: oxidized flavin adenine dicnucleotide
$FADH_2$: reduced flavin adenine dicnucleotide
FADS1: fatty acid desaturase 1
FADS2: fatty acid desaturase 2
Farnesyl-pirophosphate: Pharnesyl-PP
FASL: FAS ligand
FMN: oxidized flavin mononucleotide
$FMNH_2$: reduced flavin mononucleotide
GADPH: glyceraldehyde 3-phosphate dehydrogenase
GCF: gingivocrevicular fluid
GPX: glutathione peroxidase
GSH: reduced glutathione
GSSG: oxidized glutathione
HGM-CoA: 3-hydroxy-3-metylglutaryl-coenzyme A
Hsp: heat shock protein
ICAM-1: inter-cellular adhesion molecule-1
IFAPA: Research and Training Institute for Agricultural and Fisheries of Andalusia
IgG1: Immunoglobulin G1
IL-1: interleukin-1
IL-6: interleukin-6
IL-8: interleukin-8
IL-17: interleukin-17
IMM: inner mitochondrial membrane
iNOS: inducible nitric oxide synthase
Isopentenyl-PP: Isopentenyl-pirophostate
JNK: c-Jun N-terminal kinase
KM: Michaelis constant
LC3: light chain 3
LDL: low-density lipoprotein
LPS: lipolysaccharide
LTB_4: leukotriene B_4
MAPK: mitogen-activated protein kinase
M-CSF: macrophage colony-stimulating factor
MFI: median fluorescence intensity min: minutes
MMP: matrix metalloproteinase
mtDNA: mitochondrial DNA
mtETC: mitochondrial electron transport chain
MUFA: monounsaturated fatty acids
NAD^+: oxidized nicotin adenine dinucleotide
NADH: reduced nicotin adenine dinucleotide
$NADP^+$: oxidized nicotinamide adenine dinucleotide phosphate
NADPH: reduced ncotinamide adenine dinucleotide phosphate
NBT: Nitro blue tetrazolium
nDNA: nuclear DNA
NFATc1: nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
NF-κB: nuclear factor-κB
NF-Y: nuclear factor-Y
NHANES: National Health and Nutrition Examination Survey
NQO1: NAD(P)H-quinone oxidoreductase 1
NRF-1: nuclear respiratory factor-1
NRF-2: nuclear respiratory factor-2
OMM: outer mitochondrial membrane
OPG: osteoprotegerin
OXPHOS: oxidative phosphorilation
PA: plasminogen activator
PGC-1α: peroxisome proliferator-activated receptor gamma, coactivator-1α
PGE_2: prostglandin E_2
PMN: polymorphonuclear leukocyte
PPAR: peroxisome proliferator-activated receptor
PPRE: PPAR response element
PUFA: polyunsaturated fatty acids
QRT-PCR: quantitative real-time PCR
RAGE: receptor for advanced glycation end-products
RANK: receptor activator for nuclear factor-κB ligand

RANKL: receptor activator for nuclear factor-κB
RANTES regulated on activation, normal T cell expressed and secreted
ROS: reactive oxygen species
RQ: relative quantity
RXR: retinoid X receptor
SFA: saturated fatty acids
SIRT1: sirtuin 1
SOD: superoxide dismutase
SREBP: sterol regulatory element binding protein
TAC : total antioxidant capacity
TAOC: total antioxidant capacity
TBARS: thiobarbituric acid-reactive substances
TFAM: mitochondrial transcription factor A
TFB1M: mitochondrial transcription factor B1
TFB2M: mitochondrial transcription factor B2
TGF-β: transforming growth factor-β
TLR: toll-like receptor
TNF: tumor necrosis factor
TOS: total oxidant status
tPA: tissue plasminogen activator
TRAP: tartrate-resistant acid phosphatase
TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling
UK: United Kingdom
US: United States
UV: ultraviolet
VADC: voltage-dependent anion channel
VLDL: very low-density lipoprotein

ABSTRACT

Introduction

Aging is described as a progressive decline in endogenous and effectiveness of physiological processes after the reproductive stage of life. Traditionally, much of the research on diet and periodontal health has focused only on nutrients with well-established functions in the formation and maintenance of the structural components of the periodontal tissues. More recently, research has expanded to include nutrients that attenuate inflammatory processes or have anti-inflammatory properties, as some polyunsaturated fatty acids n-3. Similarly, the power of antioxidants has been also tested. In this regard, it has been observed that administration of CoQ patients with chronic periodontitis, as a dietary supplement or topically has improved some clinical measures. Several such studies have also been conducted in animal models, mainly in rodents, to which were induced periodontal diseases such experiental. However, these did not take into account the age-related deterioration of the host response, and bone age-related conditions alveolar. In this context, the present study reproduced an age-dependent rat model of periodontium to assess the physiological effects on periodontal tissues of diets with different fatty acid profile and the possible modulating effect may exercise the addition of CoQ_{10} to them.

Material and methods

72 Male Wistar rats (*Rattus norvegicus*) were randomly assigned to 6 different experimental treatments. These consisted of 6 semi-synthetic and isocaloric diets according to AIN93 criteria that differed only in the fat source used (virgin olive oil, sunflower or fish) supplemented or not with CoQ_{10} (50 mg/kg/day) with that they were fed from weaning until 24 months of age. 12 rats per group were sacrificed respectively at 6 and 24 months from the start of the experiment. After they sacrifice whole blood samples, serum and plasma were collected to analyze fatty acid profile, the levels circulating of bone metabolism markers and proinflammatory cytokines. The jaws of rats despielizaron and morphometrically evaluated swatches were colored for alveolar bone loss associated with age. The gingival tissue around the upper molar was excised and used to evaluate histopathological changes, lipid peroxidation degree by TBARS assay and expression of genes involved in multiple processes: inflammation, apoptosis, biogenesis, autophagy, electron transport and antioxidant defense.

Results

Age-related alveolar bone loss was higher in rats fed n-6 polyunsaturated fatty acids. However, when diets were supplied with CoQ these differences disappeared. Most of

the histopathological measures in relation to inflammation indicated that in all animal groups that process was just promoted. Gene expression analysis indicated an age-related increase in autophagy and biogenesis in animals fed on virgin olive- and fish-oil without CoQ_{10} that was accompanied by increases in the components of complex I of the mitochondrial transport chain. These changes indicating a higher rate of mitochondrial replacement as the animals aged, also were accompanied by increases in expression of antioxidant enzymes proteins measured indicating that antioxidant systems are activated with aging in these groups. In rats fed sunflower oil without CoQ_{10} these changes were absent. When CoQ_{10} was added to the diet, main effects were noted in sunflower oil-fed animals that showed increased expression of genes involved in mitochondrial biogenesis along with genes encoding complex I proteins.

Discussion and conclusions.

The main conclusion of this study is that age-associated alveolar bone loss can be modulated by an adequate dietary treatment. The mechanisms involved in this phenomenon are related to ablation of the ability of the cell to adapt to aging. So, monounsaturated fatty acids (virgin olive oil-based) or n-3 polyunsaturated (fish oil-based) would maintain a high turnover rate by mitochondrial biogenesis and autophagy processes. In turn, they would also be capable of inducing antioxidant systems, which together with the possible increase in the rate of mitochondrial replacement collaborate in reducing the oxidative stress associated with age allowing the proper functioning of mitochondrial electron transport chain. Furthermore, the addition of CoQ_{10} to the n-6 polyunsaturated fatty acid-rich diet (based on sunflower oil) appears to attenuate the effects of this age-associated alveolar bone loss. Gene expression analysis results suggests that the mechanisms involved in this effect are related to the restoring of the ability of cells to adapt the gingival tissue, at the mitochondrial level, as changes that occur as rats fed on sunflower oil age. In particular, this could be due to age-related increase in the rate of mitochondrial biogenesis and to improved oxidative and respiratory equilibrium in these animals, which could be due to the effect on biogenesis mentioned, at least in part. From a nutritional and clinical perspective, it is noteworthy that potential treatments to attenuate the alveolar bone loss associated with age (a key feature of periodontitis) may be similar to some proposed for the prevention and treatment of cardiovascular diseases, a group of pathologies associated with periodontitis and age-related.

RESUMEN

Introducción.

El envejecimiento se describe como un decaimiento endógeno y progresivo en la eficacia de los procesos fisiológicos tras la fase reproductiva de la vida. Tradicionalmente, gran parte de la investigación sobre dieta y salud periodontal se ha centrado sólo en nutrientes con funciones bien establecidas en la formación y el mantenimiento de los componentes estructurales de los tejidos peridontales. Más recientemente, la investigación se ha ampliado para incluir nutrientes que atenúan los procesos inflamatorios o que tienen propiedades anti-inflamatorias, como algunos ácidos grasos poliinsaturados n-3. Del mismo modo, el potencialde los antioxidantes ha sido probado también. En este sentido, se ha observado que la administración de CoQ a pacientes con periodontitis crónica, como suplemento dietético o de forma tópica ha mejorado algunas medidas clínicas. Varios estudios de este tipo también han sido llevados a cabo en modelos animales, principalmente en roedores, a los cuales se les inducían enfermedades periodontales de forma experiental. Sin embargo, estos no tenían en cuenta el deterioro asociado a la edad de la respuesta del hospedador, ni las condiciones óseas alveolares relacionadas con la edad. En este contexto, el presente estudio reproduce un modelo de peridonto dependiente de la edad en para evaluar los efectos fisiológicos en los tejidos periodontales de dietas con diferente perfil de ácidos grasos, así como el posible efecto modulador que pueda ejercer la adición de CoQ_{10} a las mismas.

Material y métodos.

72 ratas Wistar macho (*Rattus norvegicus*) fueron asignadas al azar a 6 tratamientos experimentales diferentes. Estos consistían en 6 dietas semi-sintéticas e isoenergéticas de acuerdo con los criterios AIN93 que diferían sólo en el fuente grasa utilizada (aceites de oliva virgen, girasol o pescado) suplementadas o no con de CoQ_{10} (50 mg/kg/día) con las que fueron alimentadas desde el destete hasta los 24 meses de edad. 12 ratas por grupo fueron sacrificadas respectivamente, a los 6 y a los 24 meses desde el inicio del experimento. Tras el sacrificio se recogieron muestras de sangre entera, suero y plasma donde se analizó el perfil de acidos grasos, y los niveles circulantes de marcadores de metabolismo óseos y citocinas proinflamatorias. Las mandíbulas de las ratas se despielizaron y tiñieron para evaluar morfométricamente la pérdida de hueso alveolar asociada a la edad. El tejido gingival alrededor de los molares superiores se excindió y se utilizó para evaluar los cambios histopatológicos, el grado de peroxidación lipídica mediante ensayo TBARS y la expresión de genes implicados en múltiples procesos: inflamación, apoptosis, biogénesis, autofagia, transporte electrónico y defensa antioxidante

Resultados.

La pérdida de hueso alveolar asociada a la edad fué mayor en las ratas alimentadas con ácidos grasos poliinsaturados n-6. Sin embargo, cuando las dietas fueron suministradas con CoQ_{10} estas diferencias desaparecieron. La mayor parte de las medidas histopatológicas en relación a la inflamación indicaron que en todos los grupos animales dicho proceso apenas fue promovido. Los análisis de expresión génica indicaron un incremento de autofagia y biogénesis asociado a la edad en los animales alimentados con aceite de oliva virgen y de pescado sin CoQ_{10}, lo fué acompaño por incrementos de los componentes del complejo I de la cadena de transporte mitocondrial. Estos cambios que indicaban una mayor tasa de recambio mitocondrial conforme los animales envejecían, también se vieron acompañados por aumentos de las enzimas antioxidantes medidas indicado que los genes relacionados con los sistemas antioxidantes se activan con el envejecimiento en estos grupos. En las ratas alimentadas con aceite de girasol sin CoQ estos cambios estuvieron ausentes. Cuando se añadió CoQ_{10} a la dieta, los principales efectos se notaron en los animales alimentados con aceite de girasol que mostraron un incremento en la expresión de los genes implicados en biogénesis junto con los genes mitocondriales que codifican para proteínas del complejo I.

Discusión y conclusiones.

La principal conclusión de este estudio es que la pérdida de hueso alveolar asociada a la edad puede ser modulada por un tratamiento dietético adecuado. Los mecanismos involucrados en este fenómeno están relacionadas con la ablación de la capacidad de la célula para adaptarse al envejecimiento. De manera que los ácidos grasos monoinsaturados (basada en aceite de oliva virgen) o poliinsaturados n-3 (basada en aceite de pescado) permitirian mantener un recambio mitocondrial elevado mediante procesos de biogénesis y autofagia. A su vez, también serian capaces de inducir sistemas antioxidantes que junto con el posible incremento de la tasa de recambio mitocondrial colaborarían en la reducción del estrés oxidativo asociado a la edad permitiendo el correcto funcionamiento cadena de transporte electrónico mitocondrial. Por otro lado, la adicción de CoQ_{10} a la dieta en rica ácido grasos poliinsaturados n-6 (basada en aceite de girasol) parece atenuar los efectos de esta sobre la pérdida de huevo alveolar asociada a la edad. Los resultados obtenidos en relación a la expresión de los genes evaluados sugiere que los mecanismos implicados en este efecto se relacinarian con la restauración de la capacidad de las células del tejido gingival para adaptarse, a nivel mitocondrial, a los cambios que irían ocurriendo a medida que las ratas alimentadas con esta fuente grasa envejecen. En particular, esto podría ser debido al aumento asociado a la edad de la tasa de la biogénesis mitocondrial, así como a una mejora del equilibrio oxidativo y respiratorio en estos animales, lo que podria deberse al efecto sobre biogénesis mencionado, al menos en parte. Desde el punto de vista nutricional y clínico, es de destacar que los potenciales tratamientos para atenuar la pérdida de hueso alveolar asociada a la edad (una característica clave de la periodontitis) podrían ser similares a algunos propuestos para la prevención y tratamiento de enfermedades cardiovasculares, un grupo de patologías asociadas con periodontitis y también relacionadas con la edad.

Part I

INTRODUCTION

Chapter 1

Aging and mitochondria

1.1 Introduction.

Those over age 65 years constitute the fastest growing segment of populations worldwide. This change in the world's age distribution and consequent demographic shift can be attributed to a large extent to reduced birth rate, greater economic development, more effective medical interventions (hence reductions in mortality), and to improvements in hygiene, nutrition, lifestyle and in general preventive care (Salomon et al., 2012).

Aging is considered a physiological process common to all multicellular organisms, where the body, over time, undergoes a progressive decline in endogenous and effectiveness of physiological processes after the reproductive stage of life, so that it becomes unable to maintain stability of body's biochemical reactions and functions against the external environment (Halliwell et al., 1999). Therefore, it is an endogenous process featured by a progressive loss of function with decreased fertility. As consequence of the decline in the effectiveness of physiological processes and the common decrease in organism's capacity to maintain homeostasis against stressful conditions, there are a greater risk for the development of many diseases, tipically cancer, cardiovascular and neurodegenerative disorders), as well as premature mortality (Camougrand and Rigoulet, 2001; Vijg and Suh, 2005; Sohal, 2002; Fraga and Esteller, 2007; M Carmen Ramírez-Tortosa and JoséL Quiles, 2007; Quiles et al., 2010; Rodríguez-Rodero et al., 2011; Barja, 2013; Kowald and Kirkwood, 2013; López-Otín et al., 2013).

The interest in aging has grown enormously in recent decades, due to social and scientific conditions. It is one of the most complex biological processes determined by genetic and environmental factors. It is estimated that 25% of the maximum life span is due to genetic factors and 75% to other types of environmental and lifestyle habits. The progressive reduction in the response capacity to physiological needs is due in part to changes in gene expression (Rodríguez-Rodero et al., 2011; Gems and Partridge, 2013). Most species of living organisms, at least those that are multicellular, have a tangible maximum age whose individuals are able to achieve, which has been called "maximum lifespan". For humans, it was established that this would be around 110-120 years. However, people rarely reach it currently, which is largely due to environmental factors such as infectious diseases or nutrition. Meanwhile, life expectancy is the expected number of years of life remaining at a given age. Although maximum life span and life expectancy are different concept, they are fairly similar numerically in most developed countries (Fontana et al., 2010; Mercken et al., 2012; Capitaine et al., 2013; Steensma et al., 2013).

If aging is deleterious for individuals and it exists all over the nature, why does it happen? The answer to that question might be that aging is the side effect of something else. Thus, the genes that delay aging could do that through the repression of the causes that generates the damage associated to aging. Reproduction appears to be a source of this type of damage. Thus when aging decreases, fertility is frequently reduced. Food could be another source of damage associated to aging due to many genes that participate in the response to the change in nutrient levels. Furthermore, it is know that the reduction in the intake of food diminishes aging in a variety of organisms from yeast to mammals (Partridge and Gems, 2002; Quiles et al., 2004; Masoro, 2005; M Carmen Ramírez-Tortosa and JoséL Quiles, 2007; Barzilai et al., 2012).

Most significantly, it has been clearly shown that the phenotype and the rate of progression of aging are highly variable in different species, in organisms within a species, in organs and tissues within an organism, in cell types within a tissue, in sub-cellular compartments within a cell type, and in macromolecules within a cell. These observations necessarily lead to the conclusion that aging has no universal cause, phenotype, and consequence, except death (Rattan, 2006).

1.2 Aging theories

We believe that the expectation of a unified theory which includes all the phenomena associated with aging is, at the present time, unrealistic. Over the years, gerontologists have become resigned to the futility of formulating a unified theory of aging which can encompass its evolutionary, biological, and sociological aspects. The main reason for the emergence of this pessimistic view is that the large body of descriptive data in gerontology underlines the multifaceted, diverse and complex nature of aging (Rattan, 2006).

In 1990 Mevdevev (Medvedev, 1990) in an excellent review stated that there were more than 300 theories of aging and the number is increasing. It is indeed possible to offer a preliminary solution to the problem of offering satisfactory theories of aging, which can guide new experiments that will of course increase our understanding of aging at the various levels of biological organization (Viña et al., 2007).

1.2.1 Stochastic theories

These theories propose that random damage accumulates over time in different biological molecules until reaching a level where the damage triggers the physiological decline known as aging (M Carmen Ramírez-Tortosa and JoséL Quiles, 2007).

- **The rate-of-living theory** (Pearl, 1928): it states that life expectancy is inversely proportional to the metabolic rate of the species. It was based on the observation that species with high metabolic rates had shorter lives, but the link beween longevity and metabolic rate was no explained.

- **Oxidative stress theory of aging** (Gomez-Cabrera et al., 2012): it is the updated name for the **theory of free radicals** (Harman, 1956) that was proposed because many reactive oxygen species (ROS) are known not to be free radicals. According to that, normal aging is the result of random damage to tissues mediated by endogenous oxidants (Harman, 1956, 2006, 2009). Initially, the existence of endogenous oxidants proposed by this theory since its first version was controversial, but with the discovery of superoxide dismutase (SOD) in 1969 (McCord and Fridovich,

1969, 2014), it was found the first evidence of *in vivo* production of O_2^{-*} by cells that need to develop their antioxidant defense mechanisms (Yu, 1994). Thus, a scientific basis for this theory was provided. .

- **Mitochondrial theory of aging** (Miquel et al., 1980): it proposes that aging resulting from the progressive damage to mitochondrial DNA (mtDNA) by ROS. To propose it, Miquel et al. (1980) was based on theory of free radicals when Harman (1972) focused it towards the mitochondria as the major source of free radicals in the organisms and the target of their damage.

- **Theories of damage accumulation**: In general, they proposes that aging is a consequence of accumulation of unrepaired damaged components in cells which affects their adequate functioning or surveillance. They could be explained by the "oxidative stress theory" due to certain similarities (Halliwell et al., 1999). It is possible to differentiate among several theories depending on the molecules or components damaged suggested as responsible for aging. These include:

  - **The crossing-over theory** (Padhukasahasram and Rannala, 2011): it indicates that aging is due to altered cell functioning as consequence of random crossing-over of proteins and DNA .
  - **The theory of the catastrophic error** (Edelmann and Gallant, 1977): it postulates that aging resulting from the accumulation of random damage in protein synthesis.
  - **The theory of glycosylation** (Tsamis et al., 2011): it point at glycosylated proteins accumulation as responsible of aging. It was based on the observation of serious disruption of cell functions after glycosylated proteins formation.
  - **The theory of longevity determinants** (Hayflick, 2007): it states that aging is caused by the products of metabolism and the capacity to protect oneself against such products determines the degree of aging.
  - **The membrane hypothesis** (Yu, 2005): it is focused on cell membrane damage since it diminishes the enzyme activity and the capacity to eliminate waste products, decreases protein synthesis and increases loss of water for the cytoplasm.
  - **The neuroendocrine theory** (Maldonado, 2013): it was based on all body part of the body can act in isolation from the nervous and endocrine systems. So, if one of these is disturbed, the other systems are also affected. However, there are organisms without nervous or endocrine systems that get older. Thus, this theory is not universal (M Carmen Ramírez-Tortosa and JoséL Quiles, 2007).

- **Theories based on mathematics and physics** (Mangel, 2001): overall, they are based on the fact that the young normal state is characterized by the existence of a large number of regulatory factors interacting in a chaotic way; while aging is characterized by the loss of complexity and the tendency to shift towards non chaotic dynamical systems, simpler, which ultimately leads to a loss of adaptive capacity of the organism. More than an explanatory theory, the utterance corresponds to a model based on the chaos theory.

  - **The entropy theory** (Gutiérrez Robledo, 1998; Gutiérrez-Robledo, 2011): some mechanisms, such as caloric restriction, reduce the degree of entropy production, releasing energy more slowly and delaying the deterioration molecular.

1.2.2 Evolutive theories

It should be noted evolutive and stochastic theories are not mutually exclusive. In fact, it seems clear that the intrinsic and extrinsic processes that lead to aging have been established, through evolution, to give a maximum lifespan in all species.

- **The theory of immediate survival** (Kirkwood and Austad, 2000; Kirkwood, 2005): it point out that aging occurs because of natural selection of genes which are important for immediate survival, even if they are deleterious long-term.

- **The immunological theory** (Walford, 1969; Effros, 2005): It says aging is a consequence of the decreasing in the capacity of immune system to produce antibodies. Because of the immune response diminishes, the capacity of the system to discriminate between its constituents and foreign elements also declines, with an increase in autoimmune reactions. However, this theory only can be applied to the immune system, and does not rule out the possibility that these changes might be secondary to other appearing earlier, such as hormonal alterations (M Carmen Ramírez-Tortosa and JoséL Quiles, 2007).

- **The reproductive-cell cycle theory** (Bowen and Atwood, 2004; Atwood and Bowen, 2011): it was based on the fact of the hormones that regulate reproduction act in an antagonistic pleiotropic manner to control aging via cell cycle signaling; promoting growth and development early in life in order to achieve reproduction. The problem appears when into adulthood the attempt to maintain reproduction is deregulated and leads to senescence. This theory also proposes a new definition of aging including not only the changes associated with the loss of function (i.e. senescence, the commonly accepted definition of aging), but also the changes associated with the gain of function (growth and development).

1.2.3 Genetic theories

Aging is programmed under the control of genes that intelligently order a series of changes in the cell, changes that ultimately lead to the decline and destruction. Its mechanism of action is based on damaging gene activation and / or deactivation of other beneficial for survival. According to these theories, aging is genetically programed, either because the original program is altered (the “somatic mutation theory”) or because the cellular changes associated with aging are included in the instructions contained in DNA from conception, so they are part of normal development. Another possibility is that genes coding for the production of a protein with useful functions become harmful at an advanced age (Catania and Fairweather, 1991; Kennedy et al., 2012).

- **The somatic mutation theory** (Catania and Fairweather, 1991; Kennedy et al., 2012): according to this theory, aging is genetically programed, either because the original program is altered (the “somatic mutation theory”) or because the cellular changes associated with aging are included in the instructions contained in DNA from conception, so they are part of normal development. Another possibility is

that genes coding for the production of a protein with useful functions become harmful at an advanced age.

- **The hypothesis of the disposable soma** (Kirkwood, 1977, 2005): the utilization of energy over a lifetime should be used preferentially for reproduction. It suggests that among the main candidates determining the life expectancy of a species from the genetic standpoint are genes that regulate the repair and maintenance of somatic cells (M Carmen Ramírez-Tortosa and JoséL Quiles, 2007). According to this hypothesis, natural selection favors those genes that act in the early stages of life to permit the species reproduction, against those ones that are in charge of preserving non-germinal cells or disposable soma (Kirkwood and Rose, 1991; Lithgow and Kirkwood, 1996; Kirkwood and Austad, 2000; Drenos and Kirkwood, 2005; M Carmen Ramírez-Tortosa and JoséL Quiles, 2007; Hammers et al., 2013). Therefore, somatic lines from all animals (contrary to germ cells) are declining and degenerate with age causing the phenotypic changes recognize as aging (Guarente and Kenyon, 2000; Barzilai et al., 2012). At cellular and molecular level, the disposable soma theory predicts that the effort to cellular maintenance and repair processes vary in direct proportion to longevity, and it is associated to an exceptional longevity with decreased reproduction (Tabatabaie et al., 2011). In this sense, Drenos and Kirkwood (2005) described a mathematical model that can make predictions about the optimal strategies for investment in somatic maintenance *versus* investments in growth and reproduction, and confirms the central prediction of the disposable soma theory that the optimum investment in somatic maintenance is less than what would be required for indefinite longevity. They also described how the optimal investment in maintenance is affected by varying the parameters that specify the times of reproduction and mortality.

- **The telomere theory of aging** (Olovnikov, 1973, 1996, 2007): according to this theory, aging could be fighted braking or blocking the process that shorten the telomeres (Mikhelson, 2001; Mikhelson and Gamaley, 2012). The shortening of the telomeres in each of the cycles of cell division is the factor responsible for the limitation in the proliferation of the cell cultures, the "Hayflick limit" (Hayflick and Moorhead, 1961). In 1998, Hayflick proposed a new hypothesis according to which telomere shortening and subsequent loss of cellular replicative capacity only determine the duration of the life of each species while aging itself is determined by the accumulation of cell damage.

1.3 The role of mitochondria in aging

1.3.1 Mitochondria: structure, function and physiology

Mitochondria are eukaryotic organelles best known for their essential role in energy transduction. In aerobic organisms, they generate most adenosine triphosphate (ATP) which is used to drive all cellular energy-consuming processes. Apart from this role, mitochondria are essential for iron/sulfur cluster biosynthesis, the synthesis of amino acids, lipid metabolism, copper homeostasis, the control of apoptosis and other processes (Osiewacz and Bernhardt, 2013). As said, oxidative phosphorilation (OXPHOS) is responsible for converting energy from macronutrients (carbohydrates, fatty acids, and amino acids) to ATP through reactions by which those macronutrients are oxidized, oxy-

gen is reduced to water, and adenosine diphosphate (ADP) is phosphorylated to ATP (Treberg and Brand, 2011).

Mitochondria are unique organelles due to its structure provides compartmentalization of metabolism. They are complex organelles with two phospholipid bilayers that divide them into 4 different segments: the outer membrane, inter-membrane space, inner membrane and matrix (Green, 1983; Neupert, 1997; Watson et al., 2000). The **outer mitochondrial membrane (OMM)** displays a composition very similar to the cytoplasmic membrane and has numerous pores and carrier proteins that allow the passage of substances through it. In turn, pores are hardly found in the **inner mitochondrial membrane (IMM)** and substances pass into the matrix through specific transporters, so that this strucure is very selective to substances passage trought it. It is probably the most interesting part of the mitochondria because it allocates the mitochondrial electron transport chain (mtETC), the responsible machinery for the generation of energy in the form of ATP needed for life processes in cells. This membrane forms folds within the mitochondrial matrix called cristae, which serves to increase the membrane surface and so to house a larger number of enzymes. Although both mitochondrial membranes are composed by lipids and proteins, they are different from the rest of biological membranes since its protein content goes beyond 80% while most of biological membranes are less than 50% (Quiles, 1995; M Carmen Ramírez-Tortosa and JoséL Quiles, 2007). As its name suggests, the **inter-membrane space** is located between both membranes. It accumulates protons derived from passage of electrons through the mtETC that later will be used by the ATP synthase to produce ATP. In addition, certain enzymes, such as creatine kinase or carnitine are responsible for transporting fatty acids from the cytosol to the mitochondrial matrix to degrade through the β-oxidation. Lastly, the **mitochondrial matrix**, the area confined to the IMM, is where Krebs cycle and the cycle of fatty acid oxidation reactions occur. It contains the enzymes implicated in such reactions but also ribosomes and enzymes involved in protein synthesis.

The protein complexes within the mtETC deserve special mention because of its importance and significance in the context of oxidative stress (M Carmen Ramírez-Tortosa and JoséL Quiles, 2007). In aerobic organisms, the mtETC produces the energy needed for life support. Basically, macronutrients are oxidized through the loss of electrons that are accepted by electronic carriers as nicotinamide adenine dinucleotide (NAD^+) and flavins (flavin mononucleotide, FMN, and flavin adenine dinucleotide, FAD). The reduced nicotinamide adenine dinucleotide (NADH) and the reduced flavins (reduced flavin mononucleotide, $FMNH_2$, and reduced flavin adenine dinucleotide, $FADH_2$) are oxidized again by oxygen, producing great amounts of ATP. Oxidation occurs with small jumps in which energy is gradually released (M Carmen Ramírez-Tortosa and JoséL Quiles, 2007; Lenaz, 2012). The mtETC is composed mainly by 5 lipoprotein complexes (Lenaz, 1998; Cadenas, 2004; Ackerman and Tzagoloff, 2005; Mulkidjanian, 2005; Ackerman and Tzagoloff, 2007; M Carmen Ramírez-Tortosa and JoséL Quiles, 2007; Clason et al., 2010; Hirst, 2013):

- **Complex I or NADH dehydrogenase complex:** this complex takes two electrons from NADH that is oxidized to NAD^+, and transfers them to FMN that is reduced to $FMNH_2$.
- **Complex II or succinate dehydrogenase:** this complex transfers electrons from succinate to CoQ, but it does not act as a H^+pump.
- **Complex III or bc1 complex:** this complex receives two electrons from CoQ and transfers each to a molecule of cytochrome c, which would reach complex IV.

- **Complex IV or cytochrome c oxidase:** this complex receives four electrons from cytochrome c and transferred them to an O_2 molecule yielding two H_2O molecules. H^+ also pumped into space inter-membrane. This tetra-electronic reduction of oxygen is not tenable in a single step but rather must be done electron by electron. Because of this gradual reduction, the protein complex must be sure that partially oxidized oxygen, highly toxic, will not leak to the medium before being transformed into water (M Carmen Ramírez-Tortosa and JoséL Quiles, 2007).
- **Complex V or ATPase:** this complex carries out the final step of mtETC. It is responsible for producing ATP from ADP using the H^+ gradient generated.

Mitochondria are the only know extra-nuclear cellular organelles in animal cells that contain their own genetic information. In contrast with nuclear DNA, **mtDNA** is structured in small circular molecules (containing 16,569 bp in length) in most species, which occurs in many copies in a single mitochondrion. Furthermore, it is not associated with histones, which leaves it in a state of true vulnerability to ROS attack, to which must be added to their defense and repair systems are also lower than the nuclear DNA, so the mutation rate is 10 times greater than the DNA nuclear (Gredilla, 2010; Desler et al., 2011). In humans a mtDNA molecule encodes different 37 genes from those encoded by nuclear DNA, 13 essential proteins of the respiratory chain and ATP synthase, a set of 22 mitochondrial tranfer RNAs (tRNAs) and the small and large subunit of the mitochondrial ribosomal RNA (Gredilla, 2010; Desler et al., 2011; Osiewacz and Bernhardt, 2013).

In spite of mitochondria possess their own DNA and protein synthesis machinery, they are semi-autonomous elements, the biogenesis of which depends on the coordinated expression of the genome located in two cellular compartments: the nucleus and the mitochondrial matrix. Only about 1% of the approximately 1,000–1,200 mitochondrial proteins are encoded by the mtDNA. The vast majority of proteins are nuclear encoded, synthesized in the cytoplasm, imported into mitochondria and sorted to the different subcompartments of their function where they may be assembled into macromolecular complexes (e.g. respiratory chain complexes). There is no *de novo* biogenesis of mitochondria, but they increase in number and mass as a result of fission of existing organelles and growth of these units by incorporation of newly synthesized molecules. Individual mitochondria can also fuse. Together, this kind of mitochondrial dynamics gives rise to constant changes in the mitochondrial morphology and is part of a hierarchical network of pathways involved in controlling the quality of a mitochondrial population. Finally, severely affected mitochondrial units can be degraded by mitophagy, a special form of selective autophagy (Weber and Reichert, 2010; Fischer et al., 2012; Osiewacz and Bernhardt, 2013).

1.3.2 Mitochondria and oxidative stress

1.3.2.1 Mitochondria is the main source of ROS in cell

There are different sources of ROS in cells. Exogenous sources include ultraviolet (UV) and visible light, ionizing radiation, drugs and environmental toxins. On the other hand, ROS are produced as by-products of aerobic metabolism in cells, and mitochondria are the major sites of ROS generation (Kowaltowski et al., 2009). In humans, more than 90% of oxygen is consumed by mitochondria, and 1–5% of the consumed oxygen is transformed into superoxide because of electron leakage of the mtETC. Superoxide generated in mitochondria is then converted to hydrogen peroxide either spontaneously or

catalyzed by SOD (Kowaltowski et al., 2009). There are endogenous sites in the cell that produces ROS, such as xanthine oxidase, cytochrome P-450 enzymes in the endoplasmic reticulum, peroxisomal flavin oxidases and plasma membrane Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) (Lenaz, 2012). Mitochondria seem to be the most important cellular site of O_2^- and H_2O_2 production in mammalian organs, and the steady state concentration of O_2^- in the mitochondrial matrix is about five-to-ten-fold higher than that in the cytosol and nucleus (Cadenas and Davies, 2000; Cadenas, 2004). However, it is widely considered that the major source of ROS is the mitochondria where have been found at least nine sites that possess the ability to produce O_2^- (Andreyev et al., 2005; Jezek and Hlavatá, 2005; Angermüller et al., 2009; Brand, 2010; Venditti et al., 2013):

- **Cytochrome b5 reductase:** it is a protein located in the OMM that oxidizes cytoplasmic NADPH and reduces cytochrome b5 in the outer membrane. It may also reduce ascorbyl free radical and, therefore, be involved in regeneration of ascorbate in mammalian liver. Mitochondrial cytochrome b5 reductase may produce superoxide with a high rate of 300 nmol/min per mg protein (Xu et al., 2011; Mukherjee et al., 2012).

- **Monoamine oxidases A and B:** they are mitochondrial bound isoenzymes located in the outer membrane which catalyze the oxidative deamination of dietary amines and monoamine neurotransmitters accompanied by release of H_2O_2. Its potential for H_2O_2 generation may far exceed that of other mitochondrial sources, even 50 times higher than complex III. Monoamine oxidase may be an important source of H_2O_2 in tissues during ischemia, aging, and oxidation of exogenous amines (Bortolato et al., 2008; Ramsay, 2012; Wang et al., 2013).

- **Dihydroorotate dehydrogenase:** it is located at the outer surface of the inner membrane and catalyzes conversion of dihydroorotate to orotate, a step in the synthesis of pyrimidine nucleotides. In the absence of its natural electron acceptor, coenzyme Q (CoQ) in the IMM, reduced dihydroorotate dehydrogenase can produce H_2O_2. Inhibition of complex II with antimycin A reduced dihydroorotate dehydrogenase activity and pyrimidine synthesis, suggesting that dihydroorotate dehydrogenase is functionally linked to complex III activity (Olgun and Akman, 2007; Fang et al., 2013).

- **α-glycerophosphate dehydrogenase:** this is a FAD-containing enzyme also located at the outer surface of the inner membrane. It catalyzes oxidation of glycerol-3-phosphate to dihydroxyacetone phosphate, utilizing mitochondrial CoQ as electron acceptor. This reaction is involved in lipid metabolism and in the glycerol phosphate shuttle that regenerates cytosolic NAD^+ from the NADH formed in glycolysis, producing H_2O_2. The rates and sites of H_2O_2 production depend upon tissue source, concentrations of glycerol 3-phosphate and calcium, and the presence of different mtETC inhibitors (Orr et al., 2012; Adam-Vizi and Tretter, 2013).

- **Succinate dehydrogenase:** this is a flavoprotein, located at the inner surface of the inner membrane, that oxidizes succinate to fumarate, with the concomitant production of $FADH_2$, using CoQ as electron acceptor. Subunits A and B of succinate dehydrogenase are bound to complex II in the IMM matrix, whereas the two other subunits, C and D, form the base of the enzyme as an intramembraneous

complex within the IMM. Succinate dehydrogenase A and B subunits are probably hydrophilic with the former involved in substrate binding and oxidation and the latter in electron transfer (Ralph et al., 2011; Ishii et al., 2013).

- **Mitochondrial aconitase:** it is localized in the mitochondrial matrix and catalyzes conversion of citrate to isocitrate as part of the tricarboxylic acid cycle. A secondary role for mitochondrial aconitase is to act as a biosensor for ROS and iron. Mammalian aconitase contain a [4Fe-4S] prosthetic group in their catalytic centers which are susceptible to inactivation by ROS, particularly O_2^-. The enzyme is inactivated upon oxidation of its iron-sulfur cluster by superoxide. Upon inactivation, isolated aconitase induces production of hydroxyl radical, most likely mediated by released Fe^{2+} (Fariss et al., 2005; Cantu et al., 2011).

- **α-ketoglutarate dehydrogenase complex:** it is composed of multiple copies of three enzymes: α-ketoglutarate dehydrogenase, dihydrolipoamide succinyltransferase and lipoamide dehydrogenase, and is associated with the matrix side of the inner membrane. This complex catalyzes oxidation of α-ketoglutarate to succinyl-CoA using NAD^+ as electron acceptor. Several studies have demonstrated that α-ketoglutarate dehydrogenase complex can generate superoxide and hydrogen peroxide. The source of ROS in α-ketoglutarate dehydrogenase complex appears to be the dihydrolipoamide dehydrogenase component. The ROS production from α-ketoglutarate dehydrogenase complex is stimulated by low availability of its natural electron acceptor, NAD^+(Andreyev et al., 2005; Shi et al., 2011).

- **mtETC:** The ROS production at the mtETC occurs when some electrons, during the pass from one complex to the other, escape and join the surrounding oxygen, generating O_2^- (Hirst, 2013; Lambert and Brand, 2009). Complex III, furthermore, is the only site that can deposit O_2^- into the inter-membrane space; the other sites deposit O_2^- into the mitochondrial matrix. Inter-membrane space ROS seems to be easier access to the cytosol that matrix ROS, because they only need to cross the OMM and not both, as matrix ROS, and that could be an advantage to the cytosol signaling capacity (Sena and Chandel, 2012). It has been reported production of ROS from nematode *Ascaris suum* (Paranagama et al., 2010) and rat (Quinlan et al., 2012) mitochondrial complex II. In addition, it has also been demonstrated that a substantial portion of H_2O_2 production is originated from electron flow into complex I and II and not from mitochondrial glycerol 3-phosphate dehydrogenase, as it was commonly attributed (Hagopian et al., 2010; Orr et al., 2012).

It is often assumed that the mitochondrial electron transport is the main biological process leading to ROS generation in most tissues in physiological conditions (Cadenas et al., 1977; Ksenzenko et al., 1983; Shimomura et al., 1985; Cross and Jones, 1991; Selivanov et al., 2008; Lambert and Brand, 2009; Brand, 2010; Pamplona and Barja, 2011; Maranzana et al., 2013). In humans, more than 90% of breathed oxygen undergoes a reduction to produce water in the mtETC, and 1–5% of the consumed oxygen is transformed into superoxide because of electron leakage of the mtETC. However it has been estimated that a 1-5% of the oxygen consumed by the mitochondria is not fully reduced to water and transformed to O_2^- (M Carmen Ramírez-Tortosa and JoséL Quiles, 2007; Kowaltowski et al., 2009; Miriyala et al., 2011) as a result of the transfer of a single electron to an oxygen molecule (Osiewacz and Bernhardt, 2013). This primary free radical can react with biomolecules in the direct vicinity or be converted to H_2O_2 either spontaneously or catalyzed by the scavenging SOD (Kowaltowski et al., 2009; Miriyala

et al., 2011). H_2O_2 can cross membranes and diffuse to different cellular compartments . It can be broken down into water by glutathione peroxidase (GPX), thioredoxin peroxidase and catalase, or undergo Fenton's reaction in the presence of divalent cations such as Fe^{2+} and Cu^{2+} to produce more aggressive hydroxyl radicals (Chen and Schopfer, 1999; Kirkinezos and Moraes, 2001; Osiewacz and Bernhardt, 2013). The products of the monovalent and bivalent reduction of oxygen are O_2^- and H_2O_2, respectively, which are usually produced during aerobic metabolism, mainly at the mitochondrial level. The mitochondrial generation of these free radicals, as well as peroxynitrite ($ONOO^-$), represent the major intracellular source of ROS under physiological conditions (Halliwell and Cross, 1994; Cadenas and Davies, 2000; Farrugia and Balzan, 2012; Matés et al., 2012). ROS physiological levels produced during electronic transport at mitochondria depend on its metabolic state. The state of mitochondrial rest (state 4), is characterized by a low respiration level and no ADP availability. This state is associated with a high rate of ROS production, probably as a consequence of the high degree of reduction of the chain components (M Carmen Ramírez-Tortosa and JoséL Quiles, 2007). On the contrary, at the active mitochondrial state (state 3), there is a high oxygen expenditure and elevated ADP availability. This state shows relatively low ROS production. In the state of anoxia (state 5), with a limitation in the oxygen delivery and absence of respiration, no ROS production is observed (Cadenas and Davies, 2000; Cadenas, 2004).

1.3.2.2 Mitochondrial structures are particularly susceptible to oxidative attack, yielding more ROS

Overproduction of ROS results in cellular damage since they can can interact with DNA, RNA, lipids and proteins, leading to destruction or irreversible alteration of the functions of the targeted molecules (Kong et al., 2014). Because of their proximity, there is a high probability that macromolecules present in mitochondrial structures react with ROS with different consequences depending of the molecule. Moreover, for many of them, there are other reasons that increase its susceptibility respect than those found in other cellular organelles.

ROS can react either directly with the side chains of amino acids, the peptidic backbone of proteins or with lipids and carbohydrates, the oxidized forms of which can generate lipid peroxidation and glycoxidation adducts on proteins, respectively (Berlett and Stadtman, 1997). Proteins containing iron-sulfur clusters in mitochondria are vulnerable to oxidative stress. Mitochondrial membrane phospholipids are extremely susceptible to ROS-induced lipid peroxidation because they have a very high content of unsaturated fatty acids (Schlame et al., 2000). Additionally, the lipoxidation-derived end products such as the carbonyl compounds can spread the membrane damage to other cellular constituents such as proteins and DNA (Pamplona et al., 2004), resulting in modifications which render them unable to be degraded and forming a variety of adducts and cross-links collectively named advanced lipoxidation end products (Thorpe and Baynes, 2003; Pamplona, 2008).

ROS-induced DNA damage, including single- or double-strand DNA breaks, DNA cross-links and modifications of purine, pyrimidine or deoxyribose, can result in alterations in transcription and signal transduction, replication errors, and genomic instability (Marnett, 2000; Cadet and Wagner, 2013). The most extensively studied DNA lesion is 8-hydroxyguanine (8-OHG) or its deoxynucleoside equivalent 8-hydroxy- 2'- deoxyguanine (8-OHdG). These lesions are highly mutagenic resulting in G:C to T:A transversions, and are widely accepted as biomarkers of oxidative damage (Dizdaroglu et al.,

2002). The mtDNA is particularly susceptible to oxidative damage because of its proximity to free radical sources and the relative lack of a protein scaffold. In addition to its proximity to the source of oxidants, mtDNA lacks protection by histones and the DNA repair capacity in mitochondria is relatively low (Linnane et al., 1989). mtDNA has been considered a major target for aging-associated somatic mutations due to the oxidative microenvironment of the mitochondria, the lack of protective histones in the mtDNA, and the limited efficiency of the mtDNA repair mechanisms compared to those of nuclear DNA (nDNA) (Linnane et al., 1989). Moreover, the mitochondrial genome lacks non-coding introns, which increases the likelihood of damage to a coding region and consequently affects the integrity of encoded RNA and proteins. These characteristics make mtDNA more vulnerable to oxidative damage than nDNA in mammalian cells (Park and Larsson, 2011).

1.3.2.3 Damaged mitochondria may accumulate with aging increasing ROS production

Progressive mitochondrial dysfunction is considered a hallmark of aging (López-Otín et al., 2013; Newgard and Pessin, 2014), and impaired mitochondrial function causes an accelerated aging phenotype, which is particularly evident in high energy demanding tissues such as brain, heart, and skeletal muscle, and in kidney and liver, two organs with essential metabolic roles (Bua et al., 2006; Coen et al., 2013; Peterson et al., 2012; Sevini et al., 2014). The generation and the damaging potential of mitochondrial derived ROS is the basis of the "mitochondrial free radical theory of aging" (Harman, 1972) which has been extensively investigated and refined during the last decades. This landmark theory initiates the molecular era of aging research. As said, in addition to being a main source of ROS, mitochondria are the prime targets of oxidative damage, which in turn reduces mitochondrial efficiency and leads to the generation of more ROS in a vicious self-destructive cycle.

Several facts illustrated the previous phenomenon. On one hand, a wide spectrum of alterations in mtDNA including point mutations, deletions and duplications have been found to accumulate in a variety of tissues during aging (Wei and Lee, 2002). The accumulation of oxidative stress-induced mtDNA mutations has been shown to correlate with a progressive decline in mitochondrial function and contribute to age-related physiological decline (Linnane et al., 1989; Wei and Lee, 2002). More importantly, damage to mtDNA can be propagated as mitochondria and cells divide, leading to the amplification of the physiological consequences of the damage (Kong et al., 2014). Reaching a critical threshold, such mutations can give rise to adverse effects particularly on mitochondria in which impaired components need to be replaced (Osiewacz and Bernhardt, 2013). On the other hand, oxidative modification and deterioration of mitochondrial iron-sulfur clusters-containing proteins could cause the loss of the biochemical and physiological functions of mitochondria in aging cells (Kong et al., 2014) since it is crucial for them to participate in redox sensing and signaling reactions (Beinert and Kiley, 1999; Veatch et al., 2009).

As an extension of the free radical theory, the mitochondrial vicious cycle theory of aging emphasizes and refines the central role of mitochondria in the aging process. This theory states that mitochondria generate ROS which lead to random damage of all kinds of molecules (Miquel et al., 1980; Miquel, 1998; Bota and Davies, 2001). Over the years, this theory has been repeatedly challenged and refuted by a "tsunami" of data showing that the mitochondrial function is a key to successful aging and aging phenotypes in

ways the Harman's theory never anticipated. As a result, the idea that mitochondria could play a central role in aging and age-related diseases has been increasingly emphasized (Gonzalez-Freire et al., 2015). In recent years the theory became challenged by accumulating controversial results (Lapointe and Hekimi, 2010). This theory has been reviewed by Barja (2013), emphasizing key aspects of the theory and including different issues, such as:

- The complex I as main ROS-generating site in the respiratory chain in relation to aging and longevity.
- The closeness or even contact between complex I and the mitochondrial DNA.
- The relationship between mitochondrial ROS production and oxygen consumption.
- The assumption that ROS are simple "by-products" of the mitochondrial respiratory chain.
- The unnecessary postulation of "vicious cycle" hypotheses of mitochondrial ROS generation which are not central to the free radical theory of aging.
- The role of DNA repair concerning endogenous versus exogenous damage. In recent years, it has been ascertained that although ROS are produced through a large number of pathways of the aerobic metabolism, the main source of these species are mitochondria (Cadenas et al., 1977; Lenaz, 1998; Sastre et al., 2000; Salvioli et al., 2001; Van Remmen and Richardson, 2001; M Carmen Ramírez-Tortosa and JoséL Quiles, 2007; Pamplona, 2011; Vendelbo and Nair, 2011; Bolisetty and Jaimes, 2013; Sassi et al., 2014).

There are some evidences about biological adaptations that determine the rate of free radical generation by mitochondria (Pamplona and Barja, 2006; Hulbert et al., 2007; Pamplona and Barja, 2007):

- To adapt the amount of the respiratory complex/es responsible for ROS generation. Thus, a decrease in the amount of complex I protein will lead to a decreased rate of ROS generation.
- To adjust the amount of uncoupling proteins. The mitochondrial superoxide production is very sensitive to the proton motive force, so it can be strongly decreased by mild uncoupling, which diminishes mitochondrial superoxide production and protects against oxidative damage.
- To regulate the degree of electronic reduction of these generators: the higher their degree of reduction, the higher will be their rate of ROS production.
- To modify by enzymatic and non-enzymatic pathways such as S-nitrosation, acetylation, and glutathionylation specific peptides of the ROS generators. Several studies have found an inverse correlation between the rate of respiratory ROS production and animal longevity. Species with short longevity could show high mitochondrial ROS production simply because their rates of mitochondrial oxygen consumption are higher, while it is generally lower in longer lived species (Page et al., 2010; Pamplona and Barja, 2011).

1.3.3 Mitochondrial mass and quality control mechanisms

In addition to an array of enzymatic and non-enzymatic antioxidant defenses (Kong et al., 2014) that protect against ROS, mitochondria and cells have quality control mechanisms to counteract the damage caused by ROS in this organelle. However, these mechanism also may be altered by aging. In that sense, it has been suggested that such alterations would led to accumulation of damaged mitochondria or a decrease in mitochondrial mass (López-Otín et al., 2013). Therefore, the reduced efficiency of mitochondrial bioenergetics with aging may result from multiple converging mechanisms beyond ROS effects. Major mechanisms implicated in mitochondrial mass regulation and quality control include mtDNA repair systems, mitochondrial chaperones and proteases, and the combination of autophagy and biogenesis processes.

1.3.3.1 mtDNA repair systems.

As the mitochondrial genome does not encode DNA repair proteins, mitochondria rely on the nuclear proteins for DNA repair, although not all nDNA repair pathways are present in the mitochondria. mtDNA lesions are repaired primarily by the base excision repair pathway (Maynard et al., 2009), which similarly to nuclear base excision repair is catalyzed by DNA glycosylase, AP endonuclease, DNA polymerase (POLG in mitochondria) and DNA ligase (Stuart and Brown, 2006; Hegde et al., 2012; Sykora et al., 2012). Alterations in mtDNA repair have been linked to oxidative damage accumulation in mammalian genomes and aging (Druzhyna et al., 2008; Ma et al., 2009)

1.3.3.2 Mitochondria posses their own chaperones and proteases to maintain protein homeostasis.

As other mitochondrial components, the proteome is continuously challenged by multiple factors such as the production of ROS (Mottis et al., 2014). This is particularly important when mitochondria become dysfunctional, since the reduction in mitochondrial ATP production would result in decreased rates of cellular protein synthesis and turnover, and the longer-lived proteins will be more susceptible to damage. Likewise, the accumulation of unfolded, misfolded, or unassembled proteins beyond mitochondrial folding capacity leads to damage and organelle/cell dysfunction (Arnould et al., 2015). To prevent unfolded and damaged protein accumulation, mitochondria possess their own arsenal of chaperones and proteases (Arnould et al., 2015). Chaperones present in mitochondria refold damaged proteins but they also are responsible for folding the newly imported proteins. All of them belong to the heat shock protein (Hsp) family and at the moment they are represented by mtHsp70, Hsp10 or Hsp60 in mitochondria. Proteases role is to guarantee the degradation of irreversibly damaged proteins. It is possible to distinct between two groups of proteases depending on its localization in mitochondria, intermembrane space or matrix. The proteases situated in the first are HtrA serine peptidase 2 and Yme1l, whereas the ATP-dependent proteases ClpP and Lon are in the second place (Mottis et al., 2014). In relation to these, it has been reported an age-associated impairment of Lon protease activity that would contribute to the buildup of oxidized proteins in mitochondria (Bota and Davies, 2001; Ugarte et al., 2010). When unfolded, misfolded, and damaged proteins accumulation is high exceeding these mechanisms capacity, other processes that also lead to degradation of protein as autophagy and mitochondrial fission (discussed below) can be initiated (Mottis et al., 2014) contributing to protein quality maintenance.

Because of most of mitochondrial proteins including those implicated in protein quality control are encoded by nuclear genome, mitochondria need to communicate with the nucleus in response to organelle dysfunction and bioenergetic impairment by retrograde communication. There is an specific retrograde signaling pathway to boost folding and degradation capacity in response to unfolded and aggregated protein accumulations in mitochondria named mitochondrial unfolded protein response, which coordinate the transcription of nuclear-encoded mitochondrial chaperones and proteases to preserve protein homeostasis (Arnould et al., 2015). It was discovered in mammals 20 years ago (Martinus et al., 1996), but the link to aging, healthy lifespan, and longevity, of this mechanism has mainly revealed by studies in the model organism *C. elegans* (Bennett and Kaeberlein, 2014; Jensen and Jasper, 2014; Jovaisaite et al., 2014).

1.3.3.3 Dysfunctional mitochondria are selectively removed by autophagy

Autophagy is a cellular recycling mechanism that sequesters cellular components into vesicles, termed autophagosomes, which subsequently deliver their cargo to lysosomes in animal systems. The role of autophagy as a quality control system is to breakdown damaged molecules or whole organelles (Szklarczyk et al., 2014). In more detail, this process begins with the formation of a membrane cisternae called isolation membrane or phagophore that encloses a portion of cytoplasm resulting in the autophagosome. Then, the outer membrane of the autophagosome fuses with the membrane of one or more primary lysosomas leading to the autolysosome or autophagolysosome where degradation of cellular components including the autophagosome inner membrane occur (Ravikumar et al., 2010).

Mitochondria are mainly degraded in cells through a selective macroautophagy called mitophagy (Kim et al., 2007; Liesa and Shirihai, 2013) maintaining homeostatic levels of this organelle (Tolkovsky, 2009). However, mitophagy has been observed to decline with aging (Cavallini et al., 2007) leading to accumulation of damaged mitochondria. Defective mitochondria fail to function properly and have an impaired oxidative capacity skewed toward increased ROS production (Ding and Yin, 2012; Chistiakov et al., 2014). As consequence, it has been suggested that this decline, in last term, is associated with advanced oxidative stress, as well as increased apoptosis as occur in muscle (Masiero and Sandri, 2010). The loss of mitophagy has been associated with aging and particularly with age-associated neurodegeneration (Baker et al., 2004; Lemasters, 2005; Batlevi and La Spada, 2011; Green et al., 2011). Dysfunctional mitochondria are selectively removed by mitophagy (Ding and Yin, 2012; Chistiakov et al., 2014). Although the cues used by autophagy machinery to identify dysfunctional mitochondria probably remain unelucidated, some advances have been made to resolve issue. One participant in autophagy, the protein Parkin, facilitates the engulfment of mitochondria by autophagosomes after recruitment based on mitochondrial low membrane potential and thus allows selective removal of damaged mitochondria (Narendra et al., 2008). The importance of the selectivity of this process mediated by Parkin in aging have been reinforced by some cases of autosomal recessive Parkinson's disease where a mutation in Parkin gene was found

1.3.3.4 Removed mitochondria are replaced by biogenesis .

Mitochondrial biogenesis is a complex process that leads to the formation of newly synthesized mitochondrial mass. It requires the synthesis, import, and incorporation of proteins and lipids to the existing mitochondrial reticulum, as well as replication of the

mtDNA (Hock and Kralli, 2009; Chistiakov et al., 2014). Because of mitochondrial proteins are encoded by nuclear and mitochondrial genomes, the coordination of transcription at both localizations is crucial. Biogenesis is regulated by different transcription factors that target distinct but overlapping sets of genes encoding mitochondrial proteins (Hock and Kralli, 2009). One of this set comprised the nuclear genes *Tfam, Tfb1m*, and *Tfb2m*. These encoded, respectively, mitochondrial proteins mitochondrial transcription factor A (TFAM) and transcription factor B1/B2, mitochondrial (TFB1M/TFB2M) that control both, the transcription and replication of mtDNA. Thereby, different transcription factors implicated in biogenesis, whose binding sites are only in nDNA, gets coordinate the expression of nuclear genes with mtDNA replication and expression (Scarpulla, 2008). Furthermore, there are coregulators able to interact with different primary transcription factors whose role is critical since they integrate signals and coordinate their actions (Hock and Kralli, 2009).

It has been identify a wide variety of physiological cues triggering biogenesis (e.g., physical activity, nutrient availability, temperature, circadian cues, exposure to infectious agents) as well as many differences in mitochondrial mass, function, and morphology among cell types (Kelly and Scarpulla, 2004). The network allows the concerted regulation of a broad mitochondrial gene set while also permitting tissue- and signal-specific expression patterns of subsets of mitochondrial genes (Hock and Kralli, 2009). Among biogenesis co-regulators identified, the peroxisome proliferator-activated receptor (PPAR)γ coactivator-1α (PGC-1α) plays a central role by integrating diverse signals that impact mitochondrial biogenesis and coordinating multiple DNA-binding factors to induce broad sets of mitochondrial genes (Hock and Kralli, 2009). Because of this ability, PGC-1α has shown to integrate physiological signals and to enhance mitochondrial biogenesis and oxidative function (Puigserver et al., 1998; Wu et al., 1999). In some tissues, as skeletal muscle, a gradually decline has been noted (Crane et al., 2010) that may be explained by a decrease in biogenesis, which has been related to an age-dependent reduction in levels of PGC-1α (Wenz et al., 2009; Bernardes de Jesus et al., 2012). The importance of PGC-1α have been supported by study in aged mice where its overexpression was associated with reduced sarcopenia and an improvement of mitochondrial function in skeletal muscles (Wenz, 2011).

1.3.3.5 Mitochondrial dynamics also is important for quality control

Mitochondria are dynamic cellular units that constantly change their morphology by fission and fusion. Along with fusion/fission events that mediates in mitochondrial connectivity, mitochondrial dynamics also include the mitochondria movement along the cytoskeleton and the regulation of mitochondrial architecture (Palmer et al., 2011). The dynamic network constituted by these processes is essential to maintain normal mitochondrial functions and it has been considered an effective mechanism to control mitochondrial quality (Szklarczyk et al., 2014).

Fission and fusion processes participate in the maintenance “healthy” population of mitochondria (Szklarczyk et al., 2014). Fission allows to separate damaged parts of the mitochondrial network from parts that are less affected because of it is an interesting mechanism for mitochondrial quality control (Twig et al., 2008). Once damage passes certain thresholds and other quality control mechanisms are not enough (Tatsuta and Langer, 2008; Figge et al., 2012; Fischer et al., 2012), mitochondrial parts with local damage accumulation can be separated from the rest fission processes. Then, the separated mitochondrial containing higher mtDNA quality formed can increased its mass

by biogenesis and divide again until generate a fully functional population of mitochondria (Figge et al., 2012). Meanwhile, the remained damaged mitochondrion is removed by autophagy (Szklarczyk et al., 2014). On the other hand, it has been suggested that fusion processes could improve overall quality of the mitochondrial population by content mixing of fully functional mitochondria with defective organelles enabling protein complementation, mtDNA repair and equal distribution of metabolites (Chen and Chan, 2009; Szklarczyk et al., 2014).

1.3.4 Mitochondrial role in other age-related processes

1.3.4.1 Mitochondria are implicated in the intrinsic apoptosis signaling pathway

Apoptosis is a "programmed cell death" process to ensure that cell contents are encapsulated and phagocitically removed by scavenging cells (Campisi, 2003) with the aim of preventing degradative enzymes release (Arends and Wyllie, 1991; Ellis et al., 1991) Membrane blebbing, cell shrinkage, chromatin condensation, DNA cleavage and fragmentation of the cell into membrane-bound apoptotic bodies are known changes that occur during this process (Estaquier et al., 2012). The role of apoptosis during development and organogenesis is essential, but it is also part of the cellular quality control network that eliminates severely damaged cells . In relation to aging, imbalances between proliferation and cell death have been observed in age-related diseases (van Vliet et al., 2014; Takeuchi et al., 2015). Mitochondria change morphology and assume a characteristic punctiform shape in apoptotic cells, suggesting that this organelle plays an important role in the process that eventually leads to cell death (Hiona et al., 2010; Seo et al., 2010).

It is possible to distinct two major signaling pathways in the initiation of apoptosis, the extrinsic and the intrinsic pathway (Degterev and Yuan, 2008; Vandenabeele et al., 2010; Tsuda et al., 2012). The first one is begins when FAS ligand (FASL) (also called death ligand) binds to the cell surface transmembrane death receptor (FAS). The receptor activates the caspase-8 (a cysteine protease) that activates downstream caspase-3 by cleaving, which triggers several mechanisms resulting in execution of apoptosis.. Meanwhile, mitochondria are implicated in the intrinsic pathway (Estaquier et al., 2012; Yacobi-Sharon et al., 2013). In this pathway, mitochondrial outer membrane permeabilization is crucial. This results in the release of different soluble factor present mitochondrial intermembrane space into cytosol (Tait and Green, 2010). One of main factors released is cytochrome c that binds to apoptotic protease-activating factor 1 (APAF1) to form a complex that will lead to apoptosome formation when it binds to caspase-9. In the apoptosome, caspase-9 is activated, and the activated caspase-9 subsequently activates the effector caspase-3, leading to the execution of apoptosis. In addition to this caspase-dependent pathway, a caspase-independent pathway have been also identified. In this last, endonuclease G and apoptosis-inducing factor (AIF) both released by mitochondria leads to nDNA degradation (Jeong and Seol, 2008).

High ROS level would decrease the mitochondrial membrane potential. This alteration of membrane potential would allow the opening of transition pores triggering apoptosis events(Rufini et al., 2013; Watanabe et al., 2013). Actually, ROS accumulation associated with loss of activity of the antioxidant enzymes, as SOD, leads p53-mediated growth arrest and apoptosis (Rufini et al., 2013; Watanabe et al., 2013). Mitochondrial deficiencies may affect apoptotic signaling by increasing the propensity of mitochondria to permeabilize in response to stress (Kroemer et al., 2007). Mis-regulated apoptosis is observed during aging and is consistent with tissue-specific outcomes for mitochondrial

maintenance failure (Shen and Tower, 2009). In contrast, a down-regulation of apoptosis is associated with both cell senescence and cancer in mammals (King and Cidlowski, 1995; Marcotte et al., 2004; Campisi, 2013) and with tissue over-growth in the aging *C. elegans* gonad (McGee et al., 2012).

1.3.4.2 Mitochondria are also implicated in other programmed cell death mechanisms

The term "programmed cell death" describe all cell deaths processes that occur in predictable places and at predictable times during development (Estaquier et al., 2012). Apoptosis is the most common form of programmed cell death but dying cells may follow other morphological types. An alternative cell death can be triggered by a phenomenon known mitochondrial permeability transition, a mechanism that depends on the protein cyclophilin D located in mitochondrial matrix. The instantaneous mitochondrial transmembrane dissipation derived from permeability transition lead to a cessation of OXPHOS, which triggers rapid necrotic cell death (Kroemer et al., 2007).

1.3.4.3 Mitochondrial production of ROS may mediate immuno-inflammatory processes

Currently, there are different evidences supporting that ROS are required for normal physiological functioning of all cells. This due in part to ROS are able to modulate activity proteins involved in signal transduction, namely phosphatases and kinases as for example mitogen-activated protein kinases (MAPKs) (Torres, 2003). However, that this is due to ROS effects on proteins with a Cys residue in the thiolate form at their active sites (as thioredoxins, peroxiredoxins and protein tyrosine phosphorylase), although their targets in this process remain unclear. Supporting ROS importance for this functional group, it has been reported that thiolates (but not thiols) are highly reactive with hydroperoxides under normal physiological conditions, after which they can participate in disulfide exchange reactions (Forman et al., 2004). Similarly, ROS also can directly affect the binding of some transcription factors to their DNA targets (Dröge, 2002; Janssen-Heininger et al., 2000a; Matt, 2002; Rovin et al., 1997). Thus, they could be needed for or involved in the modulation of different receptor-mediated signaling pathways. Its importance seems to be so high that oxidative stress by itself has the capacity to activate redox-sensitive signaling pathways in the absence of specific ligand–receptor. This can result of excessive generation of ROS within a cell or exposure to exogenous sources of ROS, but in both cases, mitochondria could be a primary source. In this context, the activation cascades of two major transcription factor implicated in inflammatory and immune responses, the nuclear factor-κB (NF-κB) and the activator protein-1 (AP-1), present multiple points that may be under redox control (Janssen-Heininger et al., 2000b). Actually, their pathways can be activated by a variety of stimuli, including oxidative stress along with many other as bacterial products, cytokines or growth factors (Makarov, 2000).

1.3.4.4 Some compounds from mitochondria are required for epigenetic changes and transcriptional processes

Mitochondria may also play an important role in epigenetic regulation by providing numerous co-substrates produced in the Krebs cycle that are required for epigenetic and transcriptional processes, such as histone modifications and chromatin remodeling (Salminen et al., 2014). Methylation of nDNA is a major component of the epi-

genetic system in mammalian cells and may strongly affect the regulation of mitochondrial function, since most of the mitochondrial proteins are encoded by nDNA. Because mitochondria are redox sensors, changes in the energy status in the mitochondria in response to alterations in the levels of metabolites, such as Fe^{2+}, succinate, or even dietary changes, could be involved in epigenetic alterations with aging. This will produce changes in the expression of several genes that regulate mitochondrial metabolism and nuclear-encoded mitochondrial genes (called the "mitochondrial retrograde pathway") (Shaughnessy et al., 2014). Shaughnessy et al. (2014) proposed that mitochondria sense stressful conditions and react by shaping the epigenetic landscape of chromatin to promote survival or trigger a senescent phenotype.

Chapter 2

Nutrition, oxidative stress and aging

2.1 Nutrition and aging.

Dietary habits are one of the most important modifiable factors that can affect the maintenance of a healthy aging phenotype (Mathers, 2013). Currently, different evidences have indicated that certain nutritional factors have relation the etiology of chronic diseases, cognitive decline, inflammation or cancer, which suggests a link between certain dietary patterns and increased longevity or the onset of such diseases. In that sense, different nutrients and other compounds present in the diet, not considered as such, have shown positive effects on age-related diseases and progression of aging. Actuallly, nutrition is probably the most important environmental factor that modulates the action of genes, which would be responsible for "maximum lifespan" and other phenotypes considered (Finch and Ruvkun, 2001; Busuttil et al., 2007; Phillips, 2013; Tucker et al., 2013).

2.2 Dietary fat, oxidative stress and aging

2.2.1 Introduction: fatty acids and minor compounds

Lipids or fats consist of numerous water-insoluble compounds that include monoglycerides, digycerides, triglycerides, phosphatides, cerebrosides, sterols, terpenes, fatty alcohols, and fatty acids (Alikhani et al., 2007). These chemical compounds represent more than 50% of plasmatic membrane and their quantity and quality are known to have an impact on the composition, characteristics and functions of the biological membrane (Fahy et al., 2005; Rohrbach, 2009). The predominant membrane lipids are phospholipids, which consist in esters of fatty acids. Additionally, fatty acids also constitute the main component of phospholipids, triglycerides, diglycerides, monoglycerides, and sterol esters (Alikhani et al., 2007). In particular, triglycerides (esters of fatty acids with glycerol) represent the foundation of oils and fats (up to 99%) (Aoun et al., 2012). Fatty acids can vary depending on the chain length, normally between 2 and 22 carbon atoms. It could be a short- (2 to 6 carbon atoms), medium- (8 to 10) or long-chain (12 or more) fatty acid. They also vary depending on the degree of unsaturation of the fatty acid, and may be: saturated fatty acids (SFA) whose bonds of the free carbon atoms are fully sat-

urated with hydrogen atoms; monounsaturated fatty acids (MUFA), with a double bond, that is, two adjacent carbon atoms that are not saturated with hydrogen; or polyunsaturated fatty acids (PUFA), with two or more double bonds. Unsaturated long-chain fatty acids can be classified into different series according to the position of the last double bond from their terminal methylcarbon that is designated as n or ω. Each of these series begins with a precursor: in the case of n-3 is α-linolenic acid (ALA); for n-6 is linoleic acid, and for n-9, oleic acid. From these precursors, by successive elongation and increases in double bonds (by elongase and desaturase enzymes) longer and more unsaturated fatty acids are formed. The serial number indicates that all fatty acids within a range have a double bond in the corresponding carbon, counting from the first one on the left and including the group called methyl (Lobb and Chow, 2008).

From the nutritional standpoint, SFA are mainly found in fats of animal origin, some ground vegetable fats (coconut and palm) and in margarine. MUFA mostly represented by the oleic acid, are present in large quantities in olive oil, rapeseed oil and in some genetically engineered seeds. Among the polyunsaturated linoleic acid, n-6 series, is the majority among seed oils (sunflower, corn, soybean, grape, etc.). Meanwhile, the polyunsaturated n-3 series are usually present in the fat of fish and the majority is eicosapentaenoic acid (EPA), which has 20 carbon atoms and five double bonds, and docosahexaenoic acid (DHA), with 22 carbon atoms and six double bonds (Calder, 2006). Of these fatty acids, there are two that are called essential because they cannot be synthesized by the body, being necessary to provide them through the diet. These are linoleic acid (n-6) and α-linolenic (n-3). The first comes from seeds and the second is present, for example, in soybeans. From α-linolenic, the aforementioned EPA and DHA are formed (Zevenbergen et al., 2009).

In addition to fatty acid constituents, fats or oils can also contain minor components which have important health benefits. In particular, much has been written about the health benefits of virgin olive oil. Because characteristics of minor components, these molecules are present almost exclusively in virgin olive oil since the process (mainly refining) involved in the production of these oils remove them. Minor components include more than 230 chemical compounds, such as non-glyceride esters, aliphatic alcohols, triterpene alcohols, sterols, hydrocarbons, pigments, lipophilic phenolic, hydrophilic phenolic and volatile compounds (Quiles, 2006). Despite the amount of minor compounds present in a daily consumption of virgin olive oil is low, its chronic ingestion could contributes to the overall pool of antioxidants, as tocopherol and carotenoids, in the body. Sterols consumption decreases levels of plasma low-density lipoprotein (LDL) cholesterol. Triterpenes, such as erythrodiol and oleanolic acid have shown anti-inflammatory and antioxidant properties in addition to vasodilatatory activity (Perez-Jimenez et al., 2005). One of the most studied polyphenols is hydroxytyrosol, which comes from the hydrolysis of oleuropein. Hydroxytyrosol has been demonstrated in numerous studies to have antiatherogenic properties with strong antioxidant power (Granados-Principal et al., 2010).

2.2.2 Roles of fatty acids in biological membranes

Biological membranes are dynamic structures that consist of bilayers of amphipathic molecules held together by non-covalent bonds (Vance and Vance, 2008). Fatty acids as components of biological membranes strongly influence membrane fluidity, which, in turn, may influence many physiological processes involved in cell death and survival such as signal transduction, protein import, membrane receptor function and metabolite

transport (Simkiss, 1998). As mentioned, membrane lipids influence several biochemical parameters, especially at the mitochondrial membrane level (Mataix et al., 1998; Uauy et al., 1999; Lobb and Chow, 2008).

2.2.2.1 Membrane fatty acids content is determinant for its susceptibility to oxidative damage

The chemical reactivity of the fatty acids from phospholipids is the main factor responsible for the susceptibility to oxidative alterations of biological membrane (Lobb and Chow, 2008). This is particularly important because of relatively high solubility of oxygen and free radicals in the lipid bilayer that makes membrane phospholipids in a primary target to be damged by oxidative stress (Simkiss, 1998).

Concerning fatty acids structure, it is known the existence of highly unstable electrons near double carbon-carbon bond from long hydrocarbon chains of phospholipids (Holman, 1954; Bielski et al., 1983; M Carmen Ramírez-Tortosa and JoséL Quiles, 2007; Gamliel et al., 2008; Milatovic et al., 2011). Consequently, biological membranes with a high degree of double carbon-carbon bond in the lipid tails of its phospholipids are especially sensitive to oxidative stress (Holman, 1954; Bielski et al., 1983; M Carmen Ramírez-Tortosa and JoséL Quiles, 2007; Gamliel et al., 2008; Milatovic et al., 2011). Along with ROS as OH^{-*} or $O_2{}^-$, some products of lipid peroxidation as highly reactive aldehydes also can cause indirectly oxidative damage to membrane lipids (Esterbauer et al., 1991). Polyunsaturated acyl chains are particularly sensitive to oxidation by ROS, increasing exponentially as a function of the number of double bonds per fatty acid molecule, since carbon-centered radicals after reaction with ROS within the membranes resulting in peroxidation of fatty acids (Halliwell et al., 1999). Thus, polyunsaturated acyl chains are easily attacked by radicals than those saturated or monounsaturated (Pamplona and Barja, 2011). Moreover, protein also can suffer covalent modifications by lipid peroxidation products (Pamplona et al., 2002). Lastly, the modification of biological membranes components by oxidative damage will lead to alterations in membrane fluidity and finally also in its function (Halliwell et al., 1999; M Carmen Ramírez-Tortosa and JoséL Quiles, 2007).

2.2.2.2 Fatty acid composition in membranes can influence immuno-inflammatory processes

Fatty acids have effects on the immune system and inflammatory processes. Thus, both primary and secondary immune responses to different factors might be also modulated by some nutritional conditions. Dietary fatty acids can affect the immune system via several mechanisms (Yaqoob et al., 2006):

- Modulation of fluidity, permeability of ion channels and functions of membranous receptors in cells participants in immune responses and inflammation.

- Effects on production of eicosanoids, cytokines. Eicosanoids are particularly important immunoregulatory molecules derivate from arachidonic acid, which is released from membrane phospholipids. Long-chain fatty acids included in n-3 and n-6 series also constitute eicosanoids (prostaglandins, prostacyclins, thromboxanes, leukotrienes, etc.) that are virtually involved in all tissues and organ systems where they perform multiple functions (Pamplona, 2008).

- Direct interaction with intracellular targets as transcription factors, which would modify gene expression.

2.2.3 Dietary fatty acids modulate membrane properties by changing its composition

The importance of dietary fatty acids resides in the fact that biological membranes, including mitochondrial membrane, adapt their lipid composition to some extent in response to dietary lipid intaked (Huertas et al., 1991, 1999; Ochoa-Herrera et al., 2001; M Carmen Ramírez-Tortosa and JoséL Quiles, 2007). In other words, dietary fatty acid intake influences in relative fatty acid composition of the membrane. In support of this, it has been reported in humans and animals that diets based on olive oil lead to membranes richer in oleic acid than diets based on sunflower oil that are associated with membranes richer in n-6 PUFA, namely linoleic acid. In addition, adaptations of the electron transport system in response to the type of dietary fat have been widely reported (Huertas et al., 1991; Quiles et al., 2001; Battino et al., 2002). In this sense, a polyunsaturated fat source will lead to membranes becoming more prone to oxidation than a saturated or a monounsaturated source, since a low level of fatty acid unsaturation in cellular membranes will decrease cellular oxidative stress. Experimental studies support this idea since their results indicate that decreases in liver and brain fatty acid unsaturation induced by diet modification also decrease oxidative damage in mtDNA (Pamplona et al., 2004). The high concentration of unsaturated fatty acids in membrane phospholipids not only makes them more sensitive to oxidation, but also enables them to participate in free radical chain reactions. Thus, biological membranes with a low degree of fatty acid unsaturation would show a decreased sensitivity to lipid peroxidation, which can even protect other molecules against lipoxidation-derived damage (Uauy et al., 1999). This has been widely demonstrated under a wide range of physiological and pathological situations using animal models (Quiles et al., 1999; Ramírez-Tortosa et al., 1999; Battino et al., 2002; Quiles et al., 2002). Regarding SFA, these acids induce a decrease of membrane fluidity and permeability. An excess of SFA affects cell membrane activity and in particular plasma membrane receptors, which play an essential role in glucose and lipid metabolism (Carluccio et al., 2007). The complexity of SFA actions and the possible differences among different individual SFA suggest that more research is necessary to better understand the role of SFA and their effects in dietary products (Astrup et al., 2011).

In spite of these findings, the role of elongase and desaturase system through the inhibition of the various components of the different series of fatty acids must be also considered in relation to fatty acid composition of biological membranes (Mataix et al., 1998; Wallis et al., 2002; Vance and Vance, 2008). As is well known, a fine control in the elongation and desaturation pathways is crucial for maintaining the membrane function through its effect on fluidity, or certain signaling cascades' mediators (Sanz et al., 2006). Several studies showed that diet rich in MUFA (from olive oil) stimulates the formation of membranes where the presence of n-3 PUFA when compared with diets rich in n-6 PUFA (based on sunflower oil as fat source) are increased (Navarro et al., 1994; Escudero et al., 1998). Likewise, a shift in the proportion of n-6 PUFA with n-3 PUFA-rich diets was observed too. There is a ratio between n-9 and n-6 series suggesting that fatty acids from n-9 series could be more easily incorporated in detriment to n-6 series. Diets rich in unsaturated fatty acids increase levels of MUFA and rich in n-3 PUFA increase n-3:n-6 ratio (Ayre and Hulbert, 1997; Pamplona and Barja, 2003). The maintenance of membrane fluidity is achieved in mammals by a homeostatic mechanism

and compensation control that maintains an optimal relationship between the proportion of saturated and unsaturated fatty acids (Gibson et al., 1984; Giron et al., 1992). This mechanism would explain that no significant changes were observed in saturation of membrane lipids since an increase in the n-6 series resulted in a decrease in the n-3 and *vice versa* (Huertas et al., 1991; Mataix et al., 1998). Regarding SFA, a tendency that their proportion remains stable has been reported as occurs with C18:0, which amonuts are largely dependent on the needs of each tissue. However, in the case of C16:0, adaptability is observed according to the composition of the diet, with a special ability to activate the Δ9 desaturase (Lobb and Chow, 2008). It was suggested, however, that high intakes of linoleic acid, even at current levels, were harmful and should be reduced (Simopoulos, 2008). This theoretical argument is based on the possibility that n-6 and n-3 PUFA in the diet could compete with each other in elongation and desaturation pathways and that longer-chain n-6 PUFA are precursors to pro-inflammatory eicosanoids, which lastly enhance oxidative molecular species production. Also, high susceptibility to oxidation of this edible oil has prompted many warnings concerning benefits to health (Arab, 2003).

Proportion of SFA and unsaturated fatty acids in membrane influence its fluidity whose maintainance is achieved in mammals by a homeostatic mechanism of control and compensation that maintains an optimal relationship among fatty acids types present in biological membranes (Gibson et al., 1984; Giron et al., 1992). This explains that no changes in membrane saturation degree occurs, which requires a decrease in n-3 PUFA content when n-6 PUFA increase and *vice versa* (Huertas et al., 1991; Quiles et al., 1999). Regarding SFA, a tendency that their proportion remains stable has been reported as occurs with C18:0, which amonuts are largely dependent on the needs of each tissue. However, for C16:0, it is has been observed an adaptability according to the diet composition, with a special ability to activate the Δ9 desaturase (Pamplona, 2008). When diets rich in fatty acids of the n-9 series are administered, mainly oleic acid, there is a increase in MUFA (Ochoa et al., 2002). In the case of PUFA, membrane levels of n-6 series components only seem to increase when administered these nutrients are largely administered in diets (Quiles et al., 2010). Likewise a high dietary administration of n-3 series PUFA indcrease thier amount in biological membranes, but also if diets are deficient in n-6 PUFA, since fatty acids belonging to both series compete for $\triangle 6$ desaturase, which determines the n3:n6 ratio (Martin, 2002).

2.2.4 Studies on dietary fat and aging

Diets with different fatty acids relative amounts has shown to diferentially affect levels of different proteins involved in multyple processes such as inflammation, homeostasis, oxidative stress, cardiovascular risk and cancer, which are of special concern in aging. In particular,this has been observed in plasma proteins levels of young or old rats lifelong fed on rats experimental diets enriched in either sunflower or virgin olive oil, where virgin olive oil diet, by providing oleic acid and minor constituents, induced significant decreases of plasma levels of acute phase protein, providing remarkable benefits on the antioxidant status, the anti-inflammatory state and the anti-atherogenic lipid profile during aging (Santos-González et al., 2012). Associations between the level of several n-3 and n-6 PUFA in serum phospholipids ad different polymorphisms of the human Δ5 and Δ6 fatty acid desaturase genes (fatty acid desaturase 1, FADS1 and fatty acid desaturase 2, FADS2) have been also described. Some response elements related with nutrients are sterol response element that are present in genes regulating the same; response elements for fatty acids and some of its metabolites in genes affecting the metabolism thereof

(Benetti et al., 2011).

Dietary fat causes diverse responses depending on the amount of fatty acids and structure of them. Essential fatty acids are nutrients that form an amazingly large array of bioactive mediators that act on a large family of selective receptors. The health consequences of specific gene-environment interactions with these nutrients are more extensive than often recognized. It also depends on derived from fatty acids such as eicosanoids, eicosanoids hydroxides, active forms of fatty acids (acyl coenzyme A [CoA]), etc (Goto et al., 2013). PUFA interact with PPARα, β and γ, which exert their action on DNA by binding to PPAR response element (PPRE) as heterodimers, after combining with retinoid X receptor (RXR). This mechanism allows the regulation of expression of genes encoding enzymes involved in fatty acid β-oxidation in both mitochondria and peroxisomes. Other transcription factors as the sterol regulatory element binding proteins (SREBP) and the nuclear factor-Y (NF-Y) also interact with PUFA. The regulation of SREBP and NF-Y activity might be a nutrient-sensitive pathway through which these fatty acids selectively and coordinately control the expression of subsets of genes involved in lipid metabolism (Benetti et al., 2011; Ihunnah et al., 2011; Jeon and Osborne, 2012). The result of the interaction between fatty acids or by-products of their metabolism and different transcription factors enables the existence of a network, through which, such nutrients may regulate different aspects lipid metabolism and carbohydrate, but also other molecules involved in cell growth and differentiation, production of various cytokines, adhesion molecules, or eicosanoids metabolism (Xu et al., 1999; Clarke, 2004; Teran-Garcia et al., 2007; Lands, 2012).

2.3 Dietary CoQ and aging

2.3.1 CoQ: Structure, localization and forms

CoQ is a naturally occurring molecule formed from the conjugation of a benzoquinone ring with a hydrophobic isoprenoid chain of varying chain length, depending on the species (Cluis et al., 2007; Prakash et al., 2010). It is mainly located in the hydrophobic domain of the phospholipid bilayer of the inner membrane system of the mitochondria CoQ but it is also present in all the other biological membranes at significant levels (Battino et al., 1990; Lenaz et al., 1999; López-Lluch et al., 2010), as well as in plasma lipoproteins (Crane, 2001). Moreover, CoQ is found in every plant and animal cell (Turunen et al., 2004; Prakash et al., 2010), because of such ubiquitous presence in nature and its quinone structure, the term ubiquinone is also used to refer to it (Gaby, 1996; Prakash et al., 2010). Ubiquinone is also referred to as "coenzyme" because of its unique ability to participate in chemical reactions but remain at steady-state levels in the cell (Littarru and Tiano, 2007). Ubiquinone molecules are classified based on the length ($_n$) of their isoprenoid side chain (CoQ_n) (Cluis et al., 2007). CoQ_9 is the predominant form in relatively short-lived species such as rats and mice whereas in humans and other long-living mammals the major homologue is CoQ_{10} (Tomasetti et al., 2001; Prakash et al., 2010). In particular, the chemical nomenclature of CoQ_{10} is 2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone that is in the trans configuration (natural) (Bhagavan and Chopra, 2006). The polyisoprenyl chain apparently facilitates the stability of the molecule within the hydrophobic lipid bilayer. In addition, the length of the CoQ isoprenoid chain seems to affect the mobility, intermolecular interaction with membrane proteins, and autoxidizability (Matsura et al., 1992; James et al., 2004).

The benzoquinone ring of CoQ can assume three alternate redox states due to the existence of different possible levels of protonation yielding three alternative CoQ forms: the fully oxidized or ubiquinone (CoQ), the fully reduced ($CoQH_2$) or ubiquinol and the partially reduced (CoQH) or ubisemiquinone (Fato et al., 1986; Matsura et al., 1992; James et al., 2004; Genova and Lenaz, 2011). Because of its extreme hydrophobicity, it is possible to found natural CoQ in 3 physical states: dissolved in lipid bilayers, forming micellar aggregates, or bound to proteins. In cells, CoQ is distributed between the two first states (Genova and Lenaz, 2011), whereas the importance of the last one is only experimental (Fato et al., 1986). All cells are able to synthesize functionally sufficient amounts of this molecule under normal physiological conditions (Bentinger et al., 2010). However, CoQ content as well as the ratios of CoQ_9 and CoQ_{10} vary in different organelles, tissues and species. For instance, lysosomes and Golgi membranes generally contain relatively higher concentrations of CoQ than mitochondrial membranes or microsomes (Dallner and Sindelar, 2000). Likewise, in mice, it has been reported higher CoQ levels in kidney and heart than in brain or liver homogenates (Lass et al., 1999; Lass and Sohal, 2000). In non-mitochondrial biological membranes, CoQ is also continuously cycling between reduced and oxidized states thanks to different enzymes with CoQ reductase activity (Ernster and Dallner, 1995a). These enzymes are NAD(P)H dehydrogenases which form part of the plasma membrane redox system, an electron transport where CoQ acts as a mediator accepting electrons from cytosolic NAD(P)H (López-Lluch et al., 2010). This system have been related to several the maintenance of intracellular redox homeostasis, the membrane antioxidant protection, the regulation of cell signaling and other functions (López-Lluch et al., 2010) that will be discussed below.

2.3.2 Physiological roles of CoQ

2.3.2.1 Mitochondrial CoQ is an essential factor for cell bioenergetics

CoQ was isolated and characterized in 1955 and only two years later it was shown to be a essential mtETC component (Mitchell, 1975). Thus, CoQ is an essential factor for cell bioenergetics (López-Lluch et al., 2010). It acts as carrier of electrons from respiratory complexes I and II to complex III (Bentinger et al., 2007). Additionally CoQ also accepts electrons from other donors including dihydroorotate dehydrogenase, and acyl-CoA dehydrogenase. Because of it has several donors but only transfer them to complex III, it may be considered a crossroad in the mitochondrial electron transport activity. Therefore, CoQ is continuously cycling between the reduced and the oxidized forms in mitochondria, but it is also bound to proteins and in a membrane free form. The sum of CoQ in any of these possible states constitute the mitochondrial CoQ total pool (Sohal and Forster, 2007). In relation to this, Lopez-LLuch et al. (2010) have proposed that an equilibrated CoQ pool may perform a better electron flow adaptation than a higher or lower CoQ pool by keeping a better mitochondrial homeostasis control. In addition, it has been reported that protein-bound CoQ participates in and is needed for the maintenance of complex III stability in yeast (Santos-Ocaña et al., 2002). According to this finding, CoQ levels in mitochondria not only would affect cell bioenergetics by its participation as coenzyme in mtETC, but also could affect protein complexes activity and structure (López-Lluch et al., 2010). Moreover, by means of plasma membrane redox system, CoQ is involved in balancing the NAD^+:NADH ratio in cells which also regulates cell bioenergetics (Larm et al., 1994).

$$QH_2 + LOO^* \longrightarrow Q + LOOH + H^+$$

Figure 2.3.1: Ubiquinol reaction with peroxyl radical

2.3.2.2 CoQ acts as lipid-soluble antioxidant in all biological membranes

One of the main functions of CoQ in biological membranes is as antioxidant (López-Lluch et al., 2010). Further, it constitutes our only lipid-soluble antioxidant endogenously synthesized that has show to efficiently prevent oxidation of proteins, lipids and DNA (Ernster, 1993). In this role, CoQ can act as antioxidant by itself or as free radicals quencher (Crane and Navas, 1997; James et al., 2004; Turunen et al., 2004) but also by participating in the regeneration of other antioxidants such as ascorbate (Gómez-Díaz et al., 1997; Santos-Ocaña et al., 1998; López-Lluch et al., 2010) and α-tocopherol (Crane and Navas, 1997; Bello et al., 2003). In particular, it has been proposed that the antioxidant effect of quinones in mitochondrial membranes is mediated by α-tocopherol recycling (Kagan et al., 1990) since it is a major liposoluble antioxidants in both, mitochondrial and the rest of cell membranes (Bindoli et al., 1985; López-Lluch et al., 2010) that have shown to be be reduced by the mitochondrial respiratory-chain substrates through ubiquinol (Bindoli et al., 1985).

By itself, reduced CoQ_{10} has also demonstrated to be an important physiological lipid-soluble antioxidant (Frei et al., 1990). In contrast to other antioxidants, ubiquinol can inhibit both the initiation and propagation of lipid peroxidation by reacting with perferryl radical and radicals generating ubisemiquinone and a non-radical lipid hydroperoxide by preventing the production of reacting with lipid peroxyl radicals, with concomitant formation of ubisemiquinone and H_2O_2 (see Figure 2.3.1)(Ernster and Dallner, 1995b; Bentinger et al., 2010). Physiological ubisemiquinones having long side chains do not react with oxygen, except in complex III under the very special conditions of the CoQ-cycle (Genova and Lenaz, 2011). On the other hand, short-chain ubiquinones have a pro-oxidant effect in complex I. Prevention of the lipid peroxidation propagation by the quenching of the initiating perferryl radicals also protects proteins from oxidation. In relation to this, is also important to point that ubiquinol effectively regenerates vitamin E from the α-tocopheroxyl radical which additionally contributes to slow the propagation step of lipid peroxidation. Lastly, CoQ also have shown to prevent DNA bases oxidation, which is particularly important in mtDNA case (Bentinger et al., 2010). Furthermore, deficiency in some nutrients considering antioxidant as vitamin E and selenium could be compensated by the induction of the CoQ-dependent antioxidant system (López-Lluch et al., 2010) as it has been observed in hepatocytes where both CoQ, levels and CoQ reductases have shown to be affected by this compensatory mechanism (Navarro et al., 1998). This last reinforce the role of CoQ as antioxidant particularly under some physiological situation as nutritional deficiencies, which particularly interesting because it is endogenously synthesized.

Explanations for the exceptionally high efficiency of CoQ as antioxidant includes its intramembraneous localization, its general and abundant distribution and its effective reduction/reactivation by a number of cellular systems (Mitchell, 1975; Bentinger et al., 2010). Firstly, its localization is of central significance, since OH^{-*} and O_2^{-*} generated in the membrane would otherwise rapidly react with neighboring lipid and protein molecules which necessitates the availability of effective protective agents close to the site of radical production (Bentinger et al., 2010). Secondly, several effective enzymatic systems catalyze CoQ reduction to achieve its active (reduced) form in eukaryotic cells

(Ernster, 1993). In mitochondria, the reduced antioxidant form of CoQ is regenerated by the respiratory chain (Bentinger et al., 2010). Besides the mitochondrial respiratory chain, other enzymes with such function have been found. These includes the NADH-cytochrome b5 reductase that can reduce CoQ through a one-electron reaction mechanism (Arroyo et al., 1998; Navarro et al., 1998) and the soluble enzyme NAD(P)H-quinone oxidoreductase 1 (NQO1) that can reduce quinones by a two-electron reaction (Beyer et al., 1996; Genova and Lenaz, 2011). This enzyme is induced under oxidative challenge (Rushmore et al., 1991) and can maintain the electron flow through the plasma membrane redox system to CoQ when this system is working mainly as antioxidant. In addition, a distinct cytosolic NADPH-CoQ reductase different from NQO1 has also been described (Takahashi et al., 1995), which seems to be a main factor for non-mitochondrial CoQ reduction (Kishi et al., 1999; Takahashi et al., 1999, 2008). However, its role and regulation under oxidative stress remains to be clarified (López-Lluch et al., 2010).

Lastly, it is important to note that ubiquinol is also present in lipoproteins, where it also exert its antioxidant activity. in particular, it has been reported that ubiquinol is the most efficient antioxidant in LDL (Stocker et al., 1991) that also contains α-tocopherol (Stocker et al., 1991; Bentinger et al., 2010). As consequence of LDL protection from oxidation, CoQ also would have anti-atherosclerotic properties . In this sense, it has been noted that it reduces the levels of lipid peroxides associated with lipoproteins in atherosclerotic lesions, as well as the size of such lesions in the aorta (Bentinger et al., 2010).

2.3.2.3 CoQ collaborates in redox state regulation by plasma membrane redox system activity

It has been proposed that plasma membrane redox system activity becomes essential in the maintenance of bioenergetics in cells when activity in mitochondria decreases as occur in aging (de Grey, 2001; López-Lluch et al., 2010). Actually, the plasma membrane redox system is up-regulated in cells lacking functional mitochondria (Larm et al., 1994; Gómez-Díaz et al., 1997; Hyun et al., 2006). In more detail, it has been reported that the amount of CoQ in plasma membrane increases and CoQ-dependent reductases are induced after mtDNA removal that induces the accumulation of cytosolic NADH. This mechanism allows cells to maintain the cytosolic NAD^+:NADH ratio in response to increase in cytosolic NADH (Larm et al., 1994; Gómez-Díaz et al., 1997; Hyun et al., 2007) which it is necessary for glycolysis occurs correctly. CoQ is also able to generate by autoxidation of ubisemiquinone form (Crane and Navas, 1997; James et al., 2004; Turunen et al., 2004). The apparently paradoxical property of CoQ to potentially act both as a prooxidant and an antioxidant would seem to suggest that it may also be a modulator of the cellular redox state under physiological and/or pathological conditions, and particularly it may play a role in the aging process (Sohal and Forster, 2007).

2.3.2.4 CoQ participates in cell signaling by plasma membrane redox system

The activity of plasma membrane redox system also plays an important role in growth and development of organisms (Crane and Navas, 1997; López-Lluch et al., 2010). In this sense, CoQ-dependent NADH oxidase of plasma membrane have shown to be involved in regulation of cell growth and differentiation. In particular, Gómez-Díaz et al. (1997) found that the activity of CoQ reductases in plasma membrane is modulated during erythrocyte differentiation. Likewise, the vitamin D_3-induced differentiation of myeloid cells to monocytes is enhanced when this system is activated by ascorbate (Quesada et al.,

1996; López-Lluch et al., 1998). Similarly, in serum-free cultures, addition of CoQ induces cell growth (Sun et al., 1992). In support of the role of plasma membrane redox system in cell signaling, that extracellular signaling molecules growth factors, insulin and pituitary extracts activated NADH oxidoreductases of plasma membrane (Brightman et al., 1992). At least in the case of myeloid cells differentiation, it has been observed that modulation of differentiation program by plasma membrane redox system activation includes modulation of intracellular second messengersand regulation of the activity of transcription factors (López-Lluch et al., 1998, 2001). Finding from studies on this topic suggest different mechanisms by means of which CoQ might exert this effects on cell signaling, these include:

- **ROS**. As previously stated, NAD(P)H dehydrogenases found in plasma membrane, cytochrome b5-reductase an other NAD(P)H dehydrogenases found in plasma membrane reduces CoQ in a one-electron mechanism yielding semiquinone forms *(Villalba et al., 1995)*. These CoQ form have pro-oxidant activity of generating O_2^{-*} or H_2O_2 that would act as second messengers that may act on cell signaling mechanisms. Consequently, these would modulate different cell responses affecting cell growth and differentiation processes (Linnane et al., 2007).

- **Tyrosine kinases**. It has been suggested that the redox state of CoQ in plasma membrane could control the activity of tyrosine kinases indirectly by generation of H_2O_2 and further inactivation of protein phosphatases, or directly by induction of redox-dependent conformational changes (Crane et al., 1994; López-Lluch et al., 2010).

- **Voltage-dependent anion channel (VDAC) proteins.** As in mitochondria, plasma membrane also contains proteins belonging to the VDAC protein family. One of the components of this family, VDAC1, can function as NADH-ferricyanide reductase, an activity associated to the plasma membrane redox system (Baker et al., 2004). Taken into consideration that CoQ is involved in regulation of VDAC/PTP in mitochondria (Papucci et al., 2003), López-Lluch et al. (2010) have suggested a putative relationship of the activity of CoQ in plasma membrane and cell signaling linked to VDAC1 to be considered in future research.

- **NAD^+-dependent deacetylases.** This group of protein implicated in genetic expression such as sirtuins, could be affected and regulated in some manner by the activity of NADH-dependent reductase in plasma membrane(Smith et al., 2000). It has been proposed that the variations in activity of CoQ-dependent NADH oxidoreductases in the different biological membranes also could regulate sirtuins because of effects on redox state (López-Lluch et al., 2010).

- **Mg^{2+}-dependent neutral sphingomyelinase**. It has been reported that CoQ-dependent plasma membrane redox system is involved in inhibition of Mg^{2+}-dependent neutral sphingomyelinase after oxidative stress (Fernández-Ayala et al., 2000; Martín et al., 2003; Villalba and Navas, 2000). This is an integral plasma membrane protein involved in the release of ceramide from plasma membrane sphingomyelin and participates in cell signaling, apoptosis, and the modulation of cell responses (Chatterjee et al., 1999; López-Lluch et al., 2010).

2.3.2.5 CoQ exerts anti-inflammatory effects through its antioxidant activity

CoQ exerts multiple anti-inflammatory effects by influencing the expression of NFκB1-dependent genes (Schmelzer et al., 2007). Likewise, H_2O_2 has been identified as an activator of the pro-inflammatory nuclear transcription factor NF-κB (Kaltschmidt et al., 1999). In view of the antioxidant properties of the reduced form of CoQ_{10} and the effective enzymatic conversion of oxidized CoQ_{10} into its reduced form, CoQ_{10} might mediate its observed anti-inflammatory effects via gene expression. For study of expression of genes implicated in inflammatory processes, *in vitro* models based on cells triggered with lipopolysaccharide (LPS) have resulted interesting since these bacterial product induces downstream signaling cascades of the transcription factor NF-κB, which in turn leads to the induction of inflammatory genes; CoQ_{10} down-regulates LPS-inducible genes in the monocytic cell line THP-1, presumably due to its antioxidant impact on gene expression (Schmelzer and Döring, 2010). Thus, as Genova and Lenaz (2011) indicated, it is likely that all effects of CoQ at the genetic level may be mediated by its antioxidant effect.

2.3.2.6 Mitochondrial CoQ prevents events leading to apoptosis

Different studies have confirmed the protective role of CoQ_{10} against apoptosis by showing inhibition of cell death independently of its antioxidant effect (Naderi et al., 2006; Papucci et al., 2003; Yamamura et al., 2001). Presumably, this occurs by inhibition of opening of the permeability transition pores (see), a high-conductance protein channel located in the IMM (Bernardi and Forte, 2007). Opening of the mitochondrial membrane transition pore depolarizes the mitochondrion (Genova and Lenaz, 2011), but also allows the translocation of molecules as large as 1500 Da in size (Papucci et al., 2003) leading to the release in the cytoplasm of proteins contained in the space between the two mitochondrial membranes, such as cytochrome c and other factors that trigger the process of apoptosis (Genova and Lenaz, 2011). Thereby CoQ is counteracting apoptotic events such as ATP depletion, release of cytochrome c into the cytosol, caspase-9 activation, depolarization of the mitochondrial membrane potential and DNA fragmentation (Fontaine et al., 1998; Walter et al., 2000). Independently of its free radical scavenging property, it has been reported that CoQ can prevent the mitochondrial depolarization and subsequent apoptotic events by inhibiting directly permeability transition pores aperture (Papucci et al., 2003). Moreover, the increase of mitochondrial CoQ levels have been related to the lack of induction of permeability transition pore opening in diabetic rats (Ferreira et al., 2003).

To elucidate the possible mechanism under this effect, it is important to point that permeability transition pore shows a ubiquinone binding site where several natural and artificial forms of CoQ interact stabilizing the pore in the closed conformation (Fontaine et al., 1998; Walter et al., 2000). In particular, quinones have also been shown to exert a direct effect on permeability transition pores. These compounds are able to modulate the permeability transition pore through a common binding site rather than through oxidation-reduction reactions. Occupancy of this site can modulate the permeability transition pore open-closed transitions, possibly through secondary changes of the Ca^{2+}-binding affinity for the pore. Permeability transition pores opening by its inducers led the cells to apoptosis (Devun et al., 2010). In these studies, the effect of hydrophobic long-chain quinones could not be investigated; Walter et al. (2000) found that three functional classes of quinone analogs could be defined in relation to permeability transition pores :

- permeability transition pores inhibitors, as CoQ_0 ,CoQ_2 , and decylubiquinone.
- permeability transition pores inducers, as idebenone (2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone).
- permeability transition pores-inactive quinones, which counteract the effects of both inhibitors and inducers, such as CoQ_1.

An indirect study (Li et al., 2005) suggests out that CoQ_{10} may be a permeability transition pores inhibitor. These authors exposed SHSY5Y neuroblastoma cells to neurotoxic β-amyloid peptides and oxygen-glucose deprivation to investigate the neuroprotective effect of CoQ_{10}. In these neuronal cells CoQ_{10} increased resistance against β-amyloid peptides-induced cell death that was related to the regulation of permeability transition pores opening. In addition, a decrease of superoxide production was also noted (Li et al., 2005). Similar studies indicated a protective effect of CoQ_{10} on permeability transition pores opening against amitriptyline toxicity (Cordero et al., 2009). However, because of ROS signaling could be implicated in permeability transition pores opening whether these CoQ_{10} effects on the transition pore depend on a direct mechanism or is mediated by the antioxidant effect is not fully clear yet (Genova and Lenaz, 2011).

2.3.2.7 CoQ present in lysosomal membrane participates in pH maintenance

The NADH-dependent CoQ reductase present in lysosomal membrane is implicated in H^+ translocation from cytosol to lysosomal lumen by means of an ATP-independent mechanism having O_2 as final electron acceptor (Gille and Nohl, 2000). As expected, this activity contributes to the maintenance of acidic pH into lysosomes. However, the exact role of this enzyme on lysosomal activity is currently under study (Crane, 2008; López-Lluch et al., 2010).

2.3.3 CoQ endogenous and exogenous sources

2.3.3.1 CoQ biosynthesis

CoQ endogenous bionsynthesis requires the synthesis of both a benzoquinone ring and an isoprenoid side chain. The precursor of the benzoquinone ring is 4-hydroxybenzoate that is synthesized from tyrosine or, at least theoretically, from phenylalanine (Bentinger et al., 2010). Meanwhile, the isoprenoid side chain is synthesized by a series of reactions starting from acetyl-CoA and ending up with farnesyl pyrophosphate (farnesyl-PP), which comprised the mevalonate pathway (Grünler et al., 1994; Ernster and Dallner, 1995b; Villalba et al., 2010). Consequently, some reactions involved in CoQ bionsynthesis are shared with cholesterol and other lipidic compounds (Grünler et al., 1994). Both the final product of this pathway, farnesyl pyrophosphate (farnesyl-PP), and the intermediary of this pathway, isopentenyl pyrophosphate (isopentenyl-PP) are utilized for the synthesis of the isoprenoid side-chain of CoQ (Bentinger et al., 2010). Then, the long isoprenoid side-chain of CoQ (which contains 6–10 isoprene units in different species) is synthesized by trans-prenyltransferase which condenses farnesyl-PP with several molecules of isopentenyl-PP, all in the trans configuration (Tran and Clarke, 2007).

Then, the 4-hydroxybenzoate-polyprenyl transferase, which is encoded by the gene *Coq-2* in humans (Forsgren et al., 2004), acts catalyzing the condensation of the isoprenoid side-chain with 4-hydroxybenzoate. After condensation, the benzoquinone ring

undergoes a sequence modifications including C-hydroxylations, decarboxylation, O-methylation and C-methylation to synthesise CoQ. This sequence has been studied mainly in bacteria and yeast. Meanwhile, in mammals, various functions of genes possibly implicated in these modifications have been established through the complementary recognition in yeast (Bentinger et al., 2010). At the moment, six genes encoding different enzymes that catalyze reactions in this sequence have been identified in humans (termed from *Coq3* to *Coq8*) (Turunen et al., 2004; Quinzii et al., 2007). However, full details of the synthesis of CoQ in animal tissues have not yet been clarified (Bentinger et al., 2010).

Endogenous CoQ_{10} synthesized using the radiolabelled precursor [14C]-pHB was first detected in mitochondria and later incorporated into mitochondria-associated membranes and endoplasmic reticulum, from where it is transported to other membranes in the cell (Fernández-Ayala et al., 2005). This suggest that CoQ biosynthesis mostly occurs in mitochondria but it is redistributed to other organelles. Although this process is mostly unknown, differences in the amounts present in different cellular organelles have been observed, with mitochondria, lysosomes and Golgi vesicles showing the highest concentration (Turunen et al., 2004). Despite all cells are able to produce CoQ, its distribution is not uniform among different organs, being present at highest concentrations in heart, kidney and liver (Aberg et al., 1992). Likewise, CoQ amount can also varies among different tissues and structures of the same organ as occurs in bovine brain where CoQ_{10} level ranges from 3 pg/g in the white matter to 25 pg/g in the striatum (Runquist et al., 1995). Moreover, interindividual variations in total CoQ_{10} and also there was a significant difference in total CoQ_{10}between healthy males and females (Molyneux et al., 2005). All these variations would indicate that CoQ_{10} concentrations are tightly distributed around a homeostatic set point in both organs and individuals. In any case, all cells should synthesize functionally sufficient amounts of CoQ under normal physiological conditions. Thus, in contrast to cholesterol, no redistribution via or uptake from the circulation is required. As said, the liver release a certain amount of CoQ that associates with very low-density lipoproteins (VLDLs), but this pool is not redistributed to other organs (Elmberger et al., 1987; Bentinger et al., 2010).

Because of CoQ_{10} is synthesized *de novo* in all tissues, it is presumed that under normal circumstances they are not dependent on an exogenous supply of CoQ_{10}. However, situations may arise in which the body's synthetic capacity is insufficient to meet CoQ_{10} requirements. Namely, susceptibility to CoQ_{10} deficiency appears to be greatest in cells that are metabolically active (such as those in the heart, immune system, gingiva and gastric mucosa), since these cells presumably have the highest requirements for CoQ_{10} (Gaby, 1996). For this reason, deepening on our understanding about CoQ synthesis regulation results interesting to deal with different pathologies. Hydroxybenzoate generally is present in excess, so that the rate of this reaction is determined by the availability of the polyisoprenoid chain (Bentinger et al., 2010). Farnesyl-PP produced by mevalonate pathway is precursor for cholesterol, CoQ, dolichol and isoprenylated proteins (Grünler et al., 1994). Moreover, the intermediary isopentenyl-PP is utilized for the synthesis of the isoprenoid side-chain of CoQ, but also for the synthesis of dolichol. It has been suggested that synthesis of all end-products of the mevalonate pathway is co-regulated since the initial sequence of reactions leading to such lipids is identical (Bentinger et al., 2010). However, terminal regulation must also occur (Bentinger et al., 2010), which would explain greatly varying synthesis rates and different amounts found in these lipids (Andersson et al., 1990). The terminal points of regulation probably involve the branch-point enzymes that has farnesyl-PP as substrate which are considered to be rate-limiting

for the terminal portion of the biosynthetic sequences (Goldstein and Brown, 1990). These are squalene synthase, trans-prenyltransferase, cis-prenyltransferase and farnesyl- or geranylgeraniol-protein transferases for cholesterol, CoQ, dolichol and isoprenylated proteins. Traditionally, it has been attributed to 3-hydroxy-3-metylglutaryl-CoA (HMG-CoA) reductase a central role in the regulation of the mevalonate pathway, but it seems that its primary regulatory role falls to cholesterol biosynthesis (Bentinger et al., 2010). Since squalene synthase Michaellis constant (KM) for farnesyl-PP is high, it is expected that the total amount of farnesyl-PP in the cell exerts its major influence on cholesterol synthesis. Therefore, when this substrate concentration decreases, the enzyme is not saturated and the cholesterol synthesis rate is reduced (Brown and Goldstein, 1980). In contrast, all of the other branch-point enzymes, i.e., trans- and cis-prenyltransferases, farnesyl- and geranylgeraniol-protein transferases, exhibit low KMs and remain saturated even when the farnesyl-PP pool is smaller.

As in other metabolic pathways, It seems that endogenous compounds also play regulatory rolls on CoQ biosynthesis (Bentinger et al., 2010). Epoxidated derivatives of certain all-trans polyisoprenoids, solanesol, tocotrienols, vitamin K_2 and CoQ up-regulate by itself CoQ synthesis in HepG2 cells. Additionally, it has been reported that several of these compounds inhibit the biosynthesis of cholesterol in the same cultures (Bentinger et al., 2008). However, none of the epoxidated poly-cis polyisoprenoids, which occur naturally without an epoxy group in large numbers, exhibit any effect on products of the mevalonate pathway in this same system (Bentinger et al., 2010). Tocotrienols exert the most profound effects on these biosynthetic pathway. It has been observed that the dolichol synthesis is also up-regulated but the importance of this phenomenon has not been clarified since the function for non-phosphorylated form in animals has not been established despite its abundance. In general, improved CoQ biosynthesis is achieved by regulating entire biosynthetic machinery, while decreasing cholesterol synthesis specifically involves the inhibition of oxidosqualene cyclase. Therefore, it has been suggested that small amounts of mono- and diepoxides polyisoprenoids as those having many organisms could be biological regulators of the mevalonate pathway (Bentinger et al., 2010). At translational level, some molecules have shown stimulate CoQ_{10} synthesis by *Coq-7* expression regulation (Aberg et al., 1996; Brea-Calvo et al., 2006). In particular, it has been shown that CoQ_{10} biosynthesis is dependent on NF-κB, which binds specifically to two binding sites present in the 5'-flanking region of the *Coq-7* gene, inducing both the *Coq-7* expression and CoQ_{10} biosynthesis (Brea-Calvo et al., 2009) .

2.3.3.2 Dietary CoQ has shown to increase CoQ levels in different body compartments

CoQ is a molecule naturally found in different dietary sources which can be uptake from intestinal lumen in similar way to other lipids (Villalba et al., 2010). According to results from different studies in rats (Reahal and Wrigglesworth, 1992; Zhang et al., 1995, 1996; Turunen et al., 1999; Bentinger et al., 2003), it has been historically considered that contribution of the dietary sources to endogenous levels of CoQ , is that although approximately 6% of the orally administered CoQ permeates the gastrointestinal tract into the blood and is transferred to liver and spleen. Consequently, the uptake in the whole body ranged between 2 and 3% of the total dose. Moreover, CoQ_{10} have been recovered in the plasma, largely in the reduced form (Zhang et al., 1995). *In vitro* studies with CaCo-2 models of absortion have suggested that CoQ_{10} is reduced to ubiquinol either during or following absorption (Bhagavan et al., 2007). In turn, CoQ uptake by other tissues

such as heart, kidney, brain and skeletal muscle was considered low or completely absent (Zhang et al., 1995; Turunen et al., 1999; Bentinger et al., 2003), unless the endogenous levels have fallen below a critical threshold.

In humans, different supplementation studies have been carried out (Svensson et al., 1999; Watts et al., 2002; Wolters and Hahn, 2003; Ikematsu et al., 2006; Hosoe et al., 2007) that varies in duration (20 days, 3 or even 6 months) and dosages (120-200mg/day), as well as in experimental groups characteristics. Despite differences, all showed a increase of CoQ_{10}levels in blood. Further, such increase seems to be dose-dependent increase according to a study where the effects of supplementation with 90, 150, and 300 mg/day of the reduced form of CoQ_{10} were compared (Ikematsu et al., 2006). In elderly women it was reported that the change was inversely associated with the baseline concentration (Wolters and Hahn, 2003), which suggests that CoQ uptake also is affected by levels present in the body. However, when CoQ_{10} concentration has been assessed in other samples (muscle homogenized and mitochondria), neither increase was noted (Svensson et al., 1999). Regarding how duration of dietary treatment affects CoQ body levels, it has been observed that plasma ubiquinol concentration had nearly reached steady-state by 2 weeks after the start of treatment, and plasma ubiquinol levels returned to that before administration 6 months after completion of treatment (Hosoe et al., 2007). In another study, maximum plasma CoQ_{10} concentration was reached in 2 weeks of supplementation with all-trans form of CoQ_{10} and then decreased to basal level after withdrawal, but CoQ_{10} levels increased a third compared with the study of Hosoe et al. using ubiquinol (Ikematsu et al., 2006).

Results from more recent studies in mice and rats firmly refuting this long-held notion that CoQ content of tissues other than plasma, liver or spleen cannot be significantly augmented by dietary administration of CoQ_{10} (Matthews et al., 1998; Lass et al., 1999; Lass and Sohal, 2000; Kwong et al., 2002; Lee et al., 2004; Ochoa et al., 2005a; Sohal et al., 2006). It was first shown by Matthews et al. (1998) that CoQ_{10} intake in their diets for 2 months by 12- or 24-months-old rats increased CoQ content in brain mitochondria. Subsequently, a series of studies conducted by Sohal et al. demonstrated that CoQ_{10} administration via food to mice (Lass et al., 1999,?; Lass and Sohal, 2000; Kwong et al., 2002; Kamzalov et al., 2003) and rats (Huertas et al., 1999; Kwong et al., 2002; Quiles et al., 2004; Ochoa et al., 2005b) caused an increase in amounts of CoQ_{10} in plasma and in tissue homogenates and mitochondria of brain, heart, kidney, skeletal muscle and liver. In all the tissues, the amount of CoQ augmentation was greater in mitochondria than in the homogenate, suggesting its preferential sequestration in mitochondria (López-Lluch et al., 2010). In brain, the increase was of a lesser magnitude and occurred primarily in mitochondria and not in the homogenate. With this background, it seems more clear that different tissues tended to vary in their capacity for CoQ accretion, with liver and skeletal muscle exhibiting the highest elevations, and brain showing the least (Sohal and Forster, 2007). The mentioned studies in animals were performed with relatively high daily dietary amounts of CoQ suggesting that higher plasma CoQ_{10} concentrations are necessary to facilitate uptake by peripheral tissues (Kwong et al., 2002; Villalba et al., 2010), which could explains differences with earlier studies. However, it has been also observed that feeding rats with very low-dosages of CoQ_{10} from weaning lead to increase of mitochondrial CoQ levels of heart and liver in 12- and 24-months-old rats. Moreover, it has been observed that this difference observed between rats fed on increases as the animals aged (Quiles et al., 2004; Ochoa et al., 2005b). Thus, age at beginning and duration of the treatment must be taken into account too. Importantly, dietary CoQ_{10} also lead to increase of the rat specific form CoQ_9 in all places where it was measured

(Lass et al., 1999; Lass and Sohal, 2000; Ochoa et al., 2005a).

Notwithstanding, it should be noted that the uptake of CoQ and other lipoidal substances is a complex process dependent upon a considerable number of different factors (Sohal and Forster, 2007). In that sense, it has been reported that intestinal absorption is threefold faster if CoQ_{10} is administrated with food intake in rats (Ochiai et al., 2007). In mice, the increase of plasma levels per 100 mg values was remarkably higher with the reduced form of CoQ_{10} when compared to the results obtained with the oxidized form CoQ_{10} (Lass et al., 1999). Additionally, other aspects of CoQ form also seems important. Villalba et al. (2010) reviewed the efficacy of a variety of commercial formulations that have been developed to solubilize CoQ_{10} and promote its better absorption *in vivo*, and its use in the therapy of pathologies associated with low CoQ levels with emphasis in the results of the clinical trials. They concluded that the relative bioavailability of CoQ_{10} is dependent on the type and amounts of oil in the formulations as well as by its delivery system, the order of decreasing bioavailability being: nanoparticulated, solubilised, oil-emulsioned and powder. Some additional dietary factors have shown to influence in CoQ contents in the body and they also could influence on the magnitude of dietary CoQ effect. In that sense, it has been reported that MUFA-rich dietary fats increased CoQ mitochondrial contents**,** whereas these decreased by diets rich in PUFA (Mataix et al., 1997). In the same sense, it has been observed that a diet rich in saturated fat for 50 days decreased CoQ levels in plasma and liver mitochondria of rabbits, which was restored by oral administration of soluble CoQ_{10} (25 mg/kg per day) over 30 days after such period (Ramirez-Tortosa et al., 2008).

2.3.4 Evidences for CoQ as antiaging compound

2.3.4.1 CoQ levels are affected by aging

It has been proposed that along the whole live of the organisms, the synthesis of CoQ changes and it is significantly reduced from the initial phases of aging (Alho and Lonnrot, 2000; Turunen et al., 2004). This statement seems contradictory with findings from *C. elegans* whose older individuals showed increases in CoQ content (Onur et al., 2014). However, measures of CoQ amount in mammals, mostly rodents, have been different. In mice, age-related decreases of CoQ levels have been observed in liver homogenates (Onur et al., 2014) as well as in mitochondrial CoQ levels of skeletal muscle, but not of kidney, brain or heart (Lass et al., 1999). As in mice, liver CoQ levels also decreases with age in pig (*Sus scrofa domestica*) (Onur et al., 2014). However, mitochondria of liver, heart and kidney from 19-month-old *ad libitum* fed rats showed lower CoQ_9than those from 4-month-old animals, although this CoQ depletion was not detectable in tissue homogenates (Kamzalov and Sohal, 2004). Authors attributed this fact to the preferential sequestration of CoQ in the mitochondrial fraction . Similarly, no changes in the levels of CoQ in rat brain after an increase during the first months of life have been reported (Zhang et al., 1996), but in this organ changes have been not observed also when only mitochondria were evaluated (Battino et al., 1995). On the other hand, an age-associated decrease of CoQ concentrations was detected in pancreas and adrenal gland, brain, heart and lung after an early increase as in brain reaching highest values at 30 days of age (Kalén et al., 1989a). In an old study, CoQ increased between 2 and 18 months and decreased significantly at 25 months in the heart and kidney, and the gastrocnemius, oblique and deep aspect (red) vastus lateralis muscles. On the other hand CoQ concentration of liver increased over the life span, while it remained relatively constant in brain, lung, and the superficial aspect (white) of the vastus lateralis muscle (Beyer et al., 1985). These

results suggest that decrease could appear relatively later in life explaining why some studies do not find clear age-associated changes.

Therefore, results of the various studies described do not support the existence of a common trend for all living organisms, although age-associated changes in CoQ content are most evident in mitochondria as was suggested by **Sohal and Forster (2007).** In mammals, CoQ levels tends to decrease with aging, but the occurrence of this depend on tissue and organism and probably many other factors. This could explain, at least in part, the differences found in aging processes among different organisms, but also why some tissues are more susceptible to aging or aging-related diseases. Studies evaluating the association between age and CoQ concentration in humans also have supplied contradictory results. In elderly women, no significant correlations were found between CoQ_{10} plasma levels and age (Wolters and Hahn, 2003). In human pancreas and adrenal gland, CoQ_{10} levels were highest at 1 year of age, and then they decreased, whereas in the brain, heart and lung, the corresponding peak value was at 20 years of age and was followed by a continuous decrease upon further aging (Kalén et al., 1989b). Age-associated decrease in brain have been confirmed by later studies (Söderberg et al., 1990; Edlund et al., 1992). In fact, it has been suggested that the mevalonate pathway, involved in the synthesis of cholesterol, dolichol and ubiquinone, is affected in the aged brain (Edlund et al., 1992).

2.3.4.2 Several age-related pathologies are associated with low levels of CoQ

Diseases such as cardiovascular diseases, neuropathies, inflammation, metabolic syndrome, arthritis, carcinogenesis, diabetes or hypercholesterolemia aggravate during aging and are considered as major age-related diseases (López-Lluch et al., 2010). Different studies have reported a beneficial effect of CoQ on them (Kagan and Quinn, 2000; Witting et al., 2000; Ayaz et al., 2008; Chew et al., 2008; Sena et al., 2008). This fact has been tougth that CoQ becomes an essential factor in the maintenance of the normal activity of cells in such conditions (López-Lluch et al., 2010).

Neurodegenerative disease have widely studied in this respect. Case-control studies have reported lower CoQ_{10} levels in serum of Lewy body disease patients (Molina et al., 2002) and plasma of subjects with amyotrophic lateral sclerosis Sohmiya et al. (2005) compared with healthy controls. Likewise, it has been found lower levels of this molecule in cerebrospinal fluid of Alzheimer´s disease patients (Isobe et al., 2010). In addition, it has been found a negative correlation between CoQ level and the duration of the illness in Amyotrophic Lateral Sclerosis (Sohmiya et al., 2005), Alzheimer's (Isobe et al., 2009) and Parkinson's disease cases (Isobe et al., 2007).The decrease of mitochondrial activity and the increase of free radical levels found in neurodegenerative diseases (Coyle and Puttfarcken, 1993; Jenner et al., 1992; Olanow, 1993; Schapira et al., 1993) highlights the importance that CoQ have in these processes. In fact, during the initial steps of Alzheimer's disease, NQO1 expression increases indicating a higher lipid peroxidation coupled to a higher necessity for CoQ-dependent antioxidant activity (Raina et al., 1999).

Some authors also have focused their studies on possible relationship of CoQ amount and some metabolic syndrome components as risk factors of cardiovascular disease. An important component associated to aging is hypercholesterolemia since it affects to significant part of aged population. In elderly women, no differences have been seen between hyperlipidemic and normolipidemic subjects in relation to serum CoQ_{10}levels. Similarly, no significant correlations have been found with body mass index, other risk factor for cardiovascular disease (Wolters and Hahn, 2003). However, as indicated above, CoQ is the main antioxidant in LDL (Stocker et al., 1991). In relation to this, it has been

total CoQ_{10}: total cholesterol ratio was reduced in diabetes mellitus patients, another component of metabolic syndrome, compared with subjects with normal glucose tolerance and impaired fasting glucose (Lim et al., 2006).

The development of certain types of cancer also could be related with low CoQ levels. Epidemiological studies in humans have shown negative associations between CoQ levels in blood and breast cancer (Folkers et al., 1997; Jolliet et al., 1998), myeloma (Folkers et al., 1997), melanoma (Rusciani et al., 2007), Graves' disease or follicular and papillary thyroid carcinomas (Mano et al., 1998). Some studies also have suggested that tumoral cells present lower CoQ levels than normal cells. In women presenting carcinomas and non-malignant-breast cancer, CoQ_{10} concentrations in breast tumor tissues significantly decreased as compared to the surrounding normal tissues (Portakal et al., 2000). Similarly, melanoma cell lines also presented low concentrations of CoQ_{10} (Rusciani et al., 2007).

2.3.4.3 Aging, development and lifespan are associated to changes in CoQ bionsynthesis

In mammals, there are at least 10 different proteins participate in the biosynthesis of CoQ that are encoded by *Coq* genes. These have both catalytic and regulatory activities. The first group are encoded by the genes *Coq-1*, *Coq-2*, *Coq-3*, and *Coq-7* whereas it has been suggested that *Coq-4*, *Coq-8* and *Coq-9* gene products have regulatory functions (López-Lluch et al., 2010; Tran and Clarke, 2007). Different studies using animals models mutants for these genes have been carried out to elucidate the role of endogenous CoQ synthesis on aging. *C. elegans* models have extensively used in this way. *clk-1* (mammals *Coq-7* orthologue gene) mutant nematodes that produce a very low amount of CoQ (Arroyo et al., 2006) showed and increased lifespan respect than wild-type animals (Jonassen et al., 2003; Wong et al., 1995). However, analysis of mutants in *Coq* genes, other than *clk-1* provided different results. In most of cases, *Coq* genes knockout showed deleterious defects that leaded to early developmental arrest (Asencio et al., 2009; Gavilán et al., 2005; Hihi et al., 2002). Namely, this have been reported for *Coq-1, Coq-3, Coq-3* and *Coq-8*. Extension of longevity by silencing *Coq* genes (Asencio et al., 2003) occurs upon moderate low levels of global CoQ content (up to 50%) but not in case of more severe CoQ depletions as the observed in *Coq-8* (Asencio et al., 2009) and *Coq-3* (Hihi et al., 2002) mutants. To explain this difference has been proposed that moderated CoQ depletion is associated to lower ROS production extending lifespan (Asencio et al., 2003), whereas a higher CoQ depletion would lead to developmental and reproductive inefficiency (Asencio et al., 2009; Hihi et al., 2002).

According to findings from the studies with C. elegans, it has been also suggested that *Coq-7* and CoQ synthesis is also related to aging in humans (Vajo et al., 1999) and other mammals. However, experimental evidences suggest that could exist important differences between mammals and *C. elegans* in this sense. In contrast to observations in nematodes, murine *Coq-7* knockout embryos arrest development at midgestation (Levavasseur et al., 2001). Similarly, it has been reported that some deletions *in Coq-7* affects mitochondrial integrity and neurogenesis (Nakai et al., 2001), which is comparable to some effects of CoQ deficiency found in humans (Boitier et al., 1998; Musumeci et al., 2001). However, heterozygous mice for such gene carrying a single functional copy show a notably increase in lifespan (Liu et al., 2005).

Mutations in genes implicated in CoQ bionsynthesis have been identified as cause of different pathologies associated with CoQ deficiencies in humans, most of which appear

early in life (López et al., 2006; Quinzii et al., 2006; Mollet et al., 2007; Lagier-Tourenne et al., 2008; Duncan et al., 2009). The most severe human CoQ_{10} deficiencies are due to autosomal recessive mutations and can be classified as primary deficiencies when mutations affect CoQ_{10} biosynthetic genes, or secondary if the cause is related to other genetic defects (DiMauro et al., 2007). Hereditary CoQ_{10} deficiencies caused by these mutations usually lead cardiomyopathies and degenerative muscle and neuronal diseases (Ogasahara et al., 1989; Boitier et al., 1998; Rötig et al., 2000; Artuch et al., 2006; Quinzii et al., 2006). The major phenotypes provoked by CoQ_{10} deficiencies are encephalomyopathy, severe infantile multisystemic disease, cerebellar ataxia, Leigh syndrome with growth retardation, ataxia and deafness, and isolated myopathy (Quinzii et al., 2008).

2.3.4.4 Studies on dietary therapies with CoQ

According to previous observations, dietary supplementation with CoQ_{10} could constitute an antiaging strategy or at least to retard some of aging effects. In humans, there is evidence, mainly indirect, that exogenous orally administered CoQ_{10} may be incorporated into mitochondria, at least in conditions of partial CoQ tissue deficiency, where it may enhance electron transfer and ATP synthesis with improvement of pathological situations as cardiac failure (Molyneux et al., 2009; Rosenfeldt et al., 2005), Parkinson's disease (Beal, 2004; Matthews et al., 1998; Shults et al., 2002; Thomas and Beal, 2010), Alzheimer disease (Dumont et al., 2010; Dumont and Beal, 2011; Yang et al., 2010) and Friedreich ataxia (Hart et al., 2005).

Despite such effects, results from animal studies are not clear about the dietary CoQ effects on longevity. In *C. elegans,* it has been reported that dietary CoQ prolonged lifespan (Asencio et al., 2003), but it has also noted with a CoQ-deficient diet (Larsen and Clarke, 2002). One possible explanation for poor diet effect, could be in the adaptability of these nematodes to stressful conditions. *C. elegans* life span of the worm is extended by the intake of antimycin A, an mETC, whereas it result toxic to most other aerobic species (Dillin et al., 2002). According to this observation, CoQ deficiency might induce a hypometabolic or a dauer-like state, which would facilitate survival under adverse conditions. However, a study focused on features of bacteria used to fed worms, has provided results that suggest that this phenomenon may be more complex. In it, a diet based on respiratory incompetent *E. coli* regardless if they were CoQ-less or CoQ-replete produces a robust life extension in wild-type *C. elegans* (Saiki et al., 2008). An explanation for these observations, that the fermentation-based metabolism of the *E. coli* diet is an important parameter of *C. elegans* longevity as it has been suggested (Saiki et al., 2008).

As in invertebrates, simple dietary CoQ supplementation has shown no direct concluding results on lifespan extension in rodents. In this studies have shown that CoQ_{10} supplementation with daily dosages ranged from 10 to 370 mg/Kg has no effect on longevity (Lönnrot et al., 1998; Alho and Lonnrot, 2000; Lee et al., 2004; Sohal et al., 2006). In spite of the absence of evidences supporting that dietary CoQ can increase lifespan in animals, some interventions in the same way has shown to retard some aging detrimental aspects in different animal models for aging or age-related disease. In SAMP mice, a mouse model for accelerated senescence and severe senile amyloidosis, life-long supplementation with $CoQH_2$ substantially decreased the senescence grading scores at different ages, although it did not alter some age-associated features of the model as the senile amyloid deposition rate. However, this intervention did not have effect on the lifespan again (Yan et al., 2006). In older mice with clear cognitive and psychomotor impairments, short-time (15 days) CoQ-supplementation improves spatial learning (Shetty

et al., 2012). A cardinal events of diabetes as diabetic neuropathies have been also found to be positively modified upon CoQ administration in diabetic rats (Ayaz et al., 2008).. In the hypercholestolemic in ApoE knockout mouse, dietary CoQ has been demonstrated an anti-atherogenic effect preventing the accumulation of lipid peroxides in aorta (Witting et al., 2000). In turn, in most of cases beneficial effects of CoQ over mitochondrial function and oxidative stress have been demonstrated (Witting et al., 2000; Sena et al., 2008; Shetty et al., 2012; Tarry-Adkins et al., 2013). However, long-term CoQ_{10} intake in health mice fed a standard diet failed to modulate mitochondrial respiratory capacity in liver or levels of oxidative stress in liver, kidney, skeletal muscle or brain (Sohal et al., 2006; Sohal and Forster, 2007). It seems clear that supplementation with CoQ is not needed when the organism is young and healthy because cell membranes seem to be near saturated at the functional level. However, these supplementations become necessary when the organism shows deficiency such as in aging.

On the other hand, the combination of dietary treatments using CoQ supplements with certain nutritional conditions associated with higher oxidative stress levels and aging detrimental effects offers interesting expectatives. From this standpoint (see), studies comparing its effects between isocaloric diets with different lipid profile are particularly interesting. In this sense, the effects long-term supplementation with CoQ_{10} daily low-dosages (0.7 mg/kg) on rats fed on MUFA-rich diets have been compared with those found in n-6 PUFA-rich diets (Quiles et al., 2004; Ochoa et al., 2005a, 2007; Quiles et al., 2010; Ochoa et al., 2011; González-Alonso et al., 2015). One of most interesting findings form such studies was that dietary CoQ_{10} produces significant increases of mean and maximum lifespan in rats fed a diet rich in n-6 PUFA (Quiles et al., 2004, 2010; Ochoa et al., 2011). At histopathological level, CoQ supplementation seems to improve endocrine pancreas structure and in particular β-cell mass resembling positive effects of virgin olive oil. When sunflower oil was the main fat in the diet, CoQ supplementation seems to improve endocrine pancreas structure and in particular β-cell mass resembling positive effects of virgin olive oil (González-Alonso et al., 2015). Dietary CoQ treatments have been also shown to be effective in counteract many of the high-fat diets consequences in animals (Ramirez-Tortosa et al., 2008; Cano et al., 2009; Ratnam et al., 2009; Safwat et al., 2009; Sohet et al., 2009; Jiménez-Santos et al., 2014; Orlando et al., 2014). In other mouse model, post-weaning dietary supplementation with CoQ_{10} rescued many of the detrimental effects of nutritional programming on cardiac aging by low birth-weight and catch-up growth (Tarry-Adkins et al., 2013).

The biochemical basis of potential beneficial effects of CoQ on life span or other aging detrimental effects may include enhancement of the cellular antioxidant protection systems in cell membranes, where CoQ sustain lipids in its reduced redox state preventing lipid peroxidation, particularly those unstable PUFA (Bello et al., 2005). In previous studies in rats, diets containing CoQ were associated with lower lipid peroxidation markers (Ochoa et al., 2005a; Quiles et al., 2010), as well as with lower oxidative damage of other macromolecules as DNA or proteins. A higher antioxidant capacity (Quiles et al., 2010; Gómez-Díaz et al., 2003) respect than those animals maintained on the same diet without additional CoQ_{10} (Quiles et al., 2004; Ochoa et al., 2005a, 2007; Quiles et al., 2010).. In addition, a lower impairment in mitochondrial function was also observed in CoQ-fed animals (Ochoa et al., 2005a). All these findings would indicate that dietary CoQ_{10} avoids, at least in part, oxidative stress linked to aging under certain conditions. Furthermore, it has been also shown that life-long dietary supplementation with CoQ_{10} attenuated a variety of changes in ezymatic activities associated with aging in rats (Gómez-Díaz et al., 2003; Bello et al., 2005). These includes increases in the

hepatic activities of Mg^{2+}-dependent nSMase (Bello et al., 2005) and of cytosolic and membrane-bound NQO1 activities (Gómez-Díaz et al., 2003), as well as decreases cytosolic glutathione-S-transferase and microsomal Se-independent glutathione peroxidase in liver plasma membrane (Bello et al., 2005). Proteomics analysis in rats under similar conditions have shown that serum albumin, which decreases with age in the rat, was significantly increased by CoQ_{10} supplementation. Additionally, it induced significant modifications of several proteins in plasma. These modifications support the beneficial role of dietary CoQ_{10} decreasing both oxidative stress and cardiovascular risk, and modulating inflammation and osteogenesis during aging (Santos-González et al., 2007).

In humans, some studies have suggested similar effects for dietary CoQ in relation to oxidative stress, at least in combination with certain dietary patterns. Short-term (4 weeks) dietary CoQ effects on aging have been tested in combination with mediterranean diet. For this proposal elderly subjects intaked a western diet rich in SFA, a mediterranean diet (rich in MUFA), and a mediterranean diet supplemented with CoQ following a cross-over desing (Yubero-Serrano et al., 2010; Gutierrez-Mariscal et al., 2011; Yubero-Serrano et al., 2012a; González-Guardia et al., 2015). CoQ_{10} addition to MUFA-rich diet reduces some postprandial oxidative stress markers levels when subjects take a breakfast with a lipid profile similar to their expiremental diets (Yubero-Serrano et al., 2011) which correlated with a lesser expression of antioxidant enzymes components (Yubero-Serrano et al., 2011; González-Guardia et al., 2015). Moreover, dietary CoQ also have shown to improve DNA repair systems (Gutierrez-Mariscal et al., 2012, 2014) and modulate inflammatory signaling cascade as well as reduce endoplamic reticulum stress (Yubero-Serrano et al., 2012b). Thus, although CoQ supplementation does not directly extend life span, it may help to prevent life span shortening due to oxidative insults (López-Lluch et al., 2010).

Chapter 3

Periodontal health, oxidative stress and aging

3.1 Peridontium antomy and function

Periodontium is the term used to name the tissues that support the teeth. These consists of gingiva, periodontal ligament, cementum, and alveolar bone (Van der Velden, 1984). Gingiva, the tissue exposed to the oral cavity, is histologically formed by epithelium and connective tissues. The fibroblasts are the main cells in the synthesis of periodontal connective tissue. There are phenotypic subpopulations of fibroblasts with different functions in the synthesis and maintenance of extracellular matrix constituents (Hou and Yaeger, 1993).

The periodontal ligament is a soft connective tissue that serves to anchor the tooth to the alveolar bone and functions as a cushion between hard tissues to mitigate the occlusal force. It is basically made up of fibroblasts, cementoblasts, osteoblasts, osteoclasts, Malassez epithelial rests, and collagen matrix (Sharpey's fibers). The periodontal ligament cells are involved in the repair of alveolar bone, cementum, and the periodontal ligament itself, being able to differentiate into osteoblasts, cementoblasts, and fibroblasts (Somerman et al., 1990).

The fibroblasts are the main cells in the synthesis of periodontal connective tissue. Gingival fibroblasts may be constantly affected by oral bacteria and its products, such as the LPS, present in their cell walls. The LPS induces gingival fibroblasts to release some inflammatory cytokines such as prostagladin E_2 (PGE_2), interleukin-1 (IL-1), and PA (Sismey-Durrant and Hopps, 1991; Abiko et al., 1998). The influence of these inflammatory mediators on both gingival and periodontal ligament fibroblasts may be an important factor in the severity of periodontal disease (Abiko et al., 1998). Periodontal ligament fibroblasts are constantly subjected to mechanical stress caused by mastication or occlusal forces (Huttner et al., 2009). This fact are particularly since a large production of PGE_2, IL-1β, and PA in response to mechanical stress in culture have been reported in periodontal ligament fibroblast cultures (Abiko et al., 1998).

The alveolar bone serves to support the teeth in association with the periodontal ligament. This tissue has a high plasticity, which under physiological conditions is maintained by the balance between osteoblastic and osteoclastic activities. Osteoblasts and osteoclasts are directly or indirectly influenced by the action of multiple molecules in-

cluging parathyroid hormone, calcitonin, vitamin D metabolites, estrogens, , some neurotransmitters, growth factors, and local cytokines, as well by the plasmatic concentrations of Ca and $PO_4{}^{3-}$(Sodek and McKee, 2000; Huttner et al., 2009). In addition, extracellular matrix surrounding osteoblasts has been shown to play an important role in bone metabolism (Abiko et al., 1998).

The remaining component, cementum, is a calcified connective tissue covering the roots of teeth. Its formation is a continuous process that occurs throughout the life of humans and animals and collagen fibers are commoly embedded in it. In these cells aging and cell death are commonly observed as part of their life cycle, which can be explained by two factors, a rapid reduction in the accessibility of nutrients and / or poor elimination of waste products (Huttner et al., 2009). For this reason, cementum is mostly acellular except at the root apices and in and in the furcation areas of multirooted teeth (Van der Velden, 1984).

3.2 Healthy periodontium features

The healthy periodontium, of which only the gingival tissues may be directly observed, is tightly adapted to the underlying tissues, with a knife edge margin where it abuts the tooth. The gingival margin is located, in the absence of pathology, at the cemento-enamel junction. It displays a scalloped edge configuration highest interdentally, where it constitutes the interdental papilla and lowest buccally and lingually. There is a gingival crevice where it abuts the tooth which in health is 1–3 mm deep. There is an absence of bleeding from the crevice on gentle probing. The crevice in health will show a small amount of interstitial fluid, gingival crevicular fluid (Brill and Krasse, 1958). The lateral wall of the crevice constitutes the free gingival margin. From the most apical extent of the free gingival to the mucogingival junction is the attached gingival which varies in width from 1-9 mm and has a stippled surface. It is an immobile tissue tightly bound down to the bone as a mucoperiostium and is a keratinized mucosa well suited to resist injury. Apical from the mucogingival junctionand continuous with the lining mucosa of the mouth is the alveolar mucosa, which is freely mobile and surmounted by a non-keratinized epithelium. It is generally thought that alveolar mucosa functions poorly as a marginal tissue and areas where there is lack of attached gingival may constitute mucogingival problems .

3.3 Age-related changes in periodontium

Different anatomical and functional changes in periodontal tissues have been associated with the aging process. At gingival level, thinning of the oral epithelium and diminished keratinization have been one of the most clear changes observed (Van der Velden, 1984). Moreover, a change from connective tissue ridges to papillae seems to ocurr since in a morphological three-dimensional study of the epithelium-connective tissue interface, connective tissue ridges were observed to be more prevalent in young individuals, whereas connective tissue papillae were predominant in elderly individuals. Additionally, epithelial cross-ridges formation was also noted with increasing age (Van der Velden, 1984). Other generalized age-related alteration affecting connective tissues of periodontium include a decrease of cellular elements number, namely of fibroblast (Van der Velden, 1984). Fibroblasts, that are the main participants in the synthesis and maintenance of extracellular matrix constituents, also have shown functional and structural

alterations in both, *in vivo* (Takatsu et al., 1999) and *in vitro* (Johnson et al., 1986; Dumas et al., 1994; Lee and McCulloch, 1997) studies. On the one hand, gingival fibroblast from old donors diplayed lower *in vitro* collagen synthesis than those from young donors (Johnson et al., 1986). This correlates with the finding that an increase aging process is associated with a increase in DNA structure methylation of collagen $\alpha - 1$ gene that would be followed by a reduction in mRNA levels and collagen type I synthesis (Takatsu et al., 1999). On the other hand, old fibroblasts also presented an increased rate of collagen intracellular phagocytosis (Lee and McCulloch, 1997). Consequently, the balance between synthesis and degradation of collagen in the connective tissue would be altered in aged people who would show dimished content of collagen. Further, other alterations in extracelular matrix components seem to occur as consequence of fibroblast alterations. In particular, proteoglycans secreted by gingival fibroblasts *in vitro* have been also observed which presented an increase in the rates of heparan sulfate and a reduction in chondroitin sulfate in old fibrobalsts in relation to young cells (Bartold et al., 1986).

Concerning periodontal ligament, its structure becomes irregular and it has been observed a decrease in fiber and cellular contents wih aging too. This could be related with the lower rates of chemotaxis and proliferation observed in periodontal ligament cells from elderly persons in comparison with those from young patients (Nishimura et al., 1997). A close finding has been also reported in relation to osteoblasts whose chemotaxis and differentiation rates were inversely related to donor's age (Groessner-Schreiber et al., 1992). The reduced ability of senescent cells to express the c-Fos ligand may be associated with the low rates of chemotaxis and proliferation of these cells (Asahara et al., 1999). In senescent periodontal ligament fibroblasts, osteocalcin expression is either reduced or ceased. This reduction may be directly related to the cell's difficulty in progressing in the cellular cycle (G1-S) and accomplishing cell respiration (Sawa et al., 2000).

Bone formation steadily declines with age, resulting in significant bone mass loss (Roholl et al., 1994). The reduction in bone formation may be due to a decrease in osteoblast-proliferating precursors or to decreased synthesis and secretion of essential bone matrix proteins (Roholl et al., 1994; Abiko et al., 1998). Moreover, possible dysfunction of extracellular matrix surrounding osteoblasts may occur concomitantly with the aging process (Abiko et al., 1998), which could have consequences on bone metabolism. On the other hand, cementum whose formation occurs throughout the whole-life, increases in width with age. In particular, there is a tendency toward greater cemental apposition in the apical region of the teeth (Van der Velden, 1984). However, with increasing age, the process of cementum formation becomes acellular. Although remodeling of cementum does not normally take place, local resorption at the cementum surface followed by cementum apposition is often observed. Therefore, the combination of increases in both, resorption and apposition, may also be responsible for an increased irregularity of the cemental surface with age (Tonna, 1976; Van der Velden, 1984).

3.4 Bone metabolism and inflammation in periodontium

As in other parts of the body, in healthy periodontium, bone formation and resorption are processes that occur continuously (Yucel-Lindberg and Båge, 2013). However, if the normal balance is shifted towards resorption, alveolar bone mass will decrease contributing to loss of teeth attachment. Such outcome can be trigger by an increased osteoclast activation and/or formation (i.e. osteoclastogenesis). Osteoclastogenesis involves dif-

ferentiation of mononucleated cells derived from hematopoietic myeloid cells present in bone marrow to cells that can fuse to mature multinucleated osteoclasts. This require progenitors cells recruitment, through the blood circulation, to the peri- and endosteal surfaces of cortical and trabecular bone surfaces where where they begin to fuse to form osteoclasts. Then, activated multinucleated osteoclasts attach to bone and start to produce acid dissolving the hydroxyapatite crystals and to release proteolytic enzymes degrading bone matrix. The rate of osteoclast formation in inflammatory diseases is dependent on the relative expression of stimulatory and inhibitory cytokines acting indirectly and directly to regulate osteoclast differentiation. Different molecules produced in the inflammatory reactions, either by the infiltrating leukocytes or by the resident cells, have been found to be able to regulate bone resorption and bone formation both *in vitro* and *in vivo* .

In healthy conditions, bone resorption is a well-regulated process which mainly depends on the differentiation of osteoclasts progenitors cells. Most of the cytokines and hormones stimulating or inhibiting osteoclast formation do so indirectly by regulating the production of osteoclastogenic molecules in osteoblasts or in resident fibroblasts. In addition, there are some examples of hormones and cytokines regulating osteoclast differentiation by directly targeting the osteoclast progenitors. It is, however, well established that stromal cells in bone marrow and late osteoblasts at bone surfaces play important roles by producing molecules crucial for osteoclast differentiation. Early progenitor cells differentiation depend on activation by certain cytokines and downstream lineage specific transcription factors. Formation of osteoclasts from osteoclast progenitors is critically dependent on activation of the receptor c-Fms by macrophage colony-stimulating factor (M-CSF). In particular, stimulation by M-CSF is necessary for proliferation and survival of the progenitor cells (Dai et al., 2002; Yoshida et al., 1990). For more step of osteoclastogenesis and subsequent bone resorption by activated osteoclasts, the interaction between two protein belonging to the superfamilies of tumor necrosis factor (TNF) receptor and TNF is needed. These are, repectively, the receptor activator of NF-κB (RANK) on osteoclast progenitor cells and its ligand (RANKL). The activation of RANK in osteoclast progenitor cells when it binds to RANKL induces their differentiation along the osteoclastic lineage and their terminal fusion to mature osteoclasts (Boyce, 2013). RANKL is a cell surface bound protein which suggests that osteclastogenesis requires cell-to-cell contact between cells expressing RANKL and progenitors cells expressing RANK. RANKL is expressed by osteoblasts in bone and stromal cells in bone marrow(Bucay et al., 1998; Guerrini et al., 2008; Simonet et al., 1997; Sobacchi et al., 2007; Souza and Lerner, 2013). However, a soluble form of RANK has been also found. Osteoclast differentiation induced by RANKL in the presence of M-CSF (Takayanagi, 2005) has shown to be mediated by TNF receptor associated factor 6 (TRAF6), c-Fos, and calcium signaling pathways. The interaction between RANK and RANKL is inhibited by osteoprotegerin (OPG), another member of TNF receptor superfamily. This is a soluble protein that as a decoy receptor for RANKL preventing interaction with it receptor RANK. When OPG expesion is inhibited, the binding of RANKL to RANK on osteoclast progenitors cells is allowed and they differentiate to osteoclasts (Blackwell et al., 2010). Given its importance in bone metabolism, deregulation of the levels of proteins involved in the RANK-RANKL-OPG axis could explain different physiological or pathological alterations that affect bone. In the context of periodontium, it has been reported high RANKL levels and reduced OPG levels in gingivocrevicular fluid (GCF) of patients with periodontitis. Consequently, RANKL:OPG ratio in GFC has been suggested as a possible biomarker test for detection of alveolar bone destruction (Kinane

et al., 2011), which has been supported by the differences found between periodontitis and healthy subjects in this ratio values (Mogi et al., 2004). Therefore, increased RANKL and/or decreased OPG seem to contribute to osteoclastic bone destruction in periodontal disease.

Along with M-CSF, among the more important pro-inflammatory citokynes and mediators reported to stimulate osteoclast activation and formation are IL-1β, TNF-α, interleukin-6 (IL-6), interleukin-17 (IL-17) and PGE2 (Pfeilschifter et al., 1989; Braun and Zwerina, 2011; Axmann et al., 2009; Schett, 2011). In addition, early publications (Hausmann et al., 1975) indicated that bacterial molecules can stimulate bone resorption independent on inflammation. Since then, several studies have shown that LPS can activate bone resorption *in vitro* and *in vivo*, it has been evident that LPS affects cellular activities through Toll-like receptors (TLRs), TLR-4 and TLR-2. Both the inflammatory mediators as PGE_2 and LPS has shown to enhance RANKL-stimulated osteoclastogenesis through direct effects on the haematopoietic cell lineage (Kaneko et al., 2007). In relation to the RANK-RANKL-OPG, pro-inflammatory cytokines such as IL-1β induce expression of some of these proteins in several cell types, including osteoclasts (Wei et al., 2005a) osteoblasts but also in resident cells as gingival fibroblasts, periodontal ligament fibroblasts and osteoblasts (Hormdee et al., 2005; Brechter and Lerner, 2007). It has been shown that IL-1 (Wei et al., 2005a) or PGE_2 (Blackwell et al., 2010) enhance expression of RANKL favoring progenitors or precursor cells differentiation to osteoclasts. In addition, the inhibition of OPG expression in osteoblastic cells by PGE_2 would have similar consequences (Blackwell et al., 2010). However, PGE_2 has been shown both to inhibit and stimulate OPG expression in human bone marrow stromal cells (Brändström et al., 1998; Hormdee et al., 2005), althouh due to it effect on on osteoclast formation, this discrepancies may beto differt incubation times (Blackwell et al., 2010)Oral pathogen sonicates also has been demonstrated to stimuate bone resorption in primary mouse osteoblasts co-cultured with bone marrow cells through the RANKL pathway (Choi et al., 2005).

3.5 Rat as model for periodontal alterations study

Animal models have been extensively used in oral disease research, particularly in periodontal diseases case, which has resulted in enormous advances in our understanding of aetiology, pathogenesis, prevention and treatment (Weinberg and Bral, 1999; Dannan and Alkattan, 2008). An optimal animal model of periodontal disease needs to be standardized, reproducible, and to share some characteristics with humans, such as periodontal anatomy, aetiology, pathophysiology, disease course, and clinical outcome. Other attractive attributes include availability and simplicity of handling. The most commonly used models are dogs and non-human primates, although other animals (rats, mice, hamsters, rabbits, miniature pigs, ferrets, and sheep) have also been used (Weinberg and Bral, 1999). Rodents present some anatomical and histological similarities with the human periodontium and periodonal disease . However, there are significant differences in oral cavity size, dental anatomy, oral microflora, inflammatory processes, and periodontal disease lesions (Genco et al., 1998; Weinberg and Bral, 1999). In particular, the use of gnotobiotic or germ-free rats/mice allows for the study of the individual effect of a particular bacterium without the interference of other microorganisms, whereas the use of knockout mouse models facilitates the exploration of new concepts regarding the pathogenesis of periodontal diseases . Rats can also be used in periodontal tissue regeneration and bone healing studies (Albuquerque et al., 2012; Struillou et al., 2010).

Several authors have pointed that rats does not show periodontitis spontaneously . However, Arai et al., 2005 compared female rats with different ages from 30 to 28 months and found a significant correlation between age and the amount of alveolar bone loss. In addition, a stronger trend was found in mandible than the maxilla (Arai et al., 2005). Aging also have additional effects on rat oral tissues including a gradual reduction of regional blood flow and in decreased levels of eluable (labile) hydroxyproline from the collagen component (Gordon et al., 1978).

3.6 Oxidative stress and periodontal disease

3.6.1 Periodontal disease is associated to high oxidative stress degree at both, local and systemic levels

In spite of the absence of studies quantifying ROS directly due to the difficulties inherent in detecting them (Chapple and Matthews, 2007), researches measuring the biomarkers generated by ROS reacting with different biomolecules have indicated a positive association between oxidative stress and periodontitis. They are exposed below:

- **Lipid oxidative damage biomarkers.** Different studies have suggested an inverse relationship between levels of lipid peroxidation and periodontal health. In humans, various case-control studies analyzing salivary levels of lipid peroxidation biomarkers have indicated higher levels of malondialdehyde (Tsai et al., 2005; Akalin et al., 2007; Guentsch et al., 2008; Wei et al., 2010; Baltacıoğlu et al., 2014) and isoprostanes (Su et al., 2009) in periodontitis patients than in their periodontally healthy counterparts (Su et al., 2009). In addition, a positive correlation with periodontal clinical measures have also been confirmed in cross-sectional studies (Wolfram et al., 2006; Su et al., 2009). GCF also have been analyzed with a similar proposal finding the same associations (Pradeep et al., 2013). This is accordance with the study of Tsai et al. (2005) where salivary and GCF levels of lipid peroxidation biomarker correlated positively. However, it is interesting to note that concentrations in GCF were 200- to 400-fold higher than in saliva. In relation to this findings, it has been suggested to reflect a substantially higher amount of ROS activity (thus lipid peroxidation) in gingival crevicular fluid than saliva, given that the two biological media do not differ greatly in total antioxidant capacity (Brock et al., 2004; Chapple and Matthews, 2007). Results from experiments in rats have shown increased levels malondialdehyde(Di Paola et al., 2005, 2004; Kim et al., 2012; Yağan et al., 2014) and thiobarbituric acid-reactive substances (TBARS) (Holanda Pinto et al., 2008) associated with ligatures placement, as well as in in the serum (Södergren et al., 1998) in experimentally-induced periodontitis models.

- **Protein oxidative damage.** Protein oxidation markers in relation to periodontal health have been evaluated in lesser extent than markers of lipid peroxidation. The most studied in humans have been the proteins carbonyl. In 2003, a cross-sectional study performed on a cohort of patients attending a routine dental check-up reavealed that poor periodontal health assessed by the Community Periodontal Index of Treatment Needs (CPITN) system was associated with increased concentrations of protein carbonyls in saliva, especially if gender influence was considered. More recently, in a case-control study analyzing GCF, this marker displays a similar association since protein carbonyl levels were higher in chronic periodontitis patients than in health controls. Likewise, they showed positive correlations

with different clinical parameters (Baltacioğlu et al., 2008). Protein oxidation have been also evaluated in another study focused specifically in three proteins, transferrin, human Immmuniglobulin G1 (IgG1) heavy chain fragment and amylase, which confirmed the negative relationship (Su et al., 2009). In support of this findings in humans, several studies analyzing gingival tissues from rats have shown positive associations between the degree of protein modification by nitrosative stress and periodontal health, since nitrotyrosine, a footprint of nitrosative stress, -positive cells presence was positively associated with experimental induction of periodontitis by ligation (Di Paola et al., 2004, 2005,?; Lohinai et al., 2001; Nishikawa et al., 2012). These changes were significantly reduced, as were tissue malondialdehyde levels, by the inducible nitric oxide synthase (iNOS) inhibitor, aminoguanidine(Di Paola et al., 2004) or a SOD mimetic that specifically removes superoxide anions (Di Paola et al., 2005). However, in two studies analyzing advanced oxidation protein and glycation end-products in saliva in relation to papillary bleeding index, it has found associations neither in adults (Celecová et al., 2013) nor in children (Tóthová et al., 2013).

- **DNA oxidative damage.** In a rat model of *E. coli* LPS-induced periodontitis , it has significant increase in gingival 8-OHdG levels, as well as in apical migration of junctional epithelium, alveolar bone resorption and number of polymorphonuclear leukocytes (PMNs) (Mousavi-Jazi et al., 2010).Studies evaluating levels of 8-OHdG, the most used biomarker of DNA oxidative damage, in subjects with chronic periodontitis have reported that levels in saliva (Takane et al., 2002; Sawamoto et al., 2005; Su et al., 2009; Sezer et al., 2012) and GCF (Dede et al., 2013) are higher in diseased subjects. 5-kbp mtDNA deletions have been also assessed as marker of DNA damage in saliva, which shows a correlation salivary 8-OHdG levels. Moreover patients with non-deleted mtDNA also shows a higher value of 8-OHdG salivary levels than those without deleted mtDNA (Canakçi et al., 2009). This supporting the role of 8-OHdG as DNA damage biomarker and support that high oxidative stress lead to DNA damage. Likewise, correlations has also been reported with some clinical outcomes (Canakçi et al., 2009; Su et al., 2009; Dede et al., 2013) including probing depth (Canakçi et al., 2009; Sezer et al., 2012), clinical attachment level (Canakçi et al., 2009; Sezer et al., 2012) and CPITN (Su et al., 2009). In one case, subjects with gingivitis were also compared those with periodontitis or periodonally healthy that present lower salivary concentration (Sezer et al., 2012). So, DNA oxidative damages may be a marker of this disease severity and increase when it advances. As with other oxidative stress biomarkers, periodontal treatments also decrease 8-OHdG levels (Takane et al., 2002; Dede et al., 2013). However, there are discrepancies between studies changes in salivary levels were not noted in one study despite this occured in GCF levels (Dede et al., 2013). According to this finding, probably GCF levels are a stronger biomarker.

3.6.2 High oxidative stress level associated with periodontal diseases have been related to low antioxidant status

A variety of studies have demonstrated a reduction in local levels of chain-breaking antioxidants and antioxidant enzyme systems in disease (Ellis et al., 1998; Brock et al., 2004; Akalin et al., 2005). A group found similar levels of SOD in the GCF, but increased

SOD activity in the gingival tissues in periodontitis patients compared with healthy subject, suggesting this might be due to increased need for antioxidant protection during disease (Su et al., 2009). Reductions in total antioxidant capacity and SOD in the GCF were observed in another sample of chronic periodontitis patients compared to healthy controls by the same group (Baltacioğlu et al., 2006). Comparing both saliva and GCF of pregnant women with periodontitis, compared to non-pregnant periodontitis patients and healthy subjects, similar reductions were observed, particularly pronounced in the third trimester of pregnancy (Akalin et al., 2009). The same investigators observed higher ROS measured by a colorimetric method based on the oxidation of ferrous iron to ferric iron in a separate cohort (Akalin et al., 2007). Reductions in total antioxidant capacity from the above-mentioned studies are consistent with what was observed in studies on UK patients (Brock et al., 2004; Chapple et al., 2007), but they are in contrast with an increase in total antioxidant capacity reported in the saliva of periodontitis patients by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay by Su et al. (2009). In a cross-sectional study on 120 individuals ranging from periodontal health to advanced periodontitis, lower antioxidant levels were detected in the GCF of patients with worse clinical periodontal scores (Sculley and Langley-Evans, 2002). Esen et al. (2012) found higher total oxidant status (TOS) and oxidative stress index (balance between free radicals and antioxidants) in the GCF of patients with chronic periodontitis and rheumatoid arthritis. An abnormal glutathione-redox balance, with a potential effect in regulating cellular immune responses, was detected in the GCF of 20 non-smoking periodontitis patients (Grant et al., 2010). GPX levels were investigated in the GCF of healthy, gingivitis and periodontitis patients and were found to increase according to periodontal disease severity (Patel et al., 2009). Serum TOS was found to be higher in periodontitis patients compared to healthy individuals in two separate studies (Akalin et al., 2007; Wei et al., 2004). An association was observed between reactive oxygen metabolites and presence of periodontitis in Japanese populations (Tamaki et al., 2008) and in a large cohort of severe periodontitis patients including chronic and aggresive periodontitis, compared to healthy subjects (D'Aiuto et al., 2010; Masi et al., 2011). Smokers were included in these studies, with the potential residual confounding despite adjustment in the analysis. In a Syrian sample of 30 non-smoking chronic periodontitis, plasma TAOC levels measured with the ABTS assay were found to be lower than in the same number of healthy controls (Abou Sulaiman and Shehadeh, 2010). TAC measured as serum concentrations of mainly uric acid, vitamin A, vitamin C, and vitamin E, was found to be inversely associated with the presence of periodontitis when data from the large Health and Nutrition Examination Survey (NHANES) III on 11,480 US adults were examined (Chapple et al., 2007). The same group, in a smaller cross-sectional study, had observed no difference in plasma TAOC between chronic periodontitis and healthy subjects as measured by enhanced chemiluminescence (Chapple et al., 2007). Systemic reductions in TAOC were also detected in the above-mentioned studies by Masi et al. (2011) and Baltacioğlu et al. (2006). Using the same assay, TAC (renamed small molecule antioxidant capacity) was found to be lower in the plasma of diabetic patients with periodontitis compared to diabetic patients with healthy periodontia (Allen et al., 2011). On the other hand, the antioxidant capacity of venous blood measured by reduction of Nitro blue tetrazolium (NBT) test was found to be higher in periodontitis patients compared to healthy subjects (Žilinskas et al., 2011). Serum SOD levels measured by the reduction of NBT by xanthine–xanthine oxidase system, were found to be higher in periodontitis patients compared to healthy individuals (Wei et al., 2004), in contrast with results of study by Baltacioğlu et al. (2006).

This suggests that any ROS generated could accumulate led to oxidative stress . In rodents, several models of experimentally-induced periodontitis have been analyzed in this sense. A study in a rat model of ligature-induced periodontal disease demonstrated increased plasma lipid peroxides, decreased hepatic reduced glutathione (GSH): oxidized glutathione (GSSG) ratio, and augmented ethanol-induced lipid peroxidation in the liver (Tomofuji et al., 2009). In addition, lipid peroxidation was increased in serum and aorta, as well as in periodontal tissue, and induction of atherosclerosis-related gene expression, histological changes, and lipid peroxidation occurred in the aorta (Ekuni et al., 2009). Other rat model where periodontitis was induced after daily topical application of LPS into the palatal gingival sulcus of maxillary molars (Ekuni et al., 2009) has proven valuable in demonstrating ROS production in the junctional epithelium during the initiation of periodontitis (Ekuni et al., 2008, 2009). Moreover, histological changes associated with disease-induced chronic inflammation such as junctional epithelial down-growth and loss of alveolar bone confirmed disease onset too(Ekuni et al., 2009). Elevated local and plasma oxidative stress as measured by 8-OHdG levels was demonstrated (Ekuni et al., 2008). Histological sections stainining have supported that periodontal alterations prsesent higher levels of ROS. Hydrogen peroxide was shown to be elevated in the disease state by 3,3´-diaminobenzidine staining of histological sections. Similarly, superoxide as measured by 3,3´-diaminobenzidine/manganese chloride staining was also found to be strongly elevated in rat LPS-treated pocket epithelium (Ekuni et al., 2009).

3.6.3 Oxidative stress contributes to periodontal tissues breakdown

Different studies have provided evidences for several mechanisms explaining how high oxidative stress levels could contribute to periodontitis pathogenesis. On one hand, ROS at high levels, or chronically produced, can result in direct damage to cells in vitro. The former includes human periodontal ligament-derived fibroblasts (Tsai et al., 2005) or epithelial cell (Altman et al., 1992) with have resulted damaged by stimulated PMNs (Tsai et al., 2005) or neutrophil myeloperoxidase (Altman et al., 1992), repectively.

Similarly, different extracelular matrix components also has shown to be affected by ROS artificially generated or produced by neutrophils *in vitro* stimulated again. The accumulation of them could disrupt both soft and calcified connective tissues of the periodontium (i.e. gums and alveolar bone). Among the different components, the protein components (core and link) of proteoglycans resulting in component glycosaminoglycans have shown to be attached to smaller peptide fragmentsha preferentially by ROS. In addition, the glycosaminoglycans themselves can be degraded, but differ in their susceptibilities to attack, with sulfated glycosaminoglycans being more resistant than non-sulfated molecules. There are also *in vitro* studies focused specifically on extracellular matrix components of periodontal tissues using proteoglycans extracted from porcine gingivae that were susceptible to degradation by ROS. Such ability of have been also demonstrated within intact frozen sections of this tissue by the same authors (Bartold et al., 1984). More recently, it has been demonstrated that all glycosaminoglycans specifically present in the soft and calcified tissues of the periodontium undergo a variable degree of chain depolymerization and residue modification. In this repect, differences have been noted according its chemical compostition being the more resistant to ROS degradation the sulfated glycosaminoglycans (Moseley et al., 1995, 1997). By contrast, chondroitin sulfate proteoglycans that are present in alveolar bone were particularly susceptible to damage by $\cdot OH$, which caused degradation of both the core proteins and glycosaminoglycan chains (Moseley et al., 1998a). H_2O_2 caused more selective damage with core proteins

being more susceptible than glycosaminoglycan chains. A similar pattern of ROS damage is said to occur with proteoglycans isolated from gingival soft tissue (Waddington et al., 2000). To this direct actions are joined possible changes in bone metabolism, it has been shown that $O_2^{-\cdot}$ and H_2O_2 activate osteoclasts (Bax et al., 1992; Hall et al., 1995) and promote osteoclast formation (Garrett et al., 1990). Furthermore, osteoclasts produce ROS at its ruffle border-bone interface, so, ROS could have more direct role in resorption (Key et al., 1994; Steinbeck et al., 1994). Regarding perdiodontitis, possible direct role of ROS in bone resorption is supported by the finding that $^{\cdot}OH$ and, to a lesser extent, H_2O_2 can degrade alveolar bone proteoglycans *in vitro* (Moseley et al., 1998b). Degradation products found in studies analyzing GCF and tissue extracts from periodontitis patients are in consistency with this. However, it is important to note that the presence of collagen metabolites in GCF could be the result, at least in part, of the presence of a high collagenase activity due to bacterial, host enzymes, or a combination. Still, damage caused by ROS may contribute directly or indirectly to this situation (Chapple and Matthews, 2007).

On the other hand, products of this oxidative damage to matrix and serum proteins can lead to further ROS-induced damage by their priming (Monboisse et al., 2000) and chemotactic actions on neutrophils (Monboisse et al., 2000) and alter different fibroblasts functions (Rittié et al., 2002). According to that, it has been suggested that oxidation-dependent changes in collagen within the periodontal connective tissues could retard neutrophil migration through the tissues and increase their potential to produce ROS (Monboisse et al., 2000; Chapple and Matthews, 2007).In addition, it has been reported that certain ROS activate osteoclasts (Bax et al., 1992; Hall et al., 1995) and promote osteoclast formation *in vitro* (Garrett et al., 1990). ROS-mediated modification of tissue fluid albumin within the periodontal tissues could contribute to the influx of neutrophils seen in disease (Petrone et al., 1980).

Lastly, some data also support that ROS directly can damage on tissue inhibitor of matrix metalloproteinases (Hadjigogos, 2003) or induce alterations in matrix metalloproteinases (MMPs) expression (Kawaguchi et al., 1996; Hemmerlein et al., 2004; Savaraj et al., 2005) as occurs in other tissues. This would be represented in the imbalance of metalloproteinases and their tissue inhibitors in GCF and in tissues associated with disease observed in some studies (Pozo et al., 2005; Soell et al., 2002; Tüter et al., 2005). In any event, ROS-related increases in matrix metalloproteinase activity could play a significant role in the destruction of both the native or oxidatively altered proteins within extracellular matrix (Chapple and Matthews, 2007).

3.6.4 Oxidative stress is thighly linked to inflammatory processes

As previously mentioned, oxidative stress is one of the main factors studied to explain the pathophysiological mechanisms of inflammatory conditions, such as periodontitis (Chapple and Matthews, 2007). As in blood, PMNs, namely neutrophils, are the predominant inflammatory cells in gums connective tissue, pocket epithelium, and within the gingival crevice where they represent the primary cellular host resistance factor against infection (Miyasaki, 1991). Together with other phagocytes, these cells posses a NOX isoform known as NOX2 able to transport electrons across the plasma membrane and to generate $O_2^{-\cdot}$ and other downstream ROS (Bedard and Krause, 2007). It is known that neurophils increase ROS production in response to different bacteria or their components (e.g. LPS). This has been reported for different plaque bacteria associated with periodontal disease including *Fusobacterium nucleatum* (Whyte et al., 1989; Asman and Bergström, 1992;

Sheikhi et al., 2000; Shin et al., 2008), *Aggregatibacter actinomycetemcomitans* (Whyte et al., 1989; Asman and Bergström, 1992) and *Treponema denticola* (Shin et al., 2008). The importance of this production for oxidative stress levels have been confirmed when biomarkers as lipid peroxidation have been measured *(Sheikhi et al., 2001)*. According to these findings, it is possible that the presence of certain types of bacteria in plaque enhance local production of ROS by neutrophils in gingival tissue increasing oxidative stress levels. Although the previous observation have been decribed *in vitro*, an enhanced lipid peroxidation caused by oxidative stress may play a role in the inflammation and destruction of the periodontium in periodontal disease (Tsai et al., 2005; Chapple and Matthews, 2007).

In addition, other factors present at high levels at diseased sites as cytokines may enhance ROS production by neutrophils at local level. Enhanced accumulation of PMNs is associated with the upregulation inter-cellular adhesion molecule 1 (ICAM-1), interleukin-8 (IL-8), IL-1β and TNF-α expression. In periodontal tissues, different cell population might contribute to increase local levels of these factors in response to bacterial products (Kim et al., 2007; Zdarilová et al., 2009). Among them, neutrophils also release different pro-inflammatory cytokines including IL-1β, TNF-α and IL-8 when they are stimulated by similar products promoting their own chemotaxis. Actually, it has been proposed that PMNs might also be important sources of IL-8, IL-1β, and TNF-α production in gingiva in addition to macrophages (Liu et al., 2001). Thus, some bacterial products also can stimulate neutrophils to infiltrate in connective tissues as gum submucosa leading to a local increase in these cells. Consequently, neutrophils accumulation further contributes to local production of ROS. Therefore, it has been proposed that increased oxidative stress generated from PMNs stimulated by chemoattractants, endotoxin, cytokine, and/or adhesion molecules might leads to cell injury in periodontal tissue (Chapple and Matthews, 2007). Simultaneously, they also produces remodeling enzymes as elastase and MMP-8 (Shin et al., 2008) contributing to peridontal tissue destruction.

Because of there are evidences for a decreased anti-oxidant capacity in persons with periodontal disease (see High oxidative stress level associated with periodontal diseases have been related to low antioxidant status) high local levels of ROS inevitably will lead to high oxidative stress levels in susceptible subjects.. The products resulting from oxidative damage in this tissue as may contribute to aggrave the pro-oxidant state and augment the damaging effects of the resultant oxidative stress. This is has been observed for advanced glycation end-products (AGEs) that are able to increase neutrophil adhesion, chemotaxis, and priming in hyper-reactive neutrophils (Brock et al., 2004; Palmer et al., 2005; Panjamurthy et al., 2005; Palmer et al., 2011). The presence of a high receptor for AGEs (RAGE) expression in periodontal tissues (Katz et al., 2005) is an important finding supporting the sensitivity of these tissues to products derived from oxidative damage. Similarly, oxidized LDL have shown to amplify neutrophil ROS generation directly as well as up-regulating adhesion molecules (Lehr et al., 1995; Scaccini and Jialal, 1994; van Tits et al., 2000).

More recently it has been proposed that other cells present in periodontal tissues are also important for oxidative stress levels associated with inflammation, namely fibroblast and keratinocytes (Chapple and Matthews, 2007). Firstly, these cells are able to release citokynes and other inflammatory factors contributing to immune and inflammatory responses and indirectly favoring local ROS production.There are several evidences *in vitro* studies indicating that human gingival fibroblasts release different inflammatory factors in response to bacterial LPS. These include the pro-inflammatory cytokines, IL-1β, TNF-α, IL-6 (Kim et al., 2007; Zdarilová et al., 2009) and IL-8 (Kim et al., 2007); but also

PGE_2 that would be due to an increase in ciclooxigenase-2 (COX-2) expression (Park et al., 2013). The pro-inflammatory transcription factors NFκB and AP-1 have shown to be involved in the changes induced by different bacterial products, but not always in the same way. For instance, it has been reported that the lipoprotein extract of *Tannerella forsythia* induced a NFκB-mediated production of IL-6 and TNF-α, probably by means of Toll-like receptor (TLR)-2 binding (Hasebe et al., 2004), whereas *P. gingivalis* LPS would cause activation of both AP-1 and NFκB pathways via CD14 and TLR-4 leading to the production of IL-1β, TNF-α, IL-6 and IL-8 (Wang and Ohura, 2002). Lastly, although there are less studies, periodontal ligament cells seem to participate in inflammatory and immune processes in a similar way. In support of this role, it has been found that these cells in primary cultures produce IL-6, IL-8, ICAM-1 against major surface proteins of oral spirochetes (*Treponema spp.*), also by activation of NFκB pathway (Lee et al., 2005). On the other hand, keratinocytes from junctional and crevicular epithelium have also shown activities which could expand its role in periodontal alterations beyond constitute a defensive "barrier" against the entry of plaque bacteria and their products into periodontal tissue. As fibroblasts, these cells also are susceptible to produce some inflammatory factors in response to plaque bacteria. In this sense, human oral epithelial cell lines have shown to produce IL-8 (Kusumoto et al., 2004), TNF-α (Krisanaprakornkit et al., 2002) in stimulation studies with different bacteria components. Again, AP-1 and NFκB would be involved in these changes and it is observed that this activation depend on stimulus nature too. For example, *P. gingivalis* presence in primary oral keratinocyte cultures results in up-regulation of c-Jun terminal kinase (JNK) and down-regulation of the MAPK extracellular signal-activated kinase (ERK)1/2, but no in activation of NFκB (Darveau et al., 1998; Watanabe et al., 2001). Moreover, downstream responses of each factor could be different, although simultaneous. This has been illustrated by primary oral epithelial cell cultures stimulated by cell wall extract of *F. nucleatum* that leads to TNF-α and β-defensin-2 production. However, the first is mediated by NFκB activation, whereas the second is due to the activation of AP-1(Krisanaprakornkit et al., 2002). The downstream different response have been also confirmed by the stimulation study with *P. gingivalis* since the absence have associated a lack of IL-8 secretion (Darveau et al., 1998). The importance of periodontal epithelial cells in inflammatory and immune responses have been extolled by an immunohistological study that shows higher presence of NFκB in the suprabasal layers of gingival epithelium from patients compared to controls (Ambili et al., 2005).

Despite its role releasing inflammatory factors, both cell types also could contibute directly to oxidative stress since it is has also reported that they also present NADPH oxidase activities due to isoforms different from phagocytes (i.e. NOX2). In fibroblast, this activiy is usually atributted to the isoform NOX4. Again, stimulation studies indicated that IL-1β, TNF-α increased $O_2^{-\cdot}$ generation by gingival fibroblasts inducing significant ROS production. Similar consequences have bee also observed for other factor that accumulate in diseased sites, TGF-β. fMetLeuPhe (Murrell et al., 1990; Skaleric and Kovac-Kavcic, 2000). Other alterations implicated iNOS and MMPs. LPS up-regulated ROS and PGE_2 production, and expression of COX-2 in human gingival fibroblasts (Park et al., 2013). These cells could be an additional local source of ROS according to results from a variety of stimulation studies (Murrell et al., 1990; Skaleric and Kovac-Kavcic, 2000; Kim et al., 2007; Yoo et al., 2015). In particular, *in vitro* studies with human gingival fibroblasts have shown high levels of ROS against *E. coli* LPS, but also from components from plaque baceria as *P. gingivalis* LPS and Group A streptococcal cell walls. In addtion, it has been reported that gingival fibroblats spontaneously release de-

tectable levels of ROS in culture media containing Ca^{2+} (Murrell et al., 1990; Skaleric and Kovac-Kavcic, 2000). The time course of the spontaneous production of ROS by such cells even would be comparable to that detected in neutrophils and unstimulated endothelial cells, both in form and magnitude (Murrell et al., 1990). The necessity of $Ca^{2+}$for detectable ROS levels in these cultures is particularly interesting since there are high *in vivo* levels of Ca^{2+}in Howship's lacunae (Silver et al., 1988). Thus, the osteoclastic activity in alveolar bone could lead to a local Ca^{2+} level increase favoring ROS production by gingival fibroblasts (Chapple and Matthews, 2007). In keratinocytes case it is known that it isoform, NOX1, have an 20-fold lower activity than NOX2, but chronic production of $O_2^{-\cdot}$ within the crevice or pocket could represent a significance source of local ROS (Chamulitrat et al., 2004). Some *in vitro* studies have supported this statement. LPS-treated periodontal porcine epithelial cell line cultures, a model established by Pan et al. (1995), showed significantly increased H_2O_2 and $O_2^{-\cdot}$ positive staining. In the case of LPS treatment, the distribution of staining for both ROS species seemed to be consistent with previous reports of H_2O_2 extracellular diffusibility (Waghray et al., 2005).

3.6.5 Age-related changes in inflammatory processes could increase periodontal damage

In 1996, Fransson et al. carried out a study comparing the effects of oral hygiene abstention in elderly (aged 65-80 years) and young (aged 20-25 years) subjects after receiving professional tooth cleaning for 4 weeks. After 3 weeks, it was noted that elderly subjects developed more gingivitis in spite of the amount of biofilm formed during this period was similar. This would indicated a higher susceptibility against inflammatory disorders in elderly subjects at periodontal level too. In that sense, the mentioned study (Fransson et al., 1996) also reported that lesions formed in the older individuals was more pronounced and contained more inflammatory cells according to the measurements and morphometric determinations made in biopsy samples of the gingival lessions. Regarding levels of cytokines related to inflammatory proccesses, it has been reported that young subjects (aged 20-22 years) presented higher levels of TNF-α, IL-8 and IL-6 in GCF than old subjects (61-65 years old), both after receiving a professional plaque control programme to establish healthy gingival conditions(Tsalikis, 2010). Similarly to the previous study from Fransson et al. (1996) clinical outcomes measured also suggest a faster worsening of periodontal health in old subjects as revealed the plaque and bleeding scores and the GCF volume. Levels of IL-6, but not IL-8 or TNF-α, but also of IL-1β were analyzed in gingival biopsies in other study where more age groups were established (prepubertal children, young adults, mature adults and elderly adults). In this case, it was found that local levels of IL-1β and IL-6, but not IL-8 or TNF-α are associated to inflammation presence in young adults, but this relationship was age-dependent (Yakovlev et al., 1996). Some systemic inflammatory markers in older people (IL-6, TNF-α) have been related to periodontal disease extent (Bretz et al., 2005). In patients with periodontitis, high levels of PGE_2 were observed to be related to the severity of periodontal disease and the increase in alveolar bone loss (Gazi et al., 1997; Tsai et al., 1998). PGE_2 may have an indirect effect on alveolar bone resorption by stimulation of osteoclasts by the action of other cytokines involved in this process (Lader and Flanagan, 1998). Age-related differences in inflammation markers or pro-inflammatory mediators levels also has been reported in animals. In rhesus monkeys (*Macaca mulatta*), it has been reported that systemic levels of PGE_2 and regulated on activation, normal T cell expressed and secreted

(RANTES) were increased with age, although $PGE_2$was significantly increased in the younger group (aged 3 years old or less) respect than those found in the older animals. In addition, serum antibody levels in response to multyple periodontal pathogens were generally lower in the young animals and were significantly related to aging in the cohort (Ebersole et al., 2008).

Results from different reports suggest that such inflammation markers and cytokines levels could be due, a least in part, to some cells present in periodontal tissues secreted higher basal levels of inflammatory factors. In this way, periodontal ligament cells from the elderly people showed an increase in the production of plasminogen activator (PA) (Mochizuki et al., 1999) PGE_2, IL-1 (Abiko et al., 1998) and IL-6 (Ogura et al., 1996) when compared to younger cells, similar to those described for stimulation with LPS (Savitt and Kent, 1991). The effect of aging on these mediators has been confirmed by gene expression analysis for IL-6 and IL-1β (Benatti et al., 2009). In addition, cells from elderly individuals showed greater activity of PA whose levels have been found particularly linked to periodontitis and greater expression of tissue PA (tPA) mRNA when compared to those from young individuals (Miura et al., 2000). The greater release of PA induced by aging might affect gingival fibroblasts and the periodontal ligament, and aggravate the inflammatory process and the degradation of the extracellular matrix in periodontal tissues of elderly people (Miura et al., 2000; Mochizuki et al., 1999). Results for animal studies extend this effect to other cells. In murine gingival fibroblast, a higher synthesis of PGE_2 and IL-1 have fond when they come from older mice (aged 20 months old) (Okamura et al., 1999; Shimizu et al., 1997). Gene expression analysis under resting conditions revealed that aging could affect synthesis of futher protein relevant for periodontal inflammation in this murine cell type (Domon et al., 2014). Moreover, several experiments have suggested an increase in some cells reactivity to bacterial products and other posible stress . Both stimulus, LPS and mechanical stress, repectively, led to increased *in vitro* productions of PGE_2 and increased COX-2 gene mRNA levels in fibroblast obtained from elderly donors (Takiguchi et al., 1996; Ohzeki et al., 1999). Fibroblasts from old rat are also particularly susceptible to activation by similar stimuli secreting higher levels of PGE_2 and IL-1β (Okamura et al., 1999; Shimizu et al., 2000). Similarly, human gingival fibroblasts stimulated by LPS from elderly donors also displayed a greater production of IL-6 when compared to young donors. Interestingly, it has been reported that surface expression of receptors that amplify inflammation was elevated in murine aged macrophages (Liang et al., 2009). According to this finding, it is possible that the increase in reactivity observed in fibroblasts was due to age-related changes in some of their recepeptors.

Lastly, menopause could be an additional risk factor in women (Reinhardt et al., 1999). The pattern of IL-1 and IL-6 secretion in menopausal women with periodontal disease have been analyzed and higher rates were found in untrated menopausal patients compared to those receiving hormonal therapy. Likewise, untreated patients with estrogen deficiency showed greater levels of IL-1 and IL-8 in GCF that subjects without estrogen deficiency (Payne et al., 1993). Therefore, it seems that there is a positive association between estrogens levels and pro-inflammatory cytokines, at least in some situations, which could aggravate periodontal health in older women.

3.7 Dietary therapies to reduce oxidative stress against periodontal aging and disease

3.7.1 Experimental Studies on Dietary fat effects on age-related changes of periodontium

studies usually evaluate the possible effect of diets with different lipid content or fatty acid profile in the diet. At the moment, it is difficult to find human studies that analyze the total dietary amount of this macronutrient on periodontitis by total intake estimates or experimental interventions. In turn, there is much information from dietary interventions in animals, particularly rats (Azuma et al., 2011; Cavagni et al., 2013) and mice (Zhou et al., 2011), although rabbits have been used too (Bullon et al., 2009). Almost all studies have increased the dietary fat amount, usually by increasing saturated fat-rich foods (Tomofuji et al., 2005; Zhou et al., 2011), adding cholesterol (Tomofuji et al., 2005; Sanbe et al., 2007a) or both (Bullon et al., 2009), to induce atherosclerosis (Bullon et al., 2009), obesity (Cascio et al., 2012) or/and diabetes, in many cases due to energy intake increase. Several studies have reported associations between consumption of those experimental diets and outcomes related to higher alveolar bone destruction (Cavagni et al., 2013; Sanbe et al., 2007b) or inflammation (Bullon et al., 2009). Others have combined dietary treatments with additional methodologies to evaluate the effect of the diet on the biological response to periodontal microorganisms. In rats, high cholesterol diets enhanced proliferation of the junctional epithelium with increasing bone resorption and cell-proliferative activity associated with application of bacterial LPSs and proteases to the gingival sulcus (Cascio et al., 2012). Excessive intake of this type of nutrients has negative effects on periodontal health, especially if they include saturated fat or cholesterol. Several markers of inflammation or oxidative stress further are elevated when measured at a local or systemic level including 8-OHdG and depletion of GSH content (represented by low GSH:GSSG ratios) (Azuma et al., 2011; Sanbe et al., 2007a; Tomofuji et al., 2006). Similarly, the effects of bacterial pathogens and their products on production of pro-inflammatory cytokines in fibroblasts were augmented (Tomofuji et al., 2006).

On the other hand, qualitative aspects of lipids in the diet also have major importance for the severity of periodontal disease. Several studies have investigated the potential effects of different fatty acids from diet or as supplements, on periodontal disease, both in humans and in animal models. The most important evidence supporting SFA negative effects derived from a longitudinal study initiated in 1998 in Niigata City (Japan), to evaluate the relationship between systemic health and dental diseases(Iwasaki et al., 2008). It assessed persons aged 70 years old non hospitalized, nor institutionalized; after five years, a dietary assessment (by self-administered diet history questionnaire) was conducted and dental health of the cohort was followed-up for one year. Eventually, a negative association was found between SFA intake and the number of periodontal disease events (Iwasaki et al., 2011), although only in non-smokers (Iwasaki et al., 2011). These results support a causal association for saturated fat intake on periodontitis progression, but just in older people, so more cohort studies are required to extend that effect to other age groups.

The effects of PUFA on periodontitis, in turn, have received more attention, and to establish conclusions requires making distinctions between n-3 and n-6 PUFA. Particularly, most studies have focused on the possible positive effects on periodontitis for polyunsaturated fatty acids belonging to the n-3 series, either as preventive or adjunc-

tive during treatment. These fatty acids include ALA and its derivatives, mainly EPA, and DHA. In a cross-sectional study, Naqvi et al. (2010) reported negative associations among dietary intakes of those three representative n-3 PUFA and periodontitis. As for longitudinal studies, results from the Niigata City study are available too. In these, an inverse independent relationship with periodontal disease progression was found just for energy-adjusted dietary intakes of DHA after controlling for several confounding factors, but not of total n-3 PUFA or EPA (Iwasaki et al., 2010, 2011). In addition, there are available data from studies evaluating fatty acids levels in serum (Figueredo et al., 2013; Ramirez-Tortosa et al., 2010) but in general they do not clarify the role of the different Fa type in periodontal disease. In one of these, it has been reported higher levels of n-6 PUFA, SFA and MUFA than in periodontally healthy persons (Ramirez-Tortosa et al., 2010). In other, serum levels were compared between patients with chronic generalized periodontitis and those displaying only gingivitis. In this, patients with chronic generalized periodontitis had higher levels of arachidonic acid as representative of n-6 PUFA but also of n-3 PUFA (DHA, DPA and EPA) (Figueredo et al., 2013). This could indicate that higher intake of all these PUFA would facilitate progression of gingivitis to generalized periodontitis. However, calculated ratios between these fatty acids, which seem more important, did not differ between groups (Figueredo et al., 2013). Moreover, gingivitis patients now could show periodontitis in the future. Therefore, to establish any causal relationship, some cohort studies are needed.

Some interventions have evaluated the utility of using supplements of n-3 PUFA alone or supplementing periodontal therapy, both in animals and humans. A combination of DHA and EPA as supplement to a regular diet was tested in rats with induced-periodontitis by LPS injections; their use was ineffective in preventing alveolar bone loss, either alone or combined with therapy (El-Sharkawy et al., 2010; Vardar et al., 2004). This is consistent with data from the transversal study using a National NHANES subset performed by Naqvi et al. (2010), who noted that associations hardly changed after accounting amounts from supplements containing DHA, EPA, or ALA or after excluding people reporting their use, except for ALA. Despite the absence of bone resorption differences, both treatments with n-3 PUFA maintained lower gingival levels of different membrane phospholipid mediators in respect to only endotoxin-treated rats (Vardar et al., 2004). When rats received prophylactic treatment, levels were similar to healthy controls for PGE_2, leukotriene B_4 (LTB_4), and PA factor (PAF), whereas in rats treated only during periodontitis induction, only PGE2 remained in similar levels although LTB4 levels were lower than in disease controls. On the other hand, serum levels of IL-1β and osteocalcin are higher in rats receiving either n-3 PUFA treatment than in only LPS-treated rats. Curiously, PGF2alfa decreased in respect to both healthy and diseased rats without treatment, and only in rats with a non-prophylactic n-3 PUFA treatment (Vardar et al., 2004). Serum C-reactive protein (CRP) levels, in turn, did not show differences among groups (Vardar-Sengül et al., 2006).

In humans, n-3 PUFA supplementation effect was investigated in the treatment of experimental gingivitis. Healthy volunteers who discontinued oral hygiene and were treated with fish oil showed a significant decrease in gingival index compared to controls (Campan et al., 1997). Additionally, in a parallel double blind clinical study, advanced chronic periodontitis patients daily treated with fish oil and aspirin after scaling and root planning demonstrated reduction in periodontal probing depth and attachment gain after three and six months in the n-3 group, compared to baseline and to the placebo-treated group. This suggests that supplementation with n-3 PUFA and aspirin resulted in a significant shift in the frequency of periodontal probing depths $\leq$4 mm. Salivary receptor

activator of NF-kappaB ligand (RANKL) and Matrix MMP-8 levels showed significant reductions in the n-3 group at three and six months, and compared to the control at six months (El-Sharkawy et al., 2010).

Regarding n-6 PUFA, those derived from linoleic acid showed harmful effects, if they were consumed at a higher proportion than n-3 polyunsaturated acids. Likewise, Iwasaki et al. (Iwasaki et al., 2011), using estimated energy-adjusted dietary intakes, found that a high n-6:n-3 PUFA ratio, but not total n-6 acids intake, was significantly associated with a greater number of periodontal disease events in the elderly. In fact, authors consider dietary n-6:n-3 PUFA ratio as the main predictor to estimate influence on periodontal disease events (Iwasaki et al., 2011). An intervention study in humans demonstrated a beneficial effect of n-3 and n-6 PUFA on periodontitis improving clinical outcomes. Adults with periodontitis received supplementation with olive oil as placebo, fish oil as a source of n-3 PUFA (mainly EPA) or borage oil as source of n-6 PUFA (mainly gamma-linolenic acid, GLA). Improvement in gingival inflammation was observed in subjects treated with borage oil, whereas improvement in probing pocket depth was seen in those subjects treated with either fish oil or borage oil (Rosenstein et al., 2003).

Animal studies in rodents also suggest beneficial effects of a diet with lower n-6:n-3 PUFA ratio. Usually, diets with high content of n-3 PUFA use fish oil as fat source, whereas n-6 acids-rich diets are more diverse although corn and sunola oil are the most common sources. Despite diet similarities, they differ according to experimental periodontitis induction. Female mice inoculated with bacteria exhibited less alveolar bone loss when fed with tuna oil than with the sunola oil treated group (Bendyk et al., 2009). Likewise, alveolar bone resorption was also reduced in female rats with induced periodontitis by bacterial inoculation treated with fish oil more than with a corn oil-containing diet (Kesavalu et al., 2007). In rats, whose teeth were subjected to a continuous force, fed diets containing refined fish oil showed less tooth movement, accompanied by less bone resorption than those fed diet containing corn oil (Iwami-Morimoto et al., 1999). Genetic expression analyses for several biomarkers (interferon-gamma, IL-1β and TNF-α) and different enzymes (COX-2, catalase and SOD) in gums have suggested that this beneficial is consequence of the modulating effect of n-3 PUFA on gingival inflammation (Kesavalu et al., 2006, 2007). Additionally, when EPA, DHA and ALA levels were analyzed in oral soft tissues, they were higher in animal fed diet rather than in fish oil (Bendyk et al., 2009). Periodontal tissue membrane lipid profile changes along with those of inflammation mediators levels in both blood and periodontal tissues, combined with improvements of some periodontal outcomes, suggests that dietary modifications of fatty acids present in biological membranes might protect periodontal tissues of local and systemic hazards.

Recently, interest in the role of MUFA on periodontitis has surfaced. According to research in rats, MUFA might offer similar or even better advantages than n-3 PUFA (Bullon et al., 2013). two nutritional interventions in humans (Campan et al., 1997; Rosenstein et al., 2003) where olive oil was used as placebo do not support this idea. Both control groups showed worst outcomes at the end than those treated with n-3 PUFA (Campan et al., 1997; Rosenstein et al., 2003) or even than n-6 PUFA-treated groups (Rosenstein et al., 2003). Differences with these trials could be to the use of non-virgin olive oil, so certain natural bioactive compounds from the minor fraction of virgin olive oil, particularly phenolic components, were not present. Among others, hydroxytyrosol has demonstrated important antioxidant and anti-inflammatory properties, which could be of interest for the prevention of diseases traditionally related to oxidative stress, such as cardiovascular diseases and cancer (Granados-Principal et al., 2010). Among other

findings, it has shown to protect LDL particles from lipid peroxidation both in vitro (Leenen et al., 2002; Salami et al., 1995) and in vivo (González-Santiago et al., 2006; Marrugat et al., 2004), as well as to inhibit PGE_2 synthesis (Maiuri et al., 2005). Another major compound present in olive oil, squalene, also has shown important antioxidant properties in vitro (Aioi et al., 1995; Kohno et al., 1995) and it has been suggested that it may have a critical role reducing sunlight-derived free radical oxidative damage to the skin (Aioi et al., 1995; Huang et al., 2009) since it is one of the main lipids in its surface (Passi et al., 2002). Results from an experimental model of atherosclerosis and stetatohepatitis in rabbits (by a 50 days' treatment with a saturated fat-rich diet with cholesterol) manifested some inflammation features also at gingival level. After development of atherosclerosis, subsequent intervention with hydroxytyrosol reduced endothelial activation and, with squalene, additionally decreased fibrosis. The results suggest that the gingival vascular changes observed after the atherosclerotic diet have been reversed by hydroxytyrosol and squalene (Bullon et al., 2009). Furthermore, it is important to note that interventions use olive oil as supplemental placebo, and it might not modify the largely lipid profile of diet. This would not markedly increase the content of MUFA in biological membranes, which could be another reason for the positive role of olive oil as a fat source.

To date, hydroxytyrosol has been demonstrated in numerous studies to have antiatherogenic, anti-inflammatory and antitumor effects, which have been widely reviewed (Granados-Principal et al., 2010). Regarding the periodontum, a study in rabbits reported a certain anti-inflammatory effect of supplementation with hydroxytyrosol. Specifically, it was reported that it reduced endothelial activation in rabbits that had developed atherosclerosis induced by a diet rich in saturated fats, but it did not improve endothelial activation and lower cellularity in gingival mucosa, which are also associated with this pathology (Bullon et al., 2009). At present, it is well established that the health benefits of olive oil are not concentrated solely in its fatty-acid content (Granados-Principal et al., 2010), since it has been noted that refined olive oil (free of phenols) lacks the antioxidant effects present in virgin olive oil (Quiles et al., 2002; Sanz et al., 2006), whereas the phenolic fraction of olive oil considerably improves the lipid peroxidation of LDL lipoproteins, which is related to an increase in antioxidant capacity (Ochoa-Herrera et al., 2001). The oil also includes several bioactive compounds with high antioxidant capacity, such as hydroxytyrosol (Pérez-Jiménez et al., 2007; Visioli and Galli, 2002).

3.7.2 Experimental Studies on CoQ effects on age-related changes of periodontium

Many clinical trials with oral administration of CoQ to patients with periodontal disease have been conducted. The results have shown that oral administration of CoQ_{10} increases the concentration of CoQ_{10} in the diseased gum and effectively suppresses periodontal inflammation (Shizukuishi et al., 1986; Wilkinson et al., 1975). Furthermore, they indicated that CoQ_{10} might reduce gingival inflammation without affecting total antioxidant levels in the GCF (Denny et al., 1999). Oral administration of CoQ combined with vitamin E has also shown a beneficial effect on the periodontal tissue, since periodontal pockets shallowed by 30% accompanied by a decrease of several clinical outcomes (including plaque index, interdental hygiene index, gingival index and sulcus bleeding index) (Matthews-Brzozowska et al., 2007). In addition to oral administration, other uses of CoQ also offer promising results. In rats, ubiquinol applied topically on the gingival surface suppresses age-related inflammatory reactions, the mechanism for this

is suggested to be the lower gene expression of caspase-1, IL-1β and Nod-like receptor protein 3 inflammasomes in the periodontal tissue. It also leads to a differentiation of osteoclasts associated with aging, probably by inhibiting oxidative stress as indicated by a decreased oxidative DNA damage (Yoneda et al., 2013). Topical application is a convenient method in the dental clinical setting, and clinical trials evaluating benefits of CoQ topical (i.e., extrasulcular) application (in gel-form) also exist in humans (Hanioka et al., 1994; Hans et al., 2012; Sale et al., 2014). Moreover, intra-pocket application was tested as well in the same trials (Hanioka et al., 1994; Sale et al., 2014). These studies showed that both types of application of a gel containing CoQ_{10} for 6 weeks improved major clinical parameters. Further, CoQ_{10} use has also been tested after a scaling and root planning treatment (Hanioka et al., 1994; Sale et al., 2014), and its application combined with mechanical treatment offered better results, but only when the gel was applied to the periodontal pockets (Hans et al., 2012).

Part II

HYPOTHESIS AND OBJECTIVES

Hypothesis

Rats life-long fed on normolipidic diets with different fat types and supplemented or not with CoQ_{10} present differences in different organs and tissues in response to the interaction between aging and diet. These have been related to mitochondrial mechanisms and oxidative stress levels due to the role of this compounds have in cellular physiology. Moreover, the development and progression of chronic pathologies has been related to this phenomenon and they can be influenced by this dietary factors. As part of organism, variations suffered by the periodontal tissues and chronic pathologies affect them, such as chronic periodontitis, shall be the same phenomenon. Thus age-associated changes in this tissue would also depend on diet and it will modulate susceptibility to disease and aging detrimental effects in periodontal tissues by means of mechanism related to oxidative stress and mitochondria.

Objetives

The present study reproduced an age-dependent rat model of periodontium to assess the physiological effects on periodontal tissues of diets with different fatty acid profile and the possible modulating effect that may exercise the addition of CoQ_{10} to them. This general objective It is developed through three specific objectives:

1. To assess the impact of the type of fat in the conditions of a morphometric and histological periodontal regarding the age level tissues.

2. To evaluate the effect of adding CoQ_{10} to diet on the physiological effects that have different types of fat in periodontal conditions for morphometric and histological age related tissue level.

3. To explore some possible mechanisms involved in these effects locally, particularly those that have to do with changes in bone metabolism and immune-inflamatory processes.

4. To explore the potential contribution that systemic changes in bone metabolism and immune-inflamatory processes may have on them

5. To explore how can contribute to oxidative stress levels locally and the possible involvement of cellular processes related to mitochondria and the defense against oxidative stress in the gingival tissue.

Part III

MATERIAL AND METHODS

Chapter 4

Material

4.1 Chemicals

Reagents and solvents used, of the highest quality came from laboratories Fluka (St. Louis, Missouri, USA), Merck Millipore (Hohenbrunn, Germany), Sigma-Aldrich (St. Louis, Missouri, USA), Panreac (Barcelona, Spain), Boehringer (Manheim, Germany), Roche (Basel, Switzerland), Bio-Rad (Hercules, California, USA), Cayman Chemical Company (Michigan, USA), Macherey-Nagel (Durën, Germany) and Thermo Scientific (Rockfor, Illinois, USA). Standards to fatty acids determinations, CoQ_9 and CoQ_{10} were supplied by Sigma-Aldrich and Fluka. HPLC quality reagents and solvents were acquired in laboratories Merck and Panreac.

Virgin olive oil came from "Venta del Llano", from Research and Training Institute for Agricultural and Fisheries of Andalusia (IFAPA); sunflower oil was bought in a local supermarket, and fish oil was acquired from Roche (Basel, Switzerland). The other components used to elaborate diets were purchased to Musal Chemical (Granada, Spain).

4.2 Equipment

- -20ºC freezer Liebher (Liebher, Biberach, Denmark). -80ºC freezer Revco VLT-1786-5-VUA (Revco, Asheville, North Carolina, USA).
- Automatic pipettes NICHIPET EX (Nichiryo, Tokyo, Japan).
- Automatic staining equipment Leica Autostainer XL (Leica, Wetzlar, Germany). Cold plate Leica EG1150C (Leica, Wetzlar, Germany).
- Computer Asus P6-P8H61E, Mini Tower, Core i3 2100 3.1 Ghz.
- Dako Automation Hematoxylin Histological Staining Reagent (Dako Denmark A/S, Denmark).
- Digital scale GRAM PRECISION (Gram Precisión, SL. Barcelona, Spain).
- Electronic micropipettes EPPENDORF RESEARCH PRO. (Eppendorf, Hamburg, Denmark).

- Eppendorfs microcentrifuge MiniSpin Rotor F45-12-11 (Eppendorf, Hamburg, Denmark).
- Ethanol 100%, partially denatured (Panreac Química SA, Barcelona, Spain).
- Fully Automated Glass Coverslipper Leica CV5030 (Leica, Wetzlar, Germany).
- Gas-liquid chromatograph model HP-5890 Series II (Hewlett Packard, Palo Alto, California, USA).
- Histological bath for inclusion in paraffin Termofin (JP Selecta, Barcelona, Spain).
- HPLC system: 3mm ODS Supelcosil column (Beckman Instruments, Fullerton, California, USASigma-Aldrich, St. Louis, Missouri, USA).
  - Analytical cell model 5011 (Thermo Scientific, Rockfor, Illinois, USA).
  - Beckman System Gold 125 pump (Beckman Instruments, Fullerton, California, USA).
  - Electrochemical detector ESA Coulochem III (Thermo Scientific, Rockfor, Illinois, USA).
  - Guard cell model 5020 (Thermo Scientific, Rockfor, Illinois, USA).
  - Water 717 plus autosampler (Milford, Massachusetts, USA).
- Ice machine SCOTSMAN AF-10 (Scotsman Ice Systems, Vernon Hills, Illinois, USA).
- Incubator (JP Selecta, Barcelona, Spain).
- Isoparaffin H, Xylene substitute (Panreac Química SA, Barcelona, Spain).
- Magnetic shaker with heater, AGIMATIC-E. (JP Selecta, S.A, (Barcelona, Spain).
- Microplate's reader SYNERGY HT, MultidetectionMicroplate Reader (BioTek Instruments, Inc. Higland Park, Vermont, USA).
- Microplate's Shaker Heidolph Titramax 100 150-1350 1/min (Heidolph Instruments GmbH & Co.KG, Schwabach, Germany).
- Microtome blades 250 µm thick (Feather Safety Razor Co, Osaka, Japan).
- Microtome Leica RM2235 (Leica, Wetzlar, Germany).
- Mounting Medium for Xylene Surrogates (Panreac Química SA, Barcelona, Spain).
- Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Rockfor, Illinois, USA).
- Optical microscope ECLIPSE E600 (Nikon Co., Tokio, Japan).
- pH-metre GLP21 (, Crison, (Barcelona, Spain).
- Platform-shaker Heidolph Polymax 1040 2-50 1/min (Heidolph Instruments GmbH & Co.KG, Schwabach, Germany).
- Potter-Elvehjem PTFE glass tube (Sigma-Aldrich, St. Louis, Missouri, USA).

- PTM 2330 F.S column (Supelco Inc, Bellefonte, Palo Alto, California, USA).
- Refrigerated centrifuge BECKMAN model J-21 (Beckman Coulter, Inc. Fullerton, Palo Alto, California, USA).
- Sterile distilled water (Fresenius Kabi AG, Sevres, France).
- Teflon pestle (Heidolph Instruments GmbH & Co.KG, Schwabach, Germany).
- Thermal Cycler of Applied Biosystems model 2720 (Life Technologies Corporation, Carlsbad, California, USA).
- Ultracut S ultramicrotome EM UC6 (Leica, Illinois, USA).
- Vortex VWR (VWR International Eurolab S.L., Barcelona, Spain).
- Washing station Fluidics Station 400 (Affymetrix, Santa Clara, California, USA).

4.3 Software

- AnaliSYS Image Processing Software (Olympus, Hamburg, Germany).
- Autostainer equipment Software DakoLink, version 1.5.2.1 (System Dynamics, New Jersey, USA).
- Fluidigm technology (www.fluidigm.com).
- Gene Chip Operating Software (GCOS 1.4, Affymetrix).
- GeNorm program (http://medgen.ugent.be/jvdsomp/genorm).
- Ingenuity Pathways Analysis (IPA) application (Ingenuity Systems, www.ingenuity.com).
- Partek Genomics Suite software. SPSS 20.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Windows 7 Home Premium.

Chapter 5

Methodology

5.1 Experimental desing

Seventy-two male Wistar rats *(Rattus norvegicus*) initially weighing 80-90 g were used for this study. The rats were randomly assigned into six experimental groups fed on different diets and match-fed from weaning until 24 months of age on a semi-synthetic and isoenergetic diet according to the AIN93 criteria described by Revees (1993; 1997), but modified in relation to the dietary fat source.

Modified AIN-93G diet was administered to rats from weaning to 2 months of age and AIN-93M was administered for the rest of the experiment. Components of AIN-93G (caloric content 401,4 Kcal/100g) and of AIN-93M (caloric content 362,4 Kcal/100g) are shown in tables 5.1.1, 5.1.2 and 5.1.3. As indicated above, AIN93 diets were modified in relation to the fat source. Original AIN93 diets are composed by soya oil. In this study, this oil was replaced by virgin olive oil (virgin olive oil fed group), sunflower oil (sunflower oil fed group) or fish oil (fish oil fed group) to formulate the experimental diets and groups. Table 5.1.4 shows the fatty acid profile of experimental oils. In three of the groups, diets were also supplemented with 50mg/kg/d of CoQ_{10}. Diets were prepared weekly and stored in darkness at 4ºC to avoid lipid peroxidation. Animals were placed

Table 5.1.1: AIN93 diets composition.

Components	AIN-93G (%)	AIN-93M (%)
Starch	38.7	46.6
Casein	20.0	14.0
Dextroxe	13.2	15.5
Sucrose	10.0	10.0
Dietary fat	8.0	4.0
Fiber (celulose)	5.0	5.0
Mineral mixture	3.5	3.5
Vitamin mixture	1.0	1.0
L-Cysteine	0.3	0.18
Methionine	-	-
Choline bitrate	0.25	0.25

Table 5.1.2: Vitamin mixture content.

Components	AIN-93G (%)	AIN-93M (%)
Nicotinic acid	0.3	0.3
Panthotenate Ca	0.16	0.16
Pirydoxine-HCl	0.07	0.07
Thiamine-HCl	0.06	0.06
Riboflavin	0.06	0.06
Folic Acid	0.02	0.02
D-Biotin	2	2
Vitamin K	0.0075	0.0075
Vitamin B-12	0.25	0.25
Vitamin A	0.08	0.08
Vitamin D	25	25
Vitamin E	1.5	1.5
Powder sucrose	97.5	97.5

Table 5.1.3: Mineral mixture content.

Components	AIN-93G (%)	AIN-93M (%)
Calcium biphasic phosphate	-	-
Calcium carbonate anhydride. 40.04% Ca	35.7	35.7
Potassium monophasic phosphate. 22.76% P; 28.73% K	19.6	25.0
Tripotassium monohydrate citrate	7.1	2.8
Sodium chloride	7.4	7.4
Potassium sulfate	4.7	4.7
Manganese oxide	2.4	2.4
Ferric citrate	$6.06x10^{-1}$	$6.06x10^{-1}$
Zinc carbonate	$1.65x10^{-1}$	$1.65x10^{-1}$
Magnesium carbonate	$6.303x10^{-2}$	$6.3x10^{-2}$
Cupric carbonate	$3x10^{-2}$	$3x10^{-2}$
Iodate potassium	$1x10^{-3}$	$1x10^{-3}$
Selenate potassium	$1.025x10^{-3}$	$1.025x10^{-3}$
Ammonium paramolybdate	$7.95x10^{-4}$	$7.95x10^{-4}$
Sodium metasilicate	145	145
Potassium chrome sulphate	$2.75x10^{-2}$	$2.75x10^{-2}$
Lithium chloride	$1.74x10^{-3}$	$8.15x10^{-3}$
Boric acid	$8.15x10^{-3}$	$6.35x10^{-3}$
Sodium fluorouridine	$6.35x10^{-3}$	$3.18x10^{-3}$
Nickel carbonate	$3.18x10^{-3}$	$1.74x10^{-3}$
Ammonium vanadate	$6.6x10^{-4}$	$6.6x10^{-4}$
Powder sucrose	22.1	21.0

Table 5.1.4: Fatty acids profile of experimental dietary fats (g/100g).

Fatty acid or index	Virgin olive oil	Sunflower oil	Fish oil
C14:0	0.0	0.1	7.2
C16:0	8.3	6.4	17.1
C16:1n-9	1.1	0.1	9.6
C18:0	3.2	4.7	2.7
C18:1n-9	77.7	24.2	15.1
C18:2n-6	3.2	62.8	2.8
C20:3n-6	0.1	0.9	0.1
C20:4n-6	0.0	0.0	2.1
C20:5n-3	0.2	0.1	18.6
C24:0	0.0	0.1	0.3
C24:1n-9	0.0	0.0	0.9
C22:6n-3	0.0	0.0	10.5
Total saturated fatty acids	12.6	11.5	30.5
Total monounsaturated fatty acids	83.7	24.4	30.1
Total n-6 polyunsaturated fatty acids	3.3	63.7	8.2
Total n-3 polyunsaturated fatty acids	0.4	0.4	31.3
Total polyunsaturated fatty acids	3.7	64.1	39.4

in a climate-controlled room (20ºC, 12 h dark/12h light cycle) for two years in collective cages, in groups of three animals per cage, with free access to water. Diet was delivered *ad libitum* for the first two months and then at 25 grams/rat/day for the rest of the experiment (in order to avoid overweight). Food intake was indirectly monitored through the weekly body weight control and daily spillage monitoring.

The animals were treated in accordance with the guidelines of the Spanish Society for Laboratory Animals and the experiment was approved by the Ethical Committee of the University of Granada (permit number 20-CEA-2004). The rats were killed by cervical dislocation followed by decapitation, at the same time of the day to avoid any circadian fluctuation.

5.2 Samples collection

At the time of animal sacrifice, blood was collected in ethylenediaminetetraacetic acid (EDTA)-coated tubes and the plasma was centrifuged at 1750 x g for 10 min. Plasma samples were stored at -80ºC until analyzed. The blood was allowed to clot for at least 30 min before centrifugation for 10 min at 1000xg. After centrifugation, serum was isolated, aliquoted and stored at -20ºC. After exsanguination, mandibles were extracted and divided into left and right side and samples taken from molar gingival mucosa (always the same for all the animals) were fixed in buffered formalin and paraffin-embedded for histologic analysis. Gingival tissue was excised from around the maxillary molars for use in quantitative real-time PCR (QRT-PCR) to assess mRNA expression of selected genes and other molecules of interest. Gingival tissues were preserved in immediately immersed in RNAlater RNA Stabilization Reagent at room temperature. RNAlater(Sigma-Aldrich, San luis, MO, USA) at -80ºC.

5.3 Fatty acid profile of plasma and dietary fat sources

fatty acid analysis of biological specimens by gas-liquid chromatography requires solvent extraction, purification, and derivatization procedures that are both lengthy and heavy. The fatty-acid profile of plasma and dietary fat sources was measured by gas-liquid chromatography with the Gas-liquid Chromatograph model HP-5890 Series II (Hewlett Packard, Palo Alto, CA, USA) equipped with a flame-ionization detector followingLepage and Roy (1986) method by which fatty acids are determined as methyl esters. This avoids most of the preparative steps since only requires in one step methylation and transesterification of samples.

For this, 100μL of each sample were dissolved into 2 ml of methanol/benzene (4:1, v/v) mixture in glass tubes and 200μL of acetyl chloride were slowly added. The tubes were covered to avoid the volatilization of the contents. Then, they were shaken and subjected to methanolysis in a bath at 100ºC for 1 h. After this time, the tubes were removed and allowed to cool at room temperature. When tubes were at room temperature, 5 ml of 0.43M K_2CO_3 solution was slowly added to stop the reaction and neutralize the mixture.Next, tubes were shaken and centrifuged at 2500 x *g* for 15 min to separate the phases. The upper benzenic phase was removed with a Pasteur pippette and transferred to other glass tube, where it were dried under vacuum. Lastly, they were resuspended to 50μL with hexane and 1μL was injected in the gas-liquid Chromatograph.

Chromatography was performed using a 60-m-long capillary column 32 mm id and 20 mm thick impregnated with Sp-2330 FS (Supelco Inc, Bellefonte, Palo Alto, CA, USA). The injector and the detector were maintained at 250 and 275ºC, respectively and nitrogen was used as the carrier gas, and the split ratio was 29:1. Temperature programming (for a total time of 40 min) was as follows:

- An initial temperature of 160ºC for 5 min,
- an increase in temperature at rate of 6ºC/min to 195ºC,
- an increase in temperature at 4ºC/min to 220ºC,
- an increase in temperature at 2ºC/min to 230ºC that was maintained for 12 min,
- and a final decrease in temperature at 14ºC/min to 160ºC

Fatty acids analyzed were: C14:0, C16:0, C16:1n9, C18, C18:1n9, C18:2n6, C20:3n6, C20:4n6, C24:0, C24:1n9, C22:6n3. Post-analytical calculations were done to determinate SFA, MUFA, PUFA, n-6 PUFA, n-3 PUFA. The results were expressed as a percentage of total fatty acids detected in the chromatogram. To perform the quantitative determination of the different fatty acid classes the following formula was applied:

$$C = ((A_x Rf x_x)/(A_{is} Rf_{is}))C_{is}$$

Where C is the concentration of a particular fatty acid, A_x is the area obtained in the chromatogram for this particular fatty acid and RF_x its response factor; Ais is the area for the internal standard in the sample and RF_{is} its response factor, C_{is} is the concentration of the internal standard.

5.4 Alveolar bone loss measurement

The left side of the mandible was defleshed by immersion in 10% H_2O_2 for 3 to 4 days at room temperature. The soft tissues were carefully removed, and the mandible was stained with methylene blue for good visual distinction between the tooth and bone.

Measurements were made at two points at mesial and distal of second premolar and first molar to quantify crestal bone level. The mandibles were photographed using an inverted microscope at x10 (Olympus SZ61). The captured image was also analyzed using , and the mean crestal bone level was calculated for each group. The mean value for each tooth was calculated. The captured image was also analyzed as above, and the mean crestal bone level around the tooth was calculated using Image J software(Rasband, 1997). Mean value obtained at 6 months was considered as baseline and the changes in bone level were calculated with 24 months data.

5.5 Histopathological analysis of gingiva

At the time of animal sacrifice, samples taken from molar gingival mucosa (always the same for all the animals) were fixed in buffered formalin and paraffin-embedded for histologic analysis. Tissue sections were cut with a microtome and stained with haematoxylin and eosin for histological examination. They were included in formol 3,9% at pH 7. After 24-48 h fixation, each of the pieces was cuted and included in a cassette for processing in paraffin according to the conventional process, after dehydration in increasing graded alcohols to absolute ethyl alcohol. Subsequently, cuts at 4µm were performed following by hematoxylin and eosin staining. Measures in the connective tissue were:

- thickness as the distance between epithelium and bone
- number of total vessels counted in 5 consecutive high power fields
- number of vessels with endothelial activation counted in 5 consecutive high power fields
- degree of fibrosis expressed with a scoring system
  - grade 1=less than 2 thick collagen bundles.
  - grade 2=between 2 and 5 thick collagen bundles.
  - grade 3=more than 5 thick collagen bundles.
- cellularity as number of fibroblast-like cells per high power field
- degree of inflammation
  - grade 1=absent.
  - grade 2=mild.
  - grade 3=moderate.

5.6 Gingival lipid peroxidation analysis

TBARS levels were determined by a method based on the reaction with thiobarbituric acid at 90–100ºC according to Harma et al. (2004). The reaction was performed at 90ºC for 15 min at pH 2–3. The sample was mixed with two volumes of cold 10% (w/v) trichloroacetic acid to precipitate protein. This precipitate was pelleted by centrifugation and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) thiobarbituric acid in a boiling water bath for 10 min. After cooling, the absorbance was read at 532 nm. Results were expressed as nmol/mL.

5.7 Measurement of bone metabolism markers and inflammatory cytokines levels in serum

Bone metabolism markers, OPG and RANKL, and IL-6, IL-1β, were measured simultaneously using the high sensitivity human cytokine multiplex immunoassay Milliplex^{TM}MAP (Merck Millipore, Billerica, MA, USA). These Kits contained two groups of microspheres (each group bearing unique fluorescence intensity and a specific cytokine antibody), biotinylated cytokine antibodies, and phycoerythrin conjugated streptavidin.

5.7.1 Preparation of serum samples:

The blood was allowed to clot for at least 30 min before centrifugation for 10 min at 1000 x *g*. After centrifugation, serum was isolated, aliquoted and stored at -20ºC. Serum samples stored at -20ºC were thawed completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.

5.7.2 Preparation of reagents for inmmunoassay

5.7.2.1 Preparation of antibody-immobilized beads

Each antibody-bead vial was sonicated for 30 s and vortexed for 1 min. Then, 150μL from each antibody-bead vial was added to the mixing bottle and it was bring to a final volume of 3 mL with the bead diluent. Next, it was vortexed well to mixed beads.

5.7.2.2 Preparation of quality controls

Before use, quality controls 1 and 2 was reconstitued with 250μL deionized water and then vials were inverted several times and vortexed to mix them. After that, vials were allowed to sit for 5-10 min and their contents were transfered to microfuge tubes.

5.7.2.3 Preparation of wash buffer

Ten X wash buffer was warmed to room temperature and mixed to bring all salts into solution. When it was ready, 30 mL from this were taken and diluited with 270 mL of deionized water to obtain 300 mL of 1X wash buffer.

5.7.2.4 Preparation of serum matrix

One mL of deionized water was added to the bottle containing lyophilized serum matrix and it was mixed well up to matrix was completetely reconstituted (at least for 10 min).

Table 5.7.1: Standard curve concentrations and preparation.

Tube	Dilution	Marker conc. (pg/ml)	H_2O vol. (μL)	Previous tube vol. (μL)
1	1	10	-	-
2	01:04:00	25	150	50
3	01:16:00	6.25	150	50
4	02:04:00	15.63	150	50
5	05:16:00	390.6	150	50
6	18:04:00	97.7	150	50
7	69:16:00	24.4	150	50
8	0	0	150	0

Abbreviations: Conc: concetration Vol.: volume.

5.7.2.5 Preparation of cytokine working standard curve

Assays were calibrated using duplicate 8-point standard curves. Prior to use, cytokine standard was reconstitued with 250μL deionized water to give a 10,000 pg/mL concentration of standard for all analytes. Vial containing it was inverted several times to mix and vortexed for 10 s. Finally, the vial was allowed to sit for 5-10 min and then it was transfered to an microfuge tube. This consisted in tube 1 from which serial dilutions were prepared. For this, 50μL from each tube was transfered to next tube containing 150μL deionized water, after mix well by vortexing. Lastly, the 0 pg/mL standard (Background) consited only in assay buffer.

5.7.3 Immunossay procedure

Before use, all reagents was allowed to warm to room temperature (20-25ºC). At the beginning, 200μL of Wash Buffer was added into each well of the plate. It was sealed and mixed on a plate shaker for 10 min at room temperature (20-25ºC). Then, Wash Buffer was decanted and the residual amount from all wells was removed by inverting the plate and tapping it smartly onto absorbent towels several times. After removing Wash Buffer, 25μL of each Standard or Control were added into their appropriate wells , whereas 25μL of Assay Buffer were added to the sample wells. Then, 25μL of Serum Matrix solution were added to all wells and 25μL of Serum Samples was added into the appropriate wells. Lastly, Mixing Bottle was vortexed and 25μL of the Mixed Beads (antibody-coated microspheres which bind to specific cytokines) was added to each well. The plate was sealed, wrapped with foil and incubated with agitation on a plate shaker overnight at 4ºC. After incubation, well contents were gently removed and plate was washed 2 times. Detection Antibodies (biotinylated cytokine antibodies, which bind to cytokines present on the microspheres) were allowed to warm to room temperature and 50μL were added into each well. Again, the plate was incubated with agitation on a plate shaker for 1 hour at room temperature (20-25ºC).When it finalized, 50μL Streptavidin-Phycoerythrin (phycoerythrin-labeled streptavidin was allowed to bind to biotinylated cytokine-antibodies present on microspheres). were added to each well and the plate was incubated with agitation on a plate shaker for 30 min at room temperature (20-25ºC) . Then, well contents were gently removed and the plate was washed plate 2 times.

Eventually, 150μL of Sheath Fluid were aded to all wells and the beads were re-suspended on a plate shaker for 5 min. After that, plate was run on LuminexX-MAP Bio-Plex 200 System Bioanalyzer (Luminex Corp., Austin, TX, USA) which quantifies

the amount of phycoerythrin fluorescence present on each of the distinct microsphere groups . At least 50 individual microspheres were counted for each cytokine, and the median fluorescence intensity (MFI) MFI data were saved and analyzed by using concentrations in samples. Assays were calibrated using duplicate 8-point standard curves. Machine performance was verified using quality control samples at low, medium and high levels for each analyte. All standard and quality control samples were analyzed in a complex matrix to match the sample background. Serum samples were analyzed at optimized dilutions.

5.8 Gene expression analysis

5.8.1 RNA extraction and purification from gingival tissue samples.

Total RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen, Düsseldorf, Germany) following manufacturer's condition. RNeasy Lipid Tissue Kits integrate phenol/ guanidine-based sample lysis and silica-membrane purification of total RNA. Kit includes QIAzol lysis reagent, a monophasic solution of phenol and guanidine thiocyanate, designed to facilitate lysis of fatty tissues and inhibit RNases. Because of RNA in harvested tissue is not protected and unwanted changes in the gene expression profile will occur, gingival tissue previously treated with RNAlater(Sigma-Aldrich, San luis, MO, USA) and preserved at -80ºC. RNAlater is an aqueous, non-toxic tissue storage reagent that rapidly permeates tissue to stabilize and protect cellular RNA *in situ* in unfrozen specimens. RNAlater eliminates the need to immediately process tissue specimens or to freeze samples in liquid nitrogen for later processing.

For RNA extraction, gingival tissue samples were weightened and thawned. Samples were pulverized in a homogenizer in the presence of liquid nitrogen. Tubes containing the homogenate were placed on the benchtop at room temperature for 5 min to promote the dissociation of nucleoprotein complexes. then, 200μL chloroform were added to tubes and these were shaked vigorously for 15 s. Next, these tubes were placed again on the benchtop at room temperature for 2–3 min. After that, they were centrifuged at 12,000 x *g* for 15 min at 4ºC. After centrifugation, 3 separated phases were present in the tubes: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. Among them, the upper, aqueous phase was transfered to a new tube and a volume of 70% ethanol was added. After mixed it thoroughly by vortexing, 700μL of the sample were transfered up to an RNeasy Mini spin column placed in a 2 ml collection tube. It was gently closed and centrifuge for 15s at 8000 x *g* at room temperature. Flow-through was dicarded and previous step was repeated. Again, the flow-through was dicarded and700μL of Buffer RW1 were added to the RNeasy spin column. It was centrifuge for 15 s at 8000 x *g* (10,000 rpm) to wash the membrane and the flow-through discarded. After, the RNeasy spin column from the collection tube was carefully removed so that the column does not contact the flow-through. 500μL of Buffer RPE were added to the RNeasy spin column and it was centrifuge for 15 s at 8000 x *g* to wash the membrane When the spin column membrane was dried, the RNeasy spin columns were placed in a new 1.5 ml collection tube and 30–50μL of RNase-free water were added directly to each spin column membrane. Finally, to elute the RNA, the columms were centrifuged for 1 min at 8000 x *g* two times and flow-through containing RNA were collected in microcentrifuge tubes.

Table 5.8.1: Reverse transcription reaction mixture

Component	Volume per sample (μL)	Final Concentration
RT Buffer (10X)	10	1X
$MgCl_2$ (25 mM)	22	5.5 mM
deoxyNTPs Mixture	20	500 μM per dNTP
Random Hexamers	5	2.5 μM
RNase Inhibitor	2	0.4 U/μL
MultiScribe Reverse Transcriptase (50 U/μL)	6.25	3.125 U/μL

5.8.2 RNA concetration and purity determination.

The quantity and purity of the RNA were determined by using absorbance values obtained at 260 and 280 nm.. To obtain these, the Nanodrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) was employed. In this, a beam of light passes through 1μL of sample and measures absorbance at 260nm (A_{260}) and RNA concentration (ng/ml) is recalculated. In addition, to determinate purity of RNA extracted, absorbance at 280nm (A_{280}) was also measured to calculate the ratio A_{260}/A_{280}. Values between 1.9 and 2.1 indicate a good purity, but a smaller value can indicate protein contamination.

5.8.3 Complementary DNA synthesis

Twenty ng of total RNA from each sample were reverse-transcribed into complementary DNA (cDNA) using the enzyme MultiScribe Reverse Transcripase (Life technologies, Carlsbad, CA, USA). Firstly, reaction mix for all total RNA samples to be reverse transcribed was prepared according to proportions indicated in 5.8.1. Then, 20ng of each total RNA sample were transfered microcentrifuge tubes. Next, each total RNA sample was diluted to a volume of 34.75μL with RNase-free, deionized water, if necessary. Tubes were capped and gently tapped to mix, and they were briefly centrifuged to eliminate air bubbles in the mixture. After that, 65.25μL of the reaction mix (already prepared) were pippeted to each MicroAmp Reaction Tube (one for each RNA sample) and 34.75μL of each dilute total RNA sample were transfered to their correponding tube. Tubes were capped and gently tapped to mix. Again, the tubes were briefly centrifuged to force the solution to the bottom and to eliminate air bubbles from the mixture. Finally, they were load into a thermal cycler and it was programmed with the following conditions: and reaction was initiated. For this, it was incubated at 48ºC for 30 min following by warm up to 95ºC maintened for 5 min to inactivate the enzyme. cDNA was obtained in a final volume of 20μL. cDNA samples were aliquoted and stored at -20ºC according to the manufacturer's protocol. When reverse transcriptio finalized, the quantity and purity of the cDNA were determined form the absorbance at 260/230 nm in the Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Rockfor, Illinois, USA).

5.8.4 QRT-PCR

Real-Time PCR system 7900HT (Applied Biosystems, Foster City, CA, USA) and real time PCR kit (TaqManGene Expression Assays, Applied Biosystems, Foster City, CA, USA) were employed based on the manufacturer's instruction.

Table 5.8.2: Assay IDs

Gene	Description	Assay ID	Reference sequence
Atg5	*autophagy related 5*	Rn01767063_m1	NM_001014250.1
Bad	*BCL2-associated agonist of cell death*	Rn00575519_m1	NM_022698.1
Bcl2	*B-cell CLL/lymphoma 2*	Rn99999125_m1	NM_001033672.1
Bax	*Bcl2-associated X protein*		
Cat	*catalase*	Rn00560930_m1	NM_012520.1
Gadph	*glyceraldehyde-3-phosphate dehydrogenase*	Rn99999916_s1	NM_017008.3
Gpx1	*glutathione peroxidase 1*	Rn00577994_g1	NM_030826.3
Il1b	*interleukin 1* beta	Rn99999009_m1	NM_031512.2
Il6	*interleukin 6*	Rn99999011_m1	NM_012589.1
Il8	*interleukin 8*	Rn00567841_m1	NM_017183.1
Map1lc3a	*microtubule-associated protein 1 light chain 3* alpha	Rn01536227_g1	NM_199500.1
mt-Nd1	*NADH dehydrogenase 1, mitochondrial*	Rn03296764_s1	
mt-Nd4	*NADH dehydrogenase 4, mitochondrial*	Rn03296781_s1	
Ndufs1	*NADH dehydrogenase (ubiquinone) Fe-S protein 1*	Rn01438310_g1	NM_001005550.1
Nfe2l2	*Mus musculus nuclear factor, erythroid derived 2, like 2*	Rn00477784_m1	NM_010902.3
Ppargc1a	*peroxisome proliferator-activated receptor gamma, coactivator 1 alpha*	Rn01511922_m1	NM_133284.2
Sirt1	*sirtuin 1*	Rn00490758_m1	NM_001153061.1
Sod1	*superoxide dismutase 1, soluble*	Rn00566938_m1	NM_017050.1
Sod2	*superoxide dismutase 2, mitochondrial*	Rn99999088_g1	NM_017051.2
Tfam	*transcription factor A, mitochondrial*	Rn00580051_m1	NM_031326.1
Tnf	*tumor necrosis factor*	Rn99999017_m1	NM_012675.3
Tnfrsf11b/Opg	*tumor necrosis factor receptor superfamily, member 11b*	Rn00563499_m1	NM_012870.2
Tnfsf11/RANKL	*tumor necrosis factor (ligand) superfamily, member 11*	Rn00589289_m1	NM_057149.1

TaqMan probes detection is based on the use of a DNA fragment (probe) complementary to an intermediate portion of the DNA to be amplified. This probe is affixed to a fluorescent molecule and another molecule inhibiting the fluorescence ("quencher"), so that only when the probe is displaced from its position by action of the DNA polymerase liberates the fluorescent molecule with "quencher" action and fluoresces when illuminated with a laser. Quantification of the fluorescence emitted during each PCR cycle will be proportional to the amount of DNA being amplified and also turn proportional to the amount of RNA that exists in the sample. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as an internal control. The probes of studied genes and GADPH are shown in Tables 5.7.1 and 5.8.1. PCR thermal cycling were development in three steps:

- Step 1 at 50ºC lasting 2 min.
- Step 2 at 95ºC lasting 10 min and
- A final step with 40 cycles at 95ºC lasting 15 seconds and at 60ºC lasting 1 min

5.8.4.1 Data analysis

Data analysis was performed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Briefly, the average threshold cycle (Ct) of 4 to 6 technical available replicates was calculated. As reference genes (housekeeping genes) *Gapdh*, *18S* and *B2m* were used. By

the use of GeNorm program (http://medgen.ugent.be/~jvdesomp/genorm/) the most appropriate housekeeping gene was selected (Schlotter et al., 2009).

5.8.5 Statistical analysis

The results shown represent the mean and the standard error of the mean of six animals. Prior to any statistical analysis, all variables were checked for normality and homogeneous variance using the Kolmogorov-Smirnoff and the Levene tests, respectively. When a variable was found not to follow normality, it was log-transformed and reanalysed. Different statistical comparison were done to test the effect of dietary treatments at 6 months, at 24 months and to test the role of age (differences between 6 and 24 months) separately for each dietary treatment. In particular, to evaluate differences in the means between groups at 6 and 24 months, one-way ANOVA analysis followed by a multiple-comparison test, adjusted by Bonferroni correction, was performed. To test differences between dietary treatments between the two age periods, Student's t-test were performed. Data were analysed using the SPSS/PC statistical software package (SPSS for Windows, 15.0.1, 2006, SPSS Inc. Chicago, IL, USA).

Part IV

RESULTS

Chapter 6

Dietary fat effects on periodontium

6.1 Body weight and food intake

The body weights of the animals were similar for all groups at 6 months (309±9 g for virgin olive oil group, 301±11 g for sunflower oil group and 315±13 g for fish oil group). At twenty four months, fish oil groups showed higher body weight (604±23 g) than virgin olive oil group (534±52 g) and sunflower oil group (508±19 g). No differences concerning food intake were found between groups or in relation to age.

6.2 Alveolar bone level and gingival histology

Animals fed on sunflower oil showed the significantly highest bone loss, followed by those fed on fish oil (Figure6.2.1). Concerning histopathological findings, results are reported in Table6.2.1. The groups did not differ significantly for thickness. At 24 months, the number of vessels was lower for all groups, compared to 6 months, particularly in the sunflower group. The endothelial cells tended to be activated at 24 months. At 24 months, only the sunflower group showed a high degree of fibrosis and a moderate degree of inflammation; both sunflower and fish showed a reduction of cellularity.

Table 6.2.1: Histological findings in rat tissue sections stained with haematoxylin and eosin.

Parameter	6 months			24 months		
	V	S	F	V	S	F
Thickness (mm)	0.25	0.23	0.21	0.24	0.22	0.23
Vessel number	9.8±1.2	9.6±1.2	10.2±1.4	8.9±1.1	8.3±1.1	9.2±1.3
Endothelial activation (vase x 5 HPF)	1	2	2	2	4	4
Fibrosis	+/-	+	+	+/-	++	+
Cellularity (cells x HPF)	<20	<20	<20	<20	<10	<10
Inflammation	-	-	+/-	+/-	+	+/-

Acronyms and Abreviations: V: rats fed on virgin olive oil; S: rats fed on sunflower oil; F: rats fed on fish oil; HPF: high power fields.

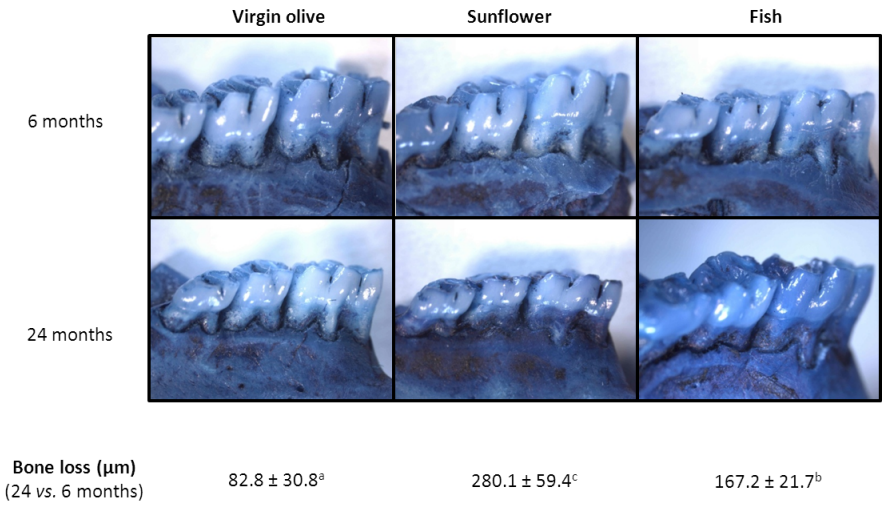

Figure 6.2.1: Effects of dietary fat type on alveolar bone loss in the mesial and distal roots of the second premolar and the first molar of young (6 months old) and old (24 months old) rats. Results are presented as mean ± EEM of bone loss between 6 and 24 months. Lower-case letters, when different, represent statistically significant differences ($P_¡$ 0.05) between the three dietary treatments. The figure also shows dental cervical area after stained with methylene blue.

6.3 Plasma fatty acid profile

Table 6.3.1 shows the plasma lipid profile of rats. For SFA, the highest proportion for C14:0 and C16:0 were found for fish oil fed animals, which showed the lowest proportion for C18:0. For MUFA, virgin olive oil fed rats showed the highest proportion for C18:1n-9 and total MUFA index. Individual and total n-6 PUFA index were the highest for sunflower oil group. For n-3 PUFA, fish oil fed animals reported the highest proportions.

6.4 Inflammatory cytokines gene expression

Figure 6.4.1 shows the relative quantity (RQ) of *Il1b, Il8, Il6* and *Tnf* mRNAs in gum. Concerning *Il1b*, changes were found only between treatments at 6 months of age, showing that fish oil fed animals had a higher RQ than sunflower oil fed animals. Similar changes were also found for *Il8*. Also differences between six and twenty four months were found for virgin olive oil fed animals, showing old animals with a higher RQ. Concerning *Il6*, changes between 6 and 24 months were found only for sunflower oil group, with higher RQ at twenty four months. *Tnf* RQ showed no age-related differences. Animals fed on fish oil presented the highest RQ both at six and at twenty four months.

6.5 Bone metabolism markers circulating levels and gene expression

Figure 6.5.1 shows circulating levels and RQ in gum mRNA for *Tnfsf11* and *Opg*. Concerning RANKL, RQ was the highest for virgin olive oil fed animals at 6 months. Age-

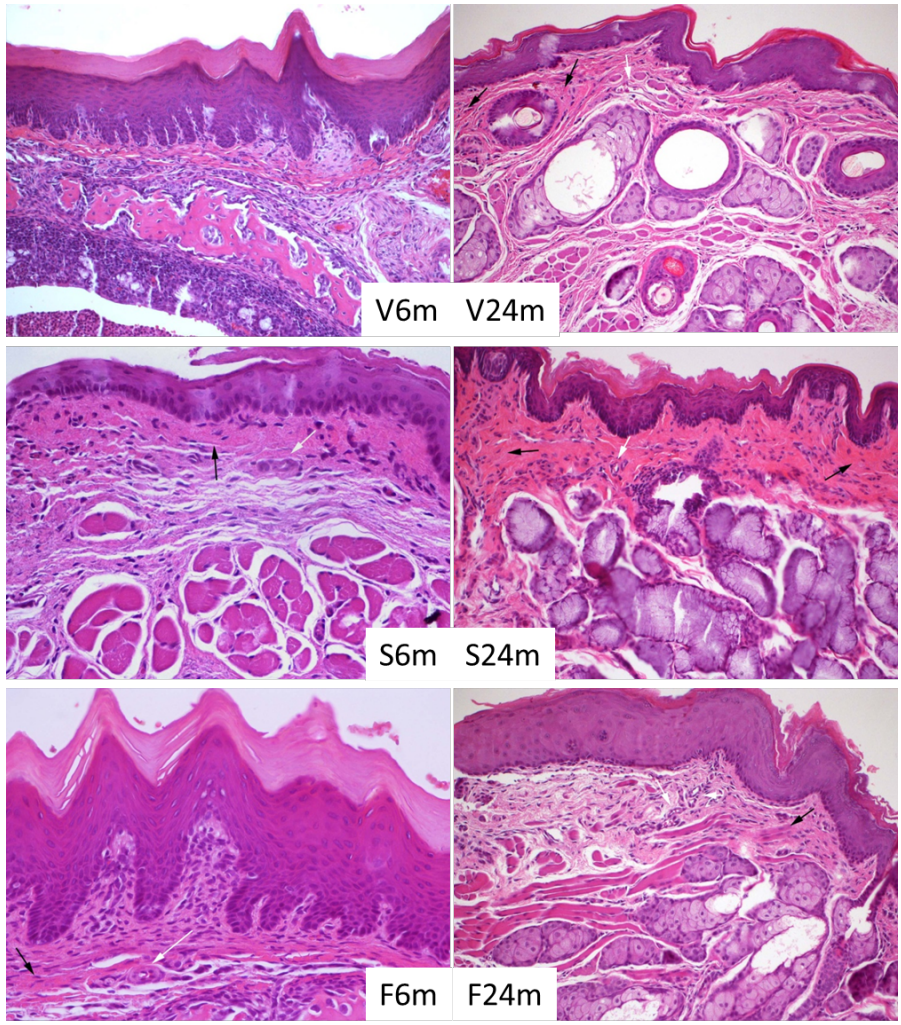

Figure 6.2.2: Histological features of gingival tissue in 6 months old (6m, left column) and 24 months old (24m, right column) rats fed on virgin olive (V), sunflower (S) or fish (F) oils. White arrows show areas with activated endothelial vessels. Black arrows show areas with fibrosis.

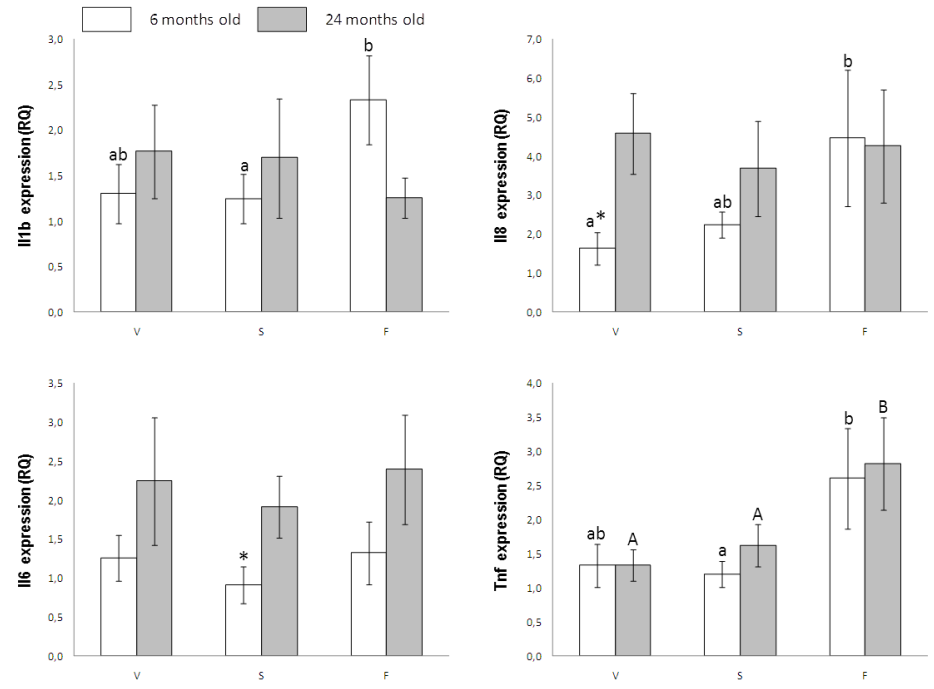

Figure 6.4.1: Effects of diets based on virgin olive (V), sunflower (S) or fish (F) oils on gingival expression of interleukins genes (*Il1b, Il8, Il6* and *Tnf*) in young (6 months old) and old (24 months old) rats measured as mRNA relative quantity (RQ) respect than 6-monts-old rats fed on virgin olive oil. Results are presented as mean ± standard error of the mean. Asterisk (*) means a statistically significant difference between the same dietary treatment for rats aged 6 and 24 months. Lower-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 6 months. Upper-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 24 months.

Table 6.3.1: Plasma fatty acids in rats fed on different dietary fat sources (g/100g).

Fatty acid or index	Virgin olive oil	Sunflower oil	Fish oil
C14:0	0.7 ± 0.1^{a}	0.6 ± 0.1^{a}	1.1 ± 0.1^{b}
C16:0	20.9 ± 1.3^{a}	20.8 ± 0.5^{a}	25.8 ± 1.3^{b}
C16:1n-9	5.5 ± 0.5^{b}	3.6 ± 0.6^{a}	$6.7 \pm 0.3c$
C18:0	12.4 ± 0.5^{b}	12.8 ± 0.5^{b}	9.9 ± 0.5^{a}
C18:1n-9	26.0 ± 5.3^{b}	10.5 ± 5.3^{a}	11.9 ± 3.7^{a}
C18:2n-6	7.3 ± 0.4^{b}	14.7 ± 2.2^{c}	2.3 ± 0.7^{a}
C20:3n-6	0.3 ± 0.1^{ab}	0.4 ± 0.1^{b}	0.2 ± 0.1^{a}
C20:4n-6	6.7 ± 0.2^{a}	15.7 ± 1.9^{b}	$5.8 \pm 0.4a$
C20:5n-3	0.2 ± 0.1^{a}	0.1 ± 0.0^{a}	8.4 ± 1.1^{b}
C24:0	0.8 ± 0.1^{a}	1.6 ± 0.4^{b}	0.8 ± 0.3^{a}
C24:1n-9	0.9 ± 0.2^{a}	1.1 ± 0.3^{ab}	1.5 ± 0.2^{b}
C22:6n-3	2.0 ± 0.2^{b}	0.6 ± 0.3^{a}	9.0 ± 0.6^{c}
Total saturated fatty acids	37.2 ± 1.5^{a}	38.5 ± 1.1^{a}	40.6 ± 1.7^{a}
Total monounsaturated fatty acids	40.6 ± 1.8^{b}	27.9 ± 3.3^{a}	31.6 ± 2.4^{a}
Total n-6 polyunsaturated fatty acids	19.8 ± 0.7^{b}	32.2 ± 2.7^{c}	9.5 ± 0.4^{a}
Total n-3 polyunsaturated fatty acids	2.4 ± 0.1^{b}	1.4 ± 0.3^{a}	18.3 ± 1.2^{c}
Total polyunsaturated fatty acids	22.2 ± 0.7^{a}	33.6 ± 2.5^{c}	27.8 ± 1.3^{b}

Results are presented as mean ± standard error of the mean. Asterisk (*) means a statistically significant difference between the same dietary treatment for rats aged 6 and 24 months. Lower-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 6 months. Upper-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 24 months.

related differences were found only for virgin olive oil group, showing higher values at six months. Concerning plasma RANKL, differences were found only associated to age, with animals at six months showing higher levels, for all dietary treatment, than those at 24 months. For *Opg* RQ mRNA, the lowest value was found for fish oil fed animals at six months. Age-related changes were found only for fish oil groups, with animals at twenty four months showing higher values than those at six months. Plasma OPG reported at six months that animals fed on virgin olive oil showed the lowest concentration. Virgin olive oil and fish oil fed rats reported higher concentrations at 24 than at 6 months.

6.6 Apoptosis, autophagy, biogenesis, mtETC and antioxidant defenses-related gene expression

Concerning apoptosis, RQ for *Bad*, age related differences were found for virgin olive- and fish-oil fed groups, with old animals showing higher values than those at 6 months of age. Concerning *Bax*, differences were found only between six and twenty four months for virgin olive oil, with the aged group showing the highest RQ. Regarding *Bcl2*, RQ at 6 months was higher for sunflower oil fed animals compared with fish oil fed animals. Age-related changes were found for virgin olive- and fish-oil fed animals, always with higher values for old animals (Figure6.6.1).

In relation to autophagy, for *Atg5*, differences between young and old animals were found for virgin olive- and fish-oil groups, with old animals showing higher values. In relation to *Maplc3a*, at 6 months, the highest RQ was found for sunflower oil group. At

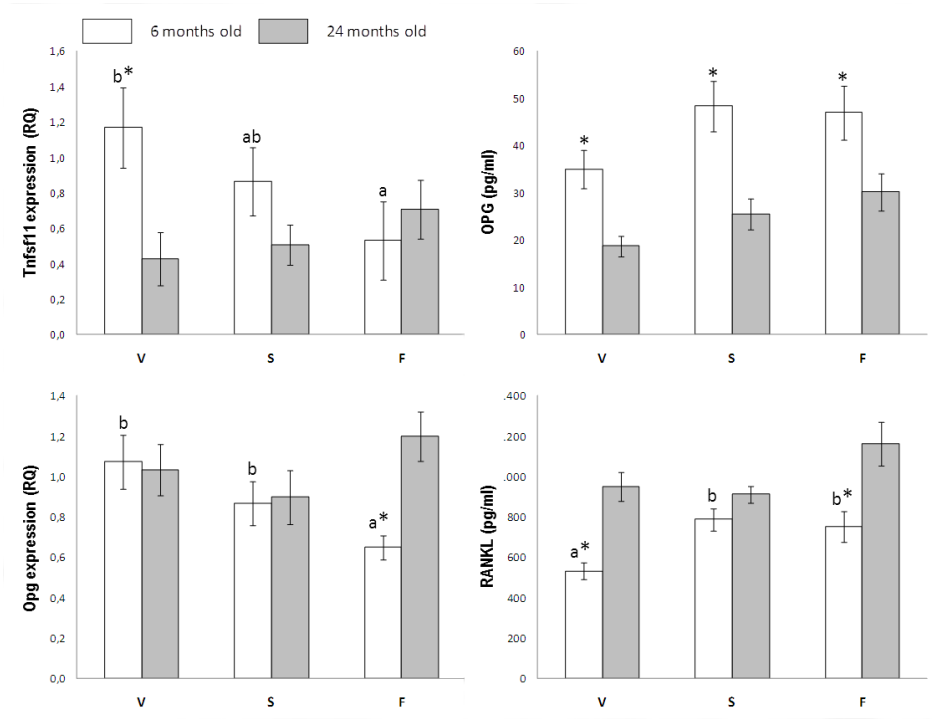

Figure 6.5.1: Effects of diets based on virgin olive (V), sunflower (S) or fish (F) oils on circulating levels of bone resorption marrkers (RANKL and OPG) in young (6 months old) and old (24 months old) rats and on gingival expression of their genes (*Opg* and *Tnfsf11*) measured as mRNA relative quantity (RQ) respect than 6-monts-old rats fed on virgin olive oil. Results are presented as mean ± standard error of the mean. Asterisk (*) means a statistically significant difference between the same dietary treatment for rats aged 6 and 24 months. Lower-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 6 months. Upper-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 24 months.

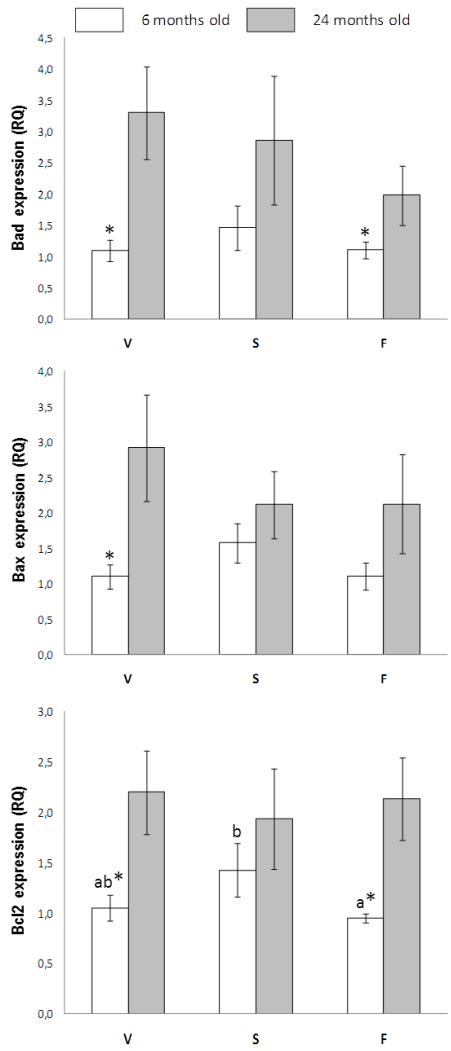

Figure 6.6.1: Effects of diets based on virgin olive (V), sunflower (S) or fish (F) oils on gingival expression of apoptosis-related genes (*Bad*, *Bax* and *Bcl2)* in young (6 months old) and old (24 months old) rats measured as mRNA relative quantity (RQ) respect than 6-monts-old rats fed on virgin olive oil. Results are presented as mean ± standard error of the mean. Asterisk (*) means a statistically significant difference between the same dietary treatment for rats aged 6 and 24 months. Lower-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 6 months. Upper-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 24 months.

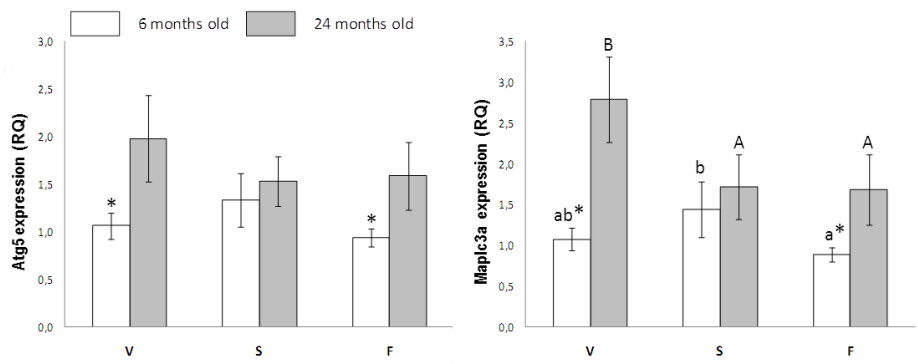

Figure 6.6.2: Effects of diets based on virgin olive (V), sunflower (S) or fish (F) oils on gingival expression of genes related to autophagy (*Atg5* and *Map11c3a*) in young (6 months old) and old (24 months old) rats measured as mRNA relative quantity (RQ) respect than 6-monts-old rats fed on virgin olive oil. Results are presented as mean ± standard error of the mean. Asterisk (*) means a statistically significant difference between the same dietary treatment for rats aged 6 and 24 months. Lower-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 6 months. Upper-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 24 months.

twenty four months, the highest RQ was found for virgin olive oil fed animals. Differences concerning age were found for virgin olive oil and fish oil groups, with old animals showing higher values than the young animals (Figure 6.6.2).

Concerning biogenesis, for *Tfam* RQ at 24 months, the highest value was found for virgin olive oil fed animals. Virgin olive oil and sunflower oil groups presented higher RQ at twenty four months than at 6 months. Similar results were found for *Ppargc1a* and *Sirt1* RQ (Figure 6.6.3).

Three genes related to complex I from the mtETC were analyzed. Two of these genes are from mtDNA origin (*mt-Nd1* and *mt-Nd4*) and the other from nuclear origin (*Ndufs1*). Concerning *mt-Nd1*, at 24 months virgin olive oil fed animals showed the highest RQ. Differences related to aging were found for virgin olive oil group, with higher RQ at twenty four months. In relation to *mt-Nd4* and *Ndufs1*, similar results were found. At 6 months the lowest RQ was found for fish oil fed animals. At twenty four months, the highest value was found for virgin olive oil group. Age-related differences were found for virgin olive and fish oil groups, being higher at 24 months (Figure6.6.4).

Regarding antioxidant enzymes, SOD genes, both cytoplasmic (*Sod2*) and mitochondrial (*Sod2*) reported almost similar RQ. Sunflower oil led to the highest RQ at 6 months. Virgin olive- and fish-oil fed animals presented higher values at 24 months than at 6 months. Similar values were found for *Nrf2* RQ (Figure 6.6.5).

6.7 Lipid peroxidation at the gums

Lipid peroxidation at the gums was evaluated through the TBARS assay (Table 6.7.1). At 24 months the highest concentration was found for sunflower oil fed animals. Age led to differences for virgin olive and fish oil fed animals, with higher concentrations at 6 months, compared with 24 months.

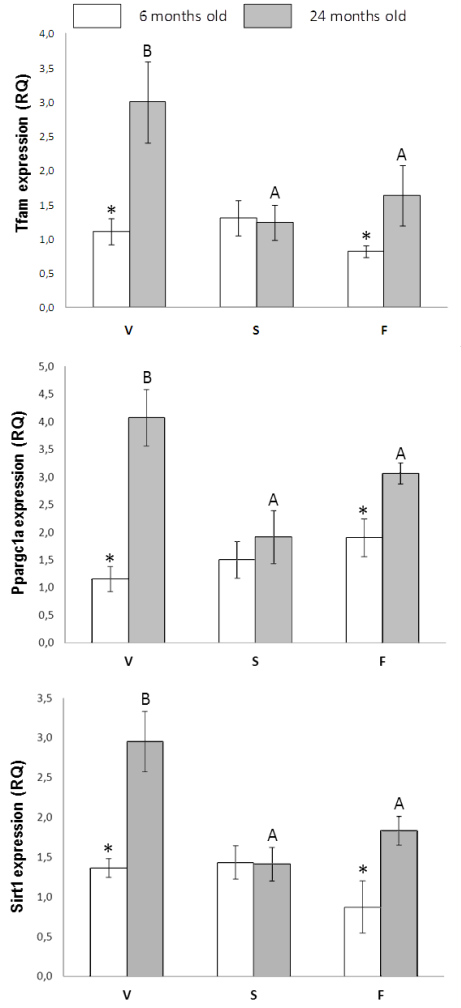

Figure 6.6.3: Effects of diets based on virgin olive (V), sunflower (S) or fish (F) oils on gingival expression of apoptosis-related genes (Tfam, *Pgc1* and *Sirt1)* in young (6 months old) and old (24 months old) rats measured as mRNA relative quantity (RQ) respect than 6-monts-old rats fed on virgin olive oil. Results are presented as mean ± standard error of the mean. Asterisk (*) means a statistically significant difference between the same dietary treatment for rats aged 6 and 24 months. Lower-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 6 months. Upper-case letters, when different, represent statistically significant differences *(*$P<0.05$) between the three dietary treatments at 24 months

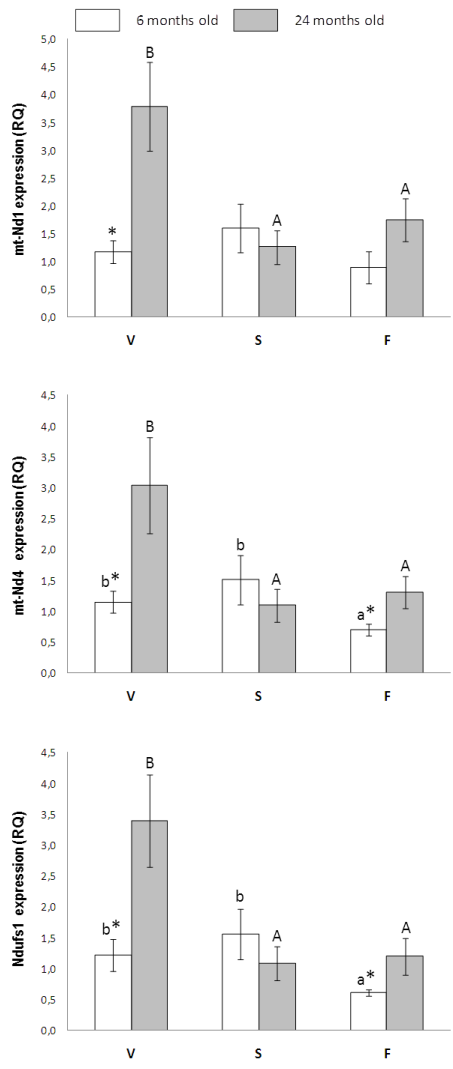

Figure 6.6.4: Effects of diets based on virgin olive (V), sunflower (S) or fish (F) oils on gingival expression of mitochondrial electron trasport chain components genes (*mt-Nd1, mt-Nd4* and *Ndfus1*) in young (6 months old) and old (24 months old) rats measured as mRNA relative quantity (RQ) respect than 6-monts-old rats fed on virgin olive oil. Results are presented as mean ± standard error of the mean. Asterisk (*) means a statistically significant difference between the same dietary treatment for rats aged 6 and 24 months. Lower-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 6 months. Upper-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 24 months

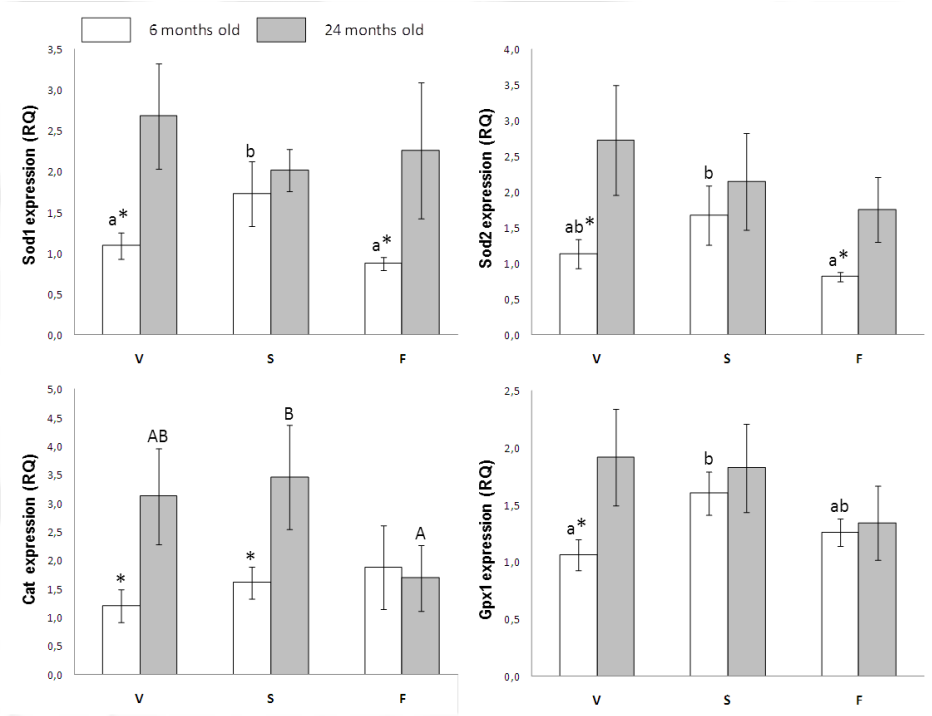

Figure 6.6.5: Effects of diets based on virgin olive (V), sunflower (S) or fish (F) oils on gingival expression of genes related to antioxidant enzymes (*Sod1, Sod2*) in young (6 months old) and old (24 months old) rats measured as mRNA relative quantity (RQ) respect than 6-monts-old rats fed on virgin olive oil. Results are presented as mean ± standard error of the mean. Asterisk (*) means a statistically significant difference between the same dietary treatment for rats aged 6 and 24 months. Lower-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 6 months. Upper-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 24 months.

Table 6.7.1: Effects of dietary fat type gum levels of thiobarbituric acid reactive substances (TBARS, nmol/mg) of young (6 months old) and old (24 months old) rats.

Age	Virgin olive oil	Sunflower oil	Fish oil
6 months	$0.13 \pm 0.01*$	0.16 ± 0.01	$0.14 \pm 0.03*$
24 months	$0.09 \pm 0.01B$	$0.16 \pm 0.02C$	$0.06 \pm 0.01A$

Results are presented as mean ± standard error of the mean. Asterisk (*) means a statistically significant difference between the same dietary treatment for rats aged 6 and 24 months. Lower-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 6 months. Upper-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 24 months. CoQ: Coenzyme Q.

Chapter 7

Dietary CoQ effects on periodontium

7.1 Body weight and food intake

Body weights of animals receiving CoQ_{10} in diet were similar for all groups at 6 months 305±3 g for animals fed on virgin olive oil ; 301±3g for rats fed on sunflower oil; and 305±3 g for animals fed on fish oil. At 24 months, fish oil fed group showed higher body weight (624±20 g) than virgin olive oil (496±20 g) and sunflower oil (499±6 g) groups. No differences concerning food intake were found between groups or in relation to age.

7.2 Alveolar bone level and gingival histology

Age-related alveolar bone loss (differences between 24 and 6 months of age) were 139±28 μm for virgin olive oil fed group, 163±38 μm for sunflower oil group and 97±57 μm for fish oil fed group. No statistically significant differences between groups were found.(see Table 7.2.1).

Table 7.2.1: Histological findings in rat tissue sections stained with haematoxylin and eosin.

Parameter	6 months			24 months		
	VQ	SQ	FQ	VQ	SQ	FQ
Thickness (mm)	0.28	0.25	0,24	0.23	0.23	0.22
Vessel number	9.5±1.2	9.3±1.1	9.8±1.3	9.1±1.3	8.2±1.2	9.5±1.6
Endothelial activation (vase x 5HPF)	<1	<1	2	2	3	3
Fibrosis	-	+	+/-	+/-	+	+
Cellularity (cells x HPF)	<20	<20	<20	<20	<10	<10
Inflammation	-	-	-	+/-	+	+/-

Acronyms and abbreviations: VQ: rats fed on virgin olive oil and coenzyme Q; SQ: rats fed on sunflower oil and coenzyme Q; FQ: rats fed on fish oil and coenzyme Q.

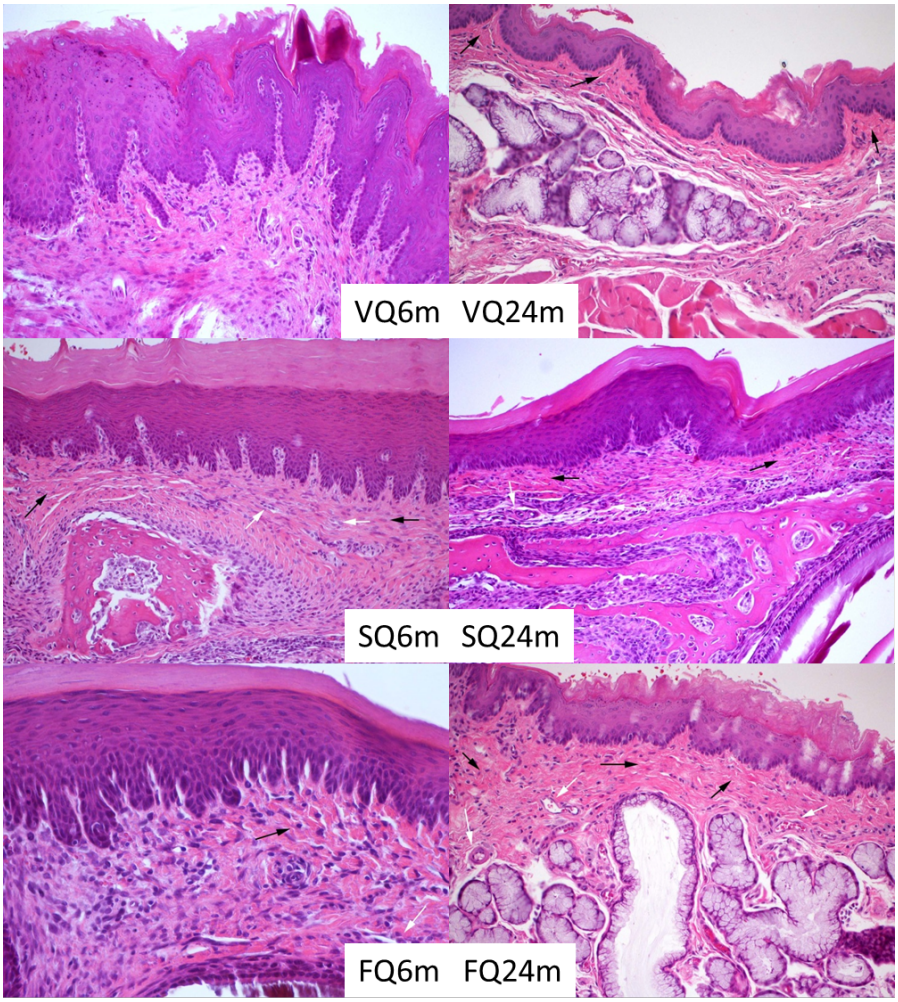

Figure 7.2.1: Histological features of gingival tissue in 6 (6m, left column) and 24 months old (24m, right column) rats fed on virgin olive (VQ), sunflower (SQ) or fish (FQ) oils supplemented with CoQ. White arrows show areas with activated endothelial vessels. Black arrows show areas with fibrosis.

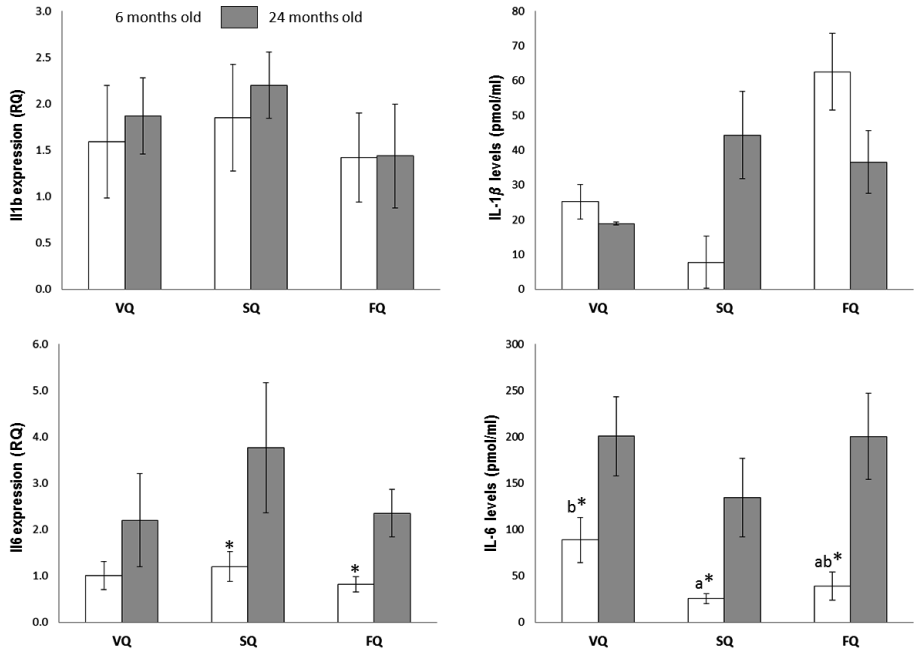

Figure 7.3.1: Effects of CoQ_{10}-rich diets based on virgin olive (VQ), sunflower (SQ) or fish (FQ) oils on circulating levels of interleucins implicated in inflmmation (IL-1β and IL-6) in young (6 months old) and old (24 months old) rats and on gingival expression of their genes (*Il1b* and *Il6*) measured as mRNA relative quantity (RQ) respect than 6-monts-old-rats fed on virgin olive oil. Results are presented as mean $\pm$ standard error of the mean. Asterisk (*) means a statistically significant difference between the same dietary treatment for rats aged 6 and 24 months. Lower-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 6 months. Upper-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 24 months.

7.3 Inflammatory citokynes circulating levels and gene expression

Figure 7.3.1 shows the gingival mRNA RQ for the genes *Il1b* and *Il6*, as well as the circulating levels of IL-1β and IL-6 in animal maintained on diets with CoQ_{10}. Concerning IL-1β, differences were found only according to circulating levels. Higher values were found for fish oil group at 24 months of age, which also showed the highest levels at 6 months. On the other hand, sunflower oil fed rats, which showed similar levels to virgin olive oil fed group at 6 months, presented higher levels at 24 months that were similar to those found for animals fed on fish oil-based diet. In turn, RQ of IL-6 mRNA changed in an age-related way, with 24 months old rats maintained with diet based on virgin olive- and fish-oil showing higher levels than their younger counterparts. Regarding circulating levels, it was found a higher level for old animals in the three dietary groups. In addition, differences among dietary groups were noted at 6 months, with rats fed on sunflower oil showing lower levels than those fed on virgin olive oil.

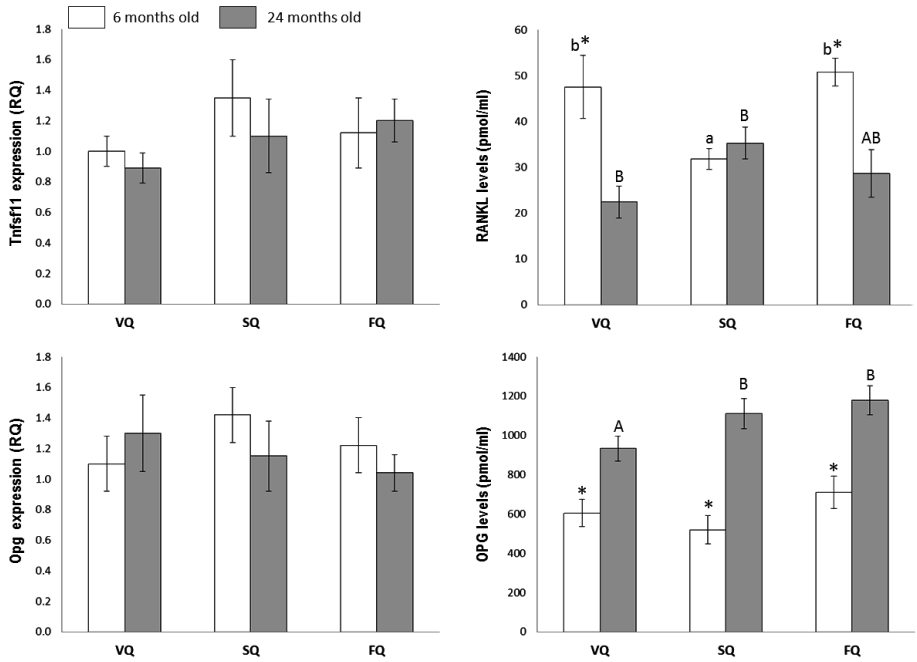

Figure 7.4.1: Effects of CoQ_{10}-rich diets based on virgin olive (VQ), sunflower (SQ) or fish (FQ) oils on circulating levels of bone resorption markers (OPG and RANKL) in young (6 months old) and old (24 months old) rats and on gingival expression of their genes *(Opg* and *Tnfsf11)* measured as mRNA relative quantity (RQ) respect than 6-monts-old rats fed on virgin olive oil. Results are presented as mean ± standard error of the mean. Asterisk (*) means a statistically significant difference between the same dietary treatment for rats aged 6 and 24 months. Lower-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 6 months. Upper-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 24 months.

7.4 Bone resorption markers circulating levels and gene expression

Figure 7.4.1 shows circulating levels of *Tnfsf11* and *Opg* and RQ in gingival mRNA for *Tnfsf11* and *Opg* genes. Concerning RANKL, groups fed on virgin olive- and fish-oil based diet with CoQ_{10} showed higher plasma levels than sunflower oil fed group at 6 months, but these differences disappeared at 24 months. Likewise, significant age-related changes only were represented by lower values in animals belonging to groups receiving CoQ_{10}-rich diets based on virgin olive- and fish-oil. For OPG, all dietary groups receiving CoQ_{10} showed similar plasma levels at 6 months, which increased significantly in relation to age. Among aged rats, those fed on sunflower- and fish-oil showed the highest levels. Despite these observations at systemic levels, no significant differences in gene expression of any of the markers were found.

Table 7.6.1: Effects of dietary fat type on gum levels of thiobarbituric acid reactive substances (TBARS, nmol/mg) of young (6 months old) and old (24 months old) rats.

Age	Virgin olive oil with CoQ	Sunflower oil with CoQ	Fish oil with CoQ
6 months	$0.13 \pm 0.01^{a}*$	$0.27 \pm 0.02^{b}*$	$0.15 \pm 0.02^{a}*$
24 months	$0.07 \pm 0.01A$	$0.17 \pm 0.01BB$	$0.08 \pm 0.02AA$

Results show means ± standard error of the mean. Asterisk (*) means statistically significant difference between the same dietary treatment for rats aged 6 and 24 months. Lower-case letters, when different, represent statistically significant differences ($P< 0.05$) between the three dietary treatments at 6 months. Upper-case letters, when different, represent statistically significant differences ($P< 0.05$) between the three dietary treatments at 24 months. CoQ: Coenzyme Q.

7.5 Autophagy, biogenesis, mtETC and antioxidant defenses-related gene expression

Concerning autophagy, at 6 months of age, *Maplc3a*, *Atg5* RQs in virgin olive- and fish-oil fed rats receiving CoQ_{10} were lower compared with the sunflower oil and CoQ_{10} fed group, whereas there were no differences among dietary groups at 24 months. Both groups also showed significantly higher *Atg5* RQs with aging. However, higher age-related *Maplc3a* only was significant in animals fed on fish oil based diet. On the other hand, mRNA RQs of Tfam was similar between dietary groups at both ages, but aged animals showed the highest levels in all groups (see Figure7.5.1).

As in the previous groups three genes related to complex I from the mitochondrial ETC were analyzed. Two of these genes are from mitochondrial origin (*mt-Nd1* and *mt-Nd4*) and the other from nuclear origin (*Ndufs1*). RQs of *mt-Nd1* and *mt-Nd4* was higher in aged groups fed on sunflower and fish oil, but in *Ndufs1* case this only was found for fish oil fed animals (see Figure 7.5.2). However, there are no significant differences among dietary groups taking CoQ_{10} at any age.

Regarding antioxidant enzymes genes (*Cat, Sod1, Sod2* and *Gpx1*), differences between CoQ_{10}-supplemented dietary groups were found in young animals, mainly between sunflower- and fish-oil fed groups, with these last showing lower RQs for all analyzed genes except *Gpx1*. This dietary group also was the only that showed age-related changes, with old animals exhibiting the highest RQs for the four genes. In addition, mRNA RQs for the transcription factor *Nrf2* in both rats fed virgin olive- and fish-oil were lower than those noted in sunflower oil fed group, but only at 6 months of age. Both groups also showed significant age-related increases of *Nrf2* (see Figures 7.5.3 and 7.5.4).

7.6 Lipid peroxidation at the gums

Lipid peroxidation at the gums was evaluated through the TBARS assay (Table 7.6.1). Sunflower oil and CoQ_{10} fed rats showed the highest concentration of malondialdehyde both, at 6 and at 24 months. Regarding age-related differences, TBARS levels were lower in 24-months-old animals in the three dietary groups receiving CoQ_{10}.

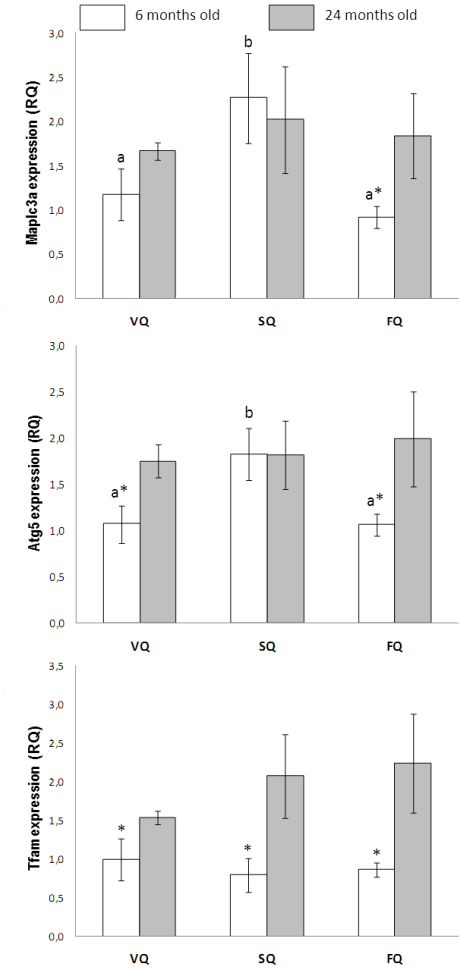

Figure 7.5.1: Effects of CoQ_{10}-rich diets based on virgin olive (VQ), sunflower (SQ) or fish (FQ) oils on gingival expression of genes related to autophagy (*Maplc3a* and *Atg5*) and biogenesis (*Tfam*) in young (6 months old) and old (24 months old) rats measured as mRNA relative quantity (RQ) respect than 6 monts old rats fed on virgin olive oil. Results are presented as mean $\pm$ standard error of the mean. Asterisk (*) means a statistically significant difference between the same dietary treatment for rats aged 6 and 24 months. Lower-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 6 months. Upper-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 24 months.

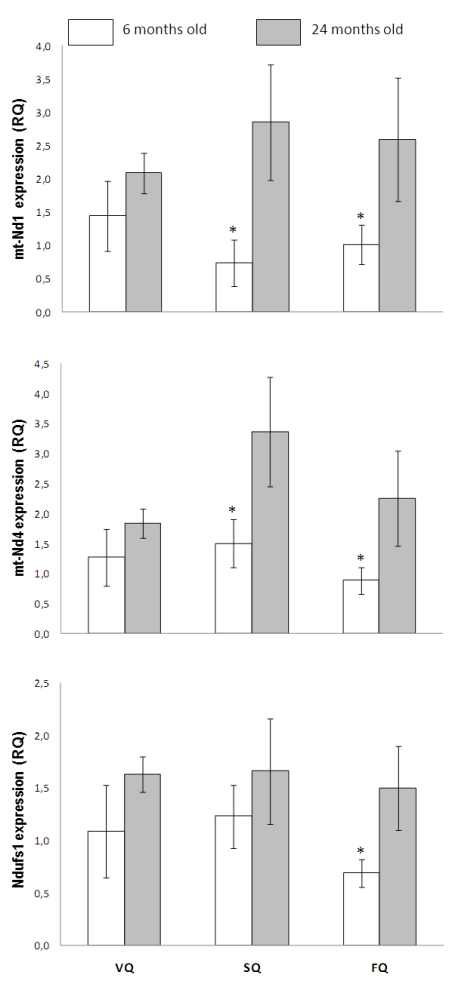

Figure 7.5.2: Effects of CoQ_{10}-rich diets based on virgin olive (VQ), sunflower (SQ) or fish (FQ) oils on gingival expression of genes related to genes related to electron transport chain (*mt-Nd1, mt-Nd4* and *Ndfus*) in young (6 months old) and old (24 months old) rats measured as mRNA relative quantity (RQ) respect than 6-monts-old rats fed on virgin olive oil. Results are presented as mean $\pm$ standard error of the mean. Asterisk (*) means a statistically significant difference between the same dietary treatment for rats aged 6 and 24 months. Lower-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 6 months. Upper-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 24 months.

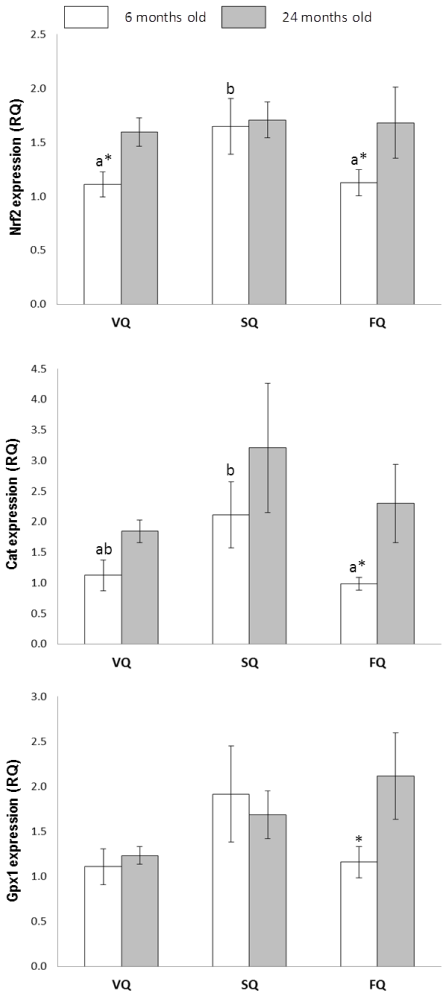

Figure 7.5.3: Effects of CoQ_{10}-rich diets based on virgin olive (VQ), sunflower (SQ) or fish (FQ) oils on gingival expression of genes related to antioxidant defense (Nrf2, Cat, Gpx1) in young (6 months old) and old (24 months old) rats measured as mRNA relative quantity (RQ) respect than 6-monts-old rats fed on virgin olive oil. Results are presented as mean $\pm$ standard error of the mean. Asterisk (*) means a statistically significant difference between the same dietary treatment for rats aged 6 and 24 months. Lower-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 6 months. Upper-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 24 months.

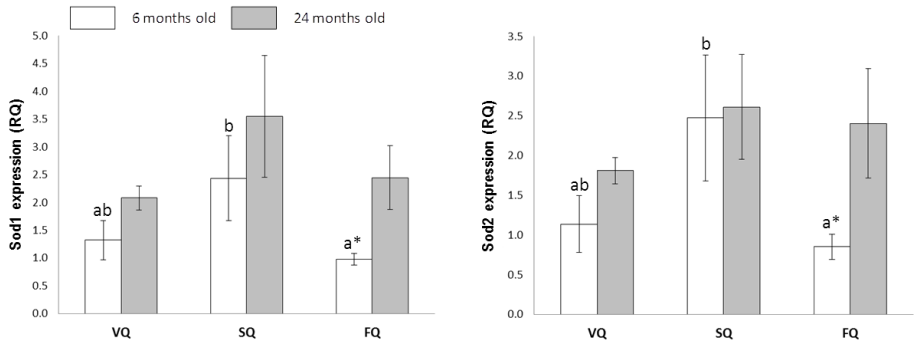

Figure 7.5.4: Effects of CoQ_{10}-rich diets based on virgin olive (VQ), sunflower (SQ) or fish (FQ) oils on gingival expression of genes related to antioxidant defense (*Sod1* and *Sod2*) in young (6 months old) and old (24 months old) rats measured as mRNA relative quantity (RQ) respect than 6-monts-old rats fed on virgin olive oil. Results are presented as mean $\pm$ standard error of the mean. Asterisk (*) means a statistically significant difference between the same dietary treatment for rats aged 6 and 24 months. Lower-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 6 months. Upper-case letters, when different, represent statistically significant differences ($P<0.05$) between the three dietary treatments at 24 months.

Part V

DISCUSSION

Chapter 8

Dietary fat effects on age-related changes of periodontium

As other body parts, oral tissues becomes histophysiologically and clinically altered during aging (Mombelli, 1998; Huttner et al., 2009), although such alterations does not neccessary include pathological conditions (Savitt and Kent, 1991; Huttner et al., 2009). Moderate loss of alveolar bone and periodontal attachment is common in the elderly people, but severe periodontitis is not a natural consequence of aging (Huttner et al., 2009). Still, age has been suggested as a factor that affect the severity and progression of periodontitis. In particular, chronic periodontitis has been defined as an infectious disease resulting in inflammation within the supporting structures of teeth and progressive loss of attachment and alveolar bone (Lindhe et al., 1999). In 1994, Burt reported that a loss of periodontal attachment and alveolar bone tended to occur in older patients. Since then, several epidemiological studies have provide information supporting that fact. A Japanese epidemiological study reported that periodontal disease is extremely widespread in the elderly population (aged 70-80 years old) with prevalences of 97.1% for moderate disease in at least one site and of 47.9% for severe disease. In addition, disease progression after a 2-year study period was confirmed in the 75.1% of subjets(Hirotomi et al., 2002). More recently, a systemic review of studies from 37 countries (Kassebaum et al., 2014) has reported that the incidence of severe periodontitis was higher with increasing age, albeit was low and fairly constant among the elderly (Gil-Montoya et al., 2015). Moreover, it has been proven that aging of the periodontal tissue participates in the development of periodontitis in elderly persons . Cross-sectional studies measuring disease experience demonstrated more attachment loss and alveolar bone loss among older age groups (Naito et al., 2006). Longitudinal studies (Sheiham et al., 2001; Hung et al., 2005; Friedlander et al., 2007) addressing potential relationships between age and attachment loss or bone loss showed a statistically significant relationship between age and incidence of periodontal disease. In addition, although chronic periodontitis is an important disease in aging because it represent the major cause of tooth loss in adults (Page and Schroeder, 1976), it also may constitute a risk factor for other pathologies such as cardiovascular diseases (Chun et al., 2005; Bahekar et al., 2007; Dietrich et al., 2008; Nesbitt et al., 2010) and metabolic syndrome (Nesbitt et al., 2010). The higher risk of coronary heart disease in periodontitis patients was confirmed by a meta-analysis using different types of epidemiological studies that indicated that both prevalence and incidence, were increased in periodontitis (Bahekar et al., 2007). In that

sense, it has been found a dose-dependent association between periodontitis and coronary heart disease incidence among men younger than 60 years of age, but no association was found among men older than 60 years of age. Among men older than 60 years of age, edentulous men tended to have a higher risk of coronary heart disease than dentate men in the lowest bone loss categories (Dietrich et al., 2008). Participants with moderate to advanced alveolar bone loss were significantly more likely to have metabolic syndrome than those with minimal or no bone loss(Nesbitt et al., 2010).

One of the aims of the present study was evaluating the effects of dietary fat type on age-related changes of rat periodontium in the context of a balanced diet. For this purpose, three different unsaturated fatty acids-rich fat sources but with fatty acid profiles clearly differentiated (see Table 5.1.4) were used, virgin olive oil that is rich in MUFA, sunflower oil that is rich in n-6 PUFA, and fish oil that is rich in n-3 PUFA. Beacuse of this, it was neccesary to check if these dietary manipulations modify animals lipid profiles in different ways to establish casual relationships between the different observations in other features and the diet. In this case, it was found that plasma lipid profile from rats fed on virgin olive oil showed the highest relative concentrations of MUFA, the rats fed on sunflower oil showed the highest relative concentrations of n-6 PUFA and the rats fed on fish oil showed the highest relative concentrations of n-3 PUFA. These finding suggest a proper adaptation of animals to dietary fat (López-Miranda et al., 2010; Uauy, 2009). This is in consistency with a previous study in rats fed on balanced diets based on virgin olive or sunflower oil, where liver, skeletal muscle, and heart adapted their membrane composition to dietary fatty acids, althought different adaptation capabilities among organs were observed (Mataix et al., 1998).

Several studies have investigated the potential effects of different fatty acids, mainly n-3 PUFA, derived from diet or as supplements, on periodontal health in both humans (Campan et al., 1997; Eberhard et al., 2002; El-Sharkawy et al., 2010; Iwasaki et al., 2010; Naqvi et al., 2010; Rosenstein et al., 2003) and animal models, namely in rodents (Bendyk et al., 2009; Kesavalu et al., 2007; Vardar-Sengül et al., 2006). In 2005, Arai et al. (2005) stated that the periodontal bone of rats has similar profiles to humans in terms of resorption with aging. For this reason, these authors suggested that the rat is a good model for periodontal research on elderly humans. However, published studies in rodents evaluating dietary fat effect on periodontal health have not taken into account the influence of age (Vardar et al., 2004; Kesavalu et al., 2006; Vardar-Sengül et al., 2006; Bendyk et al., 2009). Results from the present study showed differences regarding alveolar bone loss with aging depending on the dietary fat. Sunflower oil fed animals reported the highest bone loss, followed by those fed on fish oil and finally by animals fed on virgin olive oil. Most of studies both in animals and humans have been focused on n-3 PUFA. In animals, results have been different depending on the dietary treatment schedule, duration and on the way of inducing periodontitis. Overall, when periodontitis was induced by injection of bacterial LPS, n-3 PUFA did not lead to reductions in alveolar bone loss (Vardar-Sengül et al., 2006). However, when bacterial inoculation was used, n-3 PUFA reduced alveolar bone loss in a significant manner (Bendyk et al., 2009; Kesavalu et al., 2006). It has been proposed by the same researchers that these differences are due to the high content of n-3 PUFA of fish oil versus what happens with corn oil that is rich in acids n-6 series. Overall, the results presented here are consistent with the observed in these animals as regards the fact that n-3 PUFA are more beneficial for alveolar bone than n-6 PUFA. Concerning humans, two studies focused on gingivitis (experimentally induced by higyene abstention) have been carried out but results did not show positive effects for n-3 PUFA treatment (Campan et al., 1997; Eberhard et al.,

2002). Still, it should be highlighted that alveolar bone loss not occurs among gingivitis outcomes. Other intervention has been carried out towards the treatment of existing periodontal disease. Curiosly, borage oil that is a dietary fat rich in n-6 PUFA led to a better improvement of periodontal pocket depth and gingival inflammation in periodontitis patients than fish oil after 12 weeks of treatment (Rosenstein et al., 2003). In contrast, and despite the varying experimental conditions (patient size, periodontal measures, and others), a protective association of n-3 PUFA on periodontitis was found after treatment or with higher intake of these fatty acids in an experimental but also El-Sharkawy et al. (2010) in observantional studies (Rosenstein et al., 2003; Iwasaki et al., 2010; Naqvi et al., 2010). This has been supported by the finding of that individuals with periodontitis manifest higher values for n-6:n-3 ratio in serum (Requirand et al., 2000). On the other hand, almost irrelevant it has been the study of MUFA with one of the mentioned studies evaluating the effect of n-3 PUFA on a gingivitis models in humans (Campan et al., 1997) where olive oil was used as placebo. This could explain why no clear beneficial effect for n-3 PUFA on gingival inflammation were observed, but it is important to point than olive oil was not virgin olive oil as the dietary source used in the study presented here. Despite this observation, virgin olive oil-based diet result the most beneficial for periodontium at alveolar bone level here. Altogheter, these result establish that different fatty acid supplied in diet could influence on the damage and alterations of periodontal tissues regardless the harmfull factor.

Along with alveolar bone, gingiva also suffer other changes during the aging process in humans (Huttner et al., 2009). Likewise, inflammatory process associated to gingivitis and finally to periodontitis lead to a progressive modification of this structure. In extent, periodontal pocket formation and/or gingival recession (Lindhe et al., 1999) are clear examples. However, at the beginning, gingivitis already presents associated detectable histological features (Graves and Cochran, 2003; Garlet et al., 2006). To determine the extent to which the set of changes are modified by the diets used in this study, several histopathologic parameters were assessed in sections of gum. These include thickness, cellularity, fibrosis, number of vessels and endothelial activation. Althought little differences were found among dietary and age groups, in general, periodontal inflammation was not promoted. The lack of inflammation sings in aged rats, it has been already noted in old examinations. In 1960, Klingsberg and Butcher reported that few inflammatory cells were visible in the papillae of immature rats, and only a slight increase occurred at over 6 months of age and little additional amounts were noted 20-months-old rats. In addition, litle age-associations were observed in gingiva, these include no alteration in total thickness of the epithelium, stratum granulosum, or keratinization, although the gingival epithelium of two 20-months-old rats was found to be decreased in thickness.

Because of in the present model main differences among dietary treatments in periodontium occurred in relation to alveolar bone, it was interesting to check if this was related to aging-asociated changes in bone metabolism. The discovery of the RANK– RANKL– OPG system has brought rapid progress in the understanding of the regulatory mechanisms of osteoclast differentiation and activation exerted by the immune system (Boyle et al., 2003). Increased RANKL or decreased OPG local expression can cause bone resorption at various sites of the human skeleton. It has been demonstrated that RANKL is up-regulated, whereas OPG is down-regulated in periodontitis, compared to periodontal health, resulting in an increased RANKL:OPG ratio (Jules et al., 2010). This has been observed in GCF from periodontitis patients where it was observed elevated levels of RANKL and reduced levels of OPG (Kinane et al., 2011). RANKL:OPG ratio in GCF was higher in patients with periodontitis compared with healthy subjects(Mogi

et al., 2004). Other studies show spontaneous osteoclast formation and increased bone resorption from circulating peripheral blood mononulear cells of periodontitis patients correlating with high levels of RANKL (Reynolds and Meikle, 1997; Holt and Ebersole, 2005). Here, it was found that gingival expression of the gene encoding RANKL (*Tnfsf11*) decreased with aging, as well as circulating levels of this molecule. On the other hand, circulating OPG levels were higher for old animals, but not for those fed on sunflower oil. Something similar was also found for gingival *Opg* expression at the gingival tissue. It might be expected that aging would lead to increased concentrations and mRNA levels of *Tnfsf11* and the opposite occurred for *Opg*, resulting in a loss of bone and thereafter a pro-periodontitis scenario. However, results found in the present study for virgin olive- and fish-oil fed animals are in agreement with results from other studies that suggest that OPG acts as a defensive mechanism during aging in order to avoid an excess of bone destruction induced by an excess of RANKL stimulation. In that sense, Wada et al. (2004) found that LPS-stimulation of human fibroblasts produced more OPG than RANKL. These authors suggested that the high OPG levels may be a response of the organism in an attempt to counteract the reductions in bone mass that occur throughout adulthood. Changes in relative concentrations of RANKL and OPG observed during experimentally-induced periodontitis in a mice model support the proposed mechanism. In the early part of the study increased RANKL expression relative to OPG were noted, but when the study advanced, RANKL concentration decreases, whereas OPG concentration increased. This correlated with changes in alveolar bone loss that decreased when relative expression of OPG increased. OPG increased in response to RANKL to protect alveolar bone coud be the mechanism explaining such changes (Garlet et al., 2006). An *in vitro* study carrried out by Benatti et al. (2009) suggests that this mechanism could also exist in human against aging effects since it was found that periodontal ligament cells from aged humans expressed higher mRNA levels of *Opg* than younger counterparts, meanwhile no changes in RANKL were reported. Futher, this mechanism would depend on dietary conditions as it is suggested by results from the present study.

According to previous data, the present model is based on the development of alveolar bone resorption during aging. It provides some important data of what happens in humans, more than with those obtained by the simple inoculation of bacteria or bacterial-derived toxins. Nevertheless, and irrespective of the way periodontitis was induced, it is well known that the onset of this disease is due to bacterial infection (Bullon et al., 2009). Amplification of this initial localized response to bacteria results in the release of an array of cytokines and other mediators and propagation of inflammation through the gingival tissues (Graves and Cochran, 2003; Garlet et al., 2006). Then, the failure to encapsulate this ‘‘inflammatory front’’ within gingival tissue leads to expansion of the response adjacent to alveolar bone (Garlet et al., 2006). Inflammation may affect bone formation and resorption processes through RANK– RANKL– OPG pathway. The inflammatory cytokines produced by resident cells in peridontal tissues (i.e. queratinocytes, gingival and periodontal ligaments fibroblasts, osteoblasts, and dendritic cells) and phagocytes (mainly macrophages and neutophils) are involved in osteoclastogenesis and are responsible for the alveolar bone loss in periodontal disease (Di Benedetto et al., 2013). Several studies have confirmed the effects of main pro-inflammatory cytokines on osteoclasts and their precursor cells (i.e. osteoclastogenesis) (**Pfeilschifter et al., 1989; Wei et al., 2005b; Axmann et al., 2009**). The expression of IL-β and TNF-α has been positively correlated with bone resorption of alveolar bone level in rats (Kesavalu et al., 2007). The effects of these cytokines on alveolar bone resorption has shown to be mediated by changes in RANKL or OPG. It has been reported that IL-1β and IL-6 increased produc-

tion of both RANKL and OPG in a synergistic fahion, although the dominant outcome is a net increase in RANKL activity, so osteoclast differentiation is stimulated (Ragab et al., 2002). An *in vitro* study with rat gingival fibroblasts have suggests that *in vivo* cellular aging may affect the severity of inflammation at local level (Okamura et al., 1999). There are evidences indicating that expression of some of these pro-inflammatory cytokines, among which are IL-1β, IL-6 and TNF-α in gum rises during aging and that this increase can be related with loss of alveolar bone (Liang et al., 2010; Ogura et al., 1996; Shimizu et al., 1997). Furthermore, the content of certain fatty acids in diet appears to influence gene expression or levels of these cytokines (Kesavalu et al., 2007; Vardar-Sengül et al., 2006). In particular, it has been suggested that the fatty acids of the n-3 series, which present more fish oil, are responsible for these differences preventing inflammation and bone loss (Kesavalu et al., 2007). In the present study, gingival expression of main cytokines genes (*Il1b,Il6, Il8* and *Tnf*) were measured to elucidate if diet effects on alveolar bone is mediated by them. Overall, gene expression in gums was not different in older groups depending on treatment with the exception of *Tnf* that was higher in fish oil fed group. The lack of differences concerning cytokines could be ascribed to the fact that periodontal inflammation was not promoted. In additon, the fact of fish oil fed animals showed the highest RQs for *Il1b* at 6 months and *Tnf* at 6 and 24 months would indicate that inflammation is not the main mechanism explaning alveolar bone loss differences among dietary groups in this model.

Several studies have shown age-related changes in the levels of proteins and factors that regulate apoptosis in periodontal tissues(e.g. Campisi, 2003) and and this may be an important factor to consider for aging that can aggravate the changes, if excessive cell death of certain cell types, or prevent them since it also can act as homeostasis mechanims. In mice, it has been reported that the cellular component of the submucosal connective tissue of both gingival and junctional epithelial layers decrease with aging, which has suggested to be associated with either gingival recession or to the apical migration of the junctional epithelium with aging (Sakai et al., 1999). In the same specie, an age-dependent increase has been shown in the number of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells in the submucosal connective tissue from gingival epithelium (Sakai et al., 1999; Enoki et al., 2007) and junctional epithelium (Sakai et al., 1999), whereas no significant differences in the mean number of 5-bromo-2'-deoxyuridine (BrdU)-positive cells (Sakai et al., 1999). This would indicate that age-associted increases in apoptosis are responsible for cellularity reduction and probably for morphological changes of this tissue, at least in part (Sakai et al., 1999). The increase in apoptosis has been also noted within the periodontal ligament and gingivae from aged rats at level of molars and incisors (Pycroft et al., 2002). Nevertheless when this process was assessed by a method different from TUNEL technique some variations were found. This occurred in the previous study in rats (Pycroft et al., 2002) where electron microscopy indicated that significant numbers of apoptotic cells were only in the incisor periodontium. Along with inflammation, apoptosis has also been widely investigated in relation to periodontal disease too. In humans, Jarnbring et al. in 2002 found reduced proliferation and increased apoptosis in the most apical part of the pocket in patients with periodontitis, indicating a net loss of keratinocytes in this area. Ontology analysis of 88 genes related to apoptotic pathways was performed in gingival biopsies of healthy and periodontitis sites from young, adult, and aged non-human primates (*Macaca mulatta*). Overall, lower expression of anti-apoptotic and higher expression of pro-apoptotic genes were associated with healthy gingival tissue from young compared with aged animals. Few differences in gene expression were observed in healthy gingival

tissue between adult and aged animals. Comparison between healthy and periodontitis gingival tissues showed that the up- or down-regulated apoptotic genes in diseased gingival tissue are different in adults compared with aged animals (González et al., 2011). Importantly, age has not shown effect on apoptosis at level of epithelial layer in mice (Sakai et al., 1999) suggesting differences in this according to factor evaluated (aging or bacterial products) or specie (human or mouse). Here, three members of BCL-2 protein family participating in apoptosis intrinsic pathway were taken into account. One hand, the proapoptotic protein BAX and BAK proteins that are able to form pores into the mitochondrial membrane when they change its conformations, oligomerize, and insert themselves into the membrane. On the other hand, one of their antagonists, the anti-apoptotic protein BCL-2 that binds BAX and BAK preventing its actions (Tsuda et al., 2012). Gingival gene expression observed here would suggest that aging led to higher values for at least one of the proapoptotic genes (*Bad*), but also for *Bcl*2, only for two of the dietary treatments, virgin olive and fish oil, but not for sunflower oil treated animals. Again, diet could modulate the age-associated changes in apoptosis in this tissue. In this case, virgin olive- and fish-oil could prevent the effect of age on apoptosis in this tissue. This fact could be results usefull in humans to prevent possible changes in gums, which increase succeptibility to periodontitis or gingivitis as has been proposed and maybe to treat some periodontal disease case where apoptosis is elevated helping to mitigate some of the symptoms .

Concering mitochondrial status, biogenesis and autophagy represent two opposing, but coordinated processes that determine mitochondrial content, structure, and function (Zhu et al., 2013). Increase of biogenesis rate could compensate damaged components by ROS, although if mitochondria removal rate is not adequate, with aging, accumulation of deleterious components might lead to poor quality mitochondria that may enhance cellular oxidative stress (Zhang, 2013). A number of autophagy-related genes have been identified (Tsukada and Ohsumi, 1993; Thumm et al., 1994) whose nomenclature was unified and termed as *Atg* (Klionsky et al., 2003). Most of proteins encoded by *Atg* genes (named ATG) act during autophagosome formation. Studies in yeast revealed two ubiquitylation-like conjugation systems are required for autophagosome formation (Ohsumi, 2001) that are highly conserved in mammals . One system mediates the conjugation of ATG12–ATG5 (Mizushima et al., 1998), and the second system produces covalent linkage between LC3 (or ATG8) and phosphatidylethanolamine (Ichimura et al., 2000). These molecules are very good marker proteins for autophagic membranes. ATG12–ATG5 are specific markers for the isolation membrane, and LC3 is a general marker for autophagic membranes (Mizushima, 2004). Again, higher RQs has been found for LC3 encoding gene (*Maplc3a*) and *Atg5,* but only in animals fed on virgin olive- and fish-oil groups.

Mitochondrial biogenesis is a complex process that requires the synthesis, import, and incorporation of proteins and lipids to the existing mitochondrial reticulum, as well as replication of mtDNA. Comparison of mitochondria across different tissues has shown a significant concordance between protein levels and mRNA levels (Mootha et al., 2003) suggesting that mitochondrial mass in a cell is controlled largely, although not solely, at the level of transcription (Hock and Kralli, 2009). Although this process can be inintiated by different transcription factors that differentially regulate several subsets of genes, the coordination of the nuclear and mitochondrial genomes is achieved by nucleus-encoded mitochondrial proteins, such as TFAM, TFB1M, and TFB2M that control the transcription and replication of mtDNA and are induced in response to signals promoting mitochondrial biogenesis (Chow et al., 2007). Thus, regardless stimuli that triggers this

process, enventually it is needed to activate this factors. For this reason, TFAM has been established as a good marker of the biogenesis process (Håkansson et al., 2011). Here, gene expression analysis reported, as for apoptosis *Atg* and apoptosis-related genes, no differences for *Tfam* expression in relation to the aging process in animals fed on sunflower oil. However, old animals fed virgin olive- and fish-oil-based diets showed higher RQs for this gene than their younger counterparts. This data would indicate that virgin olive- and fish-oil presence in the diet lead to an increase in mtochondrial biogenesis with aging. In addition, PGC-1α has been identified as a transcriptional co-activator of nuclear respiratory factor 1 (NRF-1), nuclear respiratory factor 2 (NRF-2), and PPARs able to integrate physiological signals and to enhance mitochondrial biogenesis and oxidative function (Puigserver et al., 1998; Wu et al., 1999; Civitarese et al., 2007). Therefore, it assists the other factor to regulate different gene subsets are coordinately regulated. Different systems implicated in the regulation of PGC-1α activity have been described, which subsequently also regulate biogenesis due to the cental role of PGC-1α. One of these, are represented by sirtuin 1 (SIRT1), a NAD^+-dependent deacetylase that deacetylates PGC-1α reversing the effects of the acetyltransferase GCN5, which acetylates and represses PGC-1α (Rodgers et al., 2008). These genes enconding for SIRT1 and PGC-1α, reinforced *Tfam* observation. In muscle, SIRT1 promotes the deacetylation of PGC-1α and the increased expression of PGC-1α and many genes encoding mitochondrial proteins, including *Tfam* (Kelly and Scarpulla, 2004). Something similar could occur in gingival tissue from virgin olive and fish oil animals, although it possible that more factors and regulatory proteins were implicated. In an i*n vitro* study with fibroblasts, it has been noted that bacterial LPS provoked increased oxidative stress and mitochondrial dysfunction at the same time that reduced oxygen consumption and mitochondrial biogenesis (Bullón et al., 2015). Thus, it is possible that the same dietary fat act on mitochondral biogenesis in the same way as occur in aging preventing, at least in part, periodontital diseases symptoms in humans.

The mitochondrial theory of aging (Barja, 2007) postulates that cellular aging is the product of mutations in the mtDNA as a result of oxidative damage. Oxidative stress has been shown to have negative consequences on the formation of bone by *in vitro* osteoblasts and facilitates it destruction(Abiko et al., 1998). With this background, it was interesting to check if the fats used could exert their effect on bone in this way. As a result of accumulated damage to mtDNA, the mitochondrial blueprints are markedly altered, thus perpetuating the production of aberrant ETC components (Gilmer et al., 2010). According to that, different elements from the mtETC together with elements of the antioxidant system were investigated also at transcriptional level in gums . mtETC component evaluated consisted in three proteins that form part of mitochondrial complex I (MT-ND1, MT-ND4 and NDUFS1), two encoded by mitochondrial genes (*mt-Nd1* and *mt-Nd4*) and one encoded by a nuclear gene (*Ndufs1*). Concerning antioxidant defenses, gene encoding for two of main detoxyfing enzymes (SOD-1 and SOD-2) were investigated. In all situations, studied molecules reported similar results than those found for the previously discussed markers with an exception for animals fed on fish oil that did not shown an age-associated increase for *mt-Nd1*. That is expected since TFAM and/or PGC-1α are directly or inderectly implicated in the regulation of the expression of these genes . PGC-1α would colaborate on induce *Ndufs1* and *Tfam* expression. In turn, the former would bind mtDNA promoting the expression of mitochondrial genes . In addition, PGC-1α also participate in the expression of other genes implicated in oxidative metabolism beyond mitochondria. Among them, it is both mitochondrial and cytosolic antoxidant enzymes genes .

The increase in autophagy and biogenesis markers found in groups fed on virgin olive- and fish-oil together with the changes in genes encoding for mitochondrial complex I proteins would suggest, that with aging, there is an increase mitochondrial turnover. This might occurrs in response to an increased damage of mitochondrial structures probably by high oxidative stress levels but other unknwon stimuli also can act favoring this mechanism. Likewise, changes observed in antioxidant defenses-related genes would be motivated by similar causes. The higher mitochondrial turn-over and the overexpression of the genes enconding detoxifying enzymes together would contibute to decrease oxidative stress levels in old animal fed on virgin olive and fish oil. However, in those fed diet rich in n-6 PUFA, the absence of changes regarding all these markers would be related to high oxidative levels in old animals, but this fact also could be due to the oxidative damages are lower in this group and compensatory mechanisms are less needed. To evaluate possible differences among groups oxidative stress levels were evaluaed by a TBARS assay. TBARS is a marker of lipid peroxidation levels that has been widely studied in diseased periodontal tissue. According to this, oxidative stress levels decreased in virgin olive and fish oil fed groups with aging, whereas sunflower oil fed animals did not changes. In addition, old animals from the last dietary group also showed higher TBARS levels than animals from the other two groups with the same age. Therefore, the age-associated increase is efective to decrease oxidative stress levels. However, it seems that this mechanism is ablated by diet rich in n-6 PUFA or at least not induced.

Chapter 9

CoQ effects on age-related changes of periodontium

A second objetive of this study is to evaluate the effects of dietary CoQ_{10} on periodontium aging in three dietary context similar to the three above. As occurs for dietary fat, it is important to clarify if the amount of CoQ_{10} given to the rats in diet is able to modify its content in the body. Because of animals from the present research supplemented with CoQ_{10} had higher values for this molecule in blood, it might be reasonable to think that positive effects found regarding alveolar bone loss could be a consequence of CoQ_{10} feeding. In addition, it is also interesting to know how affect to the biological membranes. In humans, there are evidences, mainly indirect, that dietary supplementation with CoQ_{10} may be incorporated into mitochondria, at least in conditions of partial CoQ tissue deficiency (Rosenfeldt et al., 2005; Molyneux et al., 2009; Dumont et al., 2010; Xu et al., 2010). Moreover, some clinical trials have shown oral administration of CoQ_{10} increases the concentration of CoQ_{10} in the diseased gum (Shizukuishi et al., 1986; Wilkinson et al., 1975).

In the three experimental groups discussed above, differences regarding age-related alveolar bone loss depending on the dietary fat were found. Specifically, sunflower oil fed animals reported the highest bone loss, followed by those fed on fish- and with virgin olive-oil as the best edible oil to preserve age-related alveolar bone loss. In turn, no differences between dietary groups receiving dietary CoQ_{10} were found regarding alveolar bone loss associated to age. According to the differences observed among the three previous groups, long-term feeding on low dosages of CoQ_{10} seems to be effective reducing the age-related alveolar bone loss derived from the use of sunflower oil as dietary fat source, since animals fed on diet based on them showed similar bone loss that those maintained with supplemented diets based on virgin olive oil. In addition, other applications of CoQ different from oral administration in diet also have supported the positive role of this molecule on alveolar bone (Yoneda et al., 2013). Concerning histopathological alterations, CoQ_{10} addition to sunflower oil led to lower fibrosis at the gingival level than when this dietary fat was administered without CoQ_{10}, and something similar was found for endothelial activation, both in sunflower and fish-oil fed animals in which, CoQ_{10} addition led to lower values than when dietary oils were administered alone. Thus, these variations concerning histology might help also to explain how CoQ_{10} improves periodontium health during aging. In humans, oral administration of CoQ_{10} has shown to effectively suppresses periodontal inflammation (Shizukuishi et al., 1986; Wilkinson

et al., 1975). Again, topical application of CoQ in gel-form in humans supported the positive role of this molecule (Hanioka et al., 1994; Sale et al., 2014). However, it it important to remind that inflammation degree in this rat model was not so notably as in human periodontitis.

The ways through which CoQ might act at the level of alveolar bone preservation could be related to protective effect of this molecule against age-related oxidative stress as it has been previously described in rat brain (Ochoa et al., 2011), liver (Jiménez-Santos et al., 2014), and heart (Ochoa et al., 2005a) mitochondria; or to a different mechanism activated by it. . In a number of organisms, dietary CoQ_{10} addition has been shown to cause multiple phenotypic effects and might regulate the expression of genes implicated in multiple processes (Linnane et al., 2007; Ochoa et al., 2007). Among other, it has been reported that CoQ anti-inflammatory effects seems to be due to this molecule action on NF-κB1-dependent genes (Schmelzer et al., 2007) that could include genes implicated in different processes. As previously, gingival expresion of genes related to bone metabolism, inflammation, autophagy, mitochondrial biogenesis, mtETC, and antioxidant enzymes were analyzed in order to try to understand mechanisms under CoQ_{10} effect on age-related bone resorption associated to different dietary fats, as well as circulating levels for some of their products and oxidative stress status at the gums.

According to previous results, it seems that the increase in OPG levels noted in aged individuals (Jules et al., 2010; Wada et al., 2004) was ablated by the n-6 PUFA-rich diet (i.e. sunflower oil-based diet) since RANKL circulating levels were lower in old rats for all dietary groups, whereas OPG was higher, but only for sunflower and fish-oil fed groups (see Chapter 8). Increase in OPG levels has been suggested to be a response of the organism in an attempt to counteract the reductions in bone mass that occur throughout adulthood (Jules et al., 2010; Wada et al., 2004), so its ablation would lead to more bone loss. However, in the groups also fed on CoQ_{10}, OPG circulating levels were higher in old rats with any diet. Thus, the addition of CoQ_{10} to that diet might restore these age-related higher values for OPG, which would explain, at least in part, the absence of differences in alveolar bone loss found after CoQ_{10} addition to the dietary fat rich in n-6 PUFA (i.e., sunflower oil) in which the absence of this molecule led to significantly higher alveolar bone loss in comparison with the other unsaturated fats, namely virgin olive and fish oils. Otherwise, when looking at the gingival mRNA RQs, no changes for *Tnfsf11* or *Opg* were found. That might represent the already suggested connection between gingival tissue and systemic health of the individual in the sense that unfavorable systemic levels of inflammation might lead to altered physiology at the gingival level (Bullon et al., 2009). The effects of CoQ preserving bone have already been suggested by different *in vitro* study in part by its effect on signalig pathways regulating osteoclastogenesis, but also osteoblasts differentiation and activity. It is known that ROS acts as a secondary messenger on signal pathways implicated in osteoclast differentiation, particularly those that implies to NF-κB (Moon et al., 2012). In this context, CoQ either by its role as intracellular ROS scavenger and its participation in systems that regulate cell redox status, might act as an inhibitor of osteoclastogenesis. In rats, ubiquinol applied topically on the gingival surface leads to a differentiation of osteoclasts associated with aging (Yoneda et al., 2013). Actually, several markers of RANKL-stimulated osteoclastogenesis including the nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1), tartrate-resistant acid phosphatase (TRAP), and osteoclast-associated immunoglobulin-like receptor expression were dimished in osteoclast precursors models (bone marrow-derived monocytes and RAW 264.7 cell) by treatment with non-toxic CoQ_{10}concentration, as well as the number of multinucleated TRAP-positive cells fomed. Furthermore, it enhances the

induction of several osteoblastogenic biomarkers (alkaline phosphatase, type 1 collagen, bone sialoprotein, osteoblast-specific transcription factor Osterix, and Runt-related transcription factor and promoted matrix mineralization by enhancing bone nodule formation in a dose-dependent manner . It could be possible that CoQ also mediate *Opg* expression in response to certain stimulus regarless the exact mechanism.

As stated previously, inflammation may affect bone formation and resorption processes through RANK–RANKL–OPG pathway (Ragab et al., 2002). Among inflammatory mediators that has to shown influence on bone metabolism, only the cytokines IL-1β and IL-6 were evaluated in this part of the study, but their circulating levels were also assessed. In the groups fed on CoQ_{10}, age-related changes in gingival mRNA RQs were only found for *Il6*, which was higher with aging for fish- and sunflower-oil fed groups. High levels of IL-6 stimulate bone resorption, more physiological levels do not, unless associated with others cytokines (de la Mata et al., 1995; Linkhart et al., 1991). Thus, the difference found here should not have important consequences on alveolar bone. Furthermore, periodontal inflammation was not clearly promoted as in the groups not taking CoQ_{10}, which could be related to the absence of variations in *Il1b* mRNA levels in gums, especially in older groups. On the other hand, circulating levels changes resulting from the aging process seemed to lead to a more pro-inflammatory state in old animals, particularly in those fed fish- and sunflower-oil, since IL-1β levels were higher than in virgin olive oil fed rats. In turn, it has been noted that circulating levels of several inflammatory cytokines, including IL-1β, increased in animals fed on virgin olive oil without CoQ_{10}. This suggested that CoQ_{10} have certain anti-inflammatory effect on virgin olive oil rats. However, it is unclear whether these variations to systemic level exerted significant effects on periodontal tissues. However, the role of CoQ as an anti-inflamatory molecule at periodontal level has been suggested peviously in different interventions (Wilkinson et al., 1975; Shizukuishi et al., 1986; Hanioka et al., 1994; Sale et al., 2014) But again, it is not possible to differentiate if this is due to systemic influence or local effects on this levels because of different experimenal conditions.

As in previous groups of animals, several key issues related to mitochondrial biology were also investigated at transcriptional level according to the suggested importance of mitochondrion in aging (Barja, 2007). Concerning the studied marker of biogenesis *Tfam*, in the groups fed on dietary fats but without CoQ_{10}, aging led to higher RQs in animals fed on virgin olive- or fish-oil, but not in those fed on sunflower oil. In the other three, it has been noted that CoQ_{10} induced a higher expression of *Tfam* during aging also for animals fed on sunflower oil. The improvement of mitochondrial biogenesis by CoQ_{10} have been also reported in cultures of fibroblasts treated with LPS where CoQ_{10} activated *Tfam*, but also PGC-1α (Bullón et al., 2015). The role of CoQ enhancing biogenesis has been also found in other cell cultures as occur in astrocytes receiiving UV-B radiation (Li et al., 2005). These results might reinforce the importance of biogenesis in the correct function of mitochondrial metabolism during aging and their potential correlation with alveolar bone maintenance. In relation to the other element associated to mitochondrial turnover, that is, autophagy, data from these groups seem to indicate that this process, at least under these experimental conditions, is not as relevant as biogenesis in order to understand data about alveolar bone loss since changes associated to aging *Atg5* and *Map1lc3a* expression were similar with or without CoQ_{10}. At this point, only a relevant change was found between groups fed on diets supplemented with CoQ_{10} or without CoQ_{10}. In particular, without CoQ_{10} *Tfam*, *Atg5*, and *Map1lc3a* expression in old animals fed on virgin olive oil were higher than those observed in old animals fed on the other two dietary fats. This difference was not found after CoQ_{10} supplementation,

since these markers were similar between the three dietary groups during aging. With the present data, no explanation for this issue is possible but is not relevant in order to explain the effect of these dietary treatments on alveolar bone level.

According to this interest on mitochondria, respiratory machinery was also studied from the point of view of gene expression. In this respect, CoQ_{10} has led to a balance between dietary fats in order to counterbalance disturbances produced by some of the fat sources by themselves. In other words, when fats were delivered without CoQ_{10}, a different response of studied mtETC components (*mt-Nd1*, *mt-Nd4*, and Ndfus1) was found, with higher gene expression level for animals fed on virgin olive oil, both at 6 and at 24 months of age. In the present study, after CoQ_{10} addition, these differences between dietary fats were abolished. This is expectly because of results found in relation to TFAM at least for *mt-Nd1*, *mt-Nd4* whose expression are directly regulated by it .

As stated in the previous paragraph, oxidative stress may be related to events associated to alveolar bone loss through interactions at the mitochondrial level. In this respect, TBARS and expression of genes enconding detoxifying enzymes (*Gpx1, Cat, Sod1 and Sod2*), as well of *Nrf2* were analyzed. Regarding TBARS, it is well know how dietary fat affect lipid peroxidation, with fats more polyunsatured, particularly when polyunsaturation comes from n-6 series, leading to higher peroxidation than less polyunsaturated fats, like virgin olive oil (Ochoa et al., 2003; Quiles et al., 2006). As in non-supplemented groups, this effect persisted at 6 and at 24 months of age. In the same way, antioxidant enzyme gene expression for aged animals, led to non-different results. These finding on oxidative stress might help us to understand that, at least under these experimental conditions, the role of CoQ on oxidative stress should not be a direct mechanism through which CoQ_{10} supplementation led to a lower alveolar bone loss in sunflower oil fed animals.

Part VI

CONCLUSIONS

Conclusions

Conclusion 1: on Alveolar bone loss.

Dietary n-6 PUFA are responsible for a greater alveolar bone loss associated to age. It has been found that dietary therapy with CoQ_{10} has been effective to reduce the aging-related alveolar bone loss exacerbated by n-6 PUFA-based diets. From the nutritional and clinical point of view, long-term feeding on low dosages of CoQ_{10} could counteract the negative effects of n-6 PUFA-rich diets on alveolar bone loss in relation to age. The interest of the present study resides in the finding that the excess of alveolar bone loss (a major feature of periodontitis) associated to age may be targeted by an appropriate dietary treatment.

Conclusion 2: on bone metabolism markers.

Age-associated increases in OPG levels act as a defensive mechanism during aging in order to avoid an excess of bone destruction induced by an excess of RANKL stimulation. Circulating levels of OPG would be a key factor for effects of CoQ_{10} on alveolar bone loss in animals fed n-6 PUFA-rich diet. However, the link between CoQ_{10} and OPG production need to be clarified.

Conclusion 3: on inflammation markers and gingival histology.

Periodontal inflammation was not clearly promoted in any of the groups. This fact could be related to the absence of variations in *Il1b* mRNA RQs in gums. This suggests that alveolar bone loss in aging can be exacerbated by other mechanisms independent of inflammation.

Conclusion 4: on mitochondrial turn-over and mtETC.

Both MUFA and n-3 PUFA could allow mitochondrial maintaining turnover through biogenesis and autophagy. Results from the present study would reinforce the importance of biogenesis in the correct function of mitochondrial metabolism during aging and their potential correlation with alveolar bone maintenance. Experimental data from animals taking CoQ_{10} would indicate that autophagy, at least under these experimental conditions, is not as relevant as biogenesis in order to understand data about alveolar bone loss

since changes associated to aging for autophagy markers studied were similar with or without it. A different response of studied mtETC components was found in relation to dietary fat with higher gene expression level for animals fed on virgin olive oil, both at 6 and at 24 months of age. Dietary CoQ_{10} either by their role as electron carrier, or by its role as an antioxidant, is able to balance the effect of dietary fat on mtETC components since differences between dietary fats were abolished when animal receiving this compound in their diets.

Conclusion 7: on oxidative stress markers and antioxidant defense mechanisms.

As expected, in old animals, the highest values of TBARS levels were found for those with the highest n-6 PUFA percentage. However, under the experimental conditions of this study, the role of dietary CoQ_{10} on alveolar bone loss in these animals does not seem mediated by its effect on oxidative stress. In the same way, antioxidant enzyme gene expression for aged animals, led to non-different results. These finding on oxidative stress might help us to understand that, at least under these experimental conditions, the role of CoQ on oxidative stress should not be a direct mechanism through which CoQ_{10} supplementation led to a lower alveolar bone loss in sunflower oil fed animals. MUFA or n-3 PUFA might allow mitochondrial maintaining turnover through biogenesis or autophagy. They might are also able to induce the corresponding antioxidant systems to counteract age-related oxidative stress, and it does not inhibit mtETC machinery.

General conclusion.

The interest of the present study resides in the finding that the excess of alveolar bone loss (a major feature of periodontitis) associated to age may be targeted by an appropriate dietary treatment. The mechanisms involved in this phenomenon are related to ablation of the ability of the cell to adapt to aging. So, MUFA (virgin olive oil-based) or n-3 PUFA (fish oil-based)-rich diets would maintain a high turnover rate by mitochondrial biogenesis and autophagy processes. In turn, they also would be capable of inducing antioxidant systems, which together with the possible increase in the rate of mitochondrial replacement collaborate in reducing the oxidative stress associated with age allowing the proper functioning of mitochondrial electron transport chain. The addition of CoQ to the n-6 PUFA-rich diet (based on sunflower oil) appears to attenuate the effects of this age-associated alveolar bone loss. The mechanisms involved in this effect are related to the restoring of the ability of cells to adapt the gingival tissue, at the mitochondrial level. In particular, this could be due to age-related increase in the rate of mitochondrial biogenesis and to improved oxidative and respiratory equilibrium in these animals, which could be due to the effect on biogenesis mentioned, at least in part.

Conclusiones

Conclusión 1: sobre la pérdida de hueso alveolar.

Los ácidos grasos poliinsaturados de la serie n-6 dietéticos son responsables de una mayor pérdida de hueso alveolar mayor asociada a la edad. Se ha encontrado que la terapia dietética con CoQ_{10} ha sido eficaz para reducir la pérdida de hueso alveolar asociada a la edad exacerbada por dietas basadas en ácidos grasos poliinsaturados n-6. Desde el punto de vista nutricional y clínico, la alimentación a largo plazo con dosis bajas de CoQ_{10} podría contrarrestar los efectos negativos de las dietas ricas en ácidos grasos poliinsaturados n-6 sobre la pérdida de hueso alveolar relacionada con la edad. El interés del presente estudio reside en el hallazgo de que el exceso de pérdida ósea alveolar (una característica clave de la periodontitis) asociada a la edad puede ser objetivo de un tratamiento dietético adecuado.

Conclusión 2: sobre los marcadores del metabolismo óseo.

El aumento asociado a la edad de los niveles de osteoprotegerina actúa como un mecanismo defensivo durante el envejecimiento con el fin de evitar un exceso de la destrucción ósea inducida por una estimulación excesiva por parte de RANKL. Los niveles circulantes de osteoprotegerina serían un factor clave para los efectos de CoQ_{10} sobre la pérdida de hueso alveolar en los animales alimentados con la dieta rica en ácidos poliinsaturados de la serie n-6. Sin embargo, el vínculo entre el CoQ_{10} y la producción de osteoprotegerina necesita ser esclarecido.

Conclusión 3: sobre los marcadores de inflamación y la histología gingival.

La inflamación periodontal no fue promovida claramente en ninguno de los grupos. Este hecho podría estar relacionado con la ausencia de variaciones en la cantidades relativas de ARNm de *Il1b* en las encías. Esto sugiere que la pérdida de hueso alveolar en el envejecimiento puede ser exacerbada por otros mecanismos independientes de la inflamación.

Conclusión 4: sobre el recambio y la cadena de transporte electrónico mitocondriales.

Tanto ácidos grasos poliinsaturados n-3 como los moninsaturados podrían permitir mitocondrial mantener el recambio mitocondrial través de la biogénesis o la autofagia. Los resultados de este estudio podrían reforzar la importancia de la biogénesis en el correcto funcionamiento del metabolismo mitocondrial durante el envejecimiento y su potencial correlación con el mantenimiento del hueso alveolar. Los datos experimentales de los animales que tomaban CoQ_{10} indicarían que la autofagia, al menos en estas condiciones experimentales, no es tan relevante como la biogénesis a fin de comprender los datos sobre la pérdida de hueso alveolar ya que los cambios asociados al envejecimiento de marcadores autofagia estudiados fueron similares con o sin él. En particular, y sin CoQ_{10} las cantidades relatias de ARNm para *Tfam, Atg5,* y *Maplc3*a en los animales viejos alimentados con aceite de oliva virgen fueron más altos que en los animales viejos alimentados con las otras dos grasas de la dieta. Esta diferencia no se encontró después de la suplementación CoQ_{10}. Además se encontró una respuesta diferente de componentes de la cadena de transporte de electrones mitocondrial estudiados en relación con la grasa de la dieta con un mayor nivel de expresión génica para los animales alimentados con aceite de oliva virgen, tanto a los 6 como a os 24 meses de edad. El CoQ_{10} en la dieta, ya sea por su papel como portador de electrones, o por su papel como antioxidante, es capaz de equilibrar el efecto de la grasa de la dieta en los componentes de la cadena de transporte electrónico mitocondrial ya que las diferencias entre las grasas de la dieta se fueron abolidas cuando los animales recibieron este compuesto en sus dietas.

Conclusión 7: sobre los marcadores de estrés oxidativo y los mecanismos de defensa antioxidante.

Como era de esperar, en los animales de edad, se encontró que los valores más altos de los niveles de TBARS para aquellos con el mayor porcentaje de ácidos grasos poliinsaturados n-6. Sin embargo, bajo las condiciones experimentales de este estudio, el papel de CoQ_{10}en la dieta sobre la pérdida de hueso alveolar en estos animales no parece estar mediado por su efecto sobre el estrés oxidativo. De la misma manera, la expresión de los genes de las enzima antioxidantes en animales de edad avanzada, no mostraron lugar a resultados diferentes. Estos hallazgos sobre el estrés oxidativo podrían ayudarnos a entender que, al menos en estas condiciones experimentales, el papel de la CoQ_{10} sobre el estrés oxidativo no debe ser un mecanismo directo a través del cual los suplementos de CoQ_{10} conducen a una menor pérdida de hueso alveolar en los animales alimentados con aceite de girasol. los ácidos grasos monoinsaturados (a base de aceite de oliva virgen) y poliinsaturados n-3 podrían permitir mitocondrial mantener el volumen de negocios a través de la biogénesis o la autofagia. Podrían también son capaces de inducir a los sistemas antioxidantes correspondientes a contrarrestar el estrés oxidativo relacionado con la edad, y no inhiben la maquinaria de transporte electrónico mitocondrial.

Conclusión general:

El interés del presente estudio reside en el hallazgo de que el exceso de pérdida ósea alveolar (una característica importante de la periodontitis) asociada a la edad puede ser objeto

de un tratamiento dietético adecuado. Los mecanismos involucrados en este fenómeno están relacionadas con la ablación de la capacidad de la célula para adaptarse al envejecimiento. Así, las dietas ricas en ácidos grasos monoinsaturados (a base de aceite de oliva virgen) o ácidos grasos poliinsaturados n-3 (a base de aceite de pescado) mantendrían un alto índice de recambio mitocondrial por procesos biogénesis y autofagia. A su vez, también serían capaces de inducir sistemas antioxidantes, que junto con el posible aumento en la tasa de reemplazo mitocondrial colaborarían en la reducción del estrés oxidativo asociado con la edad lo que permite el buen funcionamiento de la cadena de transporte de electrones mitocondrial. La adición de la CoQ a la dieta rica en ácidos grasos poliinsaturados n-6 (basada en aceite de girasol) parece atenuar los efectos de esta pérdida de masa ósea alveolar asociada con la edad. Los mecanismos implicados en este efecto están relacionadas con la restauración de la capacidad de las células del tejido gingival para adaptarse al envejecimiento a nivel mitocondrial. En particular, esto podría ser debido a un aumento relacionado con la edad en la tasa de la biogénesis mitocondrial y a la mejora de equilibrio oxidativo y respiratorio en estos animales, lo que podría deberse al efecto sobre la biogénesis mencionado, al menos en parte.

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